GSK-3β at the crossroads in the signalling of heart preconditioning: implication of mTOR and Wnt pathways

François Vigneron¹, Pierre Dos Santos¹,²,³*, Sandrine Lemoine¹, Maryline Bonnet¹, Liliane Tariosse¹, Thierry Couffinhal¹,²,³, Cécile Duplaà¹, and Béatrice Jaspar-D-Vinassa¹,²

¹Inserm Unit 828, Avenue du Haut Lévêque, 33600 Pessac, France; ²University Bordeaux 2, 33000 Bordeaux, France; and ³University Hospital of Bordeaux, 33000 Bordeaux, France

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Aims
Ischaemic preconditioning (IPC) protects the heart against prolonged lethal ischaemia through a signalling cascade involving Akt, glycogen synthase kinase-3β (GSK-3β), and mitochondrial ATP-sensitive potassium channels (mitoKATP). We previously demonstrated the involvement of the Wnt pathway in IPC in vivo via GSK-3β. A downstream target might be mammalian target of rapamycin (mTOR) since Wnt can impair tuberous sclerosis complex-2 (TSC2) phosphorylation by inhibiting GSK-3β. Here, we investigate whether the mTOR pathway is involved in cardioprotection.

Methods and results
Isolated-perfused mouse hearts were subjected to IPC via four cycles of ischaemia/reperfusion or pharmacological preconditioning (PPC) by diazoxide, a selective mitoKATP activator. IPC, like PPC, induced an inhibition/phosphorylation of GSK-3β through Akt activation. Preconditioning also induced phosphorylation of mTOR, p70S6K, and 4E-BP1 that correlated with a significant reduction in infarct size after 40-min ischaemia and 120-min reperfusion when compared with non-preconditioned controls. Preconditioning was impaired in GSK3 knock-in mice. In transgenic mice hearts overexpressing secreted frizzled protein 1 (sFRP1, a Wnt/Frz antagonist), GSK-3β phosphorylation, mTOR activation, and cardioprotection were impaired. Cardioprotection and its signalling were also inhibited by rapamycin (an mTOR inhibitor), 5-HD (a mitoKATP blocker), and N-(2-mercaptopropionyl) glycine (MPG) as a reactive oxygen species (ROS) scavenger.

Conclusions
We propose that the preconditioning signalling pathway involving an amplification loop results in a downregulation of GSK-3β and a constant opening of mitoKATP with ROS generation to activate the mTOR pathway and induce cardioprotection. The disruption of the Wnt/Frz pathway by sFRP1 modulates this loop, inducing GSK-3β activation. This study provides evidence that cardioprotection involves both a pro-survival mTOR pathway and a developmental Wnt pathway targeting GSK-3β.

