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p53 down-regulation: a new molecular mechanism involved in ischaemic preconditioning

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Abstract Ischaemic preconditioning is associated with the activation of prosurvival mechanisms. Here we demonstrate that following a preconditioning protocol, the proapoptotic p53 is inactivated possibly via phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt)-murine double minute 2 (Mdm2) phosphorylation. Our data show that in preconditioned hearts Mdm2 was significantly phosphorylated, and wortmannin (a PI3K inhibitor) abrogated this effect (Western blotting). Also in preconditioned hearts p53 was shown to be bound to phospho-Mdm2 (co-immunoprecipitation). Furthermore, pifithrin α (a p53 inhibitor), administered to isolated perfused hearts prior to ischaemia, significantly attenuated the infarction. In conclusion our results suggest that p53 is implicated in ischaemia/reperfusion injury and that preconditioning counterbalances this effect via PI3K-Akt-Mdm2 phosphorylation.

Key words: Phosphatidylinositol 3-kinase-Akt pathway; Mdm2 phosphorylation; p53 downregulation; Myocardial ischemia; Ischemic preconditioning

1. Introduction

Ischaemic preconditioning is considered the most powerful form of myocardial protection known to date [1]. Preconditioning consists of short periods of sublethal ischaemia with intermittent reperfusion prior to a sustained lethal ischaemic insult. Although it has been shown that the ischaemic preconditioning is manifested as a reduction in both necrotic and apoptotic cell death [2], the molecular mechanisms responsible for this protection are still under extensive research. Recent data indicate a significant role for the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) signalling pathway in this protection. It has been demonstrated that Akt can phosphorylate a multitude of substrates, which are implicated in cell survival. Amongst them there is murine double minute 2 (Mdm2) [3], an oncogenic factor that, when activated, promotes p53 degradation. To achieve this, phospho-Mdm2 forms complexes with p53, masking its transactivation domain, promoting its nuclear export and targeting it for ubiquitination [4,5].

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Abbreviations: PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; Mdm2, murine double minute 2

p53 is a tumour suppressor protein and transcription factor present in a latent state in all cells and activated by various stresses such as hypoxia, free radicals, DNA damage and UV light [6]. It is well documented that, when activated, it induces the transcription of death proteins such as bax, Peg3, Apaf1, p53AIP1 and Fas [7,8]. However, new evidence suggests that p53 may also have immediate proapoptotic effects, independent of gene transcription, through the activation of mitochondrial apoptotic pathways [9,10].

Ischaemia/reperfusion injury has been shown to be associated with p53 activation in different organs and tissues, including the myocardium [11], and lower levels of p53 following ischaemia/reperfusion injury in preconditioned hearts were reported [12]. Furthermore, studies performed in mouse brain, both in vitro and in vivo, have shown that p53 inhibition prior to ischaemia is correlated with a reduction in neuronal infarction [13] and ischaemic preconditioning of rat brain has been associated with a reduction in p53 activation [14].

However, there are no data in the literature about the degree of implication of p53 either in the infarction induced by ischaemia/reperfusion injury or in the mechanism which may be responsible for p53 reduction following preconditioning.

With the above in mind, we hypothesised that one of the possible mechanisms by which ischaemic preconditioning reduces cell death may be due to the downregulation of p53 via Mdm2 phosphorylation, possible via PI3K-Akt activation.

For measuring Mdm2 phosphorylation we used the antibody against Ser166, as this has been shown in the literature to be the major phosphorylation site of Akt on Mdm2 [15,16]. Our results show that Mdm2 is significantly phosphorylated in preconditioned hearts. Moreover, phosphorylated Mdm2 is bound to p53 in these hearts, a phenomenon that can be correlated with significant resistance against a further lethal ischaemic insult. This assessment is further supported by evidence that in preconditioned hearts the level of p53 during reperfusion, after a lethal ischaemic insult, is significantly lower. Additional evidence for a role for p53 in ischaemia/reperfusion injury is demonstrated by the reduction in infarction observed when pifithrin α, a p53 inhibitor [17], was administered prior to the ischaemic insult or at reperfusion.

