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Plasma exosomes protect the myocardium from ischemia-reperfusion injury

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Abstract (258 words)

Background. Exosomes are nanometer-sized vesicles released from cells into the blood, where they can transmit signals throughout the body. Exosomes have been shown to act on the heart, but their composition and the signaling pathways that they activate have not been explored. We hypothesized that endogenous plasma exosomes can communicate signals to the heart and provide protection against ischemia and reperfusion injury.

Objectives. I) To isolate and characterize exosomes from rats and healthy volunteers. II) To evaluate the cardioprotective actions of plasma exosomes and to identify the molecular mechanisms involved.

Methods: The exosome-rich fraction was isolated from the blood of adult rats and human volunteers and analyzed by protein marker expression, transmission electron microscopy and nanoparticle tracking analysis. This was then used in *ex vivo, in vivo* and *in vitro* settings of ischemia-reperfusion and the protective signaling pathways activated on cardiomyocytes were identified using Western blot analyses and chemical inhibitors.

Results: Exosomes exhibited the expected size, and expressed marker proteins CD63, CD81 and HSP70. The exosome-rich fraction was powerfully cardioprotective in all tested models of cardiac ischemia-reperfusion injury. We identified a pro-survival signaling pathway activated in cardiomyocytes involving toll-like receptor 4, ERK1/2 and p38 MAPK, leading to activation of the cardioprotective protein HSP27. Cardioprotection was prevented by a neutralizing antibody against a conserved HSP70 epitope expressed on the exosome surface and by blocking TLR4 in cardiomyocytes, identifying the HSP70/TLR4 communication axis as a critical component in exosome-mediated cardioprotection.

Conclusion. Exosomes deliver endogenous protective signals to the myocardium by a pathway involving TLR4 and classic cardioprotective HSPs.

Keywords: Exosomes, ischemia, reperfusion, TLR4, HSP70, HSP27

Abbreviations: Heat Shock Protein (HSP), micro RNA (miRNA), Nanoparticle Tracking Analysis (NTA), Remote Ischaemic Preconditioning (RIC), Toll Like Receptor (TLR), transmission electron microscopy (TEM)

Introduction

In recent years, immense interest has developed regarding the potential for extracellular vesicles to be used as therapeutic agents or as biomarkers of pathological states such as cardiovascular disease or cancer (1,2). In particular, exosomes have been proposed to stimulate beneficial signaling pathways in cardiovascular disease (1), for example, potentially mediating the pro-angiogenic actions of human stem cells (3). Exosomes can ferry miRNAs and proteins through the bloodstream and represent a potential mode of intercellular communication throughout the entire circulatory system (4). They are only 50-100 nm in diameter, which renders them invisible to standard flow cytometry techniques, and necessitates ultracentrifugation for their purification.

Therapeutically, exosomes appear to mediate many of the beneficial properties of stem cells administered to the heart (5,6). These studies have mainly focused on the role of exogenous exosomes administered to the heart, but not on endogenous exosomes, which are present in the blood of humans and rodents in the order of 10^{10} exosomes per ml (7). These striking numbers raise a crucial question as to their role.

Obstruction of a coronary artery results in myocardial ischemia. The mainstay of clinical treatment is myocardial reperfusion, although this paradoxically causes further lethal injury —called myocardial reperfusion injury— for which there is, as yet, no effective clinical therapy (8). Heat shock proteins (HSPs) are powerfully cardioprotective (9-11), but clinical translation unfortunately faltered due to lack of practical techniques for their induction or delivery. After myocardial infarction, large elevations in serum HSP70 can stimulate inflammatory cytokine release via toll-like receptor 4 (TLR4) and the innate immune response (12,13). However, more mild stimulation of innate immunity via TLR ligands is necessary for myocardial preconditioning and cardioprotection (14,15). This raises the possibility that delivery of moderate levels of HSP70 could be beneficial. In addition to these effects of circulating HSP70, other families of HSP can mediate protection within the cell. For example, the small HSP27 (HSPB1), is required for optimal protection against ischemia and reperfusion injury (16,17).

We hypothesized that endogenous exosomes communicate signals to the heart to protect it against ischemia and reperfusion injury. Furthermore, since recent evidence suggests that microvesicles can transfer protection by remote ischaemic preconditioning (RIC) (18), we hypothesized that RIC would augment exosome production, and thereby stimulate cardioprotection. We purified, characterized, and quantified exosomes from the plasma of rats and humans, and showed that they protect rat cardiomyocytes or hearts subject to ischemia and reperfusion both *in vitro* and *in vivo*. Cardioprotection was found to be

mediated by HSP70 on exosomes, which activated a pathway downstream of TLR4 involving the kinases ERK1/2 and p38MAPK, and led to phosphorylation of HSP27.

Materials and Methods

Animals were treated in accordance with the Animals (Scientific Procedures) act 1986 published by the UK Home Office and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996). The study of human samples was performed according to ethics approval reference 13/LO/0222 and Declaration of Helsinki principles. Experimental details are provided in the Online Appendix.

Results

Characterization and quantification of plasma exosomes

The exosome-rich fraction was purified from the blood of adult male rats and healthy human male volunteers using a standard protocol of serial, differential centrifugation and ultracentrifugation steps. Using transmission electron microscopy (TEM) we observed the typical “cup-shaped” vesicles of exosomes that were <100 nm diameter for both rats and humans (Figure 1A).

To characterize and quantify exosomes, we used the state-of-the-art technique of nanoparticle tracking analysis (NTA), which measures the number and size distribution of particles in purified, exosome-rich preparations. By NTA, the modal size of particles purified from control rat plasma was 75 ± 2 nm (Figure 1B, D), corresponding to the expected size of exosomes and the concentration was $0.1 \pm 0.02 \times 10^{11}$ ml^{-1} plasma (n=5 rats)(Figure 1C). The concentration of particles in the plasma of human volunteers was $6 \pm 3 \times 10^{11}$ ml^{-1} (n=6)(Figure 1C), and their average modal size was 75 ± 7 nm (Figure 1D). For simplicity, we refer hereafter to the isolated particles as “exosomes”, although it should be noted that the isolated fraction also included some particles that were outside the expected size-range for exosomes.

Next, we used flow cytometry to confirm the expression of marker proteins for exosomes in the human samples. The tetraspanin molecules CD63 and CD81 (found in many exosomes), as well as Heat Shock Protein 70 (HSP70), were all detectable at high levels (Figure 1E). Isotype control antibodies were negative (Supplementary Figure 1). Interestingly, the positive signal obtained using antibody clone cmHSP70.1, which specifically recognizes an epitope of HSP70 expressed on the surface of cells and exosomes (19,20), suggested that HSP70 is exposed on the surface of human exosomes (Figure 1E).

