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**Postconditioning with Glucagon Like Peptide-2 Reduces
Ischemia/Reperfusion Injury in Isolated Rat Hearts: Role of Survival
Kinases and Mitochondrial K_{ATP} Channels**

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Running title: GLP-2 and cardioprotection

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Abstract

We recently reported that the heart expresses functional receptors for the anorexigenic glucagon-like peptide (GLP)-2. Activation of these cardiac receptors affected basal heart performance through extracellular regulated kinase (ERK1/2) activation. Since ERK1/2 is considered one of the prosurvival kinases of postconditioning cardioprotective pathways, we hypothesized that GLP-2 directly protects the heart against ischemia/reperfusion (I/R) injury *via* prosurvival kinases. Wistar rat hearts were retrogradely perfused on a Langendorff perfusion apparatus. After 40-min stabilization, hearts underwent 30-min global ischemia and 120-min reperfusion (I/R group). In GLP-2 group, hearts received 20-min GLP-2 (10^{-7} M) infusion at the beginning of the 120-min reperfusion. Perfusion pressure and left ventricular pressure (LVP) were monitored. Infarct size was evaluated by nitroblue-tetrazolium staining. Compared with the I/R group, GLP-2 treated hearts showed a significant reduction of infarct size and of postischemic diastolic LVP (index of contracture), together with a sharp improvement of developed LVP recovery (index of contractility). The protective effects were abolished by co-infusion with phosphatidylinositol 3-Kinase inhibitor, Wortmannin, the ERK1/2 inhibitor, PD98059, or the mitochondrial K_{ATP} channel blocker, 5-Hydroxydecanoate. GLP-2 effects were accompanied by increased phosphorylations of protein kinase B (PKB/Akt), ERK1/2 and glycogen synthase kinase (GSK3 β). After 7-min reperfusion, Wortmannin blocked Akt and GSK3 β phosphorylation. After 30-min reperfusion, Wortmannin inhibited phosphorylation of all kinases. In conclusion, data suggest that GLP-2, given in early reperfusion, as postconditioning, protects against myocardial I/R injury, limiting infarct size and improving post-ischemic mechanical recovery. It seems that the GLP-2-protection of rat heart involves multiple prosurvival kinases and mitochondrial K_{ATP} channels.

Key words: Cardioprotection; Glucagon-like peptide-2; Postconditioning; Prosurvival Kinases; Ischemia/Reperfusion.

Introduction

Ischemic postconditioning applied immediately after a prolonged ischemic period attenuates infarct size. It can reduce infarct size, but, unfortunately, it cannot be applied to most patients, so a *pharmacological postconditioning* mimetic that can be administered at the onset of reperfusion to protect all acute myocardial infarction patients is necessary [14-18,31,43].

Several humoral factors control the cardiovascular system and the gastrointestinal apparatus, acting both directly on heart and vessels, and indirectly by controlling appetite, fluid intake and plasma osmolarity. Among these factors, two different peptides, glucagon-like peptide 1 (GLP-1) and GLP-2, which are produced by intestinal L cells and co-released in response to food intake [10], have attracted the attention of researchers.

GLP-1 has been extensively studied [e.g. 4,10,28,50]. On the contrary, GLP-2 is now emerging as a peptide with anorexigenic properties that plays a major role in short-term regulation of appetite and satiety, also profoundly influencing many gastrointestinal functions in healthy and in diabetes [11,25]. As such GLP-1 and GLP-2 represent a new class of drug which is emerging in ischemia scenario. Although extensive research supports a cardioprotective role for GLP-1 even in diabetes [4,28,50], nobody, to our knowledge, has studied whether GLP-2 protects the myocardium against ischemia/reperfusion (I/R) injury. This is not trivial because GLP-1 and GLP-2, by acting on different receptors may have different intracellular effects, especially on calcium handling [11,25]. Moreover, it has been suggested that GLP-1(9-36) cardioprotective effects do not require the classical GLP-1 receptor [2]. Finally, GLP-2, like GLP-1, is of paramount importance in diabetes [11,25,50].

GLP-2 acts *via* a specific G protein-coupled receptor (GLP-2R), which is largely expressed in gastrointestinal tract [27] and brain [21,24,45]. It is also present in lung, cervix and vagal afferents [11]. Very recently, also the rat heart was found to express GLP-2R, whose activation depressed both basal and β -adrenergic stimulated inotropic (contractility) and

lusitropic (relaxation) performance [1]. These cardiac effects were independent from GLP-1 receptor, involved Gi/o proteins and were associated with an increase in ERK1/2 activation in normal hearts [1].

Interestingly, ERK1/2 is one of the cardioprotective elements of “reperfusion injury salvage kinase (RISK) pathway” [9,15,17,37]. Actually, the RISK acronym was first used in a study in which the pharmacological inhibitor of ERK1/2, PD98059, abolished the infarct limiting effects of urocortin administered at reperfusion as postconditioning agent [37]. The RISK pathway, which seems necessary in rodents [14,18,20,38], is activated by both ischemic and pharmacological postconditioning [14,15,17,18,20,38,40,48,49]. This pathway includes glycogen synthase kinase (GSK3 β), as a downstream target of both ERK1/2 and phosphatidylinositol 3-Kinase (PI3K)/protein kinase B (PKB/Akt). As such, GSK3 β may act as a point of convergence for a variety of prosurvival signaling pathways resulting in mitochondrial mediated cardioprotection [12,14-18].

On the basis of the above described cardiovascular effects of GLP-2, the possibility exists that this peptide might exert cardioprotective effects under I/R conditions. Therefore, we tested whether GLP-2, applied immediately after an infarcting ischemia (*i.e.* as pharmacological postconditioning), may limit infarct size and may improve the recovery of post-ischemic cardiac function. Using pharmacological antagonists we also tested the hypothesis that activation of RISK pathway plays a role in GLP-2-induced cardioprotection, conveying the cardioprotective signal to mitochondria. Hence, using a mitochondrial K_{ATP} (mitoK_{ATP}) channel antagonist we tested the involvement of these protective channels [14-16,20,31,38]. To corroborate pharmacological data, a direct involvement of Akt, ERK1/2 and GSK- β phosphorylation in GLP-2 reperfused heart was assessed by standard immunoblotting after 7- and 30-min reperfusion.

Materials and Methods

Animals

Male Wistar rats (Harlan Laboratories Srl, Udine, Italy), weighing 450-550 g, were housed (three per cage) in a ventilated cage rack system under standard conditions. Animals had food and water access ad libitum. The investigation conforms to Italian law (DL-116, January 27, 1992) and to the Guide for the Care and Use of Laboratory Animals, according to National Institutes of Health publication 85-23 (revised 1996). The project was approved by the Italian Ministry of Health, Rome, and by the ethics review board of the University of Calabria.

Isolated heart perfusion

Rats were heparinized (2.500 units i.m.) and anesthetised with urethane (1 g/kg i.p.) 10-min later. The hearts were then rapidly excised, placed in ice-cold buffer solution, and weighed. Isolated hearts were attached to the perfusion apparatus and retrogradely perfused with oxygenated Krebs-Henseleit buffer (KHs) containing (in mM) 127 NaCl, 17.7 NaHCO₃, 5.1 KCl, 1.5 CaCl₂, 1.26 MgCl₂, and 11 D-glucose, supplemented with 5 µg/ml lidocaine and gassed with 95% O₂-5% CO₂ [6,29,30,32-34]. The perfusion pressure was set to 100 mmHg and kept constant throughout the experiments. The hearts were electrically paced at 280–300 beats/min and kept in a temperature-controlled chamber (37°C).