2. Materials and methods

2.1. Materials

Sprague–Dawley male rats (350–400 g) were used in all experiments. Animal care was done in accordance with The Guidance on the Operation of the Animals (Scientific Procedure) Act 1986.

Antibodies against rat phospho-Mdm2 (Ser166) and total p53 were purchased from Cell Signalling, antibodies against rat total Mdm2

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from Upstate Technology, G-agarose beads from Roche Diagnostics, pifithrin α from Calbiochem and wortmannin (PI3K inhibitor) from Tocris.

2.2. Western blotting and immunoprecipitation

Isolated rat hearts were retrogradely perfused via the aorta (Langendorff preparation) in a constant pressure system. They were randomly divided into the following groups: group 1: perfused for 45 min (sham, $n=5$); group 2: sham hearts perfused for 45 min in the presence of 100 nM wortmannin ($n=3$); group 3: hearts preconditioned with two episodes of 5 min global normothermic ischaemia each followed by 10 min reperfusion ($n=5$); group 4: hearts preconditioned in the presence of 100 nM wortmannin ($n=3$); group 5: control hearts undergoing 35 min lethal regional ischaemia by obstruction of the left coronary artery, followed by 15 min reperfusion induced by reopening of the artery ($n=3$); group 6: preconditioned hearts (as above, with two episodes of 5 min global normothermic ischaemia each followed by 10 min reperfusion) which underwent 35 min regional ischaemia and 15 min reperfusion ($n=5$). At the end of the experiments hearts were rapidly frozen in liquid nitrogen. Experimental protocols are represented in Fig. 1A (for groups 1–4) and Fig. 3A (for groups 5 and 6). We used global ischaemia for inducing the preconditioning protection and regional ischaemia for inducing the lethal injury to avoid repeated mechanical obstructions of the coronary artery, which could induce permanent damage of the artery and therefore interfere with the infarction data. Wortmannin was used in a dose previously reported to block Akt phosphorylation [18,19] in the preconditioned hearts. The dose of pifithrin α was chosen in accordance with literature and some preliminary data we obtained using a range of concentrations (data not included) to ascertain heart function and protection.

Western blotting analysis was performed to determine the level of p53, phosphorylated Mdm2 and total Mdm2. Immunoprecipitation was performed in order to investigate the presence of the p53–Mdm2 complexes.

Frozen ventricular tissue (50 mg) was used for protein extraction. We were interested to estimate the total content of phospho-Mdm2, p53 and their association without investigating the time course of their association and migration between cytosol and nuclear fraction; therefore we used total tissue homogenate. Tissue was homogenised on ice using a IKA Labortechnik T25 basic homogeniser in either (a) 250 μl suspension buffer: (in mM) NaCl 100, Tris 10 (pH 7.6), EDTA 1 (pH 8), sodium pyrophosphate 2, sodium fluoride 2, β-glycerophosphate 2; Sigma protease inhibitor cocktail (Western blotting) or (b) 250 μl Ripa buffer containing the protease inhibitor (immunoprecipitation). Samples were centrifuged at 10 000 rpm, 10 min at 4°C on a bench centrifuge, the supernatant was collected and protein concentrations were estimated using BCA protein assay reagent (Pierce).

For Western blotting the supernatant was further diluted in 1:1 in sample buffer: (in mM) Tris 100 (pH 6.8), dithiothreitol 200, sodium dodecyl sulfate (SDS) 2%, bromophenol blue 0.2% and glycerol 20%, and subsequently boiled for 10 min at 100°C. A total protein quantity of 30 μg for each sample was separated on a 12.5% SDS–polyacrylamide gel electrophoresis gel and transferred to Hybond ECL nitrocellulose membranes (Amersham). Equal protein loading was confirmed by Ponceau red staining (Sigma) of membranes. Blots were probed with p53, Mdm2 or phospho-Mdm2 primary rabbit polyclonal antibodies and subsequently probed with horseradish peroxidase-conjugated anti-rabbit antibody according to the supplier's protocols. Proteins were detected using enhanced chemiluminescence ECL Western blotting detection reagent and bands were visualised by autoradiography.