We also performed SDS-PAGE and Western blot analyses to biochemically characterize the human samples, and were able to detect CD63, CD81 and, using either the antibody N27 or cmHsp70.1, HSP70 (Figure 1F). Similar results were obtained using rat exosomes (Supplementary Figure 2).

Plasma exosomes are cardioprotective

In order to investigate whether plasma exosomes are cardioprotective, we used four different experimental models. First, we used an *in vivo* rat model in which the entire exosome fraction isolated from a donor rat was administered to a naïve rat by i.v. tail vein injection, 15 min prior to LAD occlusion. The area at risk was the same in each group (Supplementary

Figure 3A). The infarct size relative to the area at risk was reduced from 48 ± 5% (n=6) with vehicle, to 25 ± 6% (P<0.05, n=6), with exosomes (Figure 2A). Secondly, to exclude the influence of blood, Langendorff perfused rat hearts were exposed to all of the exosomes isolated from the blood of a donor rat during 15 min perfusion, before being subject to 35 min ischemia followed by 2 h reperfusion. The area at risk was the same in each group (Supplementary Figure 3B). The size of the infarct relative to the area at risk was significantly reduced from 35 ± 3% (n=6), to 22 ± 2% (P<0.01, n=10), by pre-treatment with rat exosomes (Figure 2B).

Next, we used two *in vitro* cell models of cardiac cells to conduct simulated ischemia-reperfusion, termed hypoxia-reoxygenation. Using the HL-1 cardiomyocyte cell line, and flow cytometry we analyzed cell death using double staining with the vital dye propidium iodide (PI) and the mitochondrial transmembrane potential ($\Delta\Psi_m$)-sensitive dye $DiOC_6$(3). After hypoxia-reoxygenation, 33 ± 8% of the live cells exhibited a low $\Delta\Psi_m$ – an early indicator of cell death – compared to 6 ± 4% in normoxia. The population of dead HL-1 cells was 45 ± 9%, detected using PI, compared to 13 ± 5% in normoxia (Figure 2C). Pretreatment with rat exosomes significantly increased healthy cells from 20 ± 5% to 44 ± 7%, and reduced the population of cells with low $\Delta\Psi_m$ (24 ± 5%) as well as the PI^{+ve} population of dead cells (30 ± 6%) (Figure 2C). Protection was similar to that observed with insulin (27 ± 5% low $\Delta\Psi_m$, 31 ± 5% PI^{+ve}), which is known to be protective in this model and was used as a positive control. These *in vitro* results were confirmed using primary adult rat cardiomyocytes as a more physiological model of cardiac cells, and in a dose response analysis. Hypoxia and re-oxygenation increased cardiomyocyte cell death from 13 ± 3% to 59 ± 7% (n=5, P<0.001)(Figure 2D). The maximal tested dose of 10^9 exosomes per well was highly cardioprotective, reducing cell death to 17 ± 3%, similar to the effect of insulin, used as a positive control (14 ± 3% cell death) (Figure 2D). Significant protection remained evident down to a dose of 10^7 exosomes per well, at which dose control exosomes decreased death to 32 ± 8% (Figure 2D).

Does manipulation of exosome concentration affect cardioprotection?

If exosomes can activate cardioprotective pathways, this raises the possibility that manipulating their levels in the blood would have an impact on cardioprotection. As yet, no specific pharmacological inhibitors have been developed which can be used *in vivo* to decrease exosome production. However, it has recently been proposed that brief cycles of hind limb ischemia and reperfusion increase the number of extracellular vesicles (both exosomes and microvesicles) in the circulation (18). This manipulation, known as remote ischemic preconditioning or RIC, also renders the heart in a protected state (21,22).

We first examined whether RIC has any effect on plasma exosomes in humans subject to RIC. Exosomes were isolated from the same healthy volunteers both before and after a standard RIC procedure and characterized by NTA and TEM. RIC exosomes exhibited the same size and shape as control exosomes (Figures 1G-J), but importantly, their concentration in plasma was significantly increased after RIC (Figure 1H). Exosome concentration was similarly increased in rats after RIC, by both NTA and TEM (Supplementary Figure 4A).

The ability of exosomes from RIC-treated rats to protect the heart was then determined. We observed that administration of RIC exosomes significantly decreased infarct size, both *in vivo* (Figure 2A) and *in vitro* (Figure 2B). We performed a complete dose-response curve comparing exosomes from control and RIC rats, using the *in vitro* model of hypoxia-reoxygenation on primary cardiomyocytes. However, while there was a slight increase in protection with RIC exosomes, the difference was non-significant (Figure 2D). This suggested that, while exosomes after RIC remain cardioprotective and their numbers increase, this increase is insufficient to have a substantial effect on cardioprotection in this model.

Exosomes trigger ERK and HSP27 activation in cardiomyocytes

Next we studied the intracellular signaling pathways activated by exosomes on cardiac cells using Western blot analysis. The addition of rat exosomes to primary adult rat cardiomyocytes rapidly stimulated cardioprotective kinases. Phosphorylation of ERK1/2 was elevated by 2 min and maximal after 5 min exosome exposure, decreasing by 15 min (Figure 3A). However, no increase in AKT phosphorylation was observed after exosome treatment, (Figure 3A), despite its phosphorylation by insulin. We conducted similar experiments with human exosomes and observed a very similar time-course of ERK1/2 phosphorylation with maximal phosphorylation after 5 min (Figure 3B). Again, no phosphorylation of AKT was detected in response to exosomes at any of the time-points, in contrast to the dual activation of ERK and AKT caused by insulin (Figure 3B). These results suggest that exosomes do not activate insulin receptor signaling. To further validate our results, we used human exosomes to stimulate HL-1 cardiomyocytes and once more, observed phosphorylation of ERK1/2 after 5 min, without any effect on AKT (Supplementary Figure 5). The activation of ERK1/2 was tested in response to exosomes purified from four different human volunteers, and in each case, exosomes significantly stimulated ERK1/2 phosphorylation, by an average of 6.5 ± 1.0 fold ($P<0.05$)(Figure 3C).

To unequivocally confirm that ERK1/2 signaling was involved in the cardioprotection conferred by exosome treatment, cells were pre-incubated with 10 μM U0126 or 50 μM

PD98059, inhibitors of ERK1/2 signaling at the level of MEK. Both drugs completely inhibited ERK1/2 phosphorylation in response to human exosomes (Figure 3D), as well as eliminating cardioprotection in adult rat cardiomyocytes treated with human exosomes (Figure 3E). In control experiments the inhibitors alone had no effect (Supplementary Figure 6).