Drugs

GLP-2 (rat) peptide was purchased from Tocris Bioscience. PD98059 (PD), a specific inhibitor of ERK1/2, Wortmannin (WT), a potent phosphatidylinositol 3-Kinase (PI3K) inhibitor, 5-Hydroxydecanoate (5HD), a mitoK_{ATP} channels blocker, were purchased from Sigma Aldrich. All drug-containing solutions were freshly prepared just before the experimentation.

Experimental protocols

Each heart was allowed to stabilize for 40-min; at this time, baseline parameters were recorded. After stabilization, hearts were randomly assigned to one of the treatment groups described below and then subjected to 30-min of global, no-flow ischemia followed by 120-min of reperfusion (I/R). Pacing was discontinued at the beginning of the ischemic period and restarted after the third minute of reperfusion [29,48].

Cardiac function and Infarct size studies (Fig 1, panel A)

- 1) In the first group (I/R group; n=9), hearts were stabilized and subjected to I/R protocol only.
- 2) In the second group (GLP-2 group, n=9), GLP-2 (10^{-7} M) was infused for 20-min at the beginning of 120-min reperfusion.
- 3) In groups 3-5, hearts (n=9 for each group) were perfused with GLP-2 plus one of the following inhibitors: Wortmannin (WT, 10^{-7} M; GLP-2+WT), PD (10^{-8} M; GLP-2+PD), or 5HD (10^{-5} M; GLP-2+5HD); perfusion with inhibitors started 5-min before ischemia and continued during the early 20-min of reperfusion in the presence of GLP-2 (10^{-7} M).
- 4) In groups 6-8 hearts (n=8, for each group) were perfused with inhibitors only [WT (10^{-7} M), PD (10^{-8} M), or 5HD (10^{-5} M)], which started 5-min before ischemia and continued during the early 20-min of reperfusion.

The concentration of GLP-2 was chosen on the basis of a preliminary dose-response curve (10^{-12} - 10^{-7} M) as the dose that induced the highest infarct size reduction (data not shown). In all experiments, the antagonist concentration was selected on the basis of previous reports [16,30,32,34], which have shown the efficacy of the selected concentrations. Hemodynamic parameters were assessed using a PowerLab data acquisition system and analyzed using Chart software (both purchased by ADInstruments, Basile, Italy).

Left ventricular end diastolic pressure (LVEDP) was used as an index of the extent of *contracture* development in reperfusion. In fact, contracture can be defined as an increase in LVEDP of 4 mmHg above the baseline level [29,30,32,34]. As index of contractile activity we used the developed left ventricular pressure (dLVP), which was calculated as the difference between systolic LVP and LVEDP.

Assessment of myocardial injury

To obtain infarct areas, hearts were rapidly removed from the perfusion apparatus at the end of reperfusion, and the left ventricle was dissected into 2- to 3-mm circumferential slices. After 20-min of incubation at 37°C in 0.1% solution of nitro blue tetrazolium in phosphate buffer, unstained necrotic tissue was carefully separated from stained viable tissue by an independent observer who was not aware of the nature of the intervention. The weights of the necrotic and non necrotic tissues were then determined, and the necrotic mass was expressed as a percentage of total left ventricular mass, including septum [29,30,32,34]. Since in isolated rat hearts, both pre- and post-conditioning are known to reduce the production of lactate dehydrogenase (LDH) during reperfusion, the release of this enzyme was tested in I/R and GLP-2 groups (n= 3 for both groups), as previously described [29,34].

Western blotting (Fig 1, panel B)

To directly study whether GLP-2 is able to induce phosphorylation of upstream ERK1/2 and Akt, and downstream GSK3 β , after 40-min stabilization, rat hearts underwent 30-min global ischemia followed by 7-min or 30-min reperfusion (n = 4 for each group). Therefore, two groups of hearts underwent I/R only (7 or 30-min reperfusion), two groups received GLP-2 in the early reperfusion (for 7 or 20-min), two groups received GLP-2 plus WT as described above (for 7 or 20-min in reperfusion), and two groups were perfused with physiological solution only (Sham) for 77 or 100-min, respectively.

The supernatants, corresponding to 60 µg protein, measured with Bradford's method [5], were subjected to SDS-PAGE on acrylamide gels (10% for Akt, phospho-Akt; GSK3β, phospho-GSK3β, ERK1/2, phospho-ERK1/2) and transferred to PVDF membranes (GE Healthcare). Membranes were then incubated overnight at 4°C with the following primary antibodies: anti-Akt, anti-phospho-(Ser473)-Akt, anti-GSK3β, antiphospho-(Ser-9)-GSK3β, anti-ERK1/2, anti-phospho-(Thr202/Tyr204)-ERK1/2 (Cell Signaling). To confirm equal protein loading, membranes were incubated with an anti-vinculin antibody (Sigma). Immunoblotted proteins were visualized using an Immuno-Star HRP Substrate Kit (Bio-Rad) and quantified by Kodak Image Station 440CF. Image analyses were performed by Kodak 1D 3.5 software [30,33]. The expression of total kinases and phospho-kinases for each heart was normalized with respect to its matched loading control vinculin, and data have been presented as the ratio of phospho to total kinases.

Statistical analysis

Data are expressed as the mean ± SEM. The statistical significance of differences within group was assessed using the ANOVA test. Comparison between groups was made by using a one-way analysis of variance (ANOVA) followed by the Bonferroni correction for post-hoc t-tests. Differences were considered to be statistically significant for $p < 0.05$.

Results

In the baseline conditions hearts of all groups displayed similar cardiac function (data not shown).

Improvement of postischemic cardiac function by GLP-2 involves survival kinases of the RISK pathway and mitoK_{ATP} channels (Fig 2).

Systolic function in the post-ischemic phase is represented by the percent of developed dLVP recovery (*i.e.*, inotropic activity). As can be seen in Fig 2A, the hearts of the I/R group

presented a limited dLVP recovery; in fact, at the end of reperfusion, the percent recovery of dLVP was $41\pm9\%$ of baseline value ($p<0.01$ vs baseline). GLP-2 markedly improved the dLVP recovery during reperfusion, being it at the end of reperfusion $104\pm15\%$ of the baseline ($p<0.05$ vs I/R group; Fig 2A). The GLP-2-dependent improvement of postischemic dLVP was abolished when the hearts were co-treated with GLP-2 plus the inhibitor of PI3K or ERK1/2 (WT or PD), or with the blocker (5HD) of mitoK_{ATP} channels. In hearts perfused with inhibitors only (groups 6-8), the recovery of systolic function was not different from I/R group (Fig 2B).

Diastolic function (Fig 3) is represented by the level of contracture (*i.e.*, LVEDP 4 mmHg or more above baseline level) [2,34]. I/R markedly increased LVEDP (from 6 ± 1 mmHg in the baseline to 37 ± 10 mmHg at the end of reperfusion, $p<0.05$). During reperfusion, GLP-2 abolished contracture development; in fact, at the end of reperfusion, LVEDP was 6 ± 1 mmHg in GLP-2 group ($p<0.001$ vs I/R). The GLP-2-dependent improvement of postischemic contracture was abolished by co-treatment with GLP-2 plus the inhibitor of either PI3K or ERK1/2 (WT or PD), or with the mitoK_{ATP} channel blocker (5HD) (Fig 3A). In hearts perfused with inhibitors only (groups 6-8), the development of contracture was not different from I/R group (Fig 3B).

