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**TGFβ stimulates biglycan core protein synthesis but not
glycosaminoglycan chain elongation via
Akt phosphorylation in vascular smooth muscle**

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Keywords Akt, biglycan, transforming growth factor beta, atherosclerosis

Abstract

Transforming growth factor β (TGF- β) can mediate proteoglycan synthesis via Smad and non-Smad signalling pathways in vascular smooth muscle. We investigated whether TGF- β mediated proteoglycan synthesis is via PI3K/Akt. TGF- β induced a rapid phosphorylation of Akt that continued up to 4h. Akt phosphorylation was blocked by Akt1/2 inhibitor SN30978 however it did not block Smad2 phosphorylation at either the carboxy or linker regions indicating that TGF- β -mediated Akt phosphorylation is independent of Smad2 signalling. The role of Akt in TGF- β -mediated proteoglycan synthesis was investigated. Treatment with SN30978 showed a concentration-dependent decrease of TGF- β -mediated [35 S]-sulfate and [35 S]-Met/Cys incorporation into secreted proteoglycans however SDS-PAGE showed no change in biglycan size. In TGF- β treated cells biglycan mRNA levels increased by 40-100% by 24h and was significantly blocked by SN30978. Our findings demonstrate that Akt is a downstream signalling component of TGF- β -mediated biglycan core protein synthesis but not glycosaminoglycan chain hyperelongation in vascular smooth muscle.

Introduction

Proteoglycans including biglycan accumulate in the extracellular matrix of atherosclerotic-prone artery walls in the early stages of atherosclerotic lesion development¹. Biglycan is synthesized predominantly by vascular smooth muscle cells (VSMCs) in the vessel wall and atherogenic lipoproteins (LDL) become trapped on the negatively charged covalently attached glycosaminoglycan (GAG) chains thereby exacerbating macrophage accumulation and progression of atherosclerotic lesions. Previously it has been demonstrated that transforming growth factor- β (TGF- β) treatment of human VSMCs increases the expression of biglycan and causes marked elongation of its GAG chains with a subsequent increase in the binding of LDL^{2, 3, 4, 5}. In addition TGF- β -mediated GAG elongation on biglycan requires *de novo* protein transcription and translation⁶.

TGF- β signals via TGF- β receptors type I and type II (T β RI and T β RII) with ligand binding causing the formation of a heteromeric complex of T β RI and T β RII and resulting in the autophosphorylation and activation of T β RI⁷. T β RI then phosphorylates Smad2 and Smad3 in their C-terminal region which enables heterocomplex formation with Smad4 and subsequent translocation to the nucleus where they act as transcription factors binding to TGF- β responsive genes^{8, 9}. The canonical TGF- β -mediated Smad2/3 C-terminal phosphorylation is important in many TGF- β responses including biglycan synthesis and GAG chain elongation in VSM⁴. However it has also been established that TGF- β can mediate pathways other than the canonical Smad pathway to generate proteoglycan synthesis. TGF- β -mediated phosphorylation of Smad2 linker region, described in the literature as a non-Smad pathway despite the integral role of Smad, has been shown to lead to activation of the

ERK and p38 MAP kinase signalling pathways and results in biglycan synthesis and GAG elongation in human VSMCs ². In other contexts TGF- β -mediated phosphorylation of Smad linker region involves ERK and JNK MAP kinases ¹⁰ however in VSMCs TGF- β causes JNK phosphorylation without effect on proteoglycan synthesis ².

Recently TGF- β signalling has been shown to involve Akt via activation of the PI3K-Akt-TOR-S6 kinase pathway in cells undergoing epithelial to mesenchymal transition ¹¹. This activation has been shown to be independent of Smad2/3 in fibroblasts ¹².

