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Overexpression of the Muscle Specific Protein, Melusin, Protects from Cardiac Ischemia/Reperfusion Injury

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Short title: Cardioprotection by melusin

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Abstract

Melusin is a muscle-specific protein which interacts with β1 integrin cytoplasmic domain and acts as chaperone protein. Its overexpression induces improved resistance to cardiac overload delaying left ventricle dilation and reducing the occurrence of heart failure. Here we investigated possible protective effect of melusin overexpression against acute ischemia/reperfusion (I/R) injury with or without Postconditioning cardioprotective maneuvers.

Melusin-transgenic (Mel-TG) mice hearts were subjected to 30 minutes global ischemia followed by 60 minutes reperfusion. Interestingly, infarct size (IS) was reduced in Mel-TG mice hearts compared to wildtype (WT) hearts (40.3±3.5% Mel-TG *vs.* 59.5±3.8% WT hearts; n= 11 animals/group; $P<0.05$). The melusin protective effect was also demonstrated by measuring LDH release, which was 50% lower in Mel-TG compared to WT. Mel-TG hearts had a higher baseline level of AKT, ERK1/2 and GSK3β phosphorylation, and displayed increased phospho-kinases level after I/R compared to WT mice. Post-ischemic Mel-TG hearts displayed also increased levels of the antiapoptotic factor phospho-BAD. Importantly pharmacological inhibition of PI3K/AKT (Wortmannin) and ERK1/2 (U0126) pathways abrogated the melusin protective effect. Notably, HSP90, a chaperone known to protect heart from I/R injury, showed high levels of expression in the heart of Mel-TG mice suggesting a possible collaboration of this molecule with AKT/ERK/GSK3β pathways in the melusin-induced protection. Postconditioning, known to activate AKT/ERK/GSK3β pathways, significantly reduced IS and LDH release in WT hearts, but had no additive protective effects in Mel-TG hearts. These findings implicate melusin as an enhancer of AKT and ERK pathways and as a novel player in cardioprotection from I/R injury.

Key words: melusin; I/R injury; chaperone; Hsp90; AKT; ERK

Introduction

In the heart, ischemia followed by reperfusion (ischemia/reperfusion, I/R) induces all form of cell death, including apoptosis [39, 63], which can be inhibited by pharmacological and genetic approaches resulting in a smaller infarct size [11, 30]. Limitation of myocardial injury after I/R depends on critical adaptive responses, some of which involve the heat-shock proteins (HSPs) which acts as molecular chaperones [37, 46]. In fact, HSP gene expression is induced by virtually any kind of adaptive stress conditioning which reduces the damage of subsequent ischemic insult. The prototype of this phenomenon is known as *preconditioning* (PreC), which provides evidence for adaptive responses to stress by enhancing the cell tolerance to subsequent ischemia [7, 42, 58]. PreC can be achieved in the heart with a variety of stress responses [2, 19, 28, 43, 45], including hypothermia [53] and hyperthermia, which of course induces HSPs [33, 34, 46]. In fact, in PreC two temporary distinct protective windows have been described: a first one, which provides protection against myocardial infarction within 2–3 h after preconditioning stimuli, and a second window of protection, occurring at 24–72 h after PreC stimuli, which is characterized by a unique gene expression profile, including HSPs induction [7, 47]. The introduction of *postconditioning* (PostC) as an intervention which can be applied at the time of myocardial reperfusion, has redirected the attention to the early phase of *reperfusion* as a target for cardioprotection [40, 48, 50, 66, 71]. Studying the signaling moieties implicated in PostC *versus* PreC revealed a common cardioprotective pathway, including, among others, protein kinases such as AKT, ERK1/2 and GSK3β. These kinases are recruited at time of myocardial reperfusion and are collectively called “reperfusion injury salvage kinase” (RISK)-pathway, which may also recruit anti-apoptotic mechanistic pathways, including the phosphorylation of Bcl-2-associated death promoter (BAD) [13, 24]. The similarity of pathways between pre and postconditioning is in line with the observation that the two protections are not additive [23].

A second important cardioprotective element, common to PreC and PostC, is represented by chaperone proteins, also known as HSPs [12, 18, 41, 63]. We have previously identified melusin as a new muscle-specific chaperone protein binding to the cytoplasmic domain of β1 integrin and acting as mechanical stretch sensor of the cytoskeleton [8]. Analysis of signaling pathways in transgenic mice indicated that melusin overexpression induced increased phosphorylation of AKT, GSK3β and ERK1/2 at basal level and after pressure overload [15]. Moreover, AKT and GSK3β were under-phosphorylated in response to pressure overload in melusin null mice [8].

Therefore, since melusin overexpression enhances the phosphorylation of some RISK pathway components in *non-ischemic* conditions and since melusin has been shown to act as a co-chaperone of HSP90 [57], we hypothesized that melusin overexpression can trigger adaptive responses enhancing the heart tolerance to I/R challenging. To verify this hypothesis, in the present study, we analyzed the myocardial injury in melusin overexpressing and littermate wildtype mice after I/R insult. Moreover, the implication of the AKT and ERK1/2 signaling pathways was analyzed. Finally, the possibility of an additive protective effect by postconditioning was studied in the Mel-TG model.