Keywords
Preconditioning • GSK-3β • mTOR • Wnt • Signalling pathways

1. Introduction
Transient episodes of ischaemia/reperfusion, termed ischaemic preconditioning (IPC), confer myocardium resistance to lethal ischaemia. Although the exact mechanism of IPC remains obscure, it seems that cardioprotection results from activation of signal transduction pathways emanating from surface receptors trigged by Gi protein-coupled receptor (GPCR) agonists, such as bradykinin, opioid peptides, and adenosine, that are released by the ischaemic heart.¹ These GPCR-related signalling cascades converge and lead to opening of the mitochondrial ATP-sensitive potassium channel (mitoKATP). Pharmacological openers of mitoKATP trigger cardioprotection, whereas antagonists of this channel abrogate the protective effects of IPC.²³ Opening of mitoKATP leads to the production of reactive oxygen species (ROS), the inhibition of mitochondrial permeability transition and, ultimately, the protective effect of IPC.⁴⁻⁵ Several reports indicate that signalling pathways involved in IPC result in protein kinase C (PKC) activation and in phosphatidylinositol-3-kinase (PI3K) activation and...
translocation.6,7 Downstream targets of PI3K include proteins in the PKB/Akt pathway and glycogen synthase kinase-3β (GSK-3β). Numerous studies suggest that phosphorylation at serine 9 of GSK-3β, which results in its inhibition, is a key event in the intracellular signalling of IPC.8,9 Recent studies using rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), suggest that the mTOR pathway is involved in preconditioning, but its role remains to be elucidated. mTOR, a member of the PI3K-related kinase family, has been implicated in cardiac function and in cellular growth and proliferation through the regulation of protein translational machinery.10,11 The best known targets of mTOR are the ribosomal S6 kinase 1 (p70S6K) and the eukaryotic initiation factor 4E (eIF4E).12 mTOR directly phosphorylates p70S6K at threonine 389, which is required for its kinase activity. In turn, p70S6K phosphorylates ribosomal S6 protein and other targets, such as eIF4E, implicated in translation.13 Phosphorylation of 4E-BP1 by mTOR relieves the inhibition of eIF4E.14–16 Both phosphorylation/activation of p70S6K and phosphorylation/inhibition of 4E-BP1 are blocked by rapamycin. Data obtained by the analysis of isolated rabbit hearts suggest that protection afforded by IPC might require protein synthesis.17 Kis et al.18 proposed that mTOR and p70S6K are involved in delayed preconditioning in a rabbit in vivo model. A study of isolated rat hearts demonstrated that IPC leads to PI3K/Akt and p70S6K activation upon reperfusion.19 Interestingly, the pro-survival pathways of mTOR and PKB/Akt provide microglial cell protection through a mechanism involving inhibition of GSK-3β activity.20 Furthermore, Inoki et al.21 reported that in a tumour development model Wnt activates mTOR signalling by impairing TSC2 phosphorylation via inhibition of GSK-3β, whereas the inhibition of mTOR by rapamycin blocks Wnt-induced cell growth.

Our previous studies demonstrated that Wnt/β-catenin pathway plays a role in cardioprotection. We showed that the protective effect of IPC is abolished by overexpression of secreted frizzled protein 1 (sFRP1), an antagonist of the Wnt/β-catenin pathway, and that this effect is related to the ability of sFRP1 to decrease the phosphorylation/inhibition of GSK-3β.22 In the present study, we examined signalling events during the preconditioning phase and their effect on cardioprotection in an isolated mouse heart model. We provide evidence that cardioprotection involves a link between the mTOR pro-survival pathway and the Wnt embryonic pathway via GSK-3β. This would depend on ROS production through mitoK_{ATP} opening.

2. Methods

2.1 Generation of transgenic mice

Double-transgenic mice, called α-MHC/sFRP1, were obtained by crossing the TRE-sFRP1 mouse with the α-MHC-CreTA transactivator mouse strain.22 TRE-sFRP1 and α-MHC-CreTA transgenic littermates were used as controls. GSK-3β null knock-in (KI), GSK-3β KI, and wild-type mice were provided by Yellow23. GSK-3αβ KI mice were obtained by crossing GSK-3α KI with GSK-3β KI.

2.2 Langendorff perfusion of mouse hearts

Mice were used in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85–23, Revised 1996) and according to the recommendations of the Institutional Animal Care Committee at INSERM (Bordeaux, France). Mice hearts were Langendorff-perfused with modified Krebs–Henseleit buffer at a constant pressure of 100 mmHg (see Supplementary material online). Hearts were subjected to 35 min of stabilization in controlled aerobic conditions followed by the preconditioning protocol (see below). For western blot analysis, hearts were harvested at the end of the preconditioning protocol, trimmed of atrial tissue, and snap frozen (n ≥ 6 per group). The infarct size (IS) was determined in a separate series of hearts by 2,3,5-triphenyltetrazolium chloride (TTC) staining after 40 min global zero-flow ischaemia followed by a 120-min reperfusion (n ≥ 6 per group).