Akt, also referred to as PKB, plays a key role in regulating cell survival and apoptosis ^{13, 14}. This protein kinase is activated by insulin and various growth factors in a wortmannin-sensitive pathway involving PI3 kinase ¹⁴. Akt binds phospholipid and is activated by phosphorylation by PDK1 at Thr308 in the activation loop and by PDK2 at Ser473 at the carboxy terminus ¹⁵. Akt inhibits apoptosis by phosphorylating and inactivating Bad ¹⁶, forkhead transcription factors ¹⁷, c-Raf ¹⁸ and caspase-9. Akt also regulates glycogen synthesis through phosphorylation and inactivation of GSK-3 α and β ¹⁹. In cell cycle regulation Akt prevents GSK-3 β mediated phosphorylation and degradation of cyclin D1 ²⁰ and negatively regulates the cyclin dependent kinase inhibitors p27 Kip ²¹ and p21 Waf1/CIP1 ²². Akt also plays a critical role in cell growth by directly phosphorylating mTOR and tuberin (TSC2), an inhibitor of mTOR, in a rapamycin-sensitive complex containing raptor ²³. Inhibition of mTOR blocks protein synthesis and translation ²⁴.

Clearly Akt has key roles in EMT, cell cycle regulation, cell growth and cell survival in many cell types. TGF- β -mediated activation of PI3K/Akt pathway can complement

or antagonize Smad-mediated effects depending on cell type and context. In this study we have investigated whether TGF- β can activate Akt and its requirement for Smad in biglycan synthesis in human VSMCs.

Materials and methods

Materials

The following chemicals were purchased from Sigma, (St Louis, MO, USA): benzamidine hydrochloride, DEAE-Sephacel, Sepharose CL-6B, proteinase K, chondroitin sulfate, SB431542, PDGF, thrombin and penicillin-streptomycin. Recombinant TGF- β 1 was from R&D Systems Inc. (Minneapolis, USA). SN30978 was a kind gift from Dr. Graeme Lancaster (BakerIDI, Melbourne, Australia). Dulbecco's modified eagle medium (DMEM), glutamine, fetal bovine serum (FBS) were purchased from Invitrogen. Rainbow [^{14}C] methylated protein molecular weight standards (Amersham Pharmacia, Buckinghamshire, England). Cetyl pyridinium chloride (CPC) was from Unilab Chemicals and Pharmaceuticals, India. Whatman 3MM chromatography paper was from Biolab (Mulgrave, Australia), Instagel Plus scintillation fluid, [^{35}S]Sulfate and [^{35}S]-Met/Cys were from Perkin-Elmer (Waltham, MA, USA) and Poly-Prep columns from Bio-Rad (Hercules, CA USA)..

Culture of human VSMC

Human VSMCs were prepared by the explant method as previously described²⁵ using otherwise discarded sections of the internal mammary artery and saphenous veins from patients undergoing coronary artery bypass grafting at Alfred Health, Melbourne, Australia. The acquisition of the vessel segments was approved by the Alfred Hospital Ethics committee. VSMC were grown in Dulbecco's modified eagle medium (DMEM) with 5mM glucose, 10% FBS and 1% penicillin-streptomycin

solution. For experimentation, VSMC were seeded into 24 well plates (proteoglycan experiments) or 60 mm diameter plates (Western blotting experiments), grown until confluency then serum starved (0.1% FCS) for 48 h before experimentation as described.

Western blotting

Total cell lysates were resolved on 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% skim milk powder, incubated with primary antibodies as detailed in the text followed by HRP-anti-rabbit IgG and ECL detection (Amersham). Blots were quantified by densitometry using QuantityOne software (BioRad). Primary antibodies used in this study were anti-phosphoSmad2/3C rabbit monoclonal antibody, anti-phosphoSmad2L, antibody anti-phosphoAkt (phosphoThr308) antibody, **anti-Akt1/2/3 antibody**, anti-GAPDH antibody (Cell Signaling Technology, MA, USA)

Quantitation of proteoglycan synthesis

Quiescent cells were changed to fresh medium containing 25 $\mu\text{Ci/mL}$ of [^{35}S]sulfate in the presence or absence of TGF- β 1 for 24 hours. Media from the cell cultures was harvested with added protease inhibitors (5mM Benzamidine in 0.1M 6-aminocaproic acid). Incorporation of the radiolabel into proteoglycans was measured by CPC precipitation assay as described previously ²⁶.