Methods

Animals

Male FVB non-transgenic (wildtype, WT) and FVB transgenic littermate mice overexpressing melusin (Mel-TG) [15] received humane care in compliance with Italian law (DL-116, Jan. 27, 1992) and in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All efforts were made to minimize suffering.

Perfusion technique

Male WT and transgenic littermate FVB mice (Mel-TG) [15] weighing between 25-35 g (10-15 weeks old) were given 500 U heparin and anesthetized with sodium pentothal (50 mg/kg) by intraperitoneal injections before being culled by cervical dislocation [55, 56]. Hearts were rapidly excised and perfused retrogradely at 80 mmHg by the Langendorff technique with Krebs–Henseleit bicarbonate buffer containing (mM) NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.25, and Glucose 11. The buffer was gassed with 95% O_2:5% CO_2. The temperature of the perfusion system was maintained at 37°C. The perfusate flowing out of the heart was collected and measured. Collected coronary effluent was used for measurement of *lactate dehydrogenase* (LDH) release (see below) and the coronary flow rate was determined by the amount of perfusate measured in a specific time period.

At the end of perfusion period, hearts were divided in two parts by a transverse section (perpendicular to the long axis); while the apical part (less than 1/3 of ventricular mass) was frozen rapidly in liquid nitrogen and stored at -80°C and subsequently used for Western blot analysis, the other part was used for infarct size assessment (see below).

Experimental protocol

I/R groups (Fig 1)

Group 1 (WT_I/R, n= 11): in order to have a reference group, hearts were harvested from the WT animals and allowed to stabilize for 30-min. After a 30-min stabilization period, hearts were subjected to a protocol of I/R, which consisted in 30-min of global no-flow, normothermic ischemia and a period of 60-min of reperfusion [4].

Group 2 (WT_PostC, n= 12): after the 30-min ischemia, the WT hearts immediately underwent a protocol of PostC (i.e., 5 cycles of 10-s reperfusion and ischemia) [49, 51].

Group 3 (Mel-TG_I/R, n= 11): hearts were harvested from the transgenic animals (Mel-TG) and, as above, were perfused for a 30-min stabilization period, then global normothermic ischemia was applied by eliminating flow for 30-min, which was followed by 60-min reperfusion.

Group 4 (Mel-TG_PostC, n= 12): after the 30-min ischemia, the Mel-TG hearts immediately underwent a protocol of PostC (i.e., 5 cycles of 10-s reperfusion and ischemia).

Group 5 (Mel-TG_I/R+WM; n= 5): these hearts were treated with 0.1 μM of the PI3K inhibitor *Wortmannin* (WM) 5-min before ischemia and during the first 20-min of 60-min reperfusion.

Group 6 (Mel-TG_I/R+U0126; n= 5): these hearts were treated with 50 μM of the MEK1/2 inhibitor *U0126* five min before ischemia and during the first 20-min of 60-min reperfusion.

Groups 7-8 Sham groups (WT_SHAM, n= 3 and Mel-TG_SHAM, n= 3) consisted of hearts which after stabilization underwent 90-min buffer-perfusion only.

The dose of inhibitors WM (0.1 μM, Sigma, St. Louis, MO) and U0126 (50 μM, LC Laboratories, Woburn, MA) and the concentration of vehicle (DMSO; Sigma, St. Louis, MO) in which the inhibitors were dissolved were based on previous studies of myocardial I/R injury [10, 36, 49, 51, 52, 70]. Nevertheless, the two inhibitors were tested in hearts of WT animals (n= 3 for each condition) and the maximal DMSO concentration (43μl/100ml) was tested in hearts of both strains (n= 3 for each condition).

Assessment of myocardial injury

Infarct size assessment

Infarct areas were assessed at the end of the experiments with the nitro-blu-tetrazolium (NBT) technique in a blinded fashion, as previously described [51]. In brief, immediately after reperfusion, hearts were removed from the perfusion apparatus and, after removing and freezing the apical part (see above), the basal part of the ventricles was dissected by transverse sections into two-three slices.

Following 20-min of incubation at 37°C in 0.1% solution NBT (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffer, unstained necrotic tissue was carefully separated from stained viable tissue by an independent observer, who was unaware of the protocols. Since the ischemia was global and since we analyzed only the basal part of the ventricles the necrotic mass was expressed as a percentage of the analyzed ischemic tissue.

Lactate dehydrogenase release

Besides infarct size, myocardial injury was also assessed by measuring LDH release. The perfusion effluent was collected for 5-min immediately before ischemia and for the entire reperfusion period. LDH released from the heart was determined by spectrophotometric analysis at 340 nm, using a classic procedure [6, 49].

