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ORIGINAL ARTICLE

Inhibition of Apoptosis Signal-Regulating Kinase 1 Attenuates Myocyte Hypertrophy and Fibroblast Collagen Synthesis

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Background Cardiac remodelling is a dynamic process whereby structural and functional changes occur within the heart in response to injury or inflammation. Recent studies have demonstrated reactive oxygen species sensitive MAPK, apoptosis signal-regulating kinase 1 (ASK1) plays a critical role in cardiac remodelling. This study aims to determine the effectiveness of small molecule ASK1 inhibitors on these processes and their therapeutic potential.

Methods Neonatal rat cardiac fibroblasts (NCF) were pre-treated with ASK1 inhibitors, G2261818A (G226) and G2358939A (G235), for 2 hours before stimulated with 100 nM angiotensin II (AngII), 10 μM indoxyl sulphate (IS) or 10 ng/ml transforming growth factor β_1 (TGFβ_1) for 48 hours. Neonatal rat cardiac myocytes (NCM) were pre-treated with G226 and G235 for 2 hours before being stimulated with 100 nM AngII for 60 hours, 10 μM IS, 10 ng/ml interleukin 1β (IL-1β) or tumour necrosis factor α (TNFα) for 48 hours. ^{3}H-proline and ^{3}H-leucine incorporation was used to assess collagen turnover and hypertrophy, respectively. Pro-fibrotic, pro-hypertrophic and THP-1 inflammatory cytokine gene expressions were determined by RT-PCR.

Results Both G226 and G235 dose-dependently attenuated AngII-, IS-, IL-1β- and TNFα-stimulated NCM hypertrophy and hypertrophic gene expression, IS-, AngII- and TGFβ_1-stimulated NCF collagen synthesis and AngII- and TGFβ_1-stimulated pro-fibrotic gene expression. Inhibition of ASK1 by G226 and G235 inhibited lipopolysaccharides-stimulated inflammatory cytokine gene expression in THP-1 cells.

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Conclusions Selective ASK1 inhibition confers anti-hypertrophic and anti-fibrotic effects in cardiac cells, and anti-inflammation in monocytic cells. ASK1 inhibitors may represent novel therapeutic agents to alleviate cardiac remodelling post cardiac injury where hypertrophy, fibrosis and inflammation play critical roles.

Keywords Apoptosis signal-regulating kinase 1 • ASK1 inhibitors • Cardiac hypertrophy • Collagen synthesis • Cardiac remodelling

Introduction

Cardiac remodelling is a dynamic process whereby structural and functional changes occur within the heart in response to injury or inflammation. It is a condition characterised by neurohormonal and pro-inflammatory cytokine activation resulting in the characteristic molecular and cellular changes of the remodelled ventricle. These characteristic changes include an increase in myocyte hypertrophy, excess deposition of extracellular matrix material (pathological fibrosis) and increased apoptosis of myocardial cells.

Despite advances in anti-remodelling therapies, there remains a high mortality following conditions in which remodelling occurs. Therapeutic strategies that have been shown to slow the remodelling process have focussed primarily on the neurohormonal activation that accompanies this condition. Chief among these activated systems are the renin-angiotensin-aldosterone and the sympathetic nervous systems. Inhibitors of these systems (ACE-inhibitors, aldosterone antagonists and beta blockers) have been shown to slow progression of remodelling and this has translated into clinical benefits with improved survival, reduced hospitalisation and enhanced quality of life in post-myocardial infarction (MI) and chronic heart failure patients. In recent times, less conventional therapies for cardiovascular disease, e.g. p38 MAPK inhibitors and anti-tumour necrosis factor α (TNFα) therapy, have also shown promise in animal studies; however most have not been translated into clinical practice due to toxicity issues or lack of efficacy. Therefore, there is a great need for novel agents to be used in addition to these background therapies.

Reactive oxygen species (ROS), especially in the setting of an ischaemic myocardium, are a potent stimulus for pro-inflammatory cytokines (TNFα, interleukin 1beta (IL-1β), interleukin 6 (IL-6)) and are known to regulate cardiac cell survival and death. In fact, both can stimulate each other, and in the chronic stage, activation of matrix metalloproteases (MMPs) and collagen deposition can occur, contributing to both reparative and reactive pathological processes resulting in left ventricular dilatation and increased fibrosis.

In the post-MI setting, ROS generation is increased early, particularly in the border zone and infarct regions. Reactive oxygen species activates a ROS-sensitive MAPK, apoptosis signal-regulating kinase 1 (ASK1), which in turn activates downstream MAPKs known to be involved in cardiac remodelling (p38MAPK and JNK) [1–4]. Furthermore, ASK1 is involved in ROS activation of NFκB in cardiomyocytes and NFκB activation is known to produce TNFα and activate apoptotic pathways as well as hypertrophy [1,5,6].

The abovementioned information suggests anti-oxidant therapy would be beneficial, however, studies utilising anti-oxidant therapy in the MI setting have shown conflicting results [7]. A more direct approach that targets the signal transduction of ROS, namely ASK1, may therefore be effective [1]. In support of this, gene deletion of ASK1 in mice as well as suppression of ASK1 activity via VDUP1 DNAzyme administration have demonstrated improved cardiac function and attenuated left ventricular (LV) remodelling post-MI [1]. Use of a small molecule inhibitor may yield a more clinically relevant avenue than gene targeting.