The samples for the immunoprecipitation assay (1000 μg protein each) were incubated with 5 μl p53 antibody, on ice with shaking for 60 min, then 20 μl G-agarose spheres were added and the samples were further incubated on ice with shaking for 4 h. At the end of this period the samples were centrifuged at 7000 rpm, 4°C, 10 min, the supernatant discharged, and the pellet resuspended in Ripa buffer and recentrifuged. This last step was repeated four times. After the final centrifugation 30 μl of suspension buffer was added to the pellet and samples were boiled for 10 min at 100°C.

To rule out the possibility that the agarose beads may give unreliable results by binding to unspecific phosphorylated proteins, parallel protein extracts were incubated with agarose spheres in the absence of p53 antibody.

All the protocols were repeated in triplicate starting with the tissue homogenisation step.

2.3. Infarct data

2.3.1. Heart preparation. The animals were anaesthetised via an i.p. administration of sodium pentobarbital (55 mg/kg) and heparin (300 U). The hearts were excised, placed in ice-cold buffer and within 1 min mounted in a constant pressure Langendorff system (70 mm Hg). They were retrogradely perfused with a modified Krebs–Henseleit bicarbonate buffer containing (in mM): NaCl 118.5, $NaHCO_3$ 25, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.7 and glucose 12. All solutions were gassed with 95% O_2/5% CO_2 (pH 7.4) and maintained at 37°C. The temperature was permanently monitored by a thermocouple inserted in the right ventricle. An isovolumic latex balloon was introduced in the left ventricle through an insertion performed in the left atrial appendage and inflated to a diastolic pressure of 5–10 mm Hg. Functional monitoring was performed via a pressure transducer connected to an AD Instruments computerised system and recorded continuously. To induce ischaemia a surgical needle was inserted under the left main coronary artery and the ends of the thread were passed through a small plastic tube to form a snare. Tightening the snare induced regional ischaemia and releasing the ends of the thread allowed reperfusion to commence. At the end of reperfusion, the snare was tightened to re-occlude the coronary artery branch. A saline solution of 0.12% Evans blue was infused slowly via the aorta to delineate the non-ischaemic zone of the myocardium, which stained dark blue. The hearts were frozen, sliced into 1 mm thick transverse sections and incubated in triphenyl-tetrazolium chloride solution (1% in phosphate buffer, pH 7.4) at 37°C for 10–12 min. They were then fixed in 10% formalin, for at least 4 h. Viable tissue stains red and infarcted tissue appears pale. The risk zone and infarct areas were traced onto acetate sheets. Using computerised planimetry (Summa Sketch II, Summagraphics) the percentage of infarcted tissue within the volume of the myocardium at risk was calculated (I/R%).

2.3.2. Groups. The hearts were randomly divided into four groups: (a) controls which suffer 35 min regional ischaemia and 120 min reperfusion ($n=6$), (b) preconditioned ($n=6$) with two times 5 min global ischaemia interspaced with 10 min reperfusion prior to lethal ischaemia, (c) control hearts treated with the p53 inhibitor pifithrin α at 10 μM, for 15 min before ischaemia ($n=8$) and (d) control hearts treated with the p53 inhibitor starting 5 min prior to the end of ischaemia and continued for 10 min into reperfusion ($n=8$). The protocol is described in Fig. 4A.

2.4. Statistical analysis

Data were expressed as means ± S.E.M. and analysed using one-way ANOVA and Fisher's protected least significant difference test for multiple comparisons. Values were considered significantly different when $P<0.05$.