GLP-2 induced reduction of infarct size is also linked to survival kinases and mitoK_{ATP} channels (Fig 4).

Left ventricular (LV) mass was similar in all groups (LV weight was 927 ± 13 ; range 559–1105 mg). Total infarct size, expressed as a percentage of LV mass, was $65\pm8\%$ in I/R. The infusion of GLP-2 during the early 20-min reperfusion reduced infarct size to $31\pm5\%$ of LV mass ($p<0.001$ vs I/R). WT, PD or 5HD given in co-infusion with GLP-2 did not allow infarct size reduction; so that infarct size ($61\pm3\%$, $59\pm4\%$ and $60\pm3\%$, respectively) was not significantly different from that observed in I/R group (Fig 4A). In contrast, it resulted

significantly increased with respect to the hearts treated with GLP-2 alone ($p < 0.001$ for each antagonist). In hearts perfused with inhibitors only (groups 6-8), infarct size was not different from I/R group (Fig 4B).

To corroborate infarct size data, LDH release was analyzed in 3 hearts for I/R group and in 3 hearts for GLP-2 group. In these hearts of I/R group the cumulative LDH during reperfusion was 1197 ± 125 U/g wet wt. In GLP-2 group the release of LDH was 907 ± 96 U/g wet wt; a value significantly ($p < 0.05$) lower than that of I/R group.

GLP2 induces the phosphorylation of upstream and downstream survival kinases of the RISK pathway (Figs 5 and 6).

The involvement of kinases located upstream (Akt and ERK1/2) and downstream (GSK3 β) of the protective pathway in the GLP-2 mechanism was evaluated by Western blotting analysis in two different time points, 7th and 30th min after start of reperfusion. Representative bands and densitometric analysis of the scanned blots are presented in Figs 5 and 6. Data are normalized with respect to the mean value of single value loading of vinculin. The infusion of GLP-2 enhanced phosphorylation for all kinases analyzed (Figs 5 and 6). In particular, at the 7th min of reperfusion (Fig 5), the infusion of GLP-2 induced a remarkable phosphorylation of Akt and GSK3 β ($p < 0.001$ for both *vs* I/R), which were abolished by WT. A less evident phosphorylation of ERK1/2 was induced by GLP-2, regardless of the presence of WT ($p < 0.05$ for both *vs* I/R, $p = \text{NS}$ *vs* each other), *i.e.* early ERK1/2 activation is PI3K-independent. At the 30th min of reperfusion, while the GLP-2 induced phosphorylation of Akt and GSK3 β was less evident than that at the 7th min, the phosphorylation of ERK1/2 was similar at the two time points (Fig 6). However, the co-infusion with WT abolished the phosphorylation of the three studied kinases, including ERK1/2, *i.e.* late ERK1/2 activation is PI3K-dependent.

Discussion

This study demonstrates that GLP-2, given in the early reperfusion, has a significant protective effect against myocardial I/R injury. In particular, GLP-2 reduces infarct size and markedly improves the post-ischemic contractile function. Antagonists of upstream elements (PI3K and ERK1/2) of the RISK pathway, as well as blockade of mitochondrial K_{ATP} channels abrogate GLP-2 protective effects. This widens the influence of GLP-2 on the mammalian heart to cardioprotection, which has not been shown before.

We recently described the cardiac profile of GLP-2 by revealing, in the rat heart, the presence of a functional GLP-2R, whose activation reduces baseline and beta-stimulated contractility. The cardiac effects of GLP-2 were independent from GLP-1 receptor, being unaffected by exendin-3 (9-39) amide [1]. Here, we aimed at evaluating the potential benefit of GLP-2 on postischemic myocardial function assessed in *ex vivo* model. GLP-2 given as postconditioning agent in early reperfusion decreases infarct size and markedly improves postischemic left ventricular function. These results are particularly relevant as alteration of LV function after infarction, and the presence of myocardial viability have been shown to be major determinants of mortality and prognosis after myocardial ischemia. These results are also relevant since the archetypal ischemic postconditioning protects against the extension of infarct size, but it does not significantly and constantly improve postischemic cardiac function [31 and references therein].

In non-ischemic models of different tissues, GLP-2 effects were mediated by the activation of the prosurvival kinases ERK1/2 [1] or by PI3K/Akt-mTOR signalling pathway [39]. Several studies have demonstrated that activation of G protein-coupled receptors may lead also to PI3K/Akt activation and cardioprotective effects [8,31]. The protective mechanisms activated

in reperfusion include at least two parallel pathways, which start from both ERK1/2 and PI3K-Akt and converge during reperfusion on GSK3 β phosphorylation/inactivation with the involvement of mitoK_{ATP} channel opening [8,12,15,20,31,32]. Therefore, to evaluate whether GLP-2 improves post-ischemic cardiac function and cell survival *via* the activation of these two pathways, we inhibited the upstream kinases of the two parallel pathways (PI3K and ERK1/2). We also checked for the phosphorylation of components of the RISK pathway (Akt, ERK1/2 and GSK3 β) during the early (7th min) and later (30th min) reperfusion period. Our findings suggest that the GLP-2-induced early protection takes place only if the two pathways (one starting from PI3K and the other from ERK1/2), and one of the terminal elements, mitoK_{ATP} channels, are simultaneously activated during early reperfusion. In fact, GLP-2 protection was abrogated by blocking each of these elements. Accordingly, these kinase-dependent signal transduction pathways may converge on mitochondria in postconditioning protection [8,12,15,31].

Several authors have shown that PI3K/Akt is involved in postconditioning [9,14,15,20,38,48,49]. Intriguingly, Darling *et al.* [9] have shown the involvement of ERK1/2 rather than PI3K/Akt in the reduction of infarct size achieved with ischemic postconditioning. Here we show the strong involvement of PI3K/Akt in GLP-2 protection. In fact, Akt phosphorylation, after an initial sharp augmentation, decreases with time during reperfusion. Yet, ERK1/2 is involved in a less extent, but initially in a PI3K-independent fashion (unchanged by WT), and then persists in a PI3K-dependent manner (blocked by WT). These findings suggest a certain degree of cross-talk [49] and, perhaps, an additive protection between ERK1/2 and PI3K/Akt pathways in the early reperfusion phase. Therefore, it is likely that an adequate postconditioning stimulus can activate both pathways, thus leading to an improvement of postischemic cardiac function and to cell survival, as it is the case for GLP-2 in the present study. Since the inhibition of ERK1/2 activation by PD blocks the

protective effects of GLP-2, our results suggest that the initial ERK1/2 phosphorylation is not an epiphenomenon and that both pathways are important to mediate cardioprotection. Accordingly, a total abrogation of postconditioning-induced protection was observed either inhibiting (PI3K)-Akt or ERK1/2 activation [17,41,46].

