

# Effects of propofol on the expression of matrix metalloproteinases in rat cardiac fibroblasts after hypoxia and reoxygenation

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## Editor's key points

- Propofol induced matrix metalloproteinase activity in rat cardiac fibroblasts under conditions of hypoxia–reoxygenation.
- Cell migration, invasion, and proliferation were increased by propofol.
- This may explain the cardioprotective effects of propofol after ischaemia–reperfusion.

**Background.** Propofol is known to protect the myocardium against ischaemia/reperfusion (I/R) injury through its antioxidant and anti-inflammatory properties. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are involved in cell migration and invasion, and mediate tissue remodelling during I/R injury. They are regulated by various mechanisms including oxidative stress and AKT and ERK pathways. We investigated whether propofol affected the expression of MMPs and subsequent cell migration and invasion and the signalling pathways involved in primary rat cardiac fibroblasts undergoing hypoxia and reoxygenation.

**Methods.** The phosphorylation of ERK and AKT signalling pathways was examined by western blot analysis in rat primary cardiac fibroblasts after hypoxia and reoxygenation. mRNA expression of MMP and TIMPs was analysed by real-time PCR, and proteolytic activities of MMP-2 and -9 were assessed. The effects of propofol on migration, invasion, wound healing, and cell proliferation activity were evaluated after reoxygenation.

**Results.** Propofol induced AKT and ERK1/2 activation. Subsequent activation of MMPs resulted in increased cell migration, invasion, and wound-healing activity under hypoxia–reoxygenation, which was decreased by LY294002 (AKT inhibitor) and U0126 (ERK inhibitor) in rat cardiac fibroblasts. However, propofol had no effect on proliferation or viability of cardiac fibroblasts after hypoxia–reoxygenation.

**Conclusions.** Propofol affected the expression of MMPs and TIMPs and subsequently induced cell migration and invasive ability, through activation of the ERK and AKT signalling pathway in hypoxia–reoxygenated rat cardiac fibroblasts.

**Keywords:** ischaemia/reperfusion; matrix metalloproteinases; propofol

Accepted for publication: 5 January 2011

Matrix metalloproteinases (MMPs) are members of the zinc-dependent endopeptidase family that play an important role in the development, morphogenesis, angiogenesis, and turnover of the extracellular matrix (ECM).<sup>1,2</sup> Tissue inhibitors of metalloproteinases (TIMPs) inhibit the proteinase activities of MMPs,<sup>3</sup> and the balance between the MMPs and TIMPs controls local activities of MMPs in tissues.<sup>2,4</sup>

MMPs are important stimulators of cardiac fibroblasts, the most abundant cell type in the normal heart, playing a key role in cardiac remodelling and fibrosis after ischaemia/reperfusion (I/R) injury.<sup>5,6</sup> Accordingly, the pathological activities of the MMPs, TIMPs, and membrane-type (MT)-MMPs are closely associated with vascular remodelling, fibrosis, and left ventricular remodelling after I/R injury.<sup>7,8</sup>

Diverse signalling pathways including mitogen-activated protein kinases (MAPKs), extracellular signalling-regulated

kinase,<sup>9</sup> and AKT are responsible for the regulation of MMPs, TIMPs, and MT-MMPs.<sup>1,10</sup> In addition, phosphatidylinositol 3-kinase (PI3K)/AKT and MAPKs signalling pathways were demonstrated to be involved in cardiac disease or cardioprotective processes through regulation of expression of MMPs.<sup>11–13</sup>

Propofol (2,6-di-isopropylphenol), an i.v. anaesthetic agent, has free radical scavenging properties,<sup>14</sup> providing both vascular and cardioprotective effects against I/R injury.<sup>15</sup> Post-conditioning with propofol was shown to mitigate ischaemic brain injury through activation of the PI3K/AKT pathway<sup>16</sup> and propofol-induced ERK activation was protective in neuronal cells.<sup>17</sup> In the heart, propofol inhibited angiotensin II-mediated cardiomyocyte hypertrophy and promoted myocardial angiogenesis.<sup>18</sup>

Despite the theoretical background that propofol could modulate the activities of AKT and ERK pathways and

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protect against I/R-induced injury, there are no data regarding the effects of propofol on expression of MMPs and subsequent changes in cardiac fibroblast activities in I/R injury. This study investigated whether propofol influences MMPs and TIMPs expression and the role of AKT and ERK and subsequent proliferation, migration, and invasion activities of rat cardiac fibroblasts under conditions of hypoxia-reoxygenation.