The aim of the present study is to determine whether ASK1 inhibition has direct beneficial effects on cardiac cells as well as exploring potential mechanisms involved. This study will provide evidence of beneficial cardiovascular effects of an ASK1 inhibitor on the cardiac remodelling process including reduced inflammatory response, which may be useful in the setting of myocardial injury.

Materials and Methods

All animal usage was approved by Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee (AEC) in accordance with National Health and Medical Research Council (NHMRC) guidelines for the care and use of laboratory animals (AEC approval number: E/0981/2010/M). ASK1 inhibitors G2261818A (G226) and G2358939A (G235) were provided by GSK. Their enzyme inhibition activity for ASK1 is pKi 7.70 and 7.40, respectively with more than 20-fold selectivity over other related kinases tested by GSK.

Neonatal Rat Cardiac Myocytes and Fibroblast Culture

Neonatal rat cardiac myocyte (NCM) and fibroblast (NCF) were isolated from 1- to 2-day-old Sprague-Dawley pups with enzymatic digestion as described in detail previously [8–10]. NCFs were seeded and maintained in DMEM and 10% FBS. NCFs were used at passage 2 [11]. Purified NCMs were seeded at a density of 1000 cells/mm^2 in 12-well plates in the presence of 10% NBCS. After 24 hours, NCMs were maintained in serum-free DMEM supplemented with 10 μg/ml insulin and 10 μg/ml transferrin as described previously [11–13]. Bromodeoxyuridine (0.1 mM) was included for the first 3 days. KCl (50 mM) was added to the medium to prevent contact induced spontaneous contraction hypertrophy of the plated NCM [11–13].

Measurement of Neonatal Rat Cardiac Myocyte Hypertrophy

NCM hypertrophy was determined by ^{3}H-leucine incorporation as previously described [10–13]. Briefly, 2 hours after pre-treatment with or without ASK1 inhibitor (G226 and G235, 0.1 to 3 μM), NCMs were stimulated with either 100 nM AngII for a further 60 hours; or 10 ng/ml IL-1β or TNFα; or 10 μM IS for 48 hours before harvest. 1 μCi of ^{3}H-leucine was also added to each well. Cells were harvested with 10% TCA precipitation on ice for 30 minutes before solubilised with 1 M NaOH overnight at 4 °C. The samples were then neutralised with 1 M HCl, and ^{3}H levels were counted in scintillation fluid on a beta counter to determine the levels of ^{3}H-leucine incorporation.

Measurement of Neonatal Rat Cardiac Fibroblasts Collagen Synthesis

NCF collagen synthesis was determined by ^{3}H-proline incorporation as described previously [10–13]. NCFs were seeded at a density of 50,000 cells per well in 12-well plates and incubated overnight before serum starved with 0.5% bovine serum albumin (BSA) for 48 hours. Cells were then pre-treated in the presence or absence of ASK1 inhibitors (G226 and G235, 0.1 to 3 μM) for 2 hours before stimulation with 100 nM AngII, 10 μM IS or 10 ng/mL TGFβ_1 and addition of 1 μCi of ^{3}H-proline to each well. After 48 hours of further incubation, cells were harvested by 10% TCA precipitation and ^{3}H-proline incorporation was determined similar to cardiac myocyte hypertrophy assays as described above.

Quantitative Measurement of Pro-Fibrotic Marker mRNA Expression in Cardiac Cells

After 48 hours serum starving in 0.5% BSA, NCFs were pre-treated with or without ASK1 inhibitors (G226 and G235, 1 to 3 μM) for 2 hours before being stimulated with 100 nM AngII for a further 18 hours. Cells were then harvested and total RNA was extracted using Qiagen RNeasy kit (Qiagen, Hilden, Germany). After being reverse transcribed to cDNA with MultiScribe (Applied Biosystems, CA, USA), triplicate cDNA aliquots were amplified using sequence-specific primers (Geneworks, Adelaide, SA, Australia) and TagMan fluorogenic probe (Applied Biosystems, Foster City, CA, USA) using an ABI prism 7900 HT sequence Detection System (Applied Biosystems). Real-time polymerase chain reaction (RT-PCR) was used to quantify pro-hypertrophic and pro-fibrotic gene expression. GAPDH was used as an endogenous control in all experiments to correct for the expression of each gene.

Quantitative Measurement of Pro-Inflammatory Cytokine Gene Expression in THP-1Cells

Given that cardiac remodelling post injury is essentially an inflammatory response in the myocardium and prolonged inflammation causes maladaptive remodelling, the effects of ASK1 inhibitors on inflammatory cytokine gene expression have been investigated using monocytic cell line, THP-1 cells. THP-1 cells were maintained as per the protocol of American Type Culture Collection. After being serum starved for 48 hours, THP-1 cells were pre-treated with or without ASK1 inhibitors (G226 and G235, 1 to 3 μM) for 2 hours before stimulation with 500 ng/ml lipopolysaccharides (LPS) for further 18 hours. Cells were then harvested and total RNA was extracted as described above. RT-PCR was used to quantify pro-inflammatory cytokine gene expression (TNF-α, IL-6 and IL-1β). The primer pairs and probes were designed using Primer Express 2.0 software (Applied Biosystems) based on published sequences (http://www.ncbi.nlm.nih.gov) as previously described [12]. 18S rRNA was used as an endogenous control in all experiments to correct for the expression of each gene.