One of the classic and highly cardioprotective effector proteins is the small HSP27 (16), regulated by phosphorylation at several serine residues. We tested a panel of antibodies recognizing these phospho-serine epitopes. Exposure of cardiomyocytes to human exosomes increased phosphorylation of HSP27 at all 3 sites relative to total levels of HSP27, or when measured relative to a housekeeping protein (Figure 4A). The clearest response was observed with phosphorylation of Ser^{78}; therefore, this phosphorylation site was used in subsequent studies to define the signaling pathway leading to HSP27 phosphorylation.

HSP70 on the surface of exosomes mediates cardioprotection via TLR4/ERK/HSP27

Given the rapid activation of ERK1/2 and HSP27 we suspected that a protein carried by the exosomes was interacting directly with a cell-surface receptor. However, in some cell types exosomes fuse with the membrane and deliver their contents intracellularly. In experiments using exosomes labelled with the fluorescent dye PKH67, no rat exosomes were taken up by primary adult rat cardiomyocytes even after 24 h exposure, despite them being taken up and visible intracellularly in 24 ± 7% of HUVEC cells after 1 h exposure (Supplementary Figure 7). This suggested a direct interaction between an exosomal ligand and a cell surface receptor in cardiac myocytes.

Extracellular HSP70 is considered to be a danger associated molecular pattern (DAMP), which can activate immune responses via binding to TLR4 (13,23). Our flow cytometry experiments had suggested that HSP70 is present on the surface of exosomes. This was confirmed by immuno-gold labeling using cmHsp70.1 and TEM of rat and human exosomes (Figure 4B). To neutralize this exosomal HSP70 we pre-incubated human exosomes with cmHSP70.1. This decreased their ability to stimulate phosphorylation of both ERK1/2 and HSP27-Ser^{78} (Figure 4C). Furthermore, these exosomes were no longer able to protect primary cardiomyocytes against hypoxia and re-oxygenation (Figure 4D). By itself, the neutralizing antibody did not affect cell survival (Figure 4D).

Upstream of HSP27 phosphorylation, one of the most well established activators is the kinase p38MAPK. We used SB203580, an inhibitor of p38MAPKα/β and observed that HSP27 phosphorylation in response to exosomes was blocked, while leaving upstream ERK1/2 phosphorylation unaffected (Figure 5A). As shown above, U0126 blocked ERK1/2

phosphorylation, but also prevented phosphorylation of HSP27 (Figure 5A). These results suggest that ERK1/2 lies upstream of p38MAPK in the pathway.

In order to establish a link between extracellular HSP70 and intracellular ERK1/2 and p38MAPK activation, we used TAK-242 to block intracellular signaling from TLR4. Pre-treatment with TAK-242 decreased ERK1/2 phosphorylation in response to exosomes and blocked the phosphorylation of HSP27 (Figure 5A). Finally, we studied the effect of the whole pathway on the survival of cardiomyocytes subject to hypoxia-reoxygenation. Blocking TLR4, ERK1/2 or p38MAPK decreased the cardioprotective effect of exosome treatment (Figure 5B).

Discussion

Previously considered as extracellular debris, it is only recently that exosomes have begun to be understood as vesicles communicating between cells. Several studies have shown that exosomes from cultured stem cells are cardioprotective (5,6). The present study focused on endogenous plasma exosomes. These were isolated from healthy rats and humans, and their identity confirmed by NTA, electron microscopy and biochemical detection of specific exosomal marker proteins (CD63, CD81 and HSP70). Plasma exosomes were found to be cardioprotective in an acute setting both *in vitro* and *in vivo*. This was mediated by exosomal HSP70 which stimulated TLR4 signaling, leading to the activation of ERK1/2, p38MAPK and subsequent HSP27 phosphorylation in cardiomyocytes (Figure 6).

The origin of exosomes

Plasma exosomes may originate from platelets, endothelium, erythrocytes, lymphocytes or other blood cells, but their origin is difficult to determine experimentally since, in contrast to microvesicles, exosomes do not necessarily express the same marker proteins as their parent cells (24).

The increase in exosome number stimulated by RIC is interesting, but to date there are no tools available to prevent exosome release *in vivo*, therefore rigorous exclusion of the role of exosomes in RIC awaits the development of specific chemical inhibitors or knockout models. Alternatively, several other candidate proteins which may mediate RIC have also been proposed including SDF-1α (25) and Il-10 (26).

The effect of exosomes on cardiomyocytes

Although there is great interest of the potential for exosomes to deliver cargo inside various types of cells, there has been no prior examination of whether mature, adult cardiomyocytes are able to take up plasma exosomes. In fact, we were unable to detect any uptake of fluorescently labelled exosomes into primary cardiomyocytes even after 24 h incubation, despite their rapid uptake into endothelial cells. This, in combination with the rapidity of the signaling response activated by exosomes, and its dependence upon HSP70 on the exosome surface and on TLR4 signaling, strongly suggests that cardioprotection is mediated via direct ligand-receptor interaction on the cell surface. Since exosomes also contain other cargo such as miRNA, it will be interesting to determine whether they are also capable of delivering longer-term benefit via known cardioprotective miRNAs (27).

The protein kinase signaling pathways important for cardioprotection have been well described and include ERK1/2 and/or PI3K/AKT (8). Exosomes did not induce AKT but rapidly activated ERK1/2 phosphorylation by 5 min, and this was required for the induction of cardioprotection. There are a number of potential downstream targets of ERK1/2 including p38MAPK, which phosphorylates HSP27 (28). Inhibition of ERK1/2 with U0126 or of p38MAPK with SB203580 prevented phosphorylation of HSP27 corresponding with the loss of protection. SB203580 is specific to α and β isoforms of p38MAPK, which are also the two isoforms that are protective when transiently activated before ischemia (29).

The link between Heat Shock Proteins and cardioprotection

HSP27 is one of the most well-characterized members of the cardioprotective, small HSP family, and is highly expressed in the myocardium (16). In addition to controlling protein folding, small HSPs exert powerful protection against oxidative stress and apoptosis, and also maintain the structural integrity of the sarcomere (16,17). Strikingly, despite being well-characterized and strongly activated in response to heat stress, no practical method of stimulating HSP27 phosphorylation has been developed and translated to the clinic. Our study shows for the first time that endogenous plasma exosomes are able to activate this HSP.