## Methods

### Primary culture of rat cardiac fibroblasts

The animal procedures were approved by the committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine, and were performed in accordance with the Committee's Guidelines and Regulations for Animal Care. Isolated neonatal cardiac fibroblasts were obtained as described previously.<sup>19</sup> In brief, 1- to 2-day-old Sprague-Dawley neonatal rat pups were killed and the hearts were dissected under sterile conditions. Minced ventricular myocardium was digested consecutively for 10, 6 × 10 min each, using an enzyme mixture containing 1% (w/v) trypsin, 0.5 mM EDTA, and 0.1% collagenase in HEPES. The dissociated cells were mixed with alpha-minimum essential medium ( $\alpha$ -MEM) containing 5.5 mM glucose, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution and were centrifuged and pooled. Culture media and supplements were purchased from Hyclone (Logan, UT, USA). The resulting dissociated cell mixture was pre-plated for 1 h in a 5% CO<sub>2</sub>-containing incubator at 37°C for isolation of fibroblasts. After removal of the myocyte-enriched medium, culture medium was then added to the pre-plated fibroblasts and cultured overnight. Cardiac fibroblasts were used at the second to fourth passage.

### Gelatin zymography

Levels of secreted pro- and active MMP-2 and MMP-9 were evaluated using gelatin zymography. Fibroblasts were plated into 6-well culture plates with culture medium, incubated for 24 h, and serum-starved overnight in serum-free medium supplemented with 1% bovine serum albumin (BSA). Cells were transferred to fresh medium and incubated for 8 h in conditions of normoxia (5% CO<sub>2</sub> in air) or hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub>, 94% N<sub>2</sub>). Aliquots of conditioned media were separated by electrophoresis in 10% gelatin zymogram gels (Invitrogen Life Technologies, Carlsbad, CA, USA) after which the gels were washed twice in denaturing buffer (2.5% Triton X-100, 50 mM Tris-Cl, pH 7.5) at room temperature for 30 min followed by developing buffer (150 mM NaCl, 10 mM CaCl<sub>2</sub>, 50 mM Tris-Cl, pH 7.5) at room temperature for 30 min, and finally developing buffer at 37°C for 16–18 h. These gels were stained with 0.1% Coomassie brilliant blue in 10% methanol and 10% acetic acid. After destaining, MMP-9, pro-MMP-2, and active MMP-2 were seen as clear bands (92, 72, and 62 kDa, respectively).

### RNA isolation and real-time PCR

Expression levels of MMPs and TIMPs were examined by real-time PCR. Total RNA was isolated using RNeasy-mini kits (Qiagen, Valencia, CA, USA). cDNA was synthesized from 1  $\mu$ g of total RNA by using *Maxime* RT PreMix kit (iNtRON Biotechnology, Sungnam, Korea). Real-time PCR analysis was performed using SYBR *Premix Ex Taq*<sup>TM</sup> (TaKaRa, Otsu, Japan) and an AB 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each sample was analysed in quadruplicate and target genes were normalized to the reference housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase. Fold differences were then calculated for each treatment group using normalized C<sub>T</sub> values for the control. Rat genes and their primer sequences for real-time PCR are given in Supplementary Table S1.