Measurement of Cell Viability in Neonatal Rat Cardiac Fibroblasts Cells

NCF and NCM were seeded in 96-well plates at a density of 10,000 and 50,000 cells per well, respectively. After serum starvation for 48 hours, NCF and NCM were treated with 100 nM AngII in the presence or absence of ASK1 inhibitors (G226 and G235 0.1 to 3 μM). THP-1 cells were seeded in 96-well plates at a density of 20,000 cells per well after serum starving for 48 hours; and then treated in the presence or absence of ASK1 inhibitors (G226 and G235 0.1 to 3 μM). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were used to determine proliferation and cell viability of NCF, NCM and THP-1 cells as previously described [10–13].

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism software program version 5. All experiments have been performed at least three times in triplicate. All of the data was analysed with one-way analysis of variance (ANOVA) and Newman-Keuls multiple comparison post-hoc tests. A two-tailed value of $p < 0.05$ was considered statistically significant. Results are presented as mean ± SEM.

Results

Inhibition of Cardiac Myocyte Hypertrophy

100 nM AngII, 10 ng/ml IL-1β and TNFα, and 10 μM IS stimulated a significant increase in the level of ^{3}H-leucine incorporation in NCMs (myocyte hypertrophy)

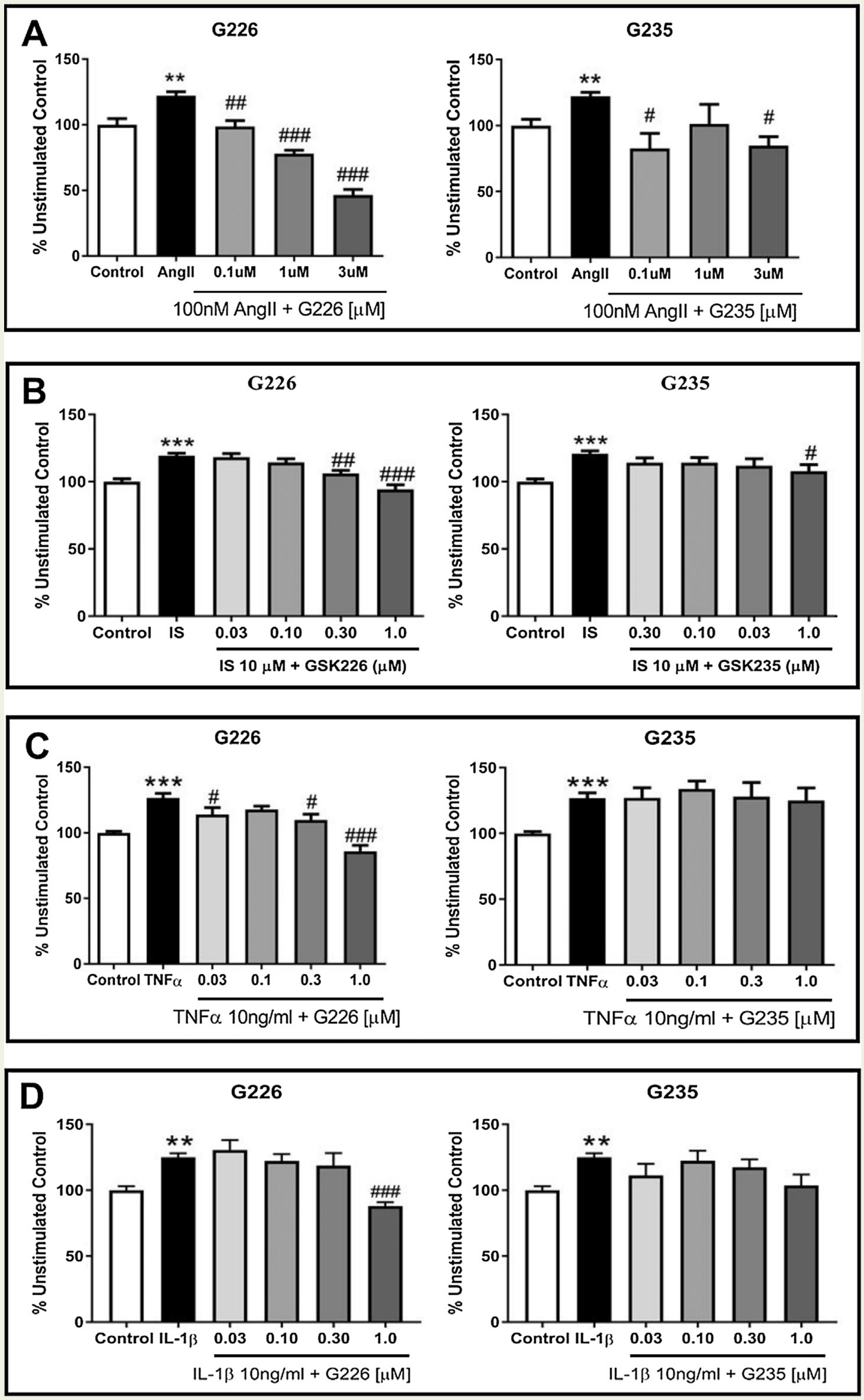

Figure 1 Inhibition of Neonatal rat cardiac myocytes (NCM) hypertrophy.
Angiotensin II, indoxyl sulphate (IS), TNFα and IL-1β-stimulated NCM hypertrophy as determined by ^{3}H-leucine incorporation is significantly inhibited by both ASK1 inhibitors G2261818A (G226) and G2358939A (G235) (**A** to **D** respectively). Results are presented as mean ± SEM. All experiments were performed in triplicates and repeated three times. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. unstimulated control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs 100 nM AngII- 10 μM IS-, 10 ng/ml TNFα or 10 ng/ml IL-1β-stimulated control.

($122.2 \pm 3.0\%$; 125.1 ± 3.0; 126.5 ± 3.5; and 119.4 ± 1.7 vs unstimulated controls and p value: 0.01, 0.01, 0.001 and 0.001 vs unstimulated controls, respectively, mean $\pm$ SEM). In the presence of ASK1 inhibitors, G226 and G235, AngII-, IS-, TNFα- and IL-1β-stimulated cardiac myocyte hypertrophy was dose-dependently inhibited by G226 (Figure 1, A to D) (treatment with 1.0 μM G226: $77.9 \pm 2.6\%$, $p < 0.001$; 94.3 ± 3.4, $p < 0.001$; 85.9 ± 4.5, $p < 0.001$ and 88.1 ± 2.8, $p < 0.001$, percentages of unstimulated control vs AngII-, IS-, TNFα- and IL-1β-stimulation, respectively), noting that G235 has less potent effects in these assays as shown by the lack of significant dose-response (when treated with 1.0 μM G235: 101.3 ± 14.8, $p > 0.05$; 107.8 ± 4.9, $p < 0.05$; 125.0 ± 9.6, $p > 0.05$ and 103.7 ± 8.3, $p > 0.05$, percentages of unstimulated control vs AngII-, IS-, TNFα- and IL-1β-stimulation; respectively, Figure 1A to D). This may be attributed to lower potency of the compound and a higher concentration may be needed to demonstrate a dose-dependent effect.