Clinical considerations

In a recent “Special Report” it was stated that “improvements in morbidity and mortality can be achieved only with the development of new adjunctive therapies coupled with reperfusion” [38]. Importantly, in both animals and humans, administration of GLP-1 exerted cardiovascular effects [4,26,28,36,50]. Although Nikolaidis *et al.* [28] were the first to obtain improvements of cardiac function with GLP-1 in humans, their study was not designed to reduce lethal *reperfusion injury*, which occurs immediately after ischemia [8,15]. In fact GLP-1 was administered several hours after reperfusion, *i.e.* far from the therapeutic window for *reperfusion injury* [8,15].

Although GLP-2 has not been tested before in cardiac I/R scenario, it was shown to exhibit promising beneficial effects in several experimental models of intestinal atrophy and injury [7,23,35], including experimental intestinal ischemia [13,51]. The GLP-2 synthetic analogue, *teduglutide* ([gly2]-hGLP-2), is currently being developed as a potential therapeutic agent for patients with intestinal diseases [22]. All these data on the beneficial effects of GLP-2, together with the striking cardioprotective effect of GLP-2 here reported for the first time, provide new insights into the possible therapeutic potential for GLP-2 agonists, which might represent a new class of drugs to be tested for reducing cardiac I/R injury. Of course future studies might ascertain the cardioprotective role of GLP-2 in the presence of comorbidities. Moreover alternative pathway(s), such as SAFE (Survivor Activating Factor Enhancement), and species differences must be taken in account [3,14,15,18,19,42-44].

In conclusion, GLP-2 reduces myocardial *reperfusion injury*, decreasing infarct size and improving post-ischemic cardiac function. It seems that in this rodent model both survival kinases and mitoK_{ATP} channels play important role in these cardioprotective effects.

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Disclosures: None

Figure legends

Fig. 1. Experimental design.

A. Cardiac function and Infarct size studies: Time-lines and protocols for all the experimental groups. After stabilization (40 min), all the hearts underwent 30-min normothermic and global ischemia followed by 120-min reperfusion, here represented with a sequence of white/black/white horizontal bars. Glucagon-like peptide 2 (GLP-2) was infused during the initial 20-min of reperfusion (gray bar). Wortmannin (WT) is a PI3K inhibitor, PD98059 (PD) is an ERK1/2 inhibitor; 5-Hydroxydecanoate (5HD) is a mitoK_{ATP} channels blocker. Antagonists (PD, WT or 5HD) were infused for 5-min prior ischemia and for 20-min after ischemia as indicated by horizontal black lines.

B. Western blotting studies: Left ventricular samples of Sham groups (77 or 100-min buffer perfusion only), I/R groups, GLP-2 groups and a GLP-2 plus WT groups were considered for Western blot analysis. For further explanation see text.

Fig. 2. Systolic function.

Percent variation of developed LVP (dLVP): data are presented as percent changes at the end of 120-min reperfusion with respect to baseline level for each group. In Panel A are reported I/R, GLP-2 and GLP-2 plus inhibitors groups. In panel B are reported inhibitor-treated controls and for comparative purposes again I/R and GLP-2 groups. *p<0.01 with respect to I/R and each antagonist group.

Fig. 3. Diastolic function.

Left ventricular end-diastolic pressure (LVEDP): data are expressed in mmHg at the end of 120-min reperfusion. In Panel A are reported I/R, GLP-2 and GLP-2 plus inhibitors groups. In panel B are reported inhibitor-treated controls and for comparative purposes again I/R and GLP-2 groups. **p<0.001 with respect to I/R and each antagonist group.

Fig. 4. Infarct size.

The amount of necrotic tissue measured after 30-min global ischemia and 120-min reperfusion is expressed as percent of the left ventricle (% IS/LV). In Panel A are reported I/R, GLP-2 and GLP-2 plus inhibitors groups. In panel B are reported inhibitor-treated controls and for comparative purposes again I/R and GLP-2 groups. **p<0.001 with respect to I/R and each antagonist group.

Fig. 5. Western blot analysis for RISK pathway at the 7th min of reperfusion.

Representative Western blots and relative densitometry showing that GLP-2 given in early reperfusion results in an increase phosphorylation of Akt (Panel A), GSK3 β (Panel B) and ERK1/2 (Panel C), with respect to I/R group. Wortmannin (WT) limits the phosphorylation of Akt, GSK3 β , but not that of ERK1/2 (p = not significant between GLP-2 and GLP-2+WT). Total kinases and phospho-kinases for each heart are normalized to vinculin, and data are presented as the ratio of phospho to total kinases. Values are mean \pm SEM. * p< 0.05, ** p<0.01 with respect to I/R; # p< 0.05 with respect to Sham; § p< 0.05 with respect to GLP-2+WT.

Fig. 6. Western blot analysis for RISK pathway at the 30th min of reperfusion.

Representative Western blots and relative densitometry showing that GLP-2 given in early reperfusion results in the phosphorylation of Akt, GSK3 β , and ERK1/2. GLP-2 induces an

increase in phospho-kinases. These phosphorylations are limited by Wortmannin (WT). Total kinases and phospho-kinases for each heart are normalized to vinculin, and data are presented as the ratio of phospho to total kinases. Values are mean \pm SEM. * $p < 0.05$ with respect to I/R; # $p < 0.05$ with respect to Sham; § $p < 0.05$ with respect to GLP-2+WT. Please note that in Panel C the data of the GLP-2 are in the last rather than in the penultimate line.

References

1. Angelone T, Filice E, Quintieri AM, Imbrogno S, Amodio N, Pasqua T, Pellegrino D, Mulè F, Cerra MC. (2010) Receptor identification and physiological characterization of glucagon-like peptide-2 in the rat heart. *Nutr Metab Cardiovasc Dis* doi:10.1016/j.numecd.2010.07.014
2. Ban K, Kim KH, Cho CK, Sauvé M, Diamandis EP, Backx PH, Drucker DJ, Husain M (2010) Glucagon-like peptide (GLP)-1(9-36)amide-mediated cytoprotection is blocked by exendin(9-39) yet does not require the known GLP-1 receptor. *Endocrinology* 151:1520-1531
3. Boengler K, Hilfiker-Kleiner D, Heusch G, Schulz R (2010) Inhibition of permeability transition pore opening by mitochondrial STAT3 and its role in myocardial ischemia/reperfusion. *Basic Res Cardiol* 105: 771-785 doi: 10.1007/s00395-010-0124-1
4. Bose AK, Mocanu MM, Carr RD, Yellon DM (2005) Glucagon like peptide-1 is protective against myocardial ischemia/reperfusion injury when given either as a preconditioning mimetic or at reperfusion in an isolated rat heart model. *Cardiovasc Drugs Ther* 19:9-11 DOI: 10.1007/s10557-007-6030-6
5. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 7:248–254
6. Cerra MC, De Iuri L, Angelone T, Corti A, Tota B (2006) Recombinant N-terminal fragments of Chromogranin-A modulate cardiac function of the Langendorff-perfused rat heart. *Basic Res Cardiol* 100:1-10 DOI: 10.1007/s00395-005-0547-2
7. Chance WT, Foley-Nelson T, Thomas I, Balasubramaniam A (1997) Prevention of parenteral nutrition-induced gut hypoplasia by coinfusion of glucagon-like peptide-2. *Am J Physiol* 273:G559- G563
8. Cohen MV, Downey JM (2011) Ischemic postconditioning: from receptor to end-effector. *Antioxid Redox Signal* 14: 821–831 doi:10.1089/ars.2010.3318.
9. Darling CE, Jiang R, Maynard M, Whittaker P, Vinten-Johansen J, Przyklenk K (2005) Postconditioning via stuttering reperfusion limits myocardial infarct size in rabbit hearts: role of ERK1/2. *Am J Physiol Heart Circ Physiol* 289:H1618- H1626 doi: 10.1152/ajpheart.00055.2005
10. Drucker DJ (1998) Glucagon-like peptides. *Diabetes* 47:159–169 doi: 10.1210/en.142.2.521
11. Dube PE, Brubaker PL (2007) Frontiers in glucagon-like peptide-2: multiple actions, multiple mediators. *Am J Physiol Endocrinol Metab* 293: E460-E465 doi: 10.1152/ajpendo.00149.2007