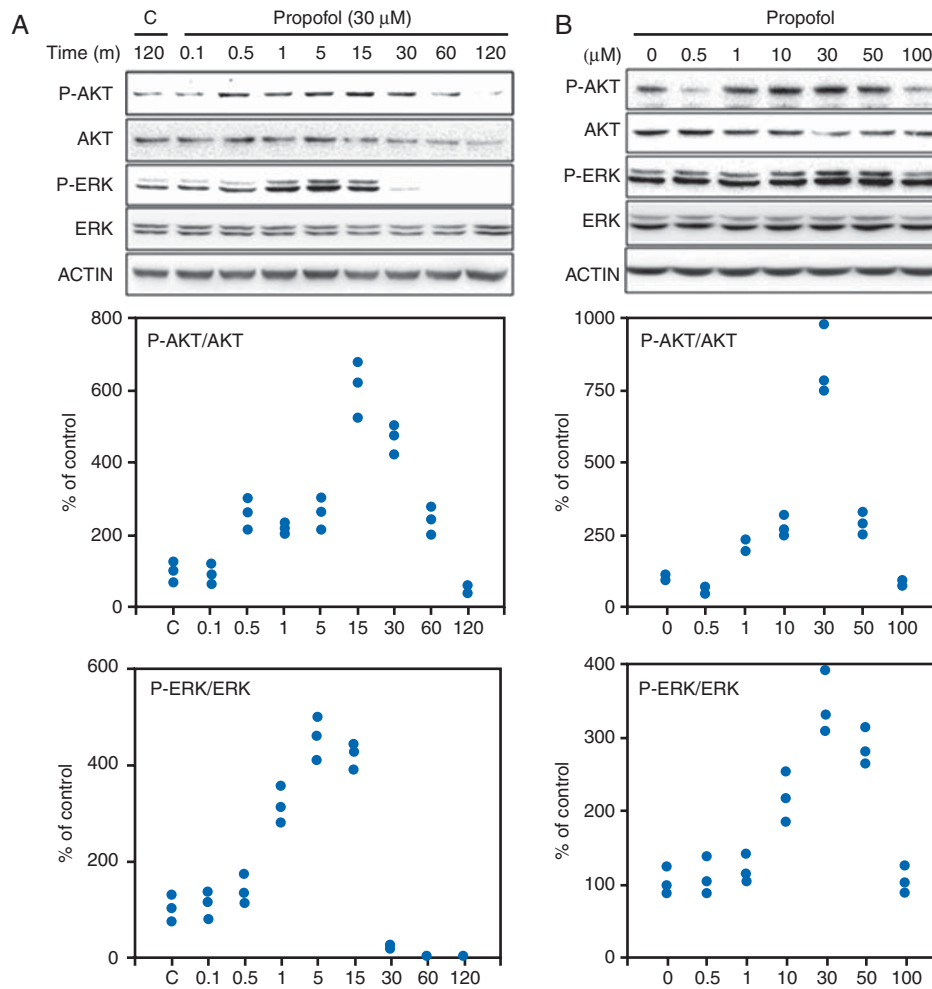
### Immunoblot analysis

Fibroblasts were seeded into 60 mm tissue culture dishes, incubated for 24 h, and serum-starved overnight. Cells were then transferred to fresh medium and incubated as previously. The cells were then washed with ice-cold PBS and lysed in cell lysis buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 mM PMSF) with a supplement of complete protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) and sonicated briefly. Protein concentrations were determined using the Bradford protein assay with BSA as a standard (Bio-Rad, Hercules, CA, USA). Samples containing equal amounts of protein (50  $\mu$ g) were subjected to SDS-polyacrylamide gel electrophoresis, after which the separated proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% non-fat, dried milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with primary antibody followed by a horseradish peroxidase conjugated secondary antibody. The following primary antibodies were used: ERK1/2, phospho-ERK1/2, AKT, phospho-AKT, and Actin (Cell Signaling, Beverly, MA, USA). The luminescence was detected using WEST-ZOL western blot detection kit (iNtRON Biotechnology, Sungnam, Korea) in a LAS4000 (Fuji PhotoFilm, Tokyo, Japan).

### Cell migration and invasion assays

Serum-starved fibroblasts were pretreated with U0126 (ERK inhibitor, 20  $\mu$ M), or LY294002 (AKT inhibitor, 10  $\mu$ M) or a vehicle control for 1 h, followed by propofol (30  $\mu$ M) or a vehicle control for 1 h, and further exposed to hypoxia for 1 h in MEM supplemented with 0.1% BSA, 2 mM CaCl<sub>2</sub>, and 2 mM MgCl<sub>2</sub>. These cells were trypsinized and seeded into the inner chamber of a 24-well migration or invasion plate followed by reoxygenation for 24 h.

Migration and invasion assays were assessed using a modified Boyden chamber technique with Matrigel basement membrane matrix-coated membranes pore size 8  $\mu$ m



**Fig 1** Propofol-induced changes in AKT and ERK signalling for the indicated times (A, upper panel) and at the indicated concentration (B, upper panel), and quantified signal (A and B, lower panel). Figure shows raw data points.

(CytoSelect™ Cell migration and invasion assay kit, Cell Biolabs, CA, USA). Chemoattractant (10% FBS in MEM) was loaded into the lower well of the migration or invasion plate. After the incubation period in hypoxia–reoxygenation conditions, migration or invasion processing was performed according to the manufacturer's instructions. Each experiment was performed at least six times.

### Cell proliferation assay

Cardiac fibroblasts were seeded into 96-well culture plates and incubated for 24 h. After propofol pretreatment for 1 h at concentrations indicated, cells were exposed to hypoxia for 1 h followed by reoxygenation for 1–2 days. After each time of incubation, cell proliferation was assessed by the Cell counting kit (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions.