Inhibition of Cardiac Fibroblasts Collagen Synthesis

100 nm AngII, 10 ng/ml transforming growth factor β_1 (TGFβ_1) or 10 μM IS stimulated a significant increase in collagen synthesis of NCFs, as determined by ^{3}H-proline incorporation ($173.0 \pm 2.3\%$, $p < 0.0001$; $218.3 \pm 8.3\%$, $p < 0.0001$; $115.2 \pm 1.9\%$, $p < 0.0001$; percentage of unstimulated controls; p value vs unstimulated controls; respectively). Collagen synthesis of NCFs stimulated by AngII and IS was significantly inhibited by both G226 and G235 at the concentrations tested (treated with 1.0 μM G226: $148.0 \pm 2.8\%$, $p < 0.05$ and $98.2 \pm 3.0\%$, $p < 0.0001$ percentage of unstimulated controls, vs AngII- and IS-stimulated groups, respectively; treated with 1.0 μM G235: $159.0 \pm 3.4\%$, $p > 0.05$ and $96.2 \pm 2.7\%$, $p < 0.0001$ percentage of unstimulated controls, vs AngII- and IS-stimulated groups, respectively; Figure 2, A and C). However, only G226 inhibited TGFβ_1-stimulated cardiac fibroblast collagen synthesis whilst G235 appeared to have little effect at the concentrations tested (treated with 1.0 μM G226: $128.1 \pm 7.1\%$ percentage of unstimulated control, $p < 0.0001$ vs TGFβ-stimulated group; treated with 1.0 μM G235: 251.3 ± 36.1 percentage of unstimulated control, $p > 0.05$ vs TGFβ-stimulated group; Figure 2B). The lack of dose-response for the results showed in Figure 2A might indicate potency and efficacy at lower concentrations in this particular setting, and further titrations may yield a better dose-response.

Inhibition of Pro-Fibrotic Gene Expression

G226, and to a lesser degree G235, attenuated the pro-fibrotic gene expression of collagen I, TGFβ_1 and connective tissue growth factor (cTGF), stimulated by AngII in cardiac fibroblasts (Figure 3). AngII stimulated a significant increase of collagen I, TGFβ1 and cTGF gene expression (1.28 ± 0.16, $p < 0.05$; 1.27 ± 0.14, $p < 0.001$ and 1.29 ± 0.12 $p < 0.001$; respectively), data were expressed as ratios of GAPDH (Figure 3), Treatment with 1.0 μM G226 or G235 significantly decreased collagen I gene expression (0.69 ± 0.05 $p < 0.05$ and 0.84 ± 0.04, $p < 0.05$ vs AngII-stimulated group, respectively)(Figure 3A). Treatment with 1.0 μM G226 significantly inhibited TGFβ1 gene expression stimulated by AngII (0.46 ± 0.06, $p < 0.01$) but there were not significant effects by G235 at both concentrations tested (0.87 ± 0.10 and 1.02 ± 0.07, both $p > 0.05$ vs AngII-stimulated group, for 3.0 and 1.0 μM respectively) (Figure 3B). Whilst treatment with 1.0 μM G226 significantly reduced cTGF gene expression (0.43 ± 0.03, $p < 0.001$ vs AngII-stimulated group), treatment with the same concentration of G235 inhibition of cTGF gene expression was not significant (1.05 ± 0.07, $p > 0.05$ vs AngII-stimulated group) but it was significant at 3.0 μM G235 (0.84 ± 0.16, $p < 0.05$ vs AngII-stimulated group) (Figure 3C).