We previously developed a neutralizing antibody against the highly conserved, exposed C-terminal of HSP70 (19), and demonstrated that it blocks the immunosuppressive signaling pathways stimulated by exosomal HSP70 in myeloid-derived suppressor cells (20). We show here that neutralizing HSP70 on exosomes prevents their protective actions in cardiomyocytes including ERK1/2 and HSP27 phosphorylation. Heat shock proteins, including HSP27 and HSP70, are intracellular chaperones important for correct protein homeostasis. HSP70 is secreted from cells by exosomes (30), and is present in the serum of healthy individuals (31). In addition to the intracellular protective role of HSP70, recognized over 20 years ago, an intriguing new, extracellular role is emerging for HSP70. For instance, it was recently demonstrated that supporting cells in the inner ear can secrete HSP70 to protect hair cells from cell death in response to ototoxic drugs (32). Plasma HSP70 is believed to play a role in the activation of the adaptive and innate immune response (33). Interestingly, circulating HSP70 is elevated in patients with heart failure and can contribute to the disease by stimulating inflammatory cytokine release (13). Although activation of the innate immune system is generally regarded as being detrimental to the heart, a small degree of activation is protective (15), which is highly reminiscent of the manner in which sub lethal episodes of ischemia are protective during ischemic preconditioning. Importantly, mice

lacking TLR4 have been found to be resistant to the beneficial effects of preconditioning (14,15). Our data suggests that exosomal HSP70 can activate protective pathways in cardiomyocytes via TLR4, part of the evolutionarily ancient, innate immune system response.

Limitations

A limitation of our study is that it is not currently possible to achieve complete purity of exosomes from plasma, and consequently, significant amount of proteins and protein aggregates may still be present in the isolated fraction. We performed TEM to confirm the presence of HSP70 on the exosomal surface, and confirmed the lack of significant endotoxin contamination, but cannot exclude that non-exosomal HSP70 may be involved, or that other known inducers of TLRs (e.g.: CRP, ceruloplasmin) are present. Since TAK-242 did not entirely block ERK1/2 phosphorylation by exosomes, it is possible either that TLR4 was incompletely inhibited by TAK-242, or that ERK1/2 can also be partially activated an alternative, parallel route such as TLR2. Furthermore, since exosome isolation from biological fluids is quite difficult (34), accurate assessment of plasma exosome concentration before and after RIC awaits improved technology permitting their direct measurement. We did not quantify the increase in exosome concentration in animals after exosome administration in order to confirm a mass-action effect of exosomes. Since a larger quantity of blood was used to isolate exosomes from humans than rats, and an additional ultracentrifugation step was included to obtain an exosome fraction of comparable purity, a direct comparison cannot be made between rat and human exosomes. All samples were compared to vehicle, which could potentially have influenced results.

Conclusion

In conclusion, given that plasma exosomes are cardioprotective, these nanoparticles are worthy of further investigation as they may represent a new frontier in our search for ways to protect the myocardium at risk of severe injury.

Perspectives

Competency in medical knowledge: Exosomes - long considered extracellular debris - are increasingly recognized as endogenous vesicles with signalling roles. Plasma exosomes are shown to activate cardioprotective signalling pathways culminating in phosphorylation of HSP27. This highlights their role as newly identified endocrine signalling molecules.

Translational outlook: Exosomes isolated from plasma have the potential to be used as therapeutic agents to activate endogenous protective mechanisms. Further work is required to develop simpler methods for their isolation and purification, and to understand the pathways regulating their release and distribution.

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Author Contributions

JMV, YZ, SD, DD, SA, C B-D, JAR, JK, VS, ARH performed experiments; GM provided reagents; JMV, SD prepared the figures; SD, DY, designed the experiments, obtained the funding, and wrote the paper.

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Figure legends

Figure 1. Characterization and quantification of plasma exosomes. (A) Electron micrographs of purified exosomes from rats and humans. Bar: 100 nm. (B) Representative results of nanoparticle tracking analysis (NTA) demonstrate similar size distribution in diluted samples of human and rat exosomes. (C, D) Average concentration and size of exosomes in plasma (n=5 rats, n=6 humans). (E) Flow cytometry analysis of microspheres coated with exosome-rich fractions isolated from humans (see Materials and Methods for details) identified the presence of exosomal markers CD63, CD81 and HSP70, both at baseline (control, black), and after remote ischemic preconditioning (RIC, red). Strong staining was also observed using antibody cmHsp70.1, specific to an external epitope of HSP70. The yellow curve indicates exosome-coated microspheres stained with secondary antibodies as a control. Isotype controls are shown in Supplementary Figure 1. (F) Western blots confirmed the presence of exosomal marker proteins CD63, CD81 and HSP70 in human samples. Levels of all proteins were increased after remote ischemic conditioning (RIC) (*P<0.05, n=3). (G) (H-K) The size distribution and concentration of human exosomes as determined by TEM (H) and NTA (I), before and after RIC. Average modal size of exosomes was unaltered by RIC (n=6). Plasma concentration of exosomes increased after RIC (**P<0.01, n=6).

Figure 2. Purified plasma exosomes are cardioprotective. (A) Exosomes purified from a control donor rat or a donor rat subject to remote ischemic preconditioning (RIC), and administered via tail vein injection into a naive rat decreased infarct size (as percentage of area at risk AAR), in an *in vivo* model of reperfusion injury. Protection was similar when RIC was applied to the rat hindlimb *in vivo* (*in vivo* RIC)(**P<0.01,n=6). (B) Exosomes purified from a control rat, or one subject to RIC, decreased infarct size (as percentage of area at risk AAR), in an isolated, *Langendorff* perfused rat heart model of reperfusion injury (**P<0.01,n=6-9). (C) Flow cytometry analysis shows that HL-1 cardiomyocytes were protected against hypoxia and reoxygenation by pre-incubation with plasma exosomes from rats, as determined by $DiOC_6(3)$ and propidium iodide staining (n=3). Insulin was used as a positive control. The quadrants include mean ± SEM percentage values from at least three independent experiments. (D) Pre-incubation with rat exosomes decreased death in primary adult rat cardiomyocytes subject to hypoxia and reoxygenation. Cells were imaged by phase contrast and red fluorescence to visualize propidium iodide. Representative images are shown from cells in conditions of normoxia, hypoxia/reoxygenation (Hypox/Reox), with the addition of 10^9 exosomes/well, or insulin as a positive control, as indicated. A dose-response curve was performed with 10^5 to 10^9 control or RIC exosomes/well and compared to insulin (ins). (*P<0.05; **P<0.01 vs. Hypox/reox alone; n=6). Scale bar 100 μm.