Western blotting groups

Frozen samples of the four I/R groups (Groups 1, 3, 5 and 6) and samples from other two additional Sham (non-ischemic) groups (Groups 7-8) were used for Western blot analysis. For Western blotting, hearts were lysed in Tris-buffered saline with 1% Triton X-100, plus Roche complete protease inhibitor cocktail containing (mM) NaF 10, PMSF 1 and Na_3VO_4 1. Protein extracts were clarified with three sequential centrifugations for 20 minutes at 20,000 *g*, at 4°C [55, 56]. Western blot band quantifications were performed with Quantity One software (Bio-Rad).

Antibodies against the following proteins were used: AKT (#9271)(1:1000, Cell Signaling), Phospho-AKT (#9272)(1:1000, Cell Signaling), GSK3β (#610202)(1:1000, BD Transduction Laboratories), Phospho-GSK3β (#9336S)(1:1000, Cell Signaling), BAD (#9292)(1:1000, Cell Signaling), Phospho-BAD (#5284S)(1:1000, Cell Signaling), HSP90 (#610419)(1:1000, BD Transduction Laboratories), GAPDH

(MAB374)(1:1000, Millipore[C1]), Phospho-p70 S6 Kinase (#9206)(1:1000, Cell Signaling), p70 S6 Kinase α (#sc-230)(1:1000, Santa Cruz Biotechnology), ERK1/2 (#sc-93)(1:1000, Santa Cruz) and Phospho-ERK1/2 (#9101)(1:1000, Cell Signaling).

Statistical analysis

All values are expressed as mean ± SE. Data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's post test. For all analyses, a minimum value of $P< 0.05$ was considered significant. Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software version 4.0).

Results

Melusin overexpression protects against myocardial ischemia/reperfusion injury.

Infarct size, after 60-min of reperfusion increased as the duration of ischemia increased from 25- to 45-min in both heart of wild type (WT) and of melusin transgenic mice (Mel-TG). A period of 30-min of ischemia was determined to be optimal for the comparison of myocardial damage studies. While infarct size was 59.5±3.8% of risk area in WT hearts subjected to 30-min ischemia and 60-min reperfusion (WT_I/R), it was significantly lower, 40.3±3.5% in Mel-TG hearts subjected to the same protocol (Mel-TG_I/R) ($P< 0.05$ *vs*. WT_I/R group) (Fig. 2A).

Moreover, total LDH release after 30-min of global ischemia and 60-min of reperfusion was almost double in WT compared to Mel-TG hearts (431.6±51.1 mU/mg in the WT_I/R and 177.9±19.5 U/mg ww Mel_I/R groups ($P< 0.01$) (Fig. 2B).

We assessed whether the protective effect of melusin overexpression can be further modified by postconditioning maneuvers. As shown in Fig. 2, while the postconditioning maneuvers reduced

significantly the infarct size and LDH release in WT hearts (infarct size 37±4% of risk area, *P*< 0.01 *vs*. WT_I/R group; LDH release 172±24 mU/mg ww; *P*< 0.01 *vs*. WT_I/R group), these maneuvers had not additive effects on reducing the I/R injury observed in the Mel-TG hearts (infarct size 35±3% of risk area, *P*= not significant (NS) *vs*. Mel-TG_I/R; LDH release 175±41 mU/mg ww; *P*=NS *vs*. Mel-TG_I/R; *P*< 0.01 *vs.* WT_I/R).

Melusin Protection depends on activation of PI3K/AKT and ERK1/2 pathways

To asses possible mechanisms involved in the melusin induced protection, major signaling pathways known to be regulated by melusin were investigated by Western blot analysis of samples collected from the same hearts used to determine cardiac injury and in the two Sham groups described in the Method section. As shown in Fig. 3, phosphorylation of ERK1/2 (WT_Sham = 0.350±0.063, Mel-TG_Sham = 1.825±0.014, *P*< 0.001; WT_I/R = 0.432±0.168; Mel-TG_I/R = 1.845±0.182 *P*< 0.001) and AKT (WT_Sham = 0.457±0.032, Mel-TG_Sham = 2.210±0.034, *P*< 0.05; WT_I/R = 0.232±0.048; Mel-TG_I/R = 1.59±0.585 *P*>0.05) was potentiated in Mel-TG hearts compared to WT both in basal condition and after I/R. In addition, GSK3β, a kinase down-stream of AKT, was also more strongly phosphorylated in Mel-TG hearts compared to WT both in basal condition and after I/R (WT_Sham = 1.390±0.016, Mel-TG_Sham = 2.130±0.043, *P*< 0.05; WT_I/R = 0.395±0.099; Mel-TG_I/R = 1.41±0.383 *P*< 0.05). Moreover, the pro-apoptotic BAD protein was significantly more phosphorylated in Mel-TG hearts subjected to I/R compared to WT (WT_I/R = 0.825±0.207, Mel-TG_I/R = 1.805±0.192, *P*< 0.05).