12. Gomez L, Paillard M, Thibault H, Derumeaux G, Ovize M (2008) Inhibition of GSK3beta by postconditioning is required to prevent opening of the mitochondrial permeability transition pore during reperfusion. *Circulation* 117:2761-2768 doi: 10.1161/CIRCULATIONAHA.107.755066
13. Guan L, Gong D, Tian N, Zou Y (2005) Uncoupling protein 2 involved in protection of glucagon-like peptide 2 in small intestine with ischemia-reperfusion injury in mice. *Dig Dis Sci* 50:554-60 doi: 10.1007/s10620-005-2474-3
14. Hausenloy DJ, Baxter G, Bell R, Bøtker HE, Davidson SM, Downey J, Heusch G, Kitakaze M, Lecour S, Mentzer R, Mocanu MM, Ovize M, Schulz R, Shannon R, Walker M, Walkinshaw G, Yellon DM (2010) Translating novel strategies for cardioprotection: the Hatter Workshop Recommendations. *Basic Res Cardiol*. 105:677-686 doi 10.1007/s00395-010-0121-4
15. Hausenloy DJ, Lecour S, Yellon DM (2011) Reperfusion Injury Salvage Kinase and Survivor Activating Factor Enhancement prosurvival signaling pathways in ischaemic postconditioning: two sides of the same coin. *Antioxid Redox Signal* 14: 893–907 doi:10.1089/ars.2010.3360.
16. Hausenloy DJ, Tsang A, Mocanu MM, Yellon DM (2005) Ischemic preconditioning protects by activating prosurvival kinases at reperfusion. *Am J Physiol Heart Circ Physiol* 288:H971-H976 , doi: 10.1152/ajpheart.00374.2004
17. Hausenloy DJ, Yellon DM (2004) New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. *Cardiovasc Res* 61:448-460. doi:10.1016/j.cardiores.2003.09.024,
18. Heusch G (2009) No risk, no ... cardioprotection? A critical perspective. *Cardiovasc Res* 84:173-175 doi: 10.1093/cvr/cvp298
19. Heusch G, Musiolik J, Gedik N, Skyschally A (2011) Mitochondrial STAT3 activation and cardioprotection by ischemic postconditioning in pigs with regional myocardial ischemia/reperfusion. *Circ Res* 109: 1302-1308 doi: 10.1161/CIRCRESAHA.111.255604
20. Hönsch A, Theuring N, Ebner B, Wagner C, Strasser RH, Weinbrenner C (2010) Postconditioning with levosimendan reduces the infarct size involving the PI3K pathway and KATP-channel activation but is independent of PDE-III inhibition. *Basic Res Cardiol* 105: 155-167 doi: 10.1007/s00395-009-0064-9

21. Hoosein NM, Gurd RS. (1984) Human glucagon-like peptides 1 and 2 activate rat brain adenylate cyclase
FEBS Lett 178:83–86 doi:10.1016/0014-5793(84)81245-4
22. Jeppesen PB, Sanguinetti EL, Buchman A, Howard L, Scolapio JS, Ziegler TR, Gregory J, Tappenden KA, Holst J, Mortensen PB. (2005) Teduglutide (ALX-0600), a dipeptidyl peptidase IV resistant glucagon-like peptide 2 analogue, improves intestinal function in short bowel syndrome patients. Gut 54:1224-1231 doi: 10.1136/gut.2004.061440
23. L'Heureux MC, Brubaker PL (2003) Glucagon-like peptide-2 and common therapeutics in a murine model of ulcerative colitis. J Pharmacol Exp Ther 306:347-354 doi: 10.1124/jpet.103.051771
24. Lovshin J, Estall J, Yusta B, Brown TJ, Drucker DJ (2001) Glucagon-like Peptide (GLP)-2 Action in the Murine Central Nervous System Is Enhanced by Elimination of GLP-1 Receptor Signaling. J Biol Chem 276:21489-21499 doi: 10.1074/jbc.M009382200
25. Lund A, Vilsbøll T, Bagger JJ, Holst JJ, Knop FK. (2011) The separate and combined impact of the intestinal hormones, GIP, GLP-1, and GLP-2, on glucagon secretion in type 2 diabetes. Am J Physiol Endocrinol Metab 300:E1038-E1046 doi: 10.1152/ajpendo.00665.2010
26. Matsubara M, Kanemoto S, Leshnower BG, Albone EF, Hinmon R, Plappert T, Gorman JH 3rd, Gorman RC. (2011) Single dose GLP-1-Tf ameliorates myocardial ischemia/reperfusion injury. J Surg Res 165:38-45 doi:10.1016/j.jss.2009.03.016
27. Munroe DG, Gupta AK, Kooshesh F, Vyas TB, Rizkalla G, Wang H, Demchyshyn L, Yang ZJ, Kamboj RK, Chen H, McCallum K, Sumner-Smith M, Drucker DJ, Crivici A (1999) Prototypic G proteincoupled receptor for the intestinotrophic factor glucagon-like peptide 2. Proc Natl Acad Sci U S A 96:1569–1573 doi: 10.1073/pnas.96.4.1569
28. Nikolaidis LA, Mankad S, Sokos GG, Miske G, Shah A, Elahi D, Shannon RP. (2004) Effects of glucagon-like peptide-1 in patients with acute myocardial infarction and left ventricular dysfunction after successful reperfusion. Circulation 109:962-965 doi: 10.1161/01.CIR.0000120505.91348.58
29. Pagliaro P, Mancardi D, Rastaldo R, Penna C, Gattullo D, Miranda KM, Feelisch M, Wink DA, Kass DA, Paolucci N. (2003) Nitroxyl affords thiol-sensitive myocardial protective effects akin to early preconditioning. Free Radic Biol Med 34:33-43 doi:10.1016/S0891-5849(02)01179-6