Figure 3. Rat plasma exosomes activate the ERK1/2 signaling pathway in cardiomyocytes. (A) Western blot analysis of adult rat cardiomyocytes shows robust and rapid activation of ERK1/2 after stimulation with rat plasma exosomes. AKT was not activated (insulin is included as a positive control). (n = 3 independent experiments). (B) Similarly, ERK1/2 but not AKT was phosphorylated in adult rat cardiomyocytes after exposure to human plasma

exosomes (n = 3 independent experiments). (C) Human plasma exosomes isolated from 4 different individuals activated ERK1/2 phosphorylation after stimulation of adult rat cardiomyocytes for 5 min (quantified on the lower graph). (D) Preincubation (15 min) with the inhibitors of ERK1/2 phosphorylation, U0126 and PD98059, prevented phosphorylation of ERK1/2 in adult rat cardiomyocytes stimulated with human exosomes for 5 min (n = 3 independent experiments). (E) Experiments using adult rat cardiomyocytes subjected to hypoxia/reoxygenation show that U0126 and PD98059 blocked protection by human exosomes, n=3. *P<0.05 and **P<0.01 vs. 0' time point (panels A-B), vs. control (panel C), or vs. normoxia (panel E).

Figure 4. HSP70 on the surface of exosomes mediates ERK1/2 and HSP27 phosphorylation, as well as cardioprotection in response to exosomes. (A) Treatment of adult rat cardiomyocytes with human exosomes activates HSP27 phosphorylation at different serine epitopes; n = 3, *P<0.05; **P<0.01 vs control conditions. (B) Electron microscopy (TEM) of rat exosomes immunostained with cmHsp70.1 demonstrates the presence of HSP70 on exosomal surface both before and after remote ischemic preconditioning (RIC) (black dots are 5 nm gold particles). (C) Neutralization of exosomal HSP70 by cmHSP70.1 blocks the phosphorylation of ERK1/2 and HSP27-Ser^{78} in adult rat cardiomyocytes stimulated with exosomes for 5 min; n = 3, **P<0.01 over untreated cells; $^{\#\#}$P<0.01 over exosomes. (D) Neutralization of exosomal HSP70 by cmHSP70.1 blocks the protection of adult rat cardiomyocytes in response to exosomes. Scale bar 100 µm. Experiments are shown as mean ± SEM, n = 3. **P<0.01, as indicated, *n.s.* = non significant.

Figure 5. Exosomes activate TLR4 to trigger ERK- and p38MAPK-dependent induction of HSP27. (A) Inhibitors of TLR4 (TAK-242), ERK1/2 (U0126), or p38MAPK (SB203580) block the phosphorylation of both ERK1/2 and HSP27-Ser^{78} in response to exosomes. On their own, TAK-242 and SB203580 increased slightly ERK1/2 phosphorylation, but there was no activation of HSP27. n = 3; **P<0.01 vs. vehicle; $^{\#\#}$P<0.01 vs. exosomes. (B) The above inhibitors blocked protection by exosomes on adult rat cardiomyocytes subjected to hypoxia/reoxygenation; n = 3; **P<0.01 as indicated, *n.s.* = non significant. Scale bar 100 µm.

Figure 6. Proposed mechanism, whereby HSP70 in exosomes stimulates TLR4 in cells, leading via ERK1/2 and p38MAPK to phosphorylation of HSP27 and cardioprotection. We hypothesize that other exosomal signals may lead alternatively to acute or delayed cardioprotection.