SDS-PAGE analysis of proteoglycan size

Proteoglycans labelled with [^{35}S]sulfate or [^{35}S]-Met/Cys were prepared for SDS-PAGE by isolation through DEAE sephacel anionic exchange mini columns. Samples

were added to pre-equilibrated columns, then washed extensively with low salt buffer (8M Urea, 0.25M NaCl, 2 mM disodium EDTA, 0.5% Triton X-100). Proteoglycans were eluted with high salt buffer (8M Urea, 3M NaCl, 2mM disodium EDTA, 0.5% Triton X-100) Aliquots (25,000 cpm) were precipitated (1.3% potassium acetate, 95% ethanol) and chondroitin sulfate was added as a cold carrier. Samples were resuspended in buffer (8M Urea, 2mM disodium EDTA, pH 7.5), to which an equal volume of sample buffer was added. Radiolabelled proteoglycans were separated on 4-13% acrylamide gels with a 3% stacking gel at 50V overnight. [¹⁴C]-protein molecular weight markers were run simultaneously. Processed and dried gels were exposed to a phosphoimaging screen (Fuji Photo Film Co, Japan) for approximately 3 days, then scanned on a Bio-imaging analyser BAS-1000 MacBas (Fuji Photo Film Co, Japan).

Determination of the expression of biglycan by RT-PCR

Human VSMCs cells were plated on 6-well culture plate pre-coated with 10 ug/ml poly-D-lysine and fed with DMEM medium supplemented with 1% FBS, plus antibiotics in 5% CO₂ at 37°C. The following day medium was replaced with DMEM alone for 1h then the cells were treated with TGFβ (2ng/ml) for different times as indicated to observe the effect of TGFβ on the expression of biglycan. In experiments using inhibitors, cells were pre-incubated with or without SN30978 (5 μM) or SB431542 (3μM) for 30min, and then treated with TGF-β (2ng/ml) for 24 hours. Total RNAs were extracted from treated cells using TRIZOL Reagent (TIANGEN Corp.). First-strand cDNA was synthesized from 1.5 μg total RNA with random primer and M-MLV RTase (Genecopoeia Corp.); Resulting cDNA were subjected to PCR amplification using specific primers (sense primer, 5'-

CTCCTCCAGGTGGTCTATC-3' and antisense primer, 5'-GCTGATGCCGTTGTAGTAG-3') for biglycan. Products of PCR were electrophoresed on a 1.5% agarose gel.

Statistical analyses

Data was analysed for statistical significance using a 1-way analysis of variance (ANOVA) or a Student's paired t-test as stated. Results were considered statistically significant at $P < 0.05$ or better as stated.

Results

TGF- β causes rapid phosphorylation of Akt in human VSMCs

To determine whether TGF- β can elicit phosphorylation of Akt in VSMCs we treated serum-deprived human VSMCs with TGF- β (2 ng/ml) for 0 – 4h and then probed a western blot of whole cell lysates with anti-pAkt antibody (Figure 1). TGF- β mediated a rapid phosphorylation of Akt Ser473 within 5 mins (Figure 1A) and elevated levels were maintained up to 4h (Figure 1B). Time-course western blots probed with antibody to pAkt Thr308 gave the same results (data not shown). PDGF (50 ng/ml) was used as a positive control because of its ability to phosphorylate Akt at both Ser473 and Thr308 in VSMCs within 5 mins and sustain elevated levels for several hours²⁷.

TGF- β activates PI3K which in turn phosphorylates Akt on Ser473 and phosphoinositide-dependent kinase 1 (PDK1) a signalling component downstream of PI3K phosphorylates Akt Thr308. Phosphorylation can be inhibited by the allosteric non-ATP competitive Akt1/2 inhibitor SN30978²⁸. Cells were treated for either 15

min or 4h with TGF- β in the absence or presence of SN30978 (Figure 2A). Akt phosphorylation was completely blocked by a 30 min pre-incubation of SN30978 (2 μ M) at both treatment times (Figure 2A). Therefore TGF- β activates PI3K signalling that leads to pAkt in VSMCs. Blocking T β RI activity with its specific inhibitor SB431542²⁹ reduced pAkt levels to basal (Figure 2B) demonstrating the requirement for T β RI upstream of Akt phosphorylation. Previously we have reported that both thrombin and PDGF mediated proteoglycan core protein synthesis in VSMCs involve PI3K activity³⁰. To determine whether Akt is phosphorylated downstream of PDGF or thrombin activation we treated cells with PDGF for 1h and observed a marked elevation of pAkt levels above basal indeed they were higher than the level generated by TGF- β treatment (Figure 2B). Thrombin stimulation of pAkt was close to basal levels at this time point. When we pre-treated cells with SN30978 and stimulated with PDGF or thrombin there was minimal generation of pAkt (Figure 2B) suggesting that PI3K/Akt are downstream of all three agonists in VSMCs but to different extents.