To demonstrate a causal role of increased AKT and ERK1/2 phosphorylation in melusin dependent protection, hearts were treated with the PI3K inhibitor *Wortmannin* (Mel-TG_I/R+WM) and the he MEK1/2 inhibitor *U0126* (Mel-TG_I/R+U0126). As shown in Figure 4A, WM treatment resulted in a strong inhibition of AKT phosphorylation as well as of the downstream kinase GSK3β. Infarct size of Mel-

TG hearts treated with PI3K inhibitor, was significantly higher ($P< 0.01$) with respect to the Mel-TG_I/R group (Figure 4C) and not significantly different from the WT_I/R group (67.2±3.9% and 66.4±8.3%, respectively). Accordingly, in Mel-TG_I/R+WM hearts total LDH release was higher ($P< 0.05$) with respect to Mel-TG_I/R group (Figure 4D) and not significantly different from the WT_I/R group (416.5±92.7 U/g ww and 396.3±66.0 U/g ww, respectively).

Similarly MEK 1/2 inhibitor U0126 strongly blunted the phosphorylation of ERK1/2 as well as of the downstream kinase p70S6K (Fig. 4B), abolished the protection in the Mel-TG heart, in fact the infarct size and LDH total release were significantly higher with respect to Mel-TG_I/R group (68±4% and 440±83 U/g ww, respectively, $P< 0.001$ respect to Mel-TG_I/R) (Fig 4C, D).

We confirmed in 6 additional WT hearts that the two inhibitors did not exacerbate I/R injury in this strain (Infarct size 61±3% and 60±3% and LDH release 446±87 U/g ww and 372±63 U/g ww for WN and U0126, respectively; P = NS *vs.* WT_IR). These data are in agreement with those reported on previous studies of myocardial I/R injury [10, 52, 70].

Finally, we confirmed in 3 WT hearts that DMSO at the used concentrations had not effect on I/R injury (Infarct size 57±2% and LDH 375±30 mU/mg, P = NS *vs.* WT_I/R) [5, 10]. We also showed in 3 Mel-TG hearts that DMSO had no effect on damage (Infarct size 37±1% and LDH 215±25 U/mg ww, P = NS *vs.* Mel_I/R).

Melusin overexpression induces HSP90 upregulation

Heat shock proteins are known to protect heart from I/R injury [3, 9, 54, 69]. Given that melusin binds to HSP90 and is co-expressed with HSPs [57], we evaluated the expression of HSP90, an ATP-dependent chaperone known to have a cardio-protective role in I/R [35]. Interestingly, as shown in Figure 3, expression of HSP90 was strongly increased in Mel-TG hearts in basal conditions (WT_Sham =

0.170±0.034, Mel-TG_Sham = 2.230±0.380, *P*< 0.01). Moreover, in Mel-TG mice, HSP90 expression remained higher after I/R (WT_I/R = 0.350±0.063; Mel-TG_I/R = 1.455±0.380 *P*> 0.05).

Discussion

Here we demonstrate, for the first time, that the overexpression of the muscle specific small molecular chaperone *melusin* confers resistance to I/R injury to the heart. Melusin over expression is characterized by increased phosphorylation of AKT/ERK/GSK3β kinases and overexpression of HSP90. This phenotype represents a protective status relevant for heart tolerance against I/R injury, as shown by the fact that the pharmacological inhibition of PI3K/AKT and MEK/ERK pathways blunted the cardioprotection observed in melusin transgenic mice. Although the involvement of RISK pathway may be species-specific [61], the importance of the PI3K/AKT/ERK pathways in protection from I/R damage has been convincingly demonstrated by a number of different laboratories [24, 27, 38, 48, 60]. Here we demonstrated a key role of the muscle specific protein melusin to regulate these pathways in the context of ischemia and reperfusion. This role is further highlighted by a recent report describing that melusin expression increases after a permanent coronary ligation [22] and that this increase improves cardiac remodeling in a mouse model of myocardial infarction [65].

In our study, due to development of necrosis in late reperfusion (60 min), we could have missed the peak of phosphorylation. In fact, in conditioned hearts maximal AKT and ERK1/2 phosphorylation were observed at 15 min of reperfusion [26]. Moreover, a role may be also played by the dynamic of development of myocardial necrosis and the loss of phospho-AKT and phospho-ERK immunoreactivity, and, specifically, by the different way of tissue sampling [14]. Nevertheless, in our Mel-TG model levels of AKT and ERK1/2 phosphorylation were higher than WT in non-ischemic conditions (Sham). Such higher levels were not reduced after I/R in Mel-TG hearts despite the development of necrosis, thus

supporting an increased process of phosphorylation in post-ischemic phase in surviving cells of melusin transgenic hearts.

The ability of melusin to sustain both the AKT and ERK1/2 pathways is likely based on its function in promoting signalosome complex assembly [20]. Notably, we have previously demonstrated the ability of melusin to bind p85 regulatory subunit of PI3Kα [68]. Given that PI3Kα is a key upstream regulator of AKT, it can be hypothesized that melusin modulate AKT activation by regulating PI3Kα activity. In addition, Melusin also organizes a signalosome complex regulating the ERK1/2 pathway [20]. We have shown, in fact, that melusin interacts with c-Raf, MEK1/2 and ERK1/2 MAP Kinases, as well as with their scaffold molecule IQGAP-1 [55]. An additional key element also present in the complex is the focal adhesion kinase FAK. Biomechanical stress leads to activation of FAK, which in turns triggers the MAPK cascade leading to ERK1/2 activation.