Figure 1

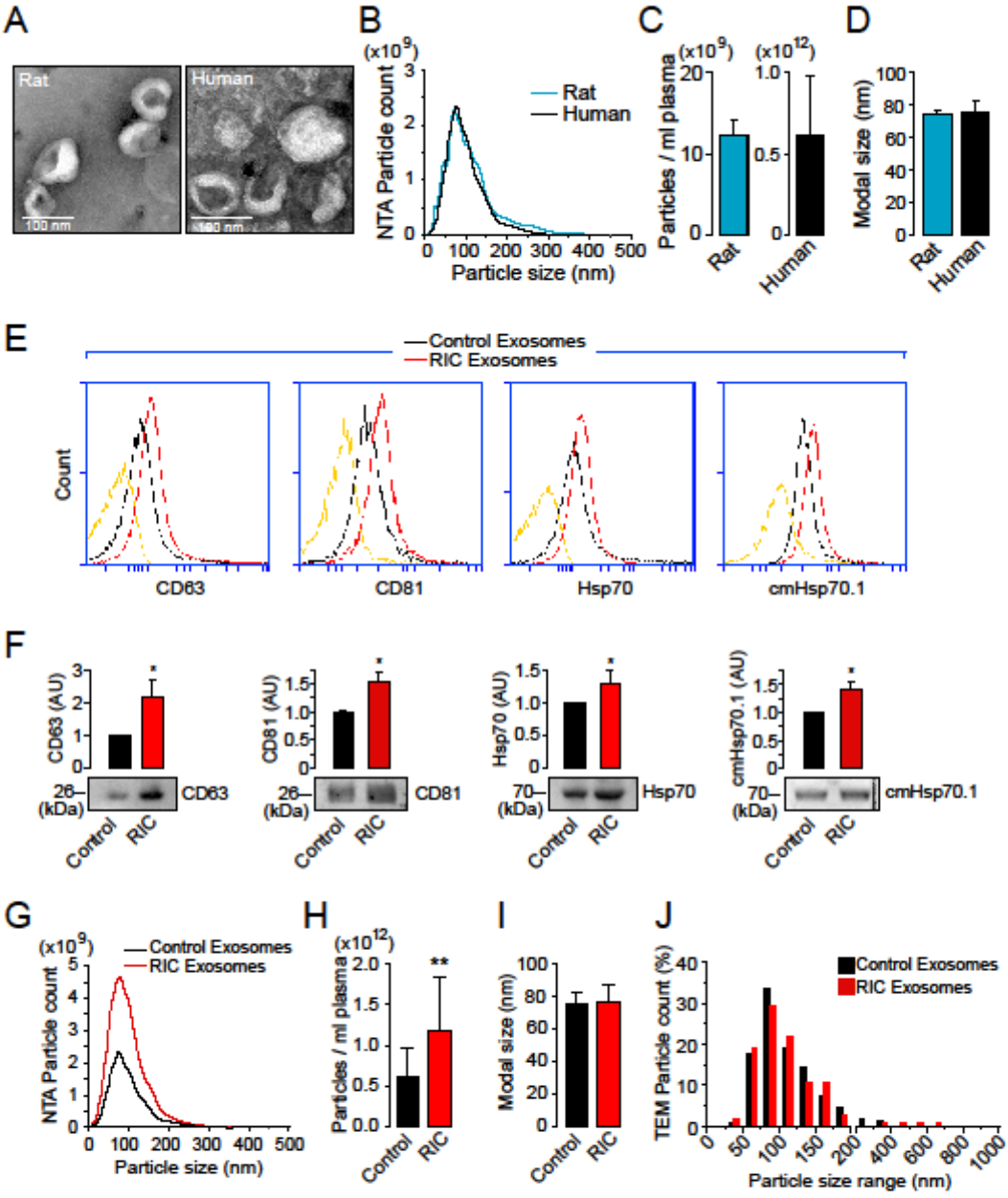

A
Rat
Human
100 nm
B
(x10^9)
NTA Particle count
Rat
Human
Particle size (nm)
C
(x10^9)
(x10^12)
Particles / ml plasma
Rat
Human
D
Modal size (nm)
Rat
Human
E
Control Exosomes
RIC Exosomes
Count
CD63
CD81
Hsp70
cmHsp70.1
F
CD63 (AU)
CD81 (AU)
Hsp70 (AU)
cmHsp70.1 (AU)
26 (kDa)
70 (kDa)
CD63
CD81
Hsp70
cmHsp70.1
Control
RIC
G
(x10^9)
Control Exosomes
RIC Exosomes
NTA Particle count
Particle size (nm)
H
(x10^12)
Particles / ml plasma
Control
RIC
I
Modal size (nm)
Control
RIC
J
Control Exosomes
RIC Exosomes
TEM Particle count (%)
Particle size range (nm)

Figure 2

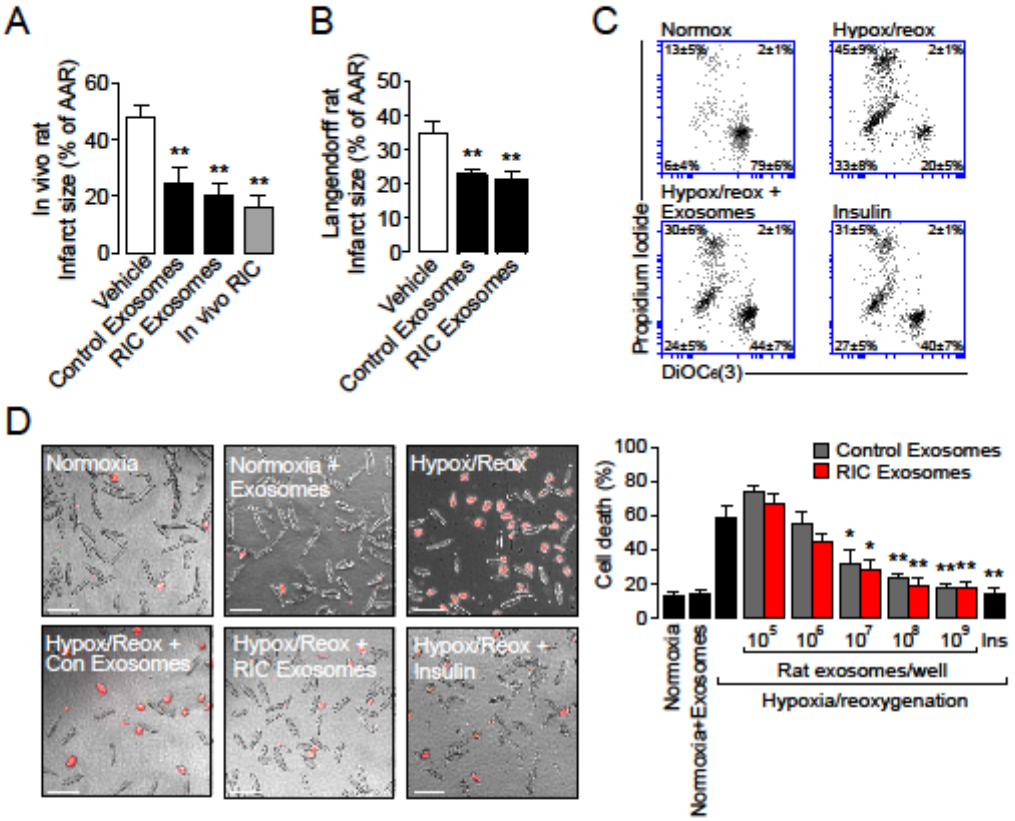

A
In vivo rat
Infarct size (% of AAR)
60
40
20
0
**
**
**
Vehicle
Control Exosomes
RIC Exosomes
In vivo RIC
B
Langendorff rat
Infarct size (% of AAR)
50
40
30
20
10
0
**
**
Vehicle
Control Exosomes
RIC Exosomes
C
Normox
Hypox/reox
13±5%
2±1%
45±9%
2±1%
6±4%
79±6%
33±8%
20±5%
Hypox/reox +
Exosomes
Insulin
30±6%
2±1%
31±5%
2±1%
24±5%
44±7%
27±5%
40±7%
Propidium Iodide
DiOCe(3)
D
Normoxia
Normoxia +
Exosomes
Hypox/Reox
Hypox/Reox +
Con Exosomes
Hypox/Reox +
RIC Exosomes
Hypox/Reox +
Insulin
Control Exosomes
RIC Exosomes
Cell death (%)
100
80
60
40
20
0
*
*
**
**
***
**
Normoxia
Normoxia+Exosomes
10^5
10^6
10^7
10^8
10^9
Ins
Rat exosomes/well
Hypoxia/reoxygenation