3. Results

3.1. p53 and Mdm2 levels in sham and preconditioned hearts

Western blot analysis and densitometric evaluation showed that there were no significant differences between the levels of p53 in either sham or preconditioned hearts (Fig. 1B). However, there was a significant increase in phosphorylated Mdm2 in the preconditioned hearts (1308 ± 140 in preconditioned vs 475 ± 70 in sham, $P<0.006$, relative densitometry). In the presence of wortmannin, the PI3K inhibitor, this increase in phosphorylated Mdm2 was abrogated (1308 ± 140 in preconditioned vs 400 ± 60 in preconditioned+wortmannin, $P<0.006$) (Fig. 1C). There were no significant differences in total Mdm2 between these groups.

3.2. p53 bound to phospho-Mdm2 in sham and preconditioned hearts

The immunoprecipitation assay data (Fig. 2A) revealed that a significant proportion of the p53 in the preconditioned hearts was already inactivated and targeted for ubiquitination,

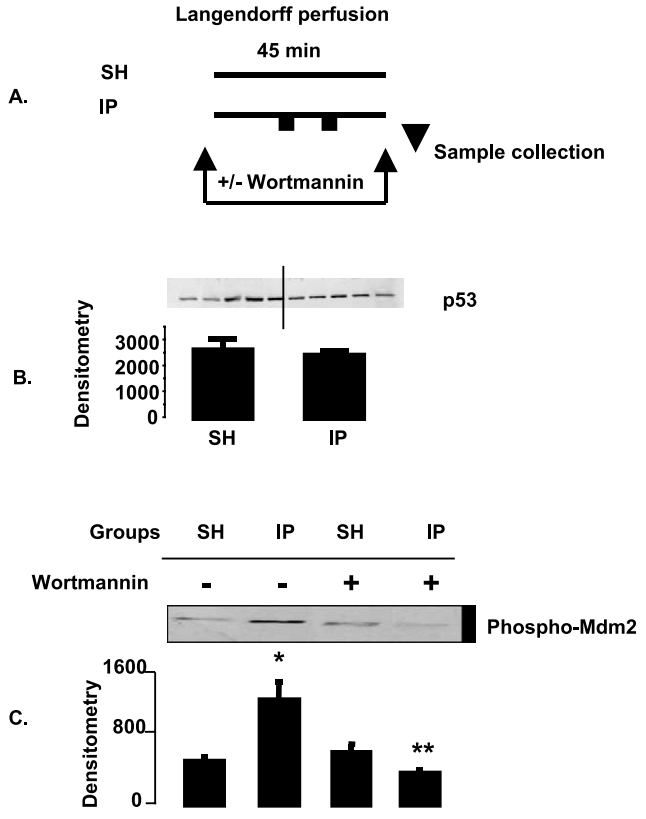

Fig. 1. Western blotting analysis of p53 and phospho-Mdm2 (P-Mdm2) in sham and preconditioned hearts. A: The experimental protocol. B: p53 level in sham (SH) and preconditioned (IP) hearts. C: P-Mdm2 in SH and IP hearts, with or without wortmannin. Small dark squares = preconditioning ischaemia (5 min); $*P < 0.006$ vs SH, $**P < 0.006$ vs IP.

being conjugated with the phosphorylated Mdm2 (73 ± 9 in sham vs 325 ± 100 in preconditioned hearts $P < 0.003$). These data may explain the slight difference, although not significant, in the p53 levels between groups, noticeable from Fig. 1B. This difference may be an indication that the degradation of p53 has already started in preconditioned hearts. No unspecific binding of phosphorylated proteins on the agarose beads in the absence of p53 was observed (Fig. 2B).

3.3. p53 level in control and preconditioned hearts which underwent ischaemia/reperfusion injury

Interestingly the level of total p53 at 15 min reperfusion after an ischaemic insult is significantly lower in preconditioned hearts which may indicate its degradation after binding with phospho-Mdm2 (relative densitometry was 2161 ± 450 in control vs 1166 ± 269 in pifithrin α group, $P < 0.05$) (Fig. 3B).