Both the AKT and ERK1/2 pathways impact importantly on cardiomyocyte survival. In fact, AKT, via GSK3β, plays an important role in the regulation of the mitochondrial permeability transition pore, thus, preventing cardiomyocyte necrosis. At the same time, via BAD phosphorylation it protects cell from apoptosis, a major cause of tissue damage in reperfusion injury [1, 17, 21, 39, 71]. In addition ERK signaling is well known to promote cell survival in different cell types including cardiomyocytes [44, 55, 59]. The MEK/ERK cascade: from signaling specificity to diverse functions [64]. Moreover, a positive crosstalk of ERK and AKT in promoting cells survival has also been reported [16].

An additional component possibly involved in melusin-dependent AKT and ERK activation is the chaperone protein HSP90. In fact, melusin, by binding to HSP90 [32, 57], acts as co-chaperone and likely directs HSP90 toward the signaling substrates, thus, protecting them from dephosphorylation and sustaining their activation state. In this context the up-regulation of HSP90 expression observed in melusin transgenic hearts could represent an important and interesting crosstalk between chaperone

and RISK signaling pathways. However, the present study was not designed to ascertain this crosstalk, which deserves future studies.

The protective effect against reperfusion injury can also be achieved by postconditioning maneuvers [13, 23-25, 27, 36, 48-51, 62, 66, 67, 71]. Our results indicated that a PostC protocol, protective in WT hearts, does not add any further protection in Mel-TG hearts, thus suggesting that the pathway(s) enhanced by melusin is similar to that triggered by PostC.

Our findings suggest potential therapeutic approaches involving the over-expression of melusin. The translation to humans might be obtained either by viral vectors to deliver exogenous melusin genes or by pharmacological treatment capable to increase the expression of the endogenous melusin gene in patients with an high risk profile for heart ischemia. However, the translation of findings on cardioprotection from rodents to humans cannot be taken for granted [29] and analysis in larger mammals, such as the pigs, may be necessary to support translational application [31].

In conclusion, here we present first evidences that melusin overexpression has an important protective effect against ischemia/reperfusion injury *via* its ability to boost RISK pathway signaling and by increasing HSP90 chaperone protein expression.

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Conflict of interest: Mara Brancaccio and Guido Tarone are scientific cofounders and consultants for Target Heart Biotec, a company that develops melusin recombinant protein as a drug to counteract heart failure.

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Figure Legends

Fig. 1 Experimental protocols. Timeline of the eight experimental groups. Experimental groups 2 and 4: vertical lines represent postconditioning protocol at the beginning of reperfusion. Experimental groups 5 and 6: Wortmaninn (0.1 μM) and U0126 (50 μM) were used as inhibitors of PI3K and MEK1/2 respectively. Inhibitors were infused 5 min before and 20 min after ischemia. Arrows indicate the sample collection for Western blot analysis. For acronyms see the text.

Fig. 2 Infarct size and LDH release. Panel A: infarct size (IS) expressed as percentage of the ischemic tissue (IT) resulted smaller in Mel-TG_I/R compared with WT_I/R mice. Panel B: the increase in LDH concentrations during reperfusion was lower in Mel-TG_I/R than in WT_I/R mice. While PostC reduced IS and LDH release in WT heart, it did not add any further protection in Mel-TG model. * $P< 0.05$; ** $P< 0.01$.

Fig. 3 Melusin overexpression induces AKT pathway activation and HSP90 upregulation. Panel A: Western blot analysis of tissue extracts from hearts subjected to ischemia reperfusion or from control hearts. GAPDH was used as loading control. Representative Western blot results are shown. Panel B: Densitometric quantification of Western blot bands. * $P< 0.05$; ** $P< 0.01$; *** $P< 0.001$

Fig. 4 Inhibition of either AKT or ERK1/2 phosphorylation blocks protection to ischemia reperfusion induced by melusin overexpression. Western blot analysis of phosphorylated AKT (P-AKT), GSK3β (P-GSK3β) (panel A), and phosphorylated ERK (P-ERK) and p70S6K (P-p70S6K) (panel B) on heart extracts from melusin overexpressing mice subjected to ischemia/reperfusion (Mel-TG_I/R) in the presence of

either PI3K inhibitor Wortmannin (WM) or MEK inhibitor U0126. Wild type hearts subjected to ischemia/reperfusion were used for comparative purpose (WT_I/R). The histogram shows the densitometric quantification of Western blot bands. Representative Western blot results are shown. Panel C: infarct size (IS) expressed as percentage of ischemic tissue (IT) resulted larger in Mel-TG treated with Wortmannin or U0126 compared to untreated Mel-TG mice. Panel D: the increase in LDH concentrations during reperfusion was higher in Mel-TG_I/R treated with Wortmannin or U0126 compared to untreated Mel-TG mice. * $P< 0.05$.