Figure 3

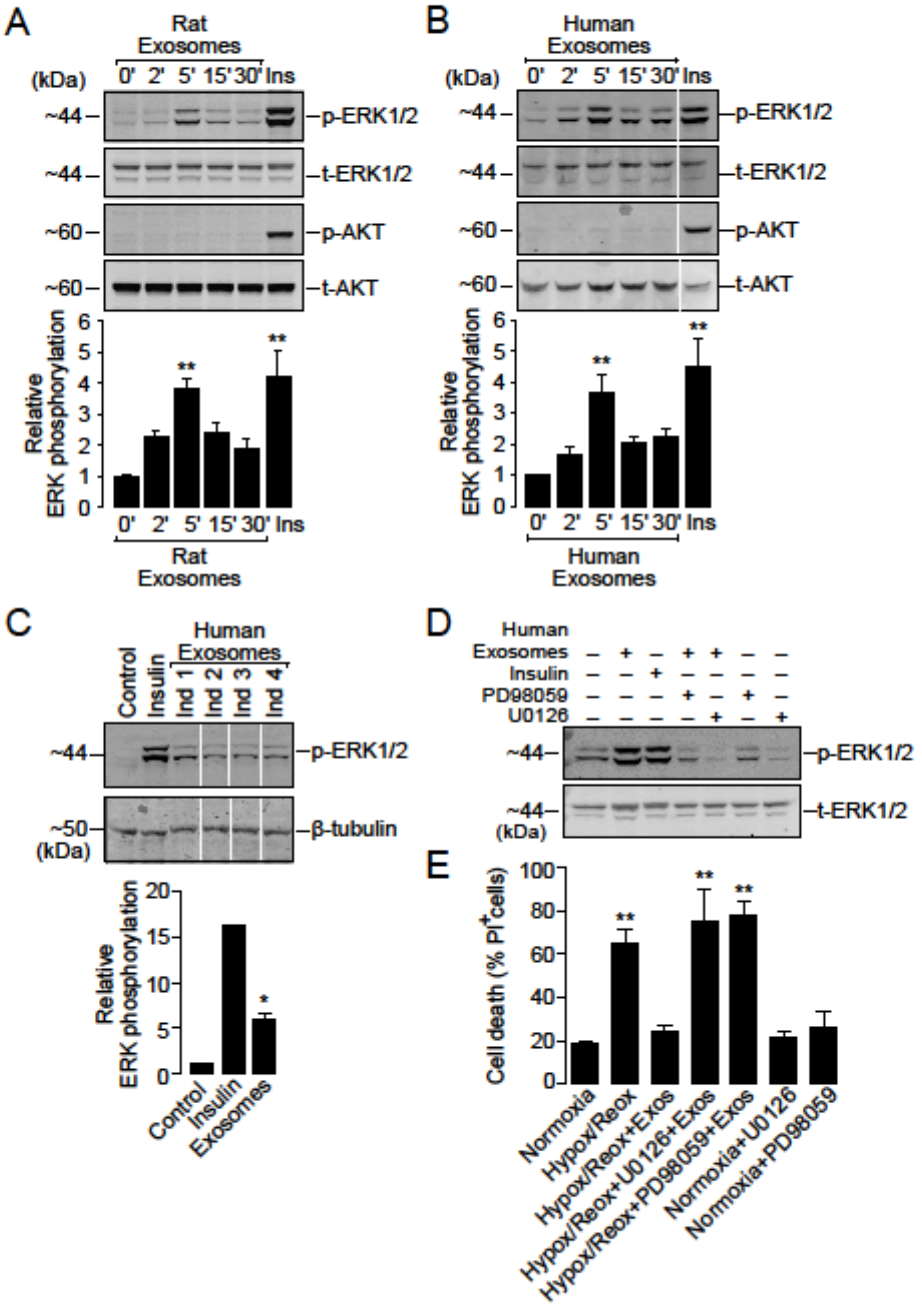

A
Rat Exosomes
(kDa)
0' 2' 5' 15' 30' Ins
~44
p-ERK1/2
~44
t-ERK1/2
~60
p-AKT
~60
t-AKT
Relative ERK phosphorylation
B
Human Exosomes
C
Control
Insulin
Ind 1
Ind 2
Ind 3
Ind 4
~50
β-tubulin
D
Human Exosomes
Insulin
PD98059
U0126
E
Cell death (% PI+cells)
Normoxia
Hypox/Reox
Hypox/Reox+Exos
Hypox/Reox+U0126+Exos
Hypox/Reox+PD98059+Exos
Normoxia+U0126
Normoxia+PD98059