30. Penna C, Alloatti G, Cappello S, Gattullo D, Berta G, Mognetti B, Losano G, Pagliaro P. (2005) Platelet-activating factor induces cardioprotection in isolated rat heart akin to ischaemic preconditioning: role of phosphoinositide 3-kinase and protein kinase C activation. *Am J Physiol Heart Circ Physiol* 288:H2512-2520 doi: 10.1152/ajpheart.00599.2004
31. Penna C, Mancardi D, Raimondo S, Geuna S, Pagliaro P (2008) The paradigm of postconditioning to protect the heart. *J Cell Mol Med* 12:435-458 doi: 10.1111/j.1582-4934.2007.00210.x
32. Penna C, Mancardi D, Rastaldo R, Losano G, Pagliaro P (2007) Intermittent activation of bradykinin B2 receptors and mitochondrial KATP channels trigger cardiac postconditioning through redox signaling. *Cardiovasc Res* 75:168-177 doi: 10.1016/j.cardiores.2007.03.001
33. Penna C, Perrelli MG, Raimondo S, Tullio F, Merlino A, Moro F, Geuna S, Mancardi D, Pagliaro P. (2009) Postconditioning induces an anti-apoptotic effect and preserves mitochondrial integrity in isolated rat hearts. *Biochim Biophys Acta* 1787:794-780 doi:10.1016/j.bbabbio.2009.03.013
34. Penna C, Rastaldo R, Mancardi D, Raimondo S, Cappello S, Gattullo D, Losano G, Pagliaro P. (2006) Post-conditioning induced cardioprotection requires signaling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K⁺ channel and protein kinase C activation. *Basic Res Cardiol* 101:180-189 doi: 10.1007/s00395-006-0584-5
35. Perez A, Duxbury M, Rocha FG, Ramsanahie AP, Farivar RS, Varnholt H, Ito H, Wong H, Rounds J, Zinner MJ, Whang EE, Ashley SW. (2005) Glucagon-like peptide 2 is an endogenous mediator of postresection intestinal adaptation. *J Parenter Enteral Nutr* 29:97-101 doi: 10.1177/014860710502900297
36. Read PA, Hoole SP, White PA, Khan FZ, O'Sullivan M, West NE, Dutka DP. (2011) A pilot study to assess whether glucagon-like Peptide-1 protects the heart from ischemic dysfunction and attenuates stunning after coronary balloon occlusion in humans. *Circ Cardiovasc Interv* 4:266-272 doi: 10.1161/CIRCINTERVENTIONS.110.960476
37. Schulman D, Latchman DS, Yellon DM (2002) Urocortin protects the heart from reperfusion injury via upregulation of p42/p44 MAPK signaling pathway. *Am J Physiol Heart Circ Physiol* 283:H1481-H1488 doi: 10.1152/ajpheart.01089.2001
38. Schwartz Longacre L, Kloner RA, Arai AE, Baines CP, Bolli R, Braunwald E, Downey J, Gibbons RJ, Gottlieb RA, Heusch G, Jennings RB, Lefer DJ, Mentzer RM, Murphy E, Ovize M, Ping P, Przyklenk K,

- Sack MN, Vander Heide RS, Vinten-Johansen J, Yellon DM (2011). New horizons in cardioprotection: recommendations from the 2010 national heart, lung, and blood institute workshop. *Circulation* 124:1172-1179 doi: 10.1161/CIRCULATIONAHA.111.032698
39. Shi X, Li X, Wang Y, Zhang K, Zhou F, Chan L, Li D, Guan X. (2011) Glucagon-like peptide-2-stimulated protein synthesis through the PI 3Kinase-dependent Akt-mTOR signaling pathway. *Am J Physiol Endocrinol Metab* 300:E554-E563 doi: 10.1152/ajpendo.00620.2010
 40. Simpkin JC, Yellon DM, Davidson SM, Lim SY, Wynne AM, Smith CC (2007) Apelin-13 and apelin-36 exhibit direct cardioprotective activity against ischemia-reperfusion injury. *Basic Res Cardiol* 102:518-528 doi: 10.1007/s00395-007-0671-2
 41. Sivaraman V, Mudalagiri NR, Di Salvo C, Kolvekar S, Hayward M, Yap J, Keogh B, Hausenloy DJ, Yellon DM (2007) Postconditioning protects human atrial muscle through the activation of the RISK pathway. *Basic Res Cardiol* 102:453-459 doi:10.1007/s00395-007-0664-1;
 42. Skyschally A, van Caster P, Boengler K, Gres P, Musiolik J, Schilawa D, Schulz R, Heusch G (2009) Ischemic postconditioning in pigs: no causal role for RISK activation. *Circ Res* 104: 15-18 doi: 10.1161/CIRCRESAHA.108.186429
 43. Skyschally A, van Caster P, Iliodromitis EK, Schulz R, Kremastinos DT, Heusch G (2009) Ischemic postconditioning: experimental models and protocol algorithms. *Basic Res Cardiol* 104: 469-483 doi: 10.1007/s00395-009-0040-4
 44. Tamarelle S, Mateus V, Ghaboura N, Jeanneteau J, Croué A, Henrion D, Furber A, Prunier F (2009) RISK and SAFE signaling pathway interactions in remote limb ischemic preconditioning in combination with local ischemic postconditioning. *Basic Res Cardiol* 106: 1329-1339 doi: 10.1007/s00395-011-0210-z
 45. Tang-Christensen M, Larsen PJ, Thulesen J, Romer J, Vrang N (2000) The proglucagon-derived peptide, glucagon-like peptide-2, is a neurotransmitter involved in the regulation of food intake. *Nat Med* 6:802–807 doi:10.1038/77535
 46. Tsang A, Hausenloy D, Mocanu M, Yellon D (2004) Postconditioning: a form of “Modified reperfusion” protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. *Circ Res* 95:230-232 doi:10.1161/01.RES.0000138303.76488.fe

47. Vinten-Johansen J, Zhao ZQ, Zatta AJ, Kin H, Halkos ME, Kerendi F (2005) Postconditioning--A new link in nature's armor against myocardial ischemia-reperfusion injury. *Basic Res Cardiol.* 100:295-310 doi: 10.1007/s00395-005-0523-x
48. Yang XM, Philipp S, Downey JM, Cohen MV (2005) Postconditioning's protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. *Basic Res Cardiol* 100:57-63 doi: 10.1007/s00395-004-0498-4
49. Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV (2004) Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. *J Am Coll Cardiol* 44:1103-1110 doi:10.1016/j.jacc.2004.05.060
50. Ye Y, Perez-Polo JR, Aguilar D, Birnbaum Y (2011) The potential effects of anti-diabetic medications on myocardial ischemia-reperfusion injury. *Basic Res Cardiol* 106:925-952 doi: 10.1007/s00395-011-0216-6
51. Zhang W, Zhu W, Zhang J, Li N, Li J (2008) Protective effects of glucagon-like peptide 2 on intestinal ischemia-reperfusion rats. *Microsurgery* 28:285-290 doi: 10.1002/micr.20491