Figure 1

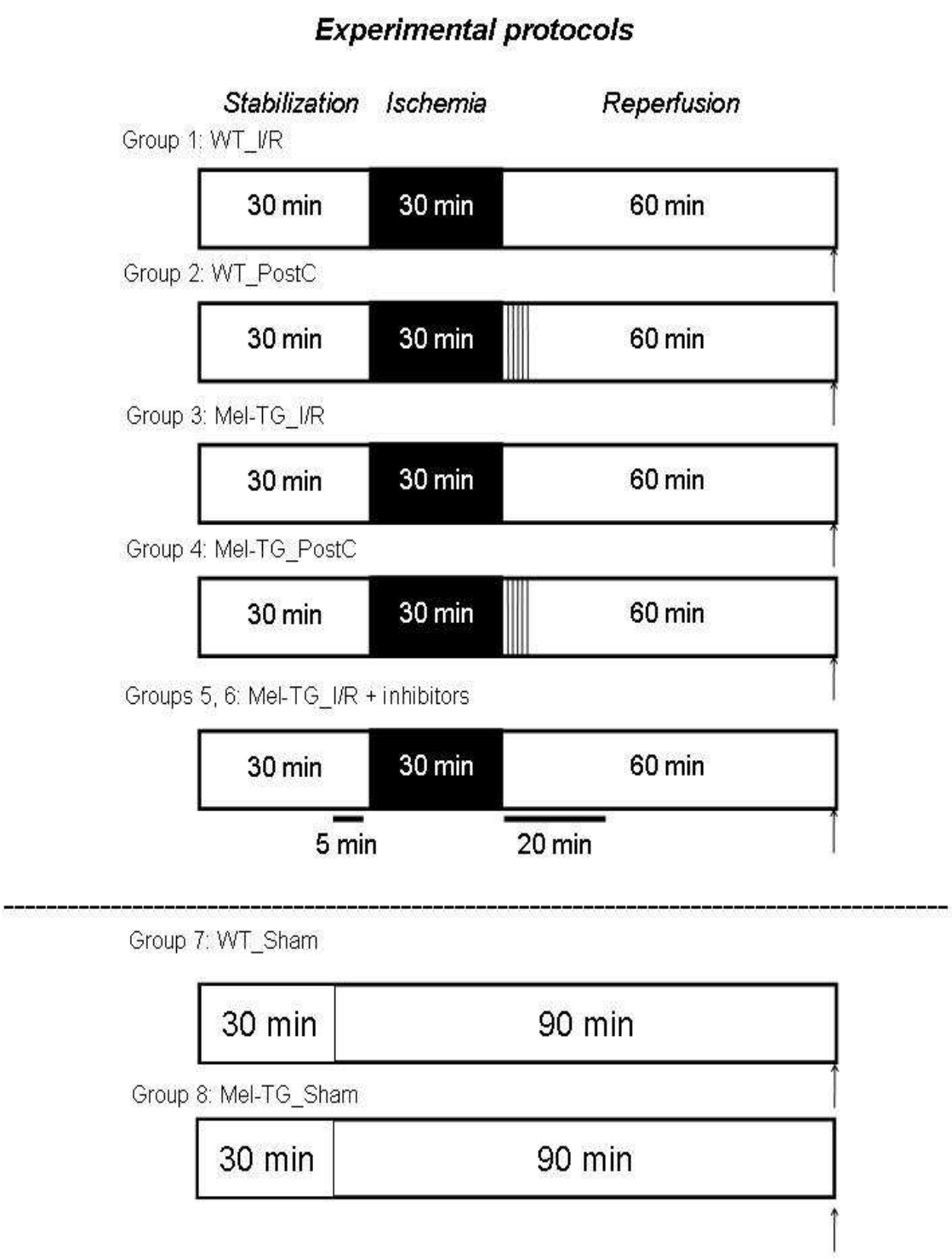
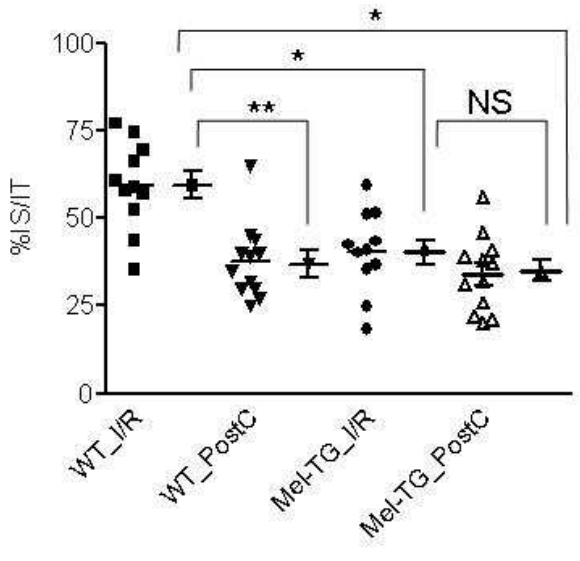

Experimental protocols
Stabilization
Ischemia
Reperfusion
Group 1: WT_I/R
30 min
30 min
60 min
Group 2: WT_PostC
30 min
30 min
60 min
Group 3: Mel-TG_I/R
30 min
30 min
60 min
Group 4: Mel-TG_PostC
30 min
30 min
60 min
Groups 5, 6: Mel-TG_I/R + inhibitors
30 min
30 min
60 min
5 min
20 min
Group 7: WT_Sham
30 min
90 min
Group 8: Mel-TG_Sham
30 min
90 min