3.4. Infarction data

To assess the role played by p53 in ischaemia/reperfusion injury we investigated whether the reduction in its active level prior to ischaemia or at reperfusion would be beneficial to the heart. For this purpose we used a p53 inhibitor (pifithrin α, 10 μM), which was administered for 15 min prior to ischaemia or 5 min at the end of ischaemia and 10 min into reperfusion. The results are expressed as percentage of infarct developed in the risk zone (*I*/*R*%). We noticed a significant decrease of infarction in the pre-treated hearts in comparison with the controls (*I*/*R*% of 50.2 ± 5 in controls vs 28.2 ± 3 in pifithrin α pre-treated hearts, $P < 0.01$) (Fig. 4B). Interestingly, administration of pifithrin α at reperfusion was also found to be associated with a degree of protection (*I*/*R*% = 37.5 ± 2, $P < 0.05$). The protection induced by p53 inhibition is much smaller than the reduction in infarction observed in the preconditioned hearts (*I*/*R*% = 18.4 ± .2) which is not surprising

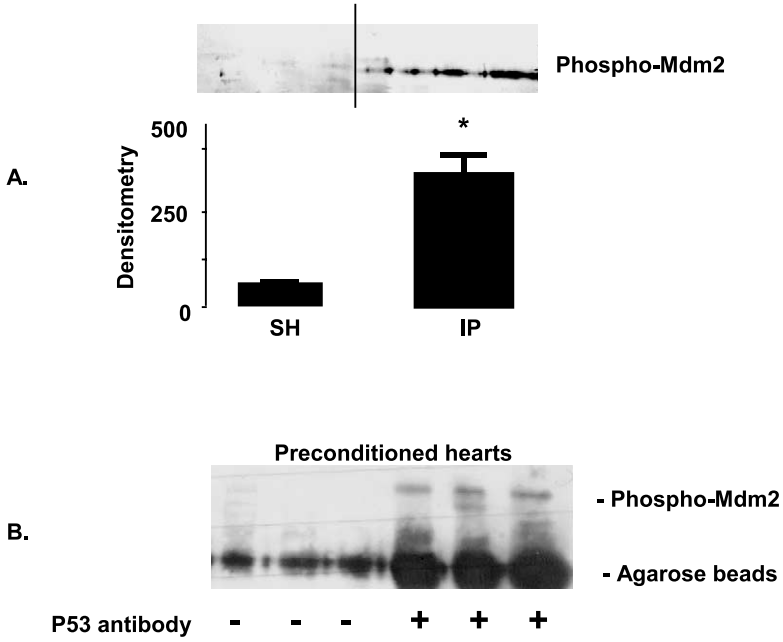

Fig. 2. Immunoprecipitation results concerning p53-phospho-Mdm2 (P-Mdm2) binding following the preconditioning protocol. A: P-Mdm2 bound to p53 in sham (SH) and preconditioned (IP) hearts; $*P < 0.01$. B: IP hearts were incubated with agarose beads with or without p53 antibody to check the unspecific ligation of phosphorylated proteins, then Western blotting was performed to measure P-Mdm2. It can be seen that there is no band for P-Mdm2 in the hearts, which were not treated with p53.