2.3 Perfusion protocols

Isolated hearts subjected to 35 min of stabilization were randomly assigned to one of the treatment protocols diagrammed in Figure 1. Non-preconditioned (NP) hearts were subjected to 20 min perfusion in aerobic conditions. IPC hearts were subjected to an IPC sequence consisting of four cycles of 5 min global zero-flow ischaemia followed by a 5-min reperfusion. Pharmacologically preconditioned (PPC) hearts were subjected to 20-min perfusion in the presence of 10 μM diazoxide. IPC and PPC hearts were also performed in the presence of inhibitors: 300 μM 5-hydroxydecanoate (5-HD, a mitoKATP antagonist), 100 nM wortmannin (WM, a specific PI3K/Akt inhibitor), 1 mM N-(2-mercaptoethyl) glycine (MPG, an ROS scavenger), or 250 μM rapamycin (a specific mTOR inhibitor). Hearts were perfused for 5 min with inhibitor prior to and also during the IPC or PPC protocol. All protocols were applied to hearts from transgenic littermates, α-MHC/sFRP1-Tg and GSK-3αβ KI mice. See Supplementary material online for chemicals and providers.

2.4 Western blot analysis

For western blot analysis, left ventricles were dissected and snap-frozen at the end of perfusion period. Tissues were homogenized in ice-cold lysis buffer and subjected to SDS-polyacrylamide gel electrophoresis as previously described.22 After transfer, PVDF membranes were probed with antibodies from Cell Signaling for Akt, phospho-Akt (ser479), phospho-GSK-3β (ser9), mTOR, phospho-mTOR (ser2448), p70S6K, phospho-p70S6K (Thr389), 4E-BP1, phospho-4E-BP1 (ser65), or 4E-BP1 (BD-Biosciences). Cytochrome c oxidase IV (Molecular Probes) was used to ensure equal protein loading. Proteins were detected by chemiluminescence using Immobilon Western HRP Substrate (Millipore). Relative densities were assessed using Scion Image software.

2.5 Assessment of infarct size

IS was determined as previously described24 by TTC staining in separate series of hearts treated with preconditioning protocols described above and followed by a 40-min zero-flow global ischaemia and a 120-min reperfusion (see Supplementary material online). IS was expressed as a percentage of total tissue mass since, during global ischaemia, area at risk (AAR) corresponds to the entire heart.

2.6 Statistical analysis

Data are expressed as mean ± SE. Data were analysed using one-way ANOVA. P-value <0.05 was considered significant.

3. Results

3.1 Preconditioning requires Akt/GSK-3β pathway

The magnitude of protection afforded by IPC in mouse hearts was similar to that observed after PPC by diazoxide (Figure 2A), as evidenced by a 42% decrease in IS/AAR in IPC group and 55% in PPC group compared with NP hearts (19.9 ± 2.1, 15.4 ± 1.6, and 34.3 ± 1.5% in IPC, PPC and NP groups, respectively, P < 0.05).
IPC and PPC (IS/AAR PI3K inhibitor, WM, which abrogated cardioprotective effects of IPC and PPC, showed low levels of Akt and GSK-3b (after IPC and PPC, respectively, compared with NP hearts). We examined the phosphorylation states of Akt (ser473) and GSK-3b with NP. The p-GSK-3b 138 and 88% increases after IPC and PPC, respectively, compared with NP hearts (Figure 2B). Under controlled aerobic perfusion conditions, hearts showed low levels of Akt and GSK-3b phosphorylation.

The importance of Akt in preconditioning was confirmed using a PI3K inhibitor, WM, which abrogated cardioprotective effects of IPC and PPC (IS/AAR = 37.2 ± 5.5 and 39.9 ± 11.2% after WM/IPC and WM/PPC, respectively, vs. 34.3 ± 1.5% in NP hearts) and increased Akt and GSK-3b phosphorylation (see Supplementary material online, Figures S1 and S2). WM without IPC or PPC did not influence phosphorylation of these proteins or IS.

Although the importance of GSK-3b in preconditioning is supported by several studies, recent evidence suggests that GSK-3b is unlikely to be the key determinant in pre- and post-conditioning in mice. To clarify the role of GSK-3b, we studied preconditioning in GSK3 KI mice. We first confirmed that phospho-GSK-3b was not detected in ventricles of KI mouse hearts by immunoblotting (see Supplementary material online, Figure S3). Cardioprotection was not induced by IPC or by PPC in GSK3 KI hearts; IS represented 36.4 ± 5.9% and 40.1 ± 6.7% of AAR, respectively, similar to that observed in NP hearts (34.3 ± 1.5%; Figure 2A).