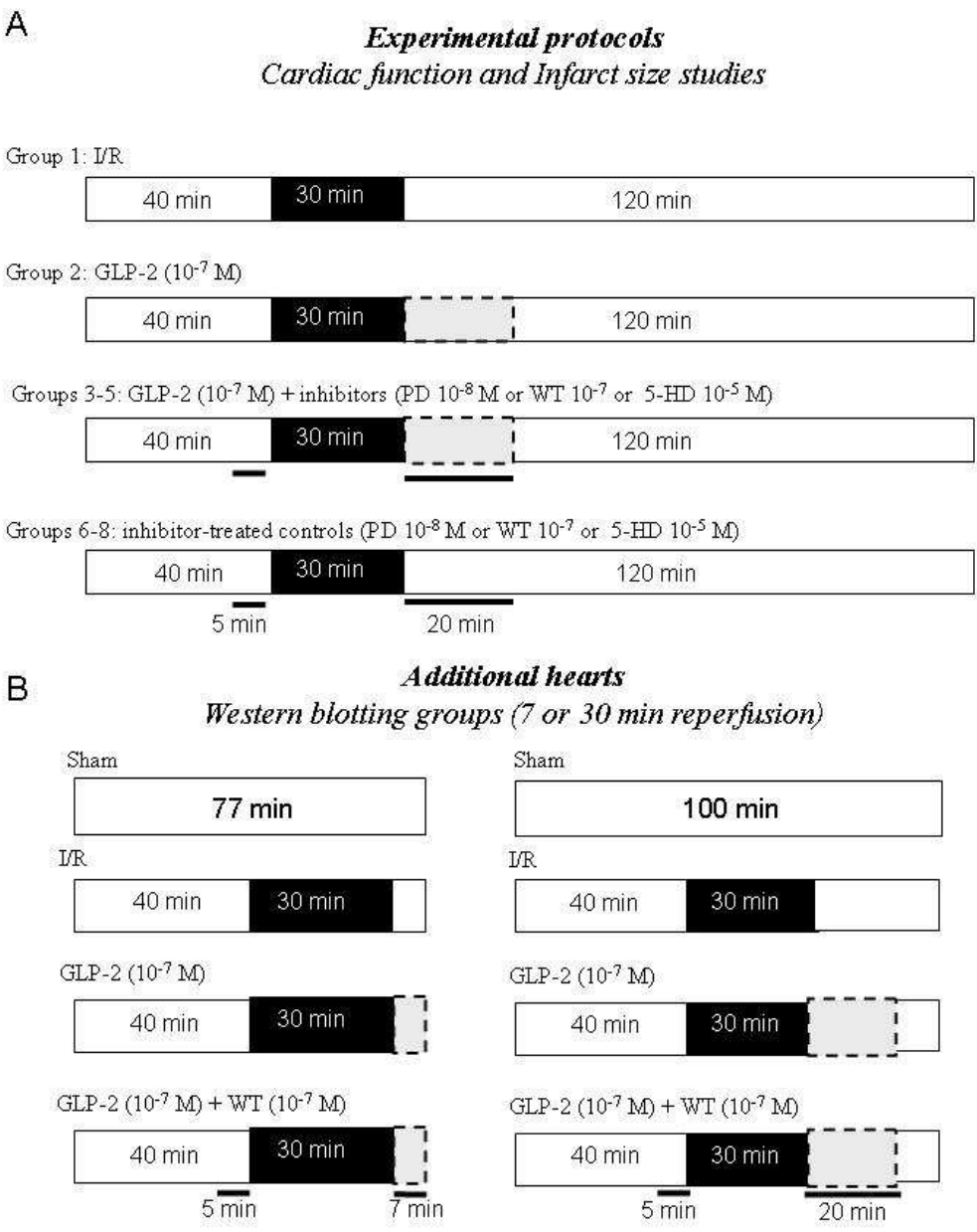


Fig.1

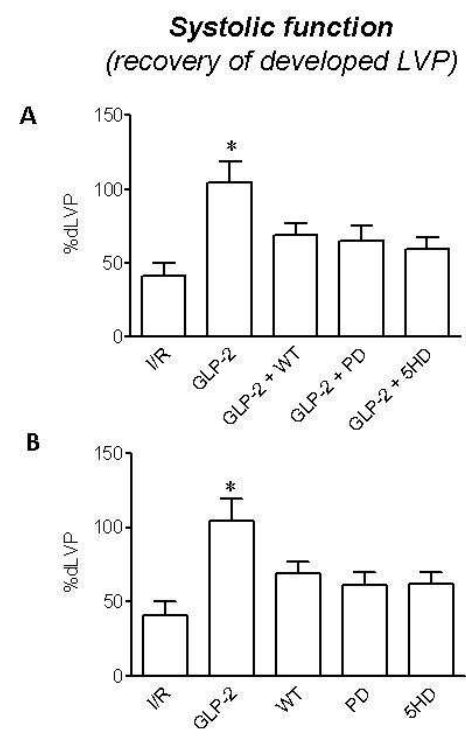


Fig.2

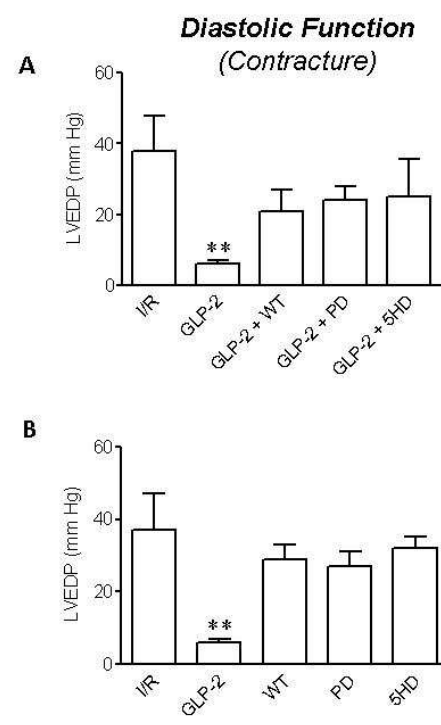


Fig.3