TGF- β -mediated Smad2 phosphorylation is independent of Akt stimulation

Smad-independent TGF- β signalling of PI3K has been reported in fibroblasts¹² and here we investigated whether blocking phosphorylation of Akt would interfere with the known pathway of TGF- β /carboxy terminal and linker region phosphorylation of Smad2^{2,4}. TGF- β induces T β RI to directly phosphorylate the two distal serines of Smad C-terminal SSXS motif and indirectly cause MAP kinases to phosphorylate serine and threonine residues in Smad linker region⁸. Western blots using anti-pSmad2C showed a significant increase (4.6-fold) in pSmad2C levels following TGF- β treatment (Figure 3A and 3B). PDGF and thrombin showed a small increase in pSmad2C levels above basal the latter similar to our published findings³¹. Blocking

Akt phosphorylation had no effect on pSmad2C levels after stimulation with any of the agonists (Figure 3A). TGF- β treatment increased the level of Smad2 linker region phosphorylation approximately 5-fold compared to a 2-fold or less increase after PDGF or thrombin stimulation respectively (Figure 3B). Similarly to pSmad2C we observed no change in the levels of pSmad2L when Akt phosphorylation was blocked with SN30978 (Figure 3B). The inability of the Akt inhibitor to block Smad phosphorylation indicates that neither carboxy nor linker region Smad2 phosphorylation are downstream of Akt and in this context are likely independent of PI3K activation.

TGF- β stimulates biglycan core protein synthesis via Akt

To determine whether Akt is a component of TGF- β -mediated proteoglycan synthesis in human VSMCs we performed experiments with the Akt1/2 inhibitor SN30978 on radiolabelled [35 S]-sulfate incorporation into proteoglycans in TGF- β treated cells. Figure 4 shows cells treated with TGF- β had a 2-3-fold increase in [35 S]-sulfate into secreted proteoglycans, in these cells predominantly biglycan, compared to controls. Treatment with SN30978 (0 – 3 μ M) showed a concentration-dependent decrease of TGF- β -mediated [35 S]-sulfate incorporation into secreted proteoglycans (Figure 4A) with a maximal 41 % decrease at 3 μ M. The T β RI inhibitor SB431542²⁹ was used as a positive control. SDS-PAGE of the secreted proteoglycans showed no change in the size of the biglycan band at any concentration of the Akt inhibitor (Figure 4B). While proteoglycan core protein size remains fixed, GAG chains can vary in length and are altered by a variety of growth factors⁶. The current findings show that Akt is not involved in TGF- β -mediated proteoglycan GAG chain hyperelongation.

To assess changes in proteoglycan core protein synthesis we labelled proteoglycans with [^{35}S]-Met/Cys for 24h and quantitated incorporated radiolabel in secreted proteoglycans. TGF- β stimulated cells showed an increase of approximately 20% in [^{35}S]-Met/Cys incorporation compared to control (Figure 4C). Treatment of cells with SN30978 showed a dose-dependent decrease in TGF- β -mediated [^{35}S]-Met/Cys incorporation with the level close to basal amounts at the maximal concentration tested (Figure 4C). To specifically examine biglycan core protein expression we measured the levels of biglycan mRNA in TGF- β treated VSMCs. Biglycan mRNA levels increased over 24h from an increase of 1.75-fold at 3h to a maximum of 2-fold at 24h (Figure 5). In the presence of SN30978 there was a significant block of TGF- β -mediated increase of biglycan mRNA (Figure 6, TGF- β , 24h) which verifies that Akt is a downstream mediator of TGF- β -stimulated biglycan core protein synthesis in VSMCs.