3.2 Preconditioning involves mTOR pathway activation

Previous studies in a tumour development model showed that Wnt activates mTOR by inhibiting GSK3 and that GSK3 inhibits mTOR pathway by phosphorylating TSC2 in a AMPK-dependent manner. To investigate the putative role of mTOR in preconditioning, we examined whether an mTOR-specific inhibitor, rapamycin, reduced cardioprotective effects of IPC and PPC. As shown in Figure 3A and Supplementary material online, Figure S1, IPC- and PPC-induced cardioprotection were blocked by rapamycin (IS/AAR = 40.0 ± 4.8 and 49.4 ± 4.2% in rapamycin/IPC and rapamycin/PPC groups, respectively, compared with rapamycin alone). We then examined the phosphorylation state of mTOR after IPC and PPC. Both preconditioning strategies lead to increased phosphorylation of mTOR (ser2448) as evidenced by a 127 and 108% increase in p-mTOR/mTOR ratio after IPC and PPC, respectively, compared with the ratio in NP hearts (Figure 3B).

The targets of mTOR pathway include several components of the translational machinery. We focused on p70S6K, which is involved in translation initiation and activated by phosphorylation on Thr389, and on 4E-BP1, which is a negative regulator of cap-dependent mRNA translation that is inhibited by hyperphosphorylation. Phosphorylation on ser65 contributes to this inhibition and would result from mTOR activation because of its high sensitivity to rapamycin. Preconditioning resulted in an increase in p70S6K phosphorylation by 91.1% after IPC and 93.8% after PPC, compared with NP group (P < 0.05, Figure 3C and Supplementary material online, Figure S4). Preconditioning also increased the phosphorylation of 4E-BP1 on ser65 by 121.3% after IPC and 150.9% after PPC (P < 0.05; Figure 3C and Supplementary material online, Figure S4).

3.3 GSK-3b plays an integral role in linking Wnt/Frz and mTOR pathways

We previously showed that sFRP1 overexpression in hearts of transgenic mice (sFRP1-Tg) impaired ser9 phosphorylation of GSK-3b. This effect was correlated with an inhibition of cardioprotection after ischaemic preconditioning in an in vivo model of myocardial infarction. Both the IPC- and PPC-induced decrease in IS were abolished in Langendorff-perfused sFRP1-Tg hearts; the IS/AARs were 28.9 ± 1.0 and 34.0 ± 4.2% of AAR after IPC and PPC, respectively, compared with 19.9 ± 2.1% after IPC and 15.4 ± 1.6% after PPC in control littermate hearts (Figure 4A). We then investigated whether sFRP1 overexpression altered the ability of preconditioning to increase Akt and/or GSK-3b phosphorylation. GSK-3b phosphorylation induced by IPC and PPC was abolished in sFRP1-Tg hearts.
with a P-GSK-3β/GSK-3β ratio of 0.62 ± 0.04 after IPC and 0.62 ± 0.04 after PPC, similar to that observed in NP hearts (0.58 ± 0.03). In contrast, the ratio of p-Akt/Akt was increased in IPC/sFRP1-Tg hearts by 107.70 ± 0.03% and in PPC/sFRP1-Tg hearts by 75.00 ± 0.14% compared with NP hearts that overexpressed sFRP1. The ratios in IPC/sFRP1-Tg (1.08 ± 0.18) and in PPC/sFRP1-Tg (0.91 ± 0.07) hearts were not significantly different than ratios observed in littermate IPC (1.18 ± 0.10) and littermate PPC (0.93 ± 0.11) hearts (Figure 4B).

In sFRP1-Tg hearts, mTOR phosphorylation was abolished after IPC and PPC and was similar to levels of phosphorylation in NP hearts. Similar levels of mTOR phosphorylation were observed in GSK3-KI hearts, indicating that GSK-3β acts upstream of the mTOR pathway in a Wnt-dependent manner to control cardioprotection against ischaemia (Figure 3B). Both p70S6K and 4E-BP1 phosphorylation were abolished in sFRP1 overexpressing hearts and in GSK3-KI hearts after preconditioning and levels were similar to those measured in NP hearts (Figure 3C and Supplementary material online, Figure S4).