Figure 2

A


*
*
**
NS
100
75
50
25
0
%IS/IT
WT_I/R
WT_PostC
Mel-TG_I/R
Mel-TG_PostC

B

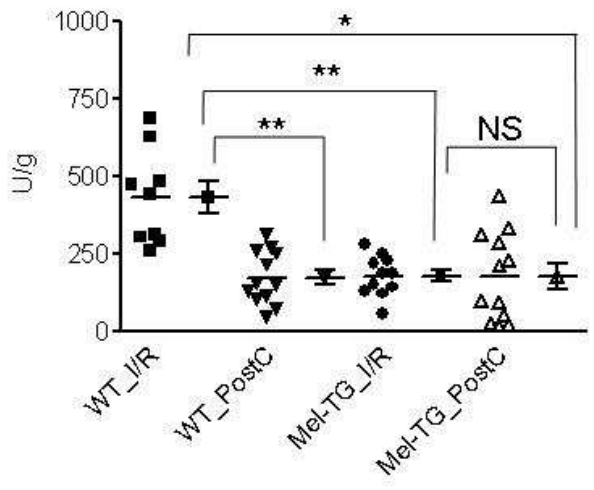

*
**
**
NS
1000
750
500
250
0
U/g
WT_I/R
WT_PostC
Mel-TG_I/R
Mel-TG_PostC

Figure 3

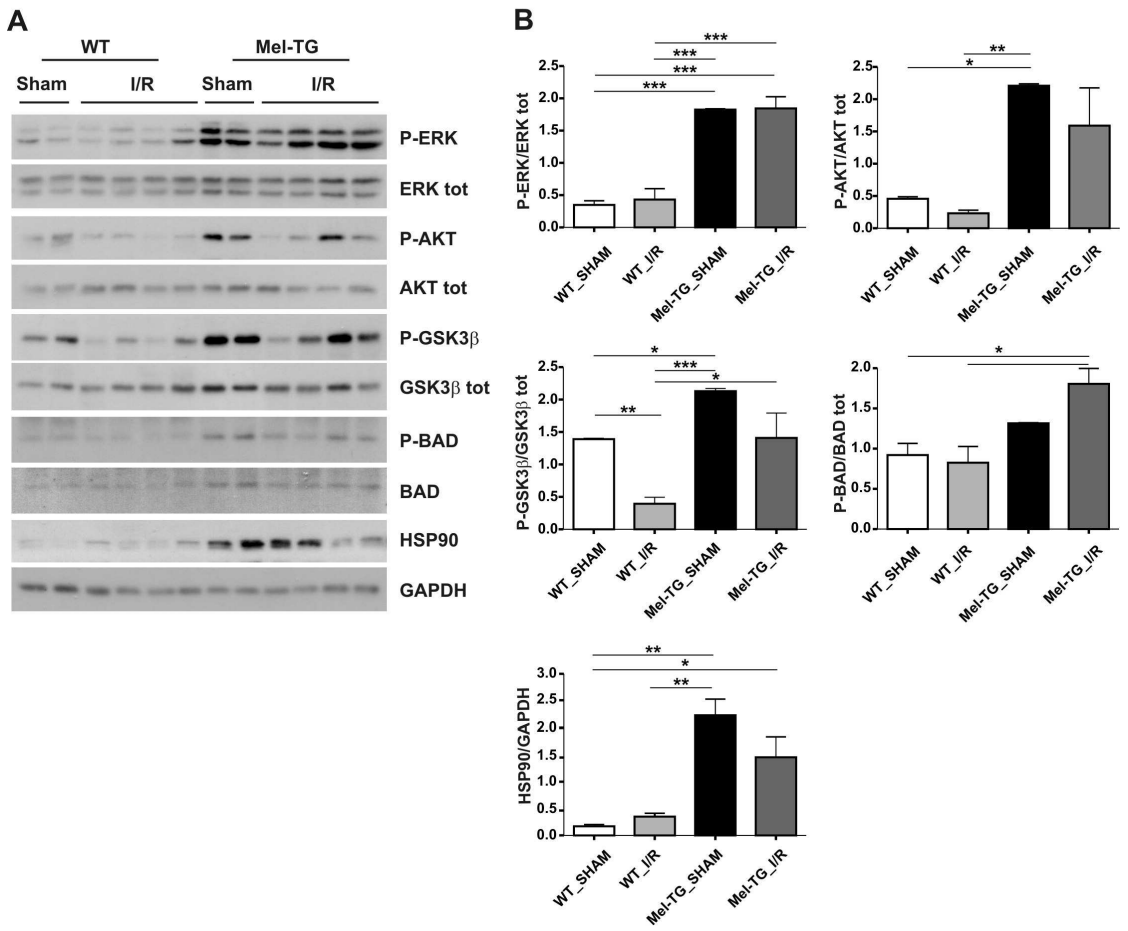
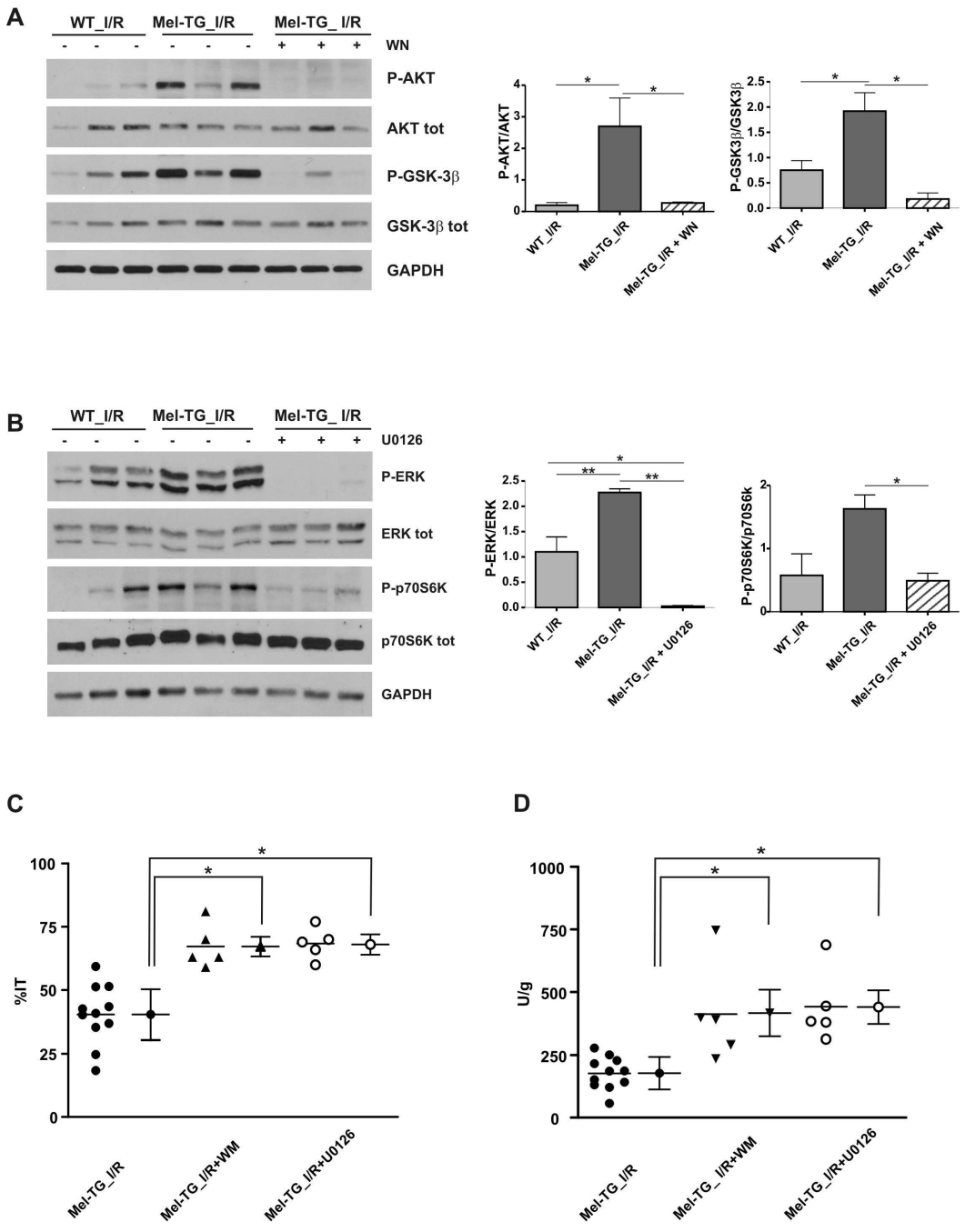

A
WT
Mel-TG
Sham
I/R
Sham
I/R
P-ERK
ERK tot
P-AKT
AKT tot
P-GSK3β
GSK3β tot
P-BAD
BAD
HSP90
GAPDH
B
P-ERK/ERK tot
P-AKT/AKT tot
P-GSK3β/GSK3β tot
P-BAD/BAD tot
HSP90/GAPDH
WT_SHAM
WT_I/R
Mel-TG_SHAM
Mel-TG_I/R

Figure 4


A
WT_I/R
Mel-TG_I/R
Mel-TG_ I/R
WN
P-AKT
AKT tot
P-GSK-3β
GSK-3β tot
GAPDH
P-AKT/AKT
P-GSK3β/GSK3β
WT_I/R
Mel-TG_I/R
Mel-TG_I/R + WN
B
U0126
P-ERK
ERK tot
P-p70S6K
p70S6K tot
GAPDH
P-ERK/ERK
P-p70S6K/p70S6k
Mel-TG_I/R + U0126
C
%IT
Mel-TG_I/R
Mel-TG_I/R+WM
Mel-TG_I/R+U0126
D
U/g