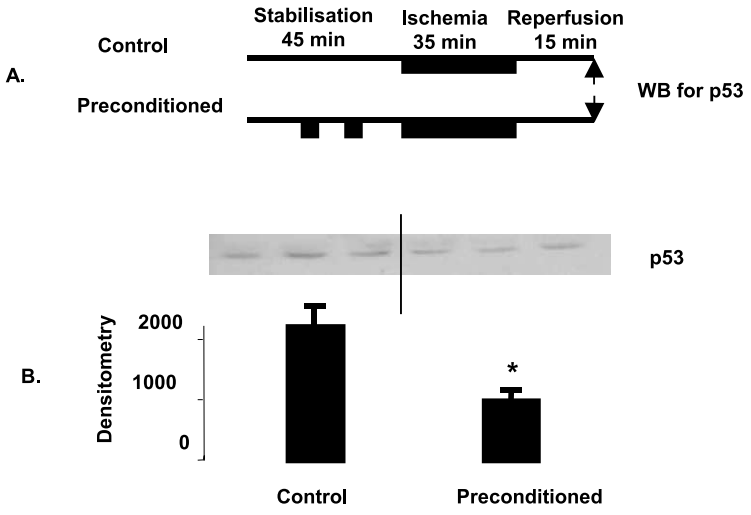

Fig. 3. Experimental protocol and Western blotting results measuring p53 levels in control and preconditioned hearts, which underwent 35 min lethal ischaemia and 15 min reperfusion. A: The experimental protocol. B: p53 levels illustrated as Western blotting images (above) and densitometric analysis (below); small dark squares = preconditioning ischaemia (5 min); black rectangle = lethal ischaemia (*$P < 0.05$).

as preconditioning is a multifactorial mechanism activating numerous survival pathways.

4. Discussions

The mechanisms implicated in ischaemic preconditioning protection are complex and not fully elucidated. It has recently been demonstrated that the PI3K-Akt pathway, which is considered the main survival pathway of the cell, plays a crucial role in ischaemic preconditioning. Inactivating this pathway using either wortmannin or LY294002 has been shown to abolish ischaemic preconditioning [18–20]. However, as yet there are not enough data regarding the possible substrates immediately downstream of Akt, which may be responsible for this protection. From the multitude of possible targets which can be phosphorylated by Akt and also linked to preconditioning, only the protective role achieved by Bad and GSK [19] phosphorylation upon the ischaemia/reperfusion injury has been demonstrated.

In this study we investigated the hypothesis that one of the mechanisms implicated in ischaemic preconditioning protection is the downregulation of p53 via activation of the PI3K-Akt prosurvival pathway.

The implication of p53 in cell death is well documented. In normal cells this tumour suppressor is present in an active form and is maintained at low level. However, after an insult which induces DNA damage, p53 is rapidly activated by phosphorylation at different sites, which makes it stable and prevents its association with Mdm2. In this state p53 induces cell death either directly by activating mitochondrial proapoptotic pathways [9,10] and/or by acting as a transcription factor for proapoptotic genes [7,8].

Mdm2 is the most important ubiquitin ligase for p53. Its role is to maintain p53 at low levels by binding to it – which means that the transcription factor cannot be activated in this state – and targeting it for proteosomal degradation. It has recently been demonstrated that Akt phosphorylates Mdm2 at Ser166 [15,16] and Ser186 [5] and it is believed that this phosphorylation regulates the affinity of Mdm2 for p53. Therefore it has been suggested that Mdm2 represents a link between two opposite processes, namely survival via the PI3K-Akt pathway and cell death via p53 [4]. The phosphorylation of Mdm2 is followed by its translocation to the nucleus [21]. In the nucleus phospho-Mdm2 forms complexes with p53, blocking its capacity to be activated. The complexes shuttle back to the cytoplasm where p53 is then targeted for degradation [22].

There is a large body of evidence implicating p53 in cell death associated with myocardial ischaemia/reperfusion injury. In a swine model it has been shown that following an ischaemia/reperfusion injury p53 level is increased even 72 h later, which demonstrates a continuous activation of death mechanisms [11]. On the contrary inhibiting p53 with a specific inhibitor (pifithrin α) proved to be protective against ischaemia/reperfusion-induced cell death not only in brain