3.4 GSK3 inhibition and mTOR activation depends on mitoKATP opening and ROS production

In order to examine the involvement of mitoKATP in the preconditioning pathway, we studied whether inhibition by 5-HD altered IPC- and PPC-induced Akt/GSK-3β phosphorylation or cardioprotection. Interestingly, perfusion of hearts with 5-HD abolished both preconditioning-induced cardioprotection (Figure 5A) and phosphorylation (Figure 5B and Supplementary material online, Figures S1 and S5). In addition, perfusion with 5-HD decreased mTOR phosphorylation by a mean of 43% in IPC and PPC hearts, when compared with control conditions (see Supplementary material online, Figure S6). 5-HD alone did not alter IS or levels of phosphorylation of Akt or GSK3β compared with the NP group.

mitoKATP opening leads to the production of small amounts of ROS.2,26,27 Therefore, we examined whether ROS, produced either after IPC or after diazoxide treatment, were involved in preconditioning effects. We observed that perfusion with MPG, an ROS scavenger, completely prevented heart protection that normally resulted from IPC or PPC (IS/AAR = 32.3 ± 3.5 and 42.7 ± 3.6% in MPG/IPC and MPG/PPC, respectively, vs. 34.3 ± 1.5% in NP hearts; Figure 5A and Supplementary material online, Figure S1) and inhibited the phosphorylation of GSK3-β (Figure 5B) as well as of Akt (see Supplementary material online, Figure S5). MPG given alone to NP hearts did not affect IS or Akt or GSK3-β phosphorylation. We then examined the effect of MPG on mTOR activation induced by PPC to ensure that the observed effects of diazoxide involved mitoKATP, mTOR phosphorylation induced by PPC was decreased by a mean of 63% if MPG was co-perfused with diazoxide (see Supplementary material online, Figure S6).
4. Discussion

In this study, we reported that ischaemic preconditioning requires GSK-3β and activation of both Wnt and mTOR pathways in an isolated-perfused mouse heart model. Our previous study in an in vivo model of heart ischaemia demonstrated that Wnt/Frz pathway was involved in IPC through activation of GSK-3β. The mechanism by which the Wnt/Frz pathway is induced during preconditioning is unknown. Since numerous Wnts and Frizzleds are expressed in the heart,28 one can speculate that brief episodes of ischaemia-reperfusion could induce Wnt release, which in turn would activate their receptors and the signalling cascade.29 The disruption of the Wnt/Frz pathway by sFRP1 induces activation of GSK-3β and abolishes the benefit of IPC.31 In addition, numerous cardioprotective drugs have been shown to converge towards GSK-3β; for example, Juhaszova et al.30 reported that diazoxide induces the phosphorylation/inhibition of GSK-3β in cardiomyocytes in culture. We demonstrated that pharmacologically activating mitoK<sub>ATP</sub> opening induced an IPC-like signalling pathway as well as protection. Indeed, as does IPC, PPC led to an increase in GSK-3β phosphorylation, dependent on PI3K/Akt activation at the end of the preconditioning phase; this was reversed by 5-HD, a mitoK<sub>ATP</sub> blocker. Although GSK-3β is thought to act at the onset of the reperfusion phase,31 our data, in concordance with those of Tong et al.,8 extend this observation to the preconditioning phase and suggest that GSK-3β inhibition is initiated at this time. Whether GSK-3β inhibition during preconditioning remains constant through the ischaemia-reperfusion protocol or follows a biphasic pattern remains to be elucidated.