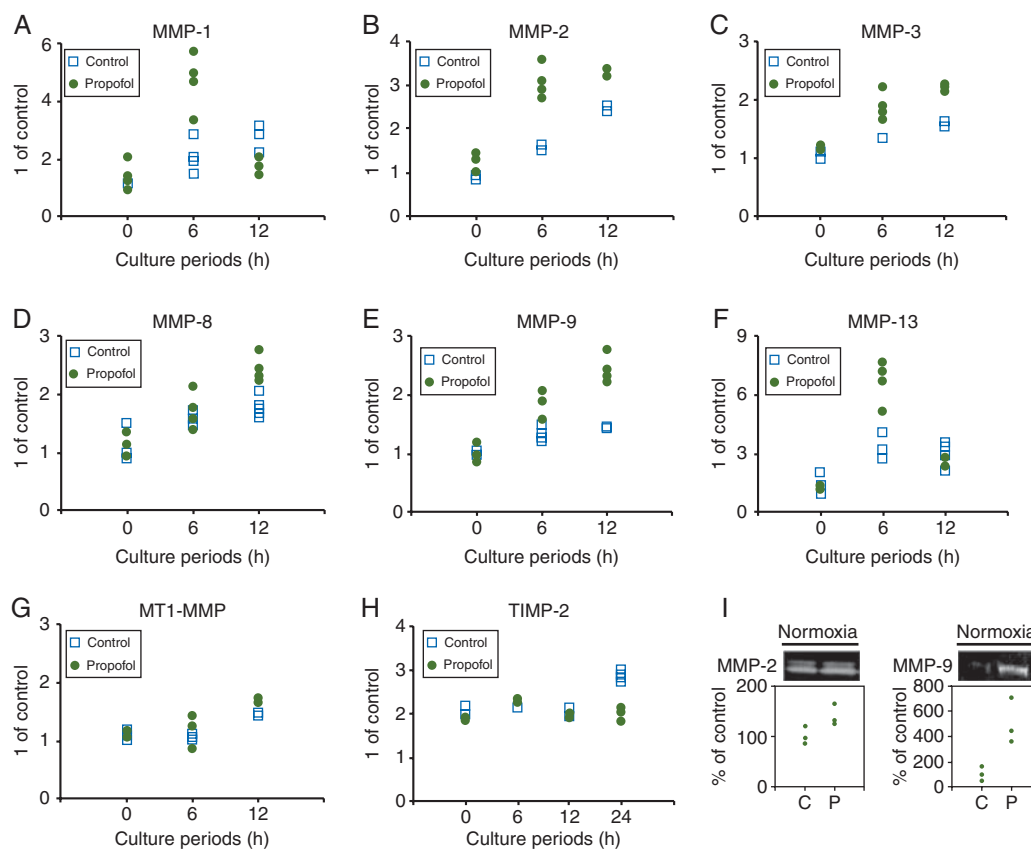
### Statistical analysis

Data are shown as raw data points apart from cell proliferation assay data which are shown as mean (SEM) and statistical differences were analysed using Student's *t*-test following the Bonferroni correction. A *P*-value of <0.05 was considered significant.

## Results

### ERK and AKT signalling pathway in primaries

Propofol induced phosphorylation of AKT and ERK within 0.5–15 and 1–15 min, respectively (Fig. 1A). Propofol treatment for 5 min at about 10–50 μM increased AKT and ERK phosphorylation in cardiac fibroblasts (Fig. 1B). In this study, commercially sold propofol solubilized in 10% intralipid emulsion was used. There was no difference in AKT and ERK activities between 10% intralipid emulsion and DMSO-treated cells (data not shown).



**Fig 2** (A–H) Propofol induced MMPs and TIMP-2 mRNA expression under normoxic conditions. (i) Effect of propofol on MMP-2 and -9 expression and quantified signal by scanning densitometry (lower panel). Figure shows raw data points. C, control; MMPs, matrix metalloproteinases; P, propofol; TIMP, tissue inhibitors of metalloproteinase.

### mRNA expression of MMP and TIMP

In normoxic condition, expression of MMPs mRNA progressively increased with time and MT1-MMP and TIMP-2 mRNA expression at 24 h was higher than baseline values. Propofol increased MMPs and MT1-MMP mRNA expression in the same pattern but not TIMP-2 mRNA (Fig. 2A–H). MMP-1 and MMP-13 mRNA expression at 6 h, and MMP-8 and -9 mRNA expression at 12 h were higher in propofol-treated cells than in the control cells. MMP-2 and -3 mRNA expression at 6 and 12 h was also higher in the propofol-treated cells. In addition, MT1-MMP mRNA expression was higher and TIMP-2 mRNA expression was lower at 12 h in propofol-treated cells. Propofol enhanced the proteolytic activity of MMP-2 and of MMP-9 about 5.8-fold (Fig. 2i).