Inhibition of Inflammatory Cytokine Gene Expression

Both G226 and G235, significantly inhibited IL-1β, IL-6 and TNFα gene expression stimulated by LPS in THP-1 cells (Figure 4, A to C) indicating the potential anti-inflammatory effects of ASK1 inhibition. LPS stimulated significant increase of IL-1β, IL-6 and TNFα gene expression (5.49 ± 0.32, $p < 0.001$; 6.72 ± 0.44, $p < 0.001$ and 7.14 ± 0.49 $p < 0.001$; respectively, p values vs unstimulated controls and data were expressed as ratios of GAPDH) (Figure 4). Treatment with 1.0 μM G226 or G235 significantly decreased IL-1β (0.70 ± 0.02, $p < 0.001$ and 3.48 ± 0.34, $p < 0.001$, vs LPS-stimulated group, respectively) (Figure 4A) and IL-6 (0.19 ± 0.09, $p< 0.001$ and 1.43 ± 0.12, $p < 0.001$, vs LPS-stimulated group, respectively) gene expression (Figure 4B). Furthermore, 1.0 μM G226 and G235 treatments significantly reduced TNFα gene expression stimulated by LPS in THP-1 cells (1.5 ± 0.11, $p < 0.001$ and 5.31 ± 0.12, $p < 0.01$, vs LPS-stimulated group, respectively) (Figure 4C).

Effects on Cellular Viability

NCF, NCM and THP-1 cellular viability were analysis with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. There were no effects on NCM, NCF and THP-1 cellular viabilities with the treatment of both ASK-1 inhibitors, G226 and G235, at the concentrations tested (0.03 to 3.0 μM) as determined by MTT assays.

Discussion

Previously, in mice models of ischaemia-reperfusion injury, administration of ASK1 inhibitor resulted in a lesser degree of myocardial infarction as well as reduction in apoptotic cardiac myocytes compared to mice without treatment [14,15]. Studies with ASK1-deficient mice have also demonstrated that the ROS-ASK1 pathway plays important roles in necrotic and apoptotic cell death, indicating that ASK1 may