Figure 4

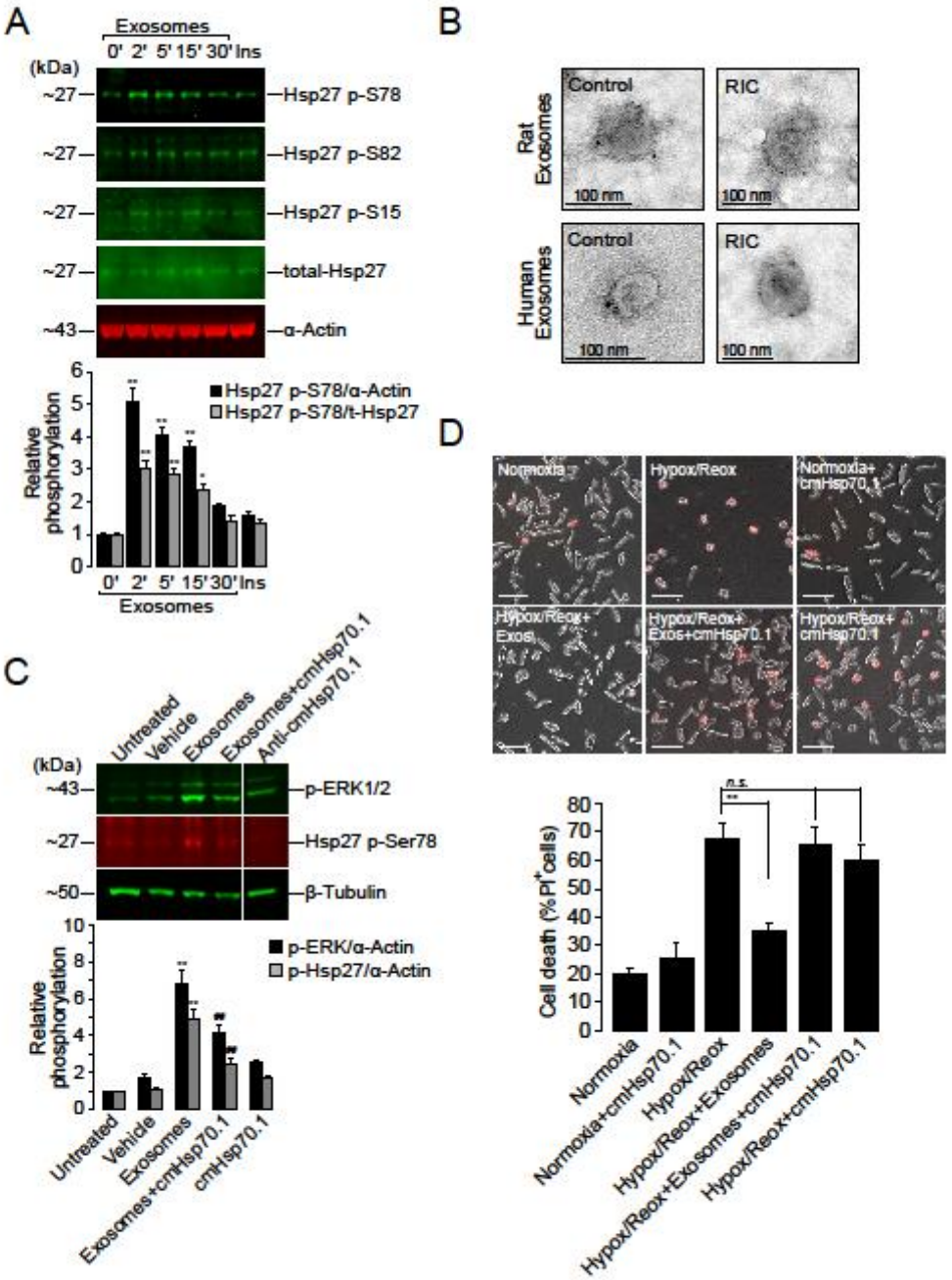

A
Exosomes
0' 2' 5' 15' 30' Ins
(kDa)
~27
Hsp27 p-S78
~27
Hsp27 p-S82
~27
Hsp27 p-S15
~27
total-Hsp27
~43
α-Actin
Relative phosphorylation
Hsp27 p-S78/α-Actin
Hsp27 p-S78/t-Hsp27
0' 2' 5' 15' 30' Ins
Exosomes
B
Rat Exosomes
Control
RIC
100 nm
Human Exosomes
Control
RIC
100 nm
C
Untreated
Vehicle
Exosomes
Exosomes+cmHsp70.1
Anti-cmHsp70.1
(kDa)
~43
p-ERK1/2
~27
Hsp27 p-Ser78
~50
β-Tubulin
Relative phosphorylation
p-ERK/α-Actin
p-Hsp27/α-Actin
Untreated
Vehicle
Exosomes
Exosomes+cmHsp70.1
cmHsp70.1
D
Normoxia
Hypox/Reox
Normoxia+ cmHsp70.1
Hypox/Reox+ Exos
Hypox/Reox+ Exos+cmHsp70.1
Hypox/Reox+ cmHsp70.1
Cell death (% PI+ cells)
n.s.
Normoxia
Normoxia+cmHsp70.1
Hypox/Reox
Hypox/Reox+Exosomes
Hypox/Reox+Exosomes+cmHsp70.1
Hypox/Reox+cmHsp70.1

Figure 5

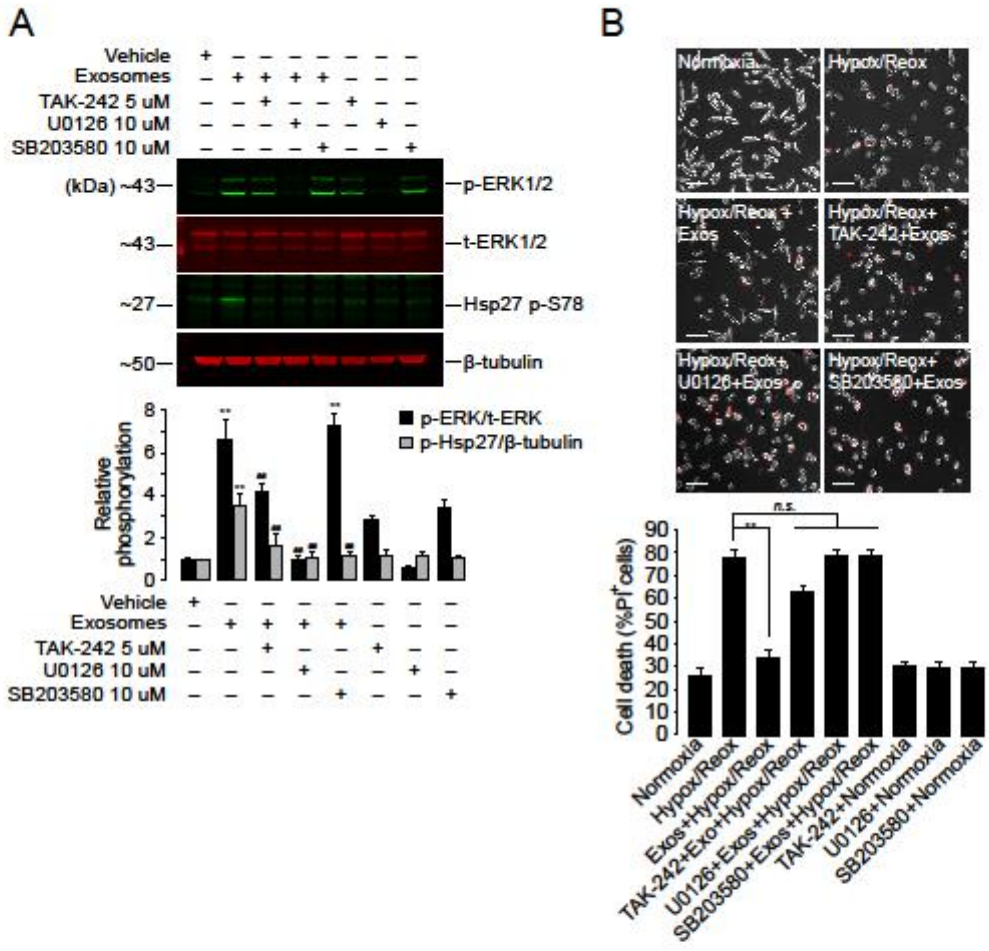
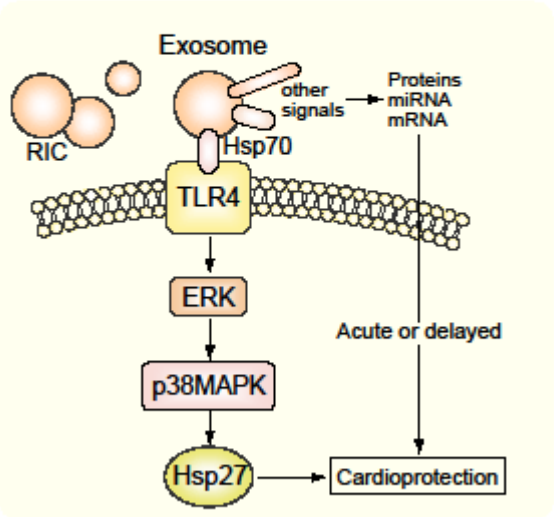

A
Vehicle
Exosomes
TAK-242 5 uM
U0126 10 uM
SB203580 10 uM
(kDa) ~43
p-ERK1/2
~43
t-ERK1/2
~27
Hsp27 p-S78
~50
β-tubulin
Relative phosphorylation
p-ERK/t-ERK
p-Hsp27/β-tubulin
B
Normoxia
Hypox/Reox
Hypox/Reox + Exos
Hypox/Reox+ TAK-242+Exos
Hypox/Reox+ U0126+Exos
Hypox/Reox+ SB203580+Exos
Cell death (%PI+ cells)
n.s.
Normoxia
Hypox/Reox
Exos+Hypox/Reox
TAK-242+Exo+Hypox/Reox
U0126+Exos+Hypox/Reox
SB203580+Exos+Hypox/Reox
TAK-242+Normoxia
U0126+Normoxia
SB203580+Normoxia

Figure 6


Exosome
RIC
other signals
Proteins
miRNA
mRNA
Hsp70
TLR4
ERK
Acute or delayed
p38MAPK
Hsp27
Cardioprotection