### Inhibition of ERK and AKT signalling pathways

Pretreatment with LY294002 (AKT inhibitor) or U0126 (ERK inhibitor) for 1 h suppressed propofol-induced MMP-1, -2, and -9 mRNA expression (Fig. 3A–C). The decrease in TIMP-2 mRNA expression mediated by propofol was also attenuated by LY294002 or U0126 (Fig. 3D). Propofol-induced increased proteolytic activity of MMP-2 was attenuated by inhibiting AKT and ERK signalling (Fig. 3E and F). LY294002

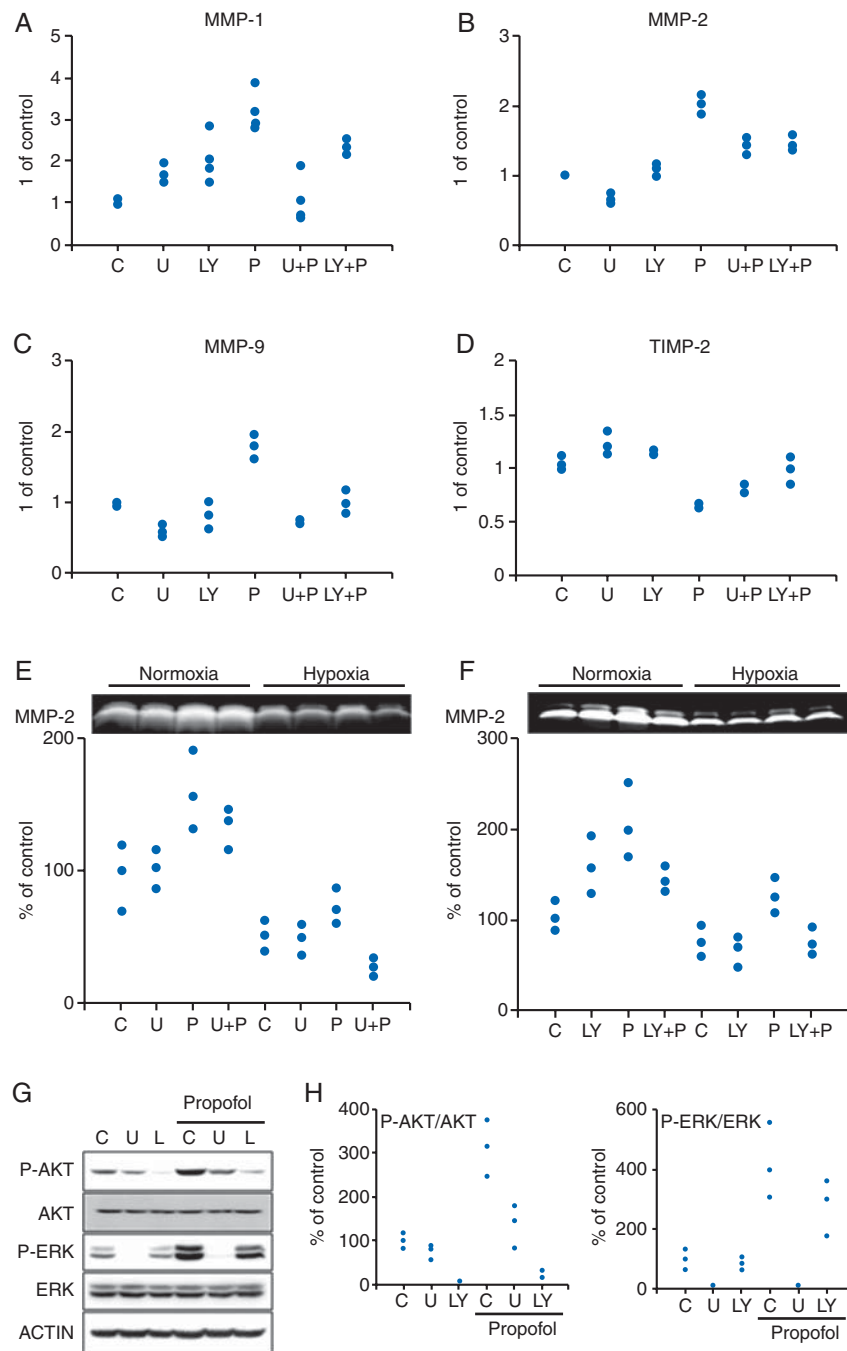
decreased propofol-induced ERK activation and U0126 also decreased propofol-induced AKT activation (Fig. 3G), demonstrating that the propofol regulated MMPs and TIMP-2 expression via AKT and ERK signalling pathways.

### mRNA expression of MMPs TIMPs under conditions of hypoxia–reoxygenation

MMP-1, -9, and -13 mRNA expression was increased by propofol after hypoxia–reoxygenation compared with that of control cells (Fig. 4). MMP-2 mRNA expression was less during hypoxia and for 3 h after reoxygenation in the propofol-treated cells but was greater at 6 and 12 h after reoxygenation in the propofol-treated cells. TIMP-1 mRNA expression 1 h after reoxygenation and TIMP-2 and -3 mRNA expression during hypoxia and early reoxygenation were higher in the propofol-treated cells than control cells. However, TIMP-1, -2, and -3 mRNA levels at 24 h after reoxygenation decreased by more than 50% in the propofol-treated cells.