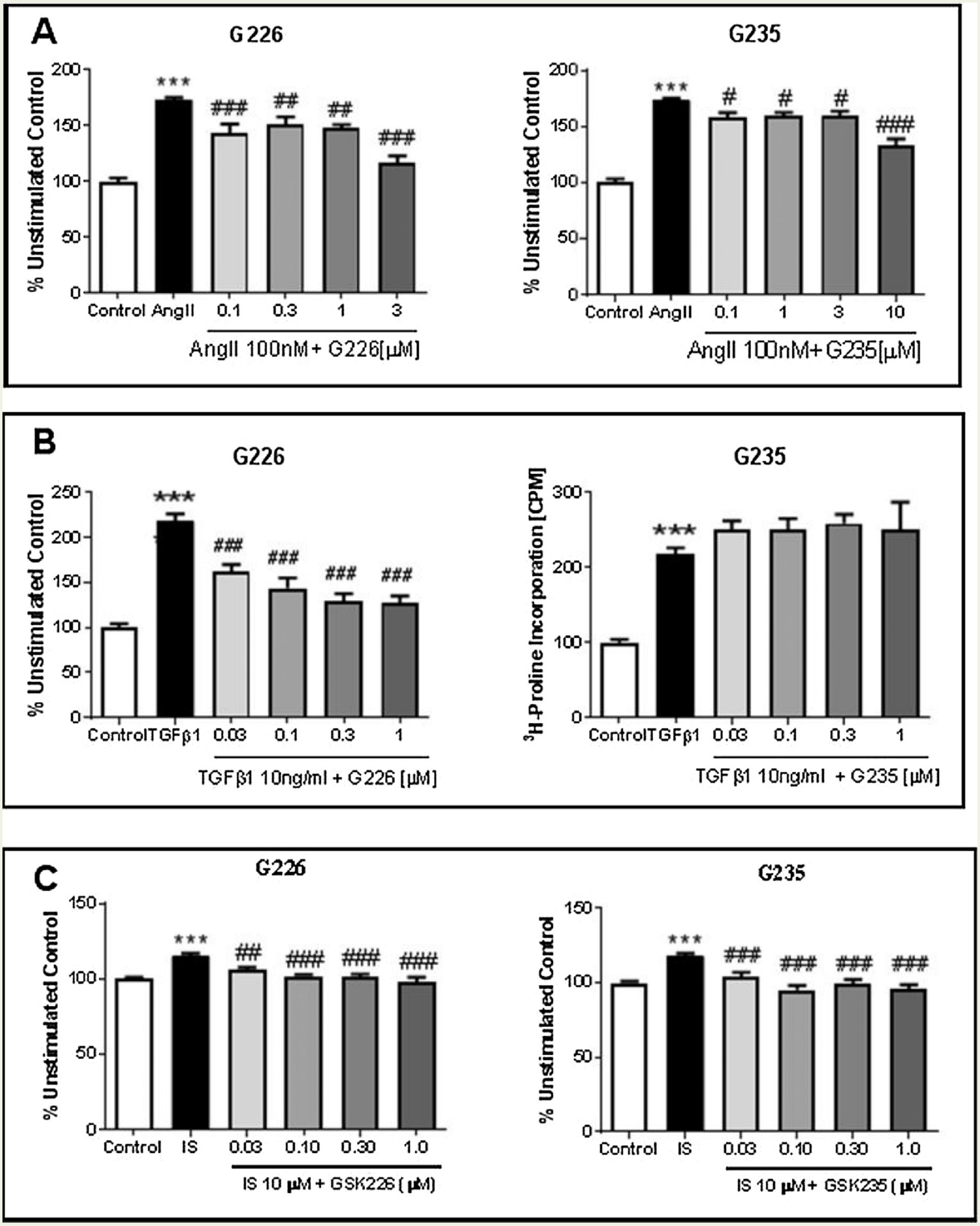

Figure 2 Inhibition of Neonatal rat cardiac fibroblasts (NCF) collagen synthesis.
Angiotensin II, TGFβ_1 and indoxyl sulphate-stimulated NCF collagen synthesis determined by ^{3}H-proline incorporation is dose-dependently inhibited by both ASK1 inhibitors G2261818A (G226) and G2358939A (G235) (**A**, **B** and **C** respectively). Results are presented as mean ± SEM. All experiments were performed in triplicates and repeated three times.
$^{***}p < 0.001$ vs. unstimulated control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs 100 nM AngII-, 10 ng/ml TGFβ_1- or 10 μM IS-stimulated control.

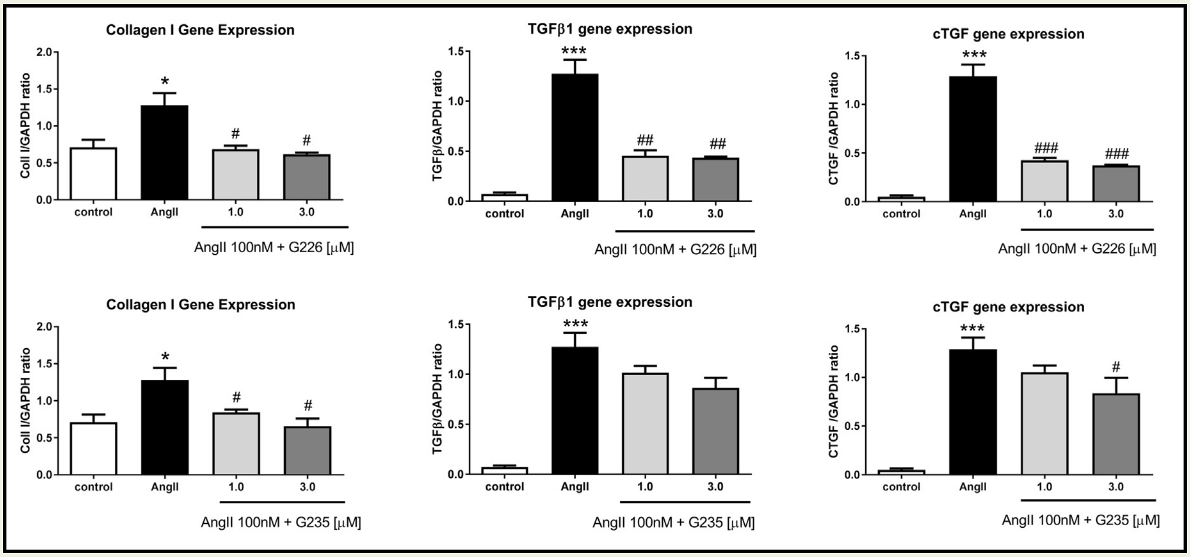

Figure 3 Inhibition of pro-fibrotic gene expression.
Angiotensin II-stimulated Neonatal rat cardiac fibroblasts (NCF) pro-fibrotic gene expression (Collagen 1, $TGF\beta_1$ and cTGF) was dose-dependently inhibited by both ASK1 inhibitors G2261818A (G226) and G2358939A (G235). Results are presented as mean ± SEM. All experiments were performed in triplicates and repeated three times.
$^*p < 0.05$, $^{***}p < 0.001$ vs. unstimulated control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs 100 nM AngII-stimulated control.

be a therapeutic target to reduce cardiac remodelling after MI [1–4]. In this study, we demonstrated that ASK1 inhibition attenuated markers of cardiac hypertrophy stimulated by AngII, IL-1β, TNFα and indoxyl sulphate (Figure 1) and inhibited cardiac fibroblast collagen synthesis stimulated by AngII and $TGF\beta_1$ (Figure 2), indicating the important roles that the apoptosis signal-regulating kinase 1 pathway plays in the pathophysiology of cardiac remodelling. A study published recently showed that selective ASK1 inhibitor, compound 19, protected rat heart from ischaemic injury in an *ex vivo* model, further supporting the therapeutic potential of selective ASK1 inhibitors in cardiac dysfunctions [16].