The importance of GSK-3β in preconditioning is supported by several studies,7,8 but recent evidence suggests that GSK-3β is unlikely to be the key determinant in pre- and post-conditioning in mice.25 Importantly, we found that the cardioprotection offered by IPC or

Figure 3 mTOR is activated during preconditioning via GSK-3β in a Wnt-dependant manner. (A) Infarct size, expressed as a percentage of the total AAR, measured after non-preconditioned ischaemia/reperfusion (ischaemia), IPC or PPC hearts with or without 250 pM rapamycin co-perfusion. Data are expressed as mean ± SEM (n > 6). *P < 0.05 vs. non-preconditioned ischaemia/reperfusion, †P < 0.05 vs. without rapamycin. (B) Representative immunoblots and quantification of pSer2448-mTOR, total mTOR, and total Cox IV in littermate, sFRP1-Tg and GSK3αβ KI hearts subjected to control aerobic perfusion (NP), IPC or PPC. Quantitative analyses of repeat experiments are expressed as the fold induction phospho/total protein ratio compared to NP. (C) Quantitative analyses of repeat experiments are expressed as the fold induction of pThr389-p70S6K/total-p70S6K protein ratio and pSer65-4E-BP1/total4E-BP1 protein ratio from littermate, sFRP1-Tg and GSK3αβ KI hearts subjected to IPC or PPC compared with NP. n > 6, *P < 0.05 vs. NP, †P < 0.05 vs. littermate corresponding group.
PPC was abolished in hearts expressing inactivation-resistant forms of GSK3. In addition, in sFRP1-Tg mice, in which GSK-3b inhibition was impaired, we observed an inhibition of IPC and PPC-induced cardioprotection independent of PI3K/Akt. Our data suggest involvement of the Wnt/Frz pathway in preconditioning phase and in cardioprotection. Even if isolated-perfused heart model is not fully representative of events occurring in vivo, these data are in good agreement with our previous studies using a model of infarction in vivo and the isolated heart provides a simpler alternative to the in vivo model. Furthermore, despite the absence of neurohumoral control or blood factors, the similarity of data obtained in this study with those obtained with an in vivo model of infarction in vivo and the isolated heart provides a simpler alternative to the in vivo model. Furthermore, the similarity of data obtained in this study with those obtained in an in vivo model of myocardial infarction suggest that the interaction between GSK-3β and the Wnt pathway was preserved in our ex vivo model. This might be due in part to the property of Wnts, whose release is tightly regulated, to accumulate near the producing cells. Indeed Wnts interact with the extracellular matrix and bind tightly to the cell surface. In this context, analysis of isolated-perfused hearts furthered our understanding of the early events of preconditioning signalling. Here, we note that in mice, PPC by diazoxide, acting on mitoKATP downstream of GSK-3β, is not able to overcome the inhibitory effect of sFRP1 or GSK3 KI on cardioprotection and that mitoKATP is not implicated as an end-effector of cardioprotection. Thus, we propose the existence of a feedback loop in which mitoKATP maintains the inhibition of GSK-3β through the activation of Akt, an upstream target in preconditioning. sFRP1 might modulate this loop through a PI3K/Akt-independent interaction (Figure 6).

We hypothesized that mTOR is a target of the GSK-3β and Wnt pathway. Wnt is known to activate the mTOR pathway in tumour models, and this possibly requires the inhibition of GSK-3β. In addition, mTOR was suspected to play a role in preconditioning. There are conflicting results regarding the role of mTOR in cardioprotection: some studies show a protective effect of rapamycin. Experiments using low doses of rapamycin ranging from pM to nM, as used here, imply a cardioprotective effect of mTOR pathway.

Interestingly, we showed that IPC, as well as PPC, induced phosphorylation of mTOR and of its downstream targets, p70S6K and 4E-BP1. All these effects were blocked in GSK3 KI mice and in sFRP1-Tg mice. In addition, we showed that rapamycin, a specific inhibitor of mTOR, abrogated the cardioprotection mediated by

**Figure 4** Ischaemic preconditioning and pharmacological preconditioning by diazoxide interfere with the Wnt/Frz pathway. (A) Photographs of sFRP1-Tg hearts and infarct size expressed as a percentage of the total AAR measured after non-preconditioned ischaemia/reperfusion (ischaemia), IPC or PPC of littermate or sFRP1-Tg hearts. One graduation indicates 1 mm. Data are expressed as mean ± SEM (n = 6). *P < 0.05 vs. littermate. (B) Representative immunoblots and quantification of pSer473-Akt, total Akt, pSer9-GSK-3β, total GSK-3β, and total Cox IV in littermate and sFRP1-Tg hearts subjected to IPC or PPC. Quantitative analyses of repeat experiments are expressed as the ratio of phospho-Akt or phospho-GSK-3β over total Akt or total GSK-3β, respectively. n > 8. *P < 0.05 vs. littermate corresponding group.
preconditioning. Our data demonstrate the existence of a cross-talk between the Wnt pathway and the mTOR pathway during preconditioning. We suggest that mTOR integrates signals from the Wnt pathway to control the development of the cardioprotection.