Discussion

TGF- β is a potent regulator of extracellular matrix synthesis including proteoglycan synthesis in the blood vessel wall^{2, 3, 4, 5, 32}. In vascular smooth muscle TGF- β upregulates biglycan expression and hyperelongation of its chondroitin sulfate GAG chains leading to increased binding to LDL with consequent increased lipid retention and accelerated atherosclerosis^{5, 33}. The signalling pathways from TGF- β to the synthesis of proteoglycans are only partially described and are cell and context dependent. In this study we examined whether TGF- β -mediated activation of the PI3K/Akt pathway in human VSMCs and to what extent the pathway is involved in proteoglycan synthesis. We demonstrated that TGF- β stimulated Akt phosphorylation within 5 mins and continued for at least 24h (see Figure 1) comparable to the response

observed recently in cardiac fibroblasts³⁴. TGF- β mediates a slower time course of Akt phosphorylation in hypoxic pulmonary artery smooth muscle cells³⁵, mesangial cells³⁶ and fibroblasts³⁷ and reflects the different cell contexts of TGF- β signalling.

Our previous studies have shown that in VSMCs TGF- β causes proteoglycan synthesis via both Smad-dependent⁴ and MAP kinase/Smad linker-dependent pathways². Our results in the current study showed that blocking Akt phosphorylation did not alter the level of Smad2 phosphorylation at either the C-terminal SXSS motif or the linker region serine residues (see Figure 3) and Smad2 phosphorylation appears independent of PI3K/Akt activation. Our findings are in agreement with reports of TGF- β regulating Smad2 and Smad3 and PI3K responses independently¹². Other reports suggest the PI3K and Smad signalling pathways may be connected with Smad3 inhibition reducing TGF- β -mediated activation of Akt³⁴ and PI3K inhibition decreasing Smad3 activity³⁸. While we cannot ignore the possibility that Smad3 may signal differently to Smad2 our work has focussed on the latter transcription factor because to date we have no evidence to suggest a role for involvement of Smad3 in proteoglycan synthesis in VSMCs (unpublished observation). The exact mechanism of TGF- β induced Akt phosphorylation in human VSMCs warrants further investigation.

Growth factor activation of the PI3K/Akt pathway upregulates extracellular matrix components including collagen via TGF- β ³⁴ and proteoglycans via IGF1³⁹ and aggrecan and versican via IGF2⁴⁰ and GAG synthesis in fetal lung fibroblasts via PDGF⁴¹. In VSMCs TGF- β mediates proteoglycan synthesis via the Akt pathway (see Figure 4) as demonstrated by dose-dependently blocking [³⁵S]-sulfate and [³⁵S]-

Met/Cys incorporation into proteoglycans with SN30978. We determined that Akt signalling does not affect GAG synthesis as there is no detectable change in biglycan GAG length when this pathway is inhibited (Figure 4). Biglycan core protein synthesis is dependent on Akt phosphorylation demonstrated by the ability of the Akt inhibitor to block TGF- β mediated increase in biglycan mRNA (see Figure 6) and incorporation of [35 S]-Met/Cys into core protein (see Figure 4). **The T β RI inhibitor SB431542 blocks proteoglycan chain elongation independently of core protein synthesis⁴. Here we saw SN30978 only able to block Akt-mediated biglycan core protein synthesis not chain elongation demonstrating that at least two signalling pathways may be involved in biglycan core protein synthesis.** Interestingly in human chondrocytes IGF2 upregulates aggrecan and versican mRNA levels via Akt⁴⁰ however IGF1 has no effect via Akt on aggrecan, decorin or biglycan core protein mRNA levels³⁹. Similarly Akt signalling results in different outcomes in GAG synthesis with our results showing TGF-mediated phosphorylation of Akt not altering biglycan GAG synthesis but PDGF-mediated Akt activation reported to have an integral role in GAG regulation in fetal lung fibroblasts⁴². The diversity of effects suggests differences and complexity reflecting not only cell type but also the activating growth factor and its downstream outputs.

Conclusions

We have demonstrated that Akt is an active component of TGF-mediated signalling to biglycan core protein synthesis in human vascular smooth muscle cells. In contrast TGF- β -induced proteoglycan GAG chain hyperelongation does not require Akt phosphorylation. Akt signalling pathways are important in many cellular contexts and

further investigations are warranted to determine whether Akt instigates changes in other proteoglycan related cellular processes and in other cell types.

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Declarations of Interest

No conflicts of interest to declare.