It is worth noting that previous reports in a gene overexpression mice model have shown ASK1 only regulates cardiomyocyte death, but not hypertrophy [7]. The discrepancy of effect on hypertrophy and myocyte cell death may be due to the difference in experimental settings, where the reported ASK1 overexpression may in fact produce non-physiological effects. This is in contrast to our current study, in which experiments were performed with normal isolated primary cardiac myocytes without disturbance of ASK1 gene overexpression. Our study was also conducted in culture conditions without other interfering factors, using a single stimulant. Another previous study in cultured cardiac myocytes has shown that ASK1 overexpression instigated cardiac hypertrophy and such effects were attenuated in myocytes with dominant negative ASK1 overexpression [17]. Thus, the possible effect of ASK1 on hypertrophy cannot be readily dismissed.

Additionally, the ASK1 signalling cascade has been demonstrated to promote renal fibrosis in animal models of kidney obstruction [18]. Furthermore, the use of ASK1 inhibitor was shown to attenuate renal pathological changes in mice with ischaemia-reperfusion injury [19]. Thus, ASK1 inhibitors may represent a novel therapeutic approach to the attenuation of not only cardiac remodelling in the setting of cardiovascular disease (post-MI, heart failure), but also in cardiorenal syndrome, where in all of these conditions, cardiac hypertrophy and fibrosis play critical roles.

The protein bound uraemic toxin indoxyl sulphate stimulated cardiac myocyte hypertrophy and cardiac fibroblast collagen synthesis, and these effects were abrogated by ASK1 inhibition (Figures 1 and 2). This finding suggests that, in addition to activation of p38MAPK, ERK1/2, and NFκB pathways as we have reported previously [12], it is likely that indoxyl sulphate also exerts its cardiac effects, at least in part, *via* the activation of the upstream MAPK apoptosis signal-regulating kinase 1 signal pathway. These findings therefore suggest a potential therapeutic benefit of ASK1 inhibition in both kidney and heart in the setting of cardiorenal disorders, especially in chronic kidney disease patients undergoing dialysis where uraemic toxins are inadequately removed.

It is well established that AngII is one of the main regulators of the neurohormonal system, and ASK1 has been suggested as crucial in ROS-induced cardiac remodelling mediated by AngII [20]. In this study, we showed that ASK1 inhibitor attenuated the expression of pro-fibrotic genes (Collagen I, $TGF\beta_1$ and cTGF) in cardiac fibroblasts stimulated with AngII (Figure 3). Our findings indicate that the ASK1 cascade may be pivotal in regulating gene expressions that lead to pathological cardiac fibrosis, particularly under the influence of AngII.