### Cell migration, invasion, proliferation, and viability

Cell migration and invasion were enhanced by propofol-treated cells compared with control cells and were

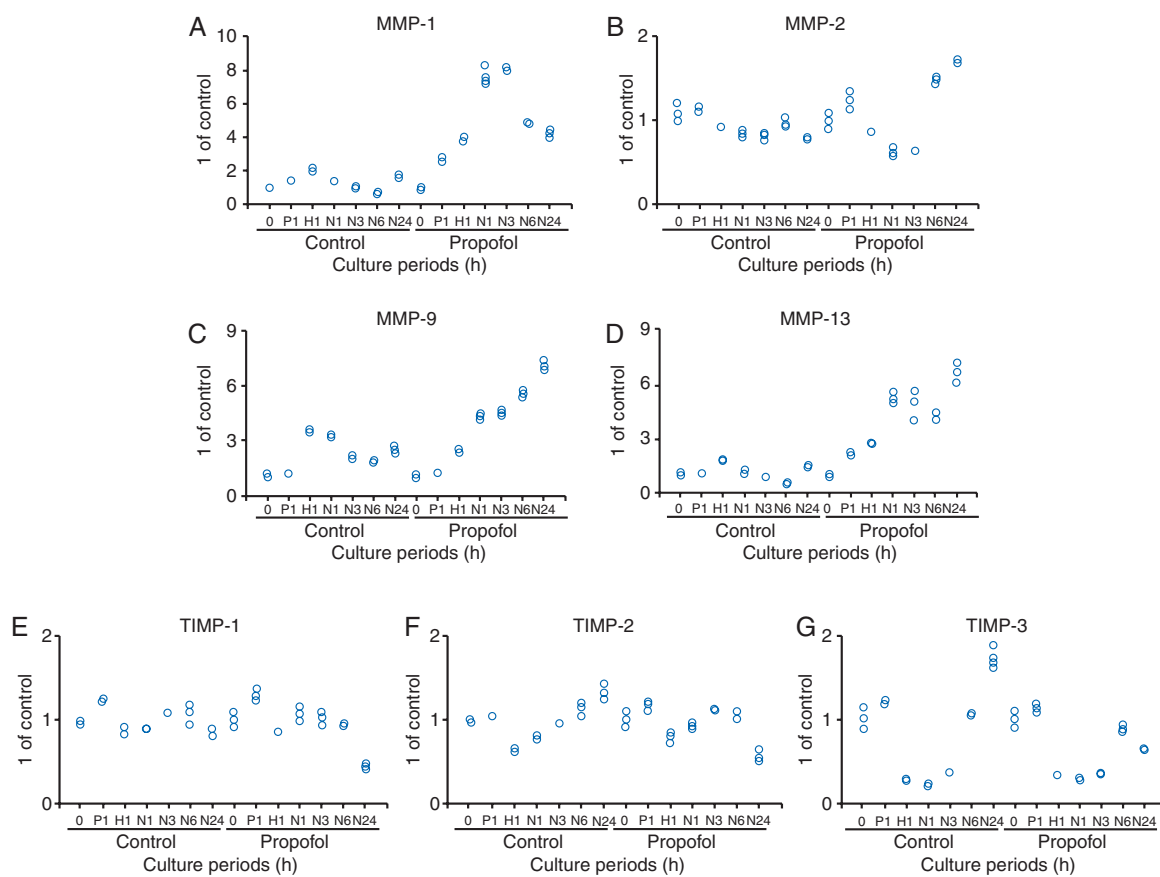


**Fig 3** (A–D) Effect of inhibiting ERK and AKT signalling pathway on propofol-induced MMPs and TIMPs gene expression. (E and F) The levels of pro- and active MMP-2 in conditioned media and quantified signal (lower panel). (G) Effect of U0126 (U), or LY294002 (L) on ERK and AKT activation, and (H) quantified signal. Figure shows raw data points. C, control; MMPs, matrix metalloproteinases; P, propofol; TIMP, tissue inhibitors of metalloproteinase.

attenuated by AKT and ERK inhibitors (Fig. 5A and B). Cell proliferation was not affected by lower concentrations of propofol under hypoxia–reoxygenation conditions, although a significant decrease in cell proliferation was seen after exposure to 200  $\mu$ M propofol for 2 days (Fig. 5C).

## Discussion

We showed that propofol regulated mRNA expression of MMPs and TIMPs through ERK and AKT signalling pathways, leading to enhanced cell migration and invasive ability without affecting cell proliferation, in rat cardiac fibroblasts.



**Fig 4** Propofol induced changes in MMP-1, -2, -9, and -13 and TIMP-1, -2, and -3 for the indicated time. Figure shows raw data points. H, hypoxia; MMPs, matrix metalloproteinases; N, normoxia; P, pre-treatment; TIMP, tissue inhibitors of metalloproteinase.