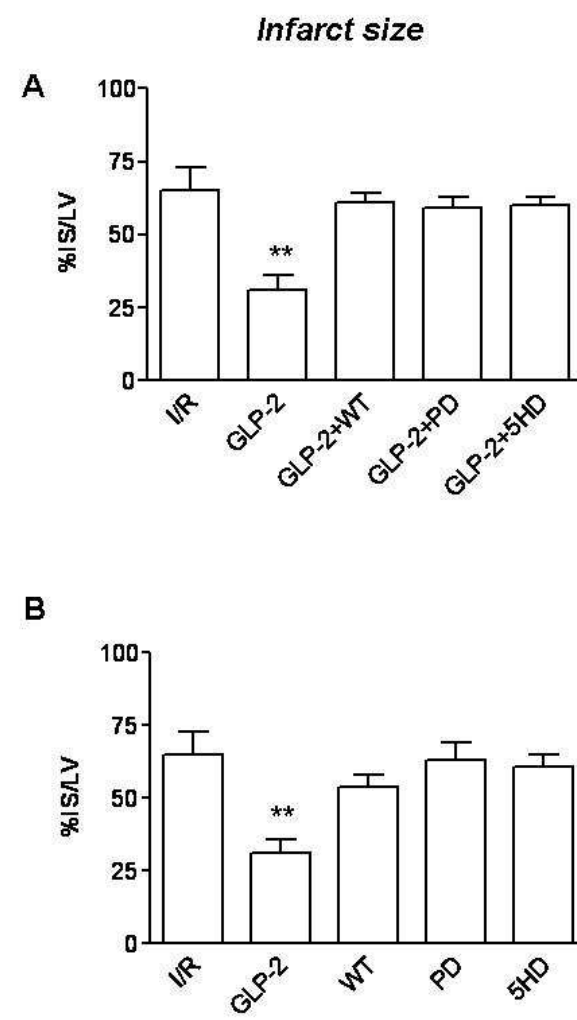


Fig.4

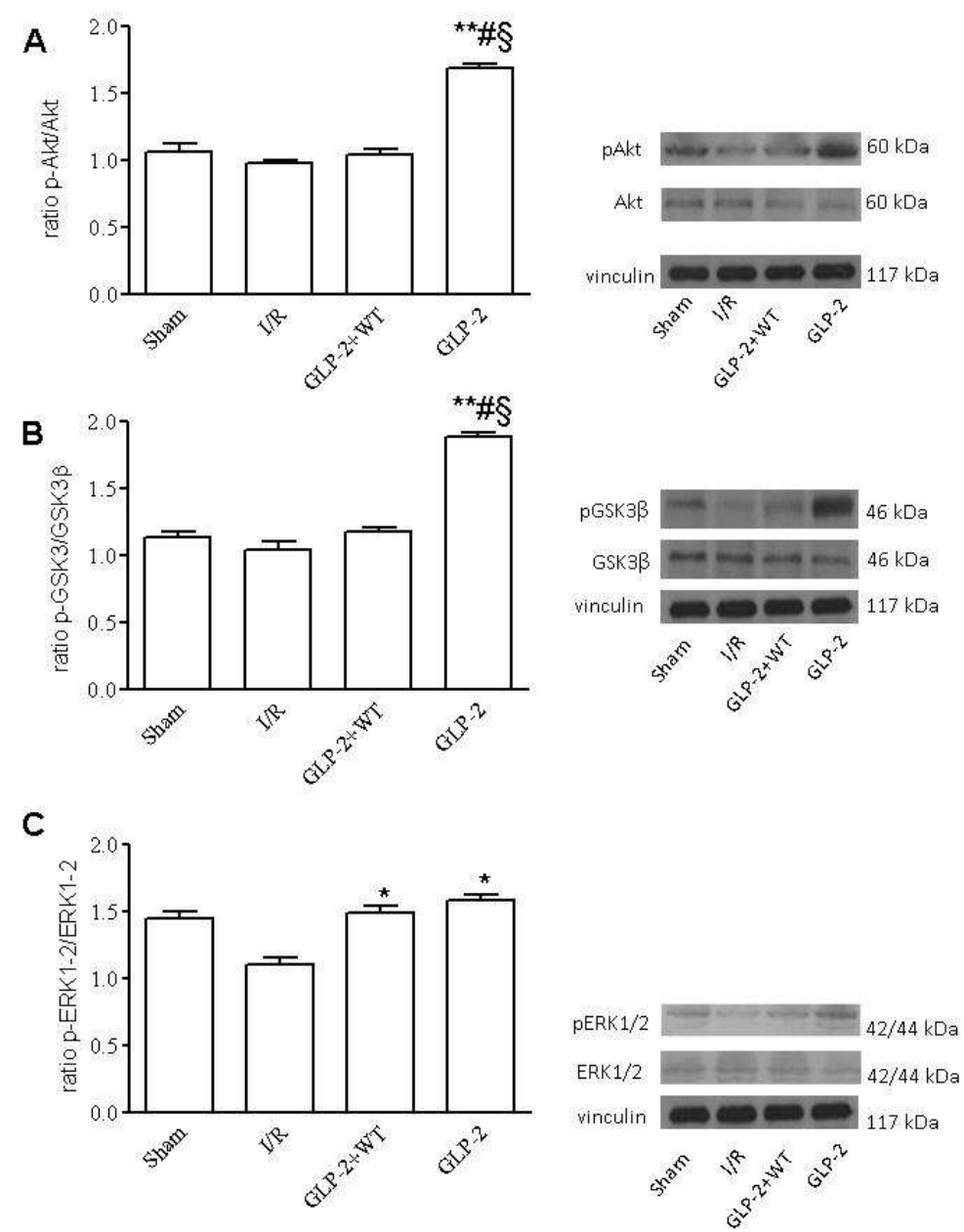


Fig.5

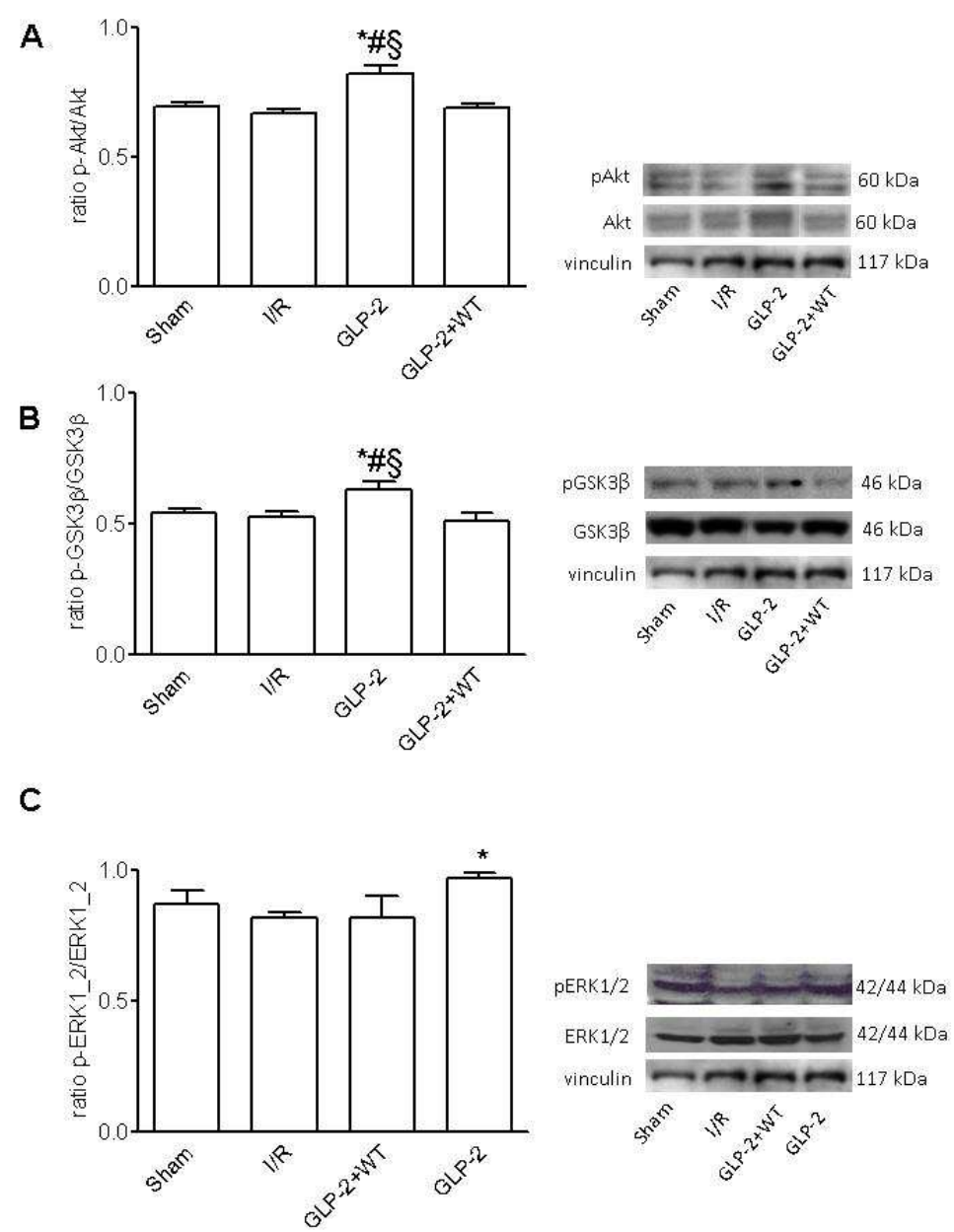


Fig.6