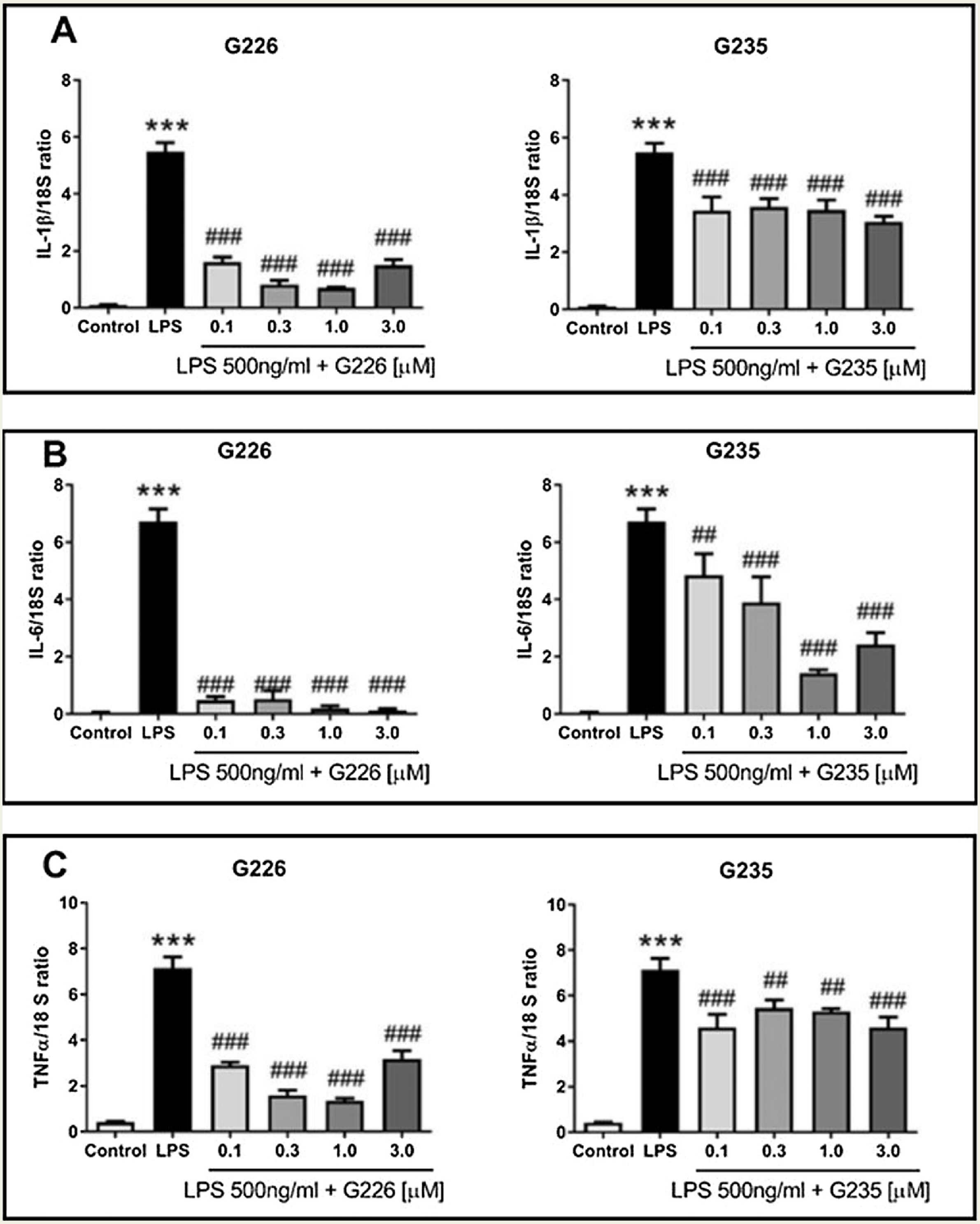

Figure 4 Inhibition of inflammatory gene expression.
LPS-stimulated THP-1 pro-inflammatory gene expression (Il-6, Il-1β and TNFα) was dose-dependently inhibited by both ASK1 inhibitors G2261818A (G226) and G2358939A (G235). Results are presented as mean ± SEM. All experiments were performed in triplicates and repeated three times.
$^{***}p < 0.001$ vs. unstimulated control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs 100 nM LPS-stimulated control.

Elevated levels of plasma IL-1β, IL-6 and TNFα were shown to promote LV remodelling and dysfunction in post-MI patients [21]. Our study showed that inhibition of the ASK1 pathway attenuated pro-inflammatory cytokine gene expression (IL-1β, IL-6 and TNFα) by monocytic THP-1 cells stimulated by LPS (Figure 4), suggesting a potential anti-inflammatory role that the ASK1 pathway may possess. A previous finding also indicates that the production of inflammatory cytokines from splenocytes and dendritic cells cultured from mice deficient in ASK1 was less than those derived from mice with ASK1 [22]. Knockout of ASK1 also resulted in mice that are more resistant to sepsis shock induced by LPS [23]. Furthermore, this study also demonstrated that inhibition of ASK1 prevents the myocyte hypertrophy stimulated by inflammatory cytokines: IL-1β and TNFα (Figure 1). Taken together, these findings suggest a potential therapeutic role for ASK1 inhibitors in cardiovascular diseases, such as post-MI, ischaemic heart disease and heart failure, where inflammation is a major contributing factor.

The results from the present study may have some clinical relevance. There is a large unmet need in the management of post-MI patients with LV dysfunction and in those with established chronic heart failure and kidney diseases. Current therapies mostly target key regulators of the neurohormonal system along with diuretics, which accounts for the current pathophysiological knowledge of cardiorenal dysfunction [21]. As ASK1 inhibitors act via pathways alternative to that of conventional anti-remodelling agents, the possibility exists of additive or even synergistic effects of ASK1 inhibitor with these agents. However, the compound's efficacy required further validation in relevant *in vivo* models of cardiac remodelling in order to translate the outcome into clinical application using drug-like inhibitor/s with good pharmacokinetics and metabolic properties.

There are several limitations in our study. Although we were able to evaluate the effectiveness of selective ASK1 inhibitors at the cellular level, other parameters such as drug toxicity and interactions with other factors can only be determined in *in vivo* studies and in clinical trials. We also noticed a difference in the potency of the two ASK1 inhibitors, G226 and G235, tested in our assays (Figures 1–4). This cannot be fully ascribed to ASK1 enzyme inhibition potency. G226 and G235 are chemically different class compounds, which may have different effectiveness within the cells. Further investigation is needed to fully clarify such discrepancy. Altogether, with increasing evidence of a favourable outcome in the cardiac impairment milieu, development of more potent and efficacious ASK1 inhibitors may realise clinical trials of this class of drug in the future.

Conclusion

In conclusion, selective inhibition of ASK1 in cardiac cells has shown potent anti-hypertrophic and anti-fibrotic effects, as well as anti-inflammatory effects in monocytic cells. ASK1 inhibitors may be novel therapeutic agents to diminish cardiac remodelling post cardiac injury by attenuating cardiac hypertrophy, fibrosis and inflammation if they are validated in suitable *in vivo* animal models and properly tested in a clinical setting.

Conflicts of Interest

There is no conflict of interest to disclose.

Declaration

All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

Acknowledgements

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