Some studies have demonstrated that IPC alters protein abundance. Matsuyama et al. suggest that protein synthesis during IPC results in the development of an adaptive tolerance to ischaemia/reperfusion in an isolated heart model. Wong et al. proposed that changes in protein expression are likely to be due to degradation or post-translational modifications, such as phosphorylation. mTOR regulates the phosphorylation and function of several proteins involved in mRNA translation. Phosphorylation of p70S6K, which was activated during IPC, leads to S6 protein phosphorylation that is thought to promote the translation of mRNAs containing a 5′ terminal oligopyrimidine tract (5′-TOP) that encode ribosomal proteins and other translation factors. S6 protein activation, by up-regulating the translation of these mRNAs, may lead to increased cellular capacity for protein synthesis. Other studies have suggested that a rapamycin-sensitive target other than S6, like eIF4B, must regulate 5′-TOP mRNA translation. mTOR stimulates translation by directly phosphorylating the initiation factor 4E-BP1. This decreases the affinity of 4E-BP1 for the cap-binding translational factor eIF4E and leads to its dissociation, allowing eIF4E to form complexes with eIF4G and others partners required for cap-dependent translation. Whatever the mediator, the ultimate effect of mTOR induction is the activation of the translational machinery. A recent study based on mTOR knock-down experiments provided evidence for the involvement of mTOR as a positive regulator of TOP mRNA translation.

Our data indicate that protein synthesis might be initiated during the preconditioning phase and we suggest that protein synthesis itself could contribute to cardioprotection after 2 h of reperfusion in agreement with data from Matsuyama et al. Finally, another mechanism for mTOR induced-cardioprotection could involve its role as negative regulator of apoptosis. Activated mTOR negatively regulates apoptosis through S6K-dependent phosphorylation of BH3-only domain protein and was shown to contribute to the anti-apoptotic effect of insulin. Our model suggests that mTOR pathway activation depends on the Wnt pathway and results from a signalling loop (Figure 6) involving PI3K/Akt, GSK-3β, and mitoK_ATP. The opening of mitoK_ATP may result in a feedback control of these kinases leading to an amplification of signalling response. To get an insight into this mechanism, we examined the role of ROS in preconditioning. Many studies have shown that mitoK_ATP opening results in ROS production, which activates PKCe and may amplify cardioprotective signalling. In our previous study, we reported that preconditioning-induced PKCe activation is inhibited by sFRP1, suggesting it might play a role in the preconditioning loop we propose. The present study suggests that preconditioning-induced phosphorylation of GSK-3β might result from ROS generation related to mitoK_ATP opening. Indeed, MPG was able to abolish both phosphorylation of Akt and GSK-3β and cardioprotection induced by preconditioning. In addition, MPG-inhibited mTOR activation mediated by mitoK_ATP opening. Qin et al. previously reported that hydrogen peroxide was able to induce PI3K and Akt activation, which was achieved through PI3K membrane recruitment to its substrate site thereby increasing its catalytic efficiency. We propose that ROS production acts as a second messenger to induce the activation of Akt and thereby contributes to the
inhibition of GSK-3β and mTOR pathway activation. Since inhibition of PI3K/Akt by WM blocks GSK-3β phosphorylation after preconditioning by diazoxide, it appears that ROS produced following mitoKATP opening are not able to directly modulate GSK-3β.

In summary, we showed for the first time that preconditioning involves Wnt and mTOR pathways linked through GSK-3β. Signalling through these pathways appears to amplify the cardioprotective signalling initiated before the onset of ischaemia.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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