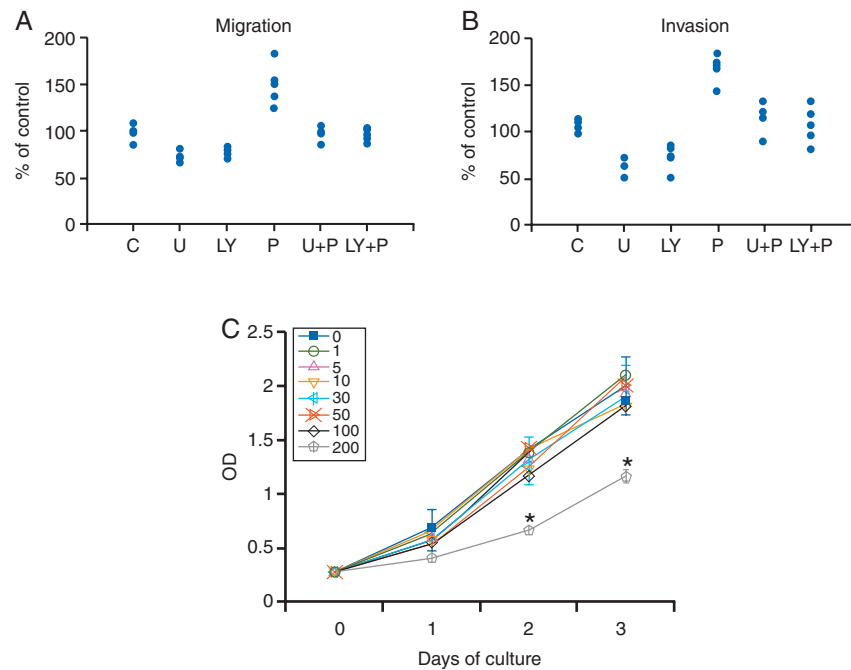
Notwithstanding the restoration of coronary blood flow, I/R injury triggers stress-signalling processes that eventually result in pathological damage to the myocardium.<sup>20 21</sup> Pre-conditioning with subclinical ischaemia or pharmacological agents including anaesthetics has been shown to provide cardioprotective effects through diverse signal transduction pathways.<sup>22</sup> PI3K/AKT and MAPK signalling pathways are known to play an important role in controlling survival and function of cardiomyocytes and cardiac fibroblasts.<sup>23</sup> Antioxidants exert cardioprotective effects by decreasing production of reactive oxygen species (ROS) during early reperfusion.<sup>24 25</sup>

Propofol, a well-known i.v. anaesthetic agent, was reported to exert significant protective activity against I/R-induced cardiac injury, partly by reducing the generation of ROS.<sup>26 27</sup> Furthermore, propofol was demonstrated to reduce damage from hydrogen peroxide-induced injury through the PI3K/AKT signalling pathway in cardiac H9c2 cells,<sup>28</sup> and to attenuate angiotensin II-induced cardiomyocyte hypertrophy,<sup>18</sup> which is an important process of remodelling after I/R injury.

In the remodelling process, cardiac fibroblasts play the most prominent role in the development of cardiac fibrosis after I/R injury and thus, these cells have been considered

as a potential therapeutic target after I/R injury in cardiac disease. Cardiac fibroblasts constitute more than two-thirds of the total cells in the normal human heart.<sup>29</sup> Pathological stimuli, such as I/R, inflammation, and induction of TGF- $\beta$ , contribute to the increase in collagen type I and III mRNA expression and MMPs activity including MMP-2 during the development of cardiac fibrosis.<sup>30</sup> Despite the evidence that cardiac fibroblast activity during remodelling is closely related to MMPs, which is partly regulated by AKT and ERK pathways, and propofol could modify the activities of AKT and ERK pathway, the effect of propofol cardiac fibroblast function under conditions mimicking I/R injury have not been investigated.

As the results of the current study indicate, propofol stimulated AKT and ERK phosphorylation rapidly in rat cardiac fibroblasts under normoxic condition. U0126, an ERK inhibitor, decreased propofol-induced AKT activation, which shows the relationship between AKT and ERK. Propofol also weakly increased MMP-1, -2, -3, -8, -9, -13, and -14 (MT1-MMP) mRNA expression and enhanced the proteolytic activities of MMP-2 and -9, whereas TIMP-2 mRNA expression was decreased compared with cells not exposed to propofol under normoxic conditions. These propofol-induced changes



**Fig 5** (A and B) Effect of propofol on cell migration and invasion. Figure shows raw data points. (C) Effect of propofol on cell proliferation assay. Data are means (SE). \*Significant difference from the control ( $P < 0.05$ ). C, control; LY, LY294002; P, propofol; U, U0126.

were mitigated by AKT or ERK inhibitors, confirming the role of AKT and ERK.