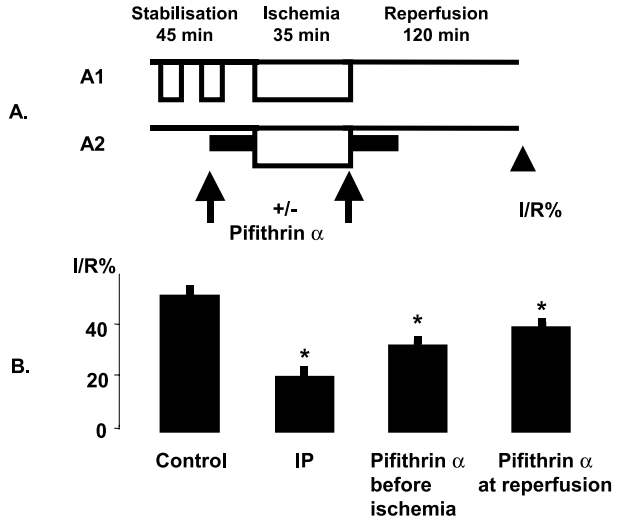

Fig. 4. Experimental protocol and infarct data demonstrating the influence of p53 inhibition upon infarction when administered prior to ischaemia or at reperfusion. Comparison with the protection afforded by ischaemic preconditioning. A: Experimental protocol. A1: Preconditioning protocol. A2: Control protocol without/with the addition of pifithrin α either prior to ischaemia or at reperfusion. Clear delineated rectangles represent ischaemic periods, black rectangles represent the timing and length of pifithrin α administration. B: Infarction developed in the risk zone in control and preconditioned hearts and in hearts treated with 10 μM pifithrin α before ischaemia or at reperfusion (*$P < 0.01$ vs control).

[13] but also in heart, as we demonstrated for the first time in the present study.

This protection is present when the inhibitor is administered either before (as a preconditioning mimetic) or at reperfusion (independent of preconditioning). The results are not surprising as p53 is activated by stresses related either to ischaemia (hypoxia) or to reperfusion injury (free radical release, DNA damage). We think that the phosphorylation of Mdm2 due to preconditioning followed by its binding to p53 has the initial effect of blocking the capacity of p53 to be activated when the above stresses take place, without changing the actual cellular level of the transcription factor. Subsequently ubiquitination will determine a decrease in the p53 level at reperfusion as others [12] and we have reported. This protection is mimicked by pifithrin α administered prior to ischaemia and at reperfusion. The achieved protection is manifested against acute, immediate, non-transcriptional proapoptotic effects of activated p53 [9,10] but more research in the field is needed to provide a clear answer.

It is also necessary to underline that preconditioning is a complex phenomenon in which protection is achieved by a number of mechanisms, amongst which there is the process of the reduction in p53 availability. Therefore pifithrin α may well induce a less marked protection compared to ischaemic preconditioning when administered prior to ischaemia. In addition it must never be forgotten that reperfusion, although a prerequisite to tissue salvage, is also associated with a paradoxical injury which is presumably small but biologically significant. Therefore any agent which will reduce infarction when given at reperfusion will never reach the level of protection achieved by preconditioning.

Our data indicate that ischaemic preconditioning plays a role in activating Mdm2. Indeed there is a significant increase in phospho-Mdm2 (Ser166) in hearts undergoing the preconditioning protocol in comparison with sham hearts. Furthermore we showed that formation of Mdm2–p53 complexes occurs prior to the sustained ischaemia. In this state p53 cannot be activated and it is tagged for ubiquitination. This effect will offer a greater chance of survival to the myocardium undergoing an ischaemia/reperfusion insult. Interestingly, after 15 min of reperfusion following a lethal ischaemic injury, the level of p53 is significantly smaller in preconditioned hearts compared to control ones. At present we do not know if this difference is due to the initial Mdm2 phosphorylation or is a consequence of the reactivation of PI3K-Akt at reperfusion followed by a subsequent phosphorylation of Mdm2.

In conclusion, we have demonstrated for the first time that ischaemic preconditioning is associated with a reduction in the availability of p53 to be activated, an effect that is due to its binding to phospho-Mdm2. This effect is a consequence of an increase in Mdm2 phosphorylation in preconditioned hearts, probably via the PI3K-Akt pathway. This may represent one of the mechanisms through which ischaemic preconditioning protection is achieved.

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