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Figure Legends

Figure 1 Time-course of TGF- β mediated phosphorylation of Akt in human

VSMCs. Human VSMC were or untreated (-) or treated with TGF- β (2 ng/ml) for (A) 0 – 30 mins and (B) 0 - 360 mins. PDGF (50 ng/ml)-treated VSMCs were used as a positive control. Whole cell protein lysates were resolved by 10% SDS-PAGE gels and transferred to PVDF membranes. Membranes were probed with primary anti-phospho-Akt (pAkt, Ser473) antibody and visualised with ECL. Blots were stripped and re-probed for **total Akt (Akt)** and GAPDH for loading controls.

Figure 2 Akt1/2 inhibitor completely blocks TGF- β mediated phosphorylation of Akt.

Cells were untreated (-) or pre-treated for 30 min with the Akt1/2 inhibitor SN30978 (2 μ M) and then stimulated with (A) TGF- β (2 ng/ml) for either 15 min or 4 h, or PDGF (50 ng/ml) for 1 h (B) Thrombin (10 Units/ml), PDGF (50 ng/ml) or TGF- β (2 ng/ml) for 1 h. Cells pre-treated for 30 min with T β RI kinase inhibitor SB431542 (3 μ M) (SB) were stimulated with TGF- β (2 ng/ml) for 1 h. Cell lysates were processed and probed for phospho-Akt, **Akt** and GAPDH as described. Density analysis using Biorad Quantity One software is shown to the right of each blot, ## P<0.01 vs control, ** P<0.01 vs agonist, # P<0.05 vs control

Figure 3 Role of Akt in TGF- β -mediated Smad pathway

Cells were untreated (-) or pre-treated for 30 min with the Akt1/2 inhibitor SN30978 (2 μ M) and then stimulated with Thrombin (10 Units/ml), PDGF (50 ng/ml) or TGF- β (2 ng/ml) for 1 h. Cells pre-treated for 30 min with T β RI kinase inhibitor SB431542 (3 μ M) (SB) were stimulated with TGF- β (2 ng/ml) for 1 h. Cell lysates were

processed as described (see Figure 1 legend) and probed with (A) anti-phospho-Smad2 carboxy terminal antibody (pSmad2C) or (B) anti-phospho-Smad2 linker region antibody (pSmad2L) and GAPDH. Density analysis using Biorad Quantity One software is shown for each blot (B) pSmad2C and (C) pSmad2L, ## P<0.01 vs control, ** P<0.01 vs agonist.

Figure 4 Role of Akt in TGF- β -mediated proteoglycan synthesis in human VSMCs.

VSMCs were pre-treated for 30 min with SN30978 (0 – 3 μ M) or SB431542 (3 μ M) (SB) and then stimulated with TGF- β (2 ng/ml) for 24 h in the presence of [35 S]-sulfate or [35 S]-Met/Cys for 24 h. (A) [35 S]-sulfate incorporation (B) SDS-PAGE analysis of [35 S]-sulfate incorporated samples (C) [35 S]-Met/Cys incorporation into proteoglycan core protein synthesis. Histogram values are expressed as percentage of untreated control value, ## P<0.01 vs control, ** P<0.01 vs agonist.

Figure 5 TGF- β -induced changes in biglycan mRNA levels in human VSMCs.

Human VSMCs were treated with TGF- β (2 ng/ml) for various times (0 – 24 h), and the expressions of biglycan were determined by RT-PCR as described in Material and Method. RPL is ribosomal protein mRNA. TGF- β time-dependently induced the expression of biglycan in human VSMCs cells. The relative mRNA level was normalised to the level of untreated control cells. ## P<0.01 vs control. Results are from four independent experiments.

Figure 6 Effects of Akt inhibitor SN30978 on biglycan mRNA levels.

VSMCs pre-treated with or without SN30978 (5 μ M) or SB431542 (3 μ M) for 30 min were stimulated with TGF- β (2 ng/ml) for 24 h, and levels of biglycan mRNA measured. Both SN30978 and SB431542 blocked TGF- β -induced expression of biglycan in VSMCs. The relative mRNA level was normalised to the level of untreated control cells (-). ** P<0.01 vs agonist, *P<0.05 vs agonist, # P<0.05 vs control. Results are from three independent experiments.

A **B**

A

B

A **B** **C**

A **B** **C**