Hypoxia is one of the numerous influences on cardiac matrix remodelling, via ECM turnover and induction of MMPs and TIMPs. In addition, I/R injury is also a critical modulator of MMP expression through alternative mechanisms.<sup>9</sup> The effect of hypoxia was maximal between 1 and 2 h of hypoxic exposure,<sup>31 32</sup> and thus serum-starved fibroblasts were exposed to hypoxia for 1 h followed by reoxygenation for 24 h in our study. Under these conditions, propofol increased MMP-1, -9, and -13 mRNA expression, and attenuated TIMP-1, -2, and -3 mRNA expression more than seen during normoxia. These changes were more obvious as the duration of reoxygenation was prolonged. These results implicate that the influence of propofol on MMP expression might be amplified during the late period of reoxygenation after hypoxia than under normoxic or chronic hypoxic conditions (data not shown) in these cells. Subsequently, propofol enhanced cell migration and invasion of hypoxia-reoxygenated rat cardiac fibroblasts, which was attenuated by LY204002 or U0126. The results of the current study are the first to report that propofol influences the activities of these cells through modulation of MMP and TIMP expression partly via AKT and ERK pathways, especially after hypoxia-reoxygenation, which implies a role for propofol as a modulator of matrix regulation.

Angiotensin II, one of the major stimuli of cardiac fibrosis, increases collagen type I and MMP-2 expression through induction of oxidative stress.<sup>33</sup> Oxidative stress regulates

collagen metabolism and stimulates remodelling of the myocardium in various cell types, such as lung and skin fibroblasts and endothelial cells. Considering the well-known antioxidant properties of propofol, increased MMP expression upon exposure to propofol was unexpected. The observed ability of propofol to increase migration and invasiveness of cardiac fibroblasts after hypoxia-reoxygenation in the current study could adversely affect cardiac remodelling in a clinical setting. However, diverse and different mechanisms are involved in regulation of MMP depending on pathological conditions and cell type. In diabetic cardiomyopathy, cardiac fibrosis has been demonstrated to be associated with reduced MMP-2 activities and MT1-MMP mRNA expression, and increased TIMP-2 expression.<sup>34</sup> Also, angiotensin II and glucose-induced reduction of MMPs activity has been described in diabetic cardiomyopathy.<sup>35</sup> Moreover, considering that proliferation of cardiac fibroblasts play a key role in cardiac fibrosis and diastolic dysfunction, the finding that propofol did not affect proliferation seems promising although the net effect on cardiac fibrosis still needs to be validated.

Different results have been observed regarding cell proliferation and AKT activation in a previous study addressing the effects of propofol on angiotensin II-induced proliferation of rat cardiac fibroblasts.<sup>36</sup> In that study, propofol was reported to mitigate angiotensin II-induced proliferation of cardiac fibroblasts partly by activation of the AKT-endothelial nitric oxide synthase-nitric oxide pathway. However, in contrast to our study, that study was performed under normoxic conditions with a known powerful stimulant

of fibroblast proliferation, angiotensin II and thus it is difficult to compare the results of the two studies directly. In the absence of angiotensin II, propofol did not affect cell proliferation *per se* in that study, as in our study. Although we did observe significant decreases in cell proliferation after exposure to propofol at high doses, this dose is well above the clinically applied doses since 30  $\mu\text{M}$  used in this study corresponds to the usual target blood concentration of 2–4  $\mu\text{g ml}^{-1}$ .

Migration is an important cellular function during cell development and organogenesis including angiogenesis, and also wound healing and stem or progenitor cell therapy.<sup>10</sup> MT1-MMP promotes granulocyte colony-stimulating factor-induced haematopoietic stem or progenitor cell mobilization.<sup>37</sup> In keratinocytes, MMP-3, -9, and -13 play a crucial role in skin wound healing in the mouse.<sup>38</sup> The role of MMPs and TIMPs in cell migration and invasion both *in vivo* and *in vitro* has been well documented in various cell types.<sup>39–40</sup> Therefore, the ability of propofol to regulate MMP and TIMP expression may exert positive effects in several clinical conditions, such as stem cell therapy for I/R-induced organ damage, but it is beyond the scope of this study and merits further evaluation.

In conclusion, propofol affected MMP and TIMP activities partly through AKT and ERK pathways in normoxic conditions in rat cardiac fibroblasts and potentiated MMP expression under conditions of hypoxia–reoxygenation, resulting in increased cell migration and invasion. These results implicate propofol as a modulator of matrix regulation.

## Supplementary material

Supplementary material is available at *British Journal of Anaesthesia* online.

## Conflict of interest

None declared.

## Funding

This study was supported by a faculty research grant (6-20090069) of Yonsei University College of Medicine 2009.

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