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Plasma Exosomes Protect the Myocardium From Ischemia-Reperfusion Injury

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ABSTRACT

BACKGROUND Exosomes are nanometer-sized vesicles released from cells into the blood, where they can transmit signals throughout the body. Shown to act on the heart, exosomes' composition and the signaling pathways 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 This study sought to isolate and characterize exosomes from rats and healthy volunteers, evaluate their cardioprotective actions, and identify the molecular mechanisms involved.

METHODS The exosome-rich fraction was isolated from the blood of adult rats and human volunteers and was 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, with the protective signaling pathways activated on cardiomyocytes identified using Western blot analyses and chemical inhibitors.

RESULTS Exosomes exhibited the expected size and expressed marker proteins CD63, CD81, and heat shock protein (HSP) 70. 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 (TLR) 4 and various kinases, leading to activation of the cardioprotective 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.

CONCLUSIONS Exosomes deliver endogenous protective signals to the myocardium by a pathway involving TLR4 and classic cardioprotective HSPs. (J Am Coll Cardiol 2015;65:1525–36) © 2015 by the American College of Cardiology Foundation.

In recent years, the potential for extracellular vesicles to be used as therapeutic agents or as biomarkers of pathological states has generated immense interest (1,2). 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 microribonucleic acid (miRNA) and proteins through the bloodstream, representing a potential mode of intercellular communication (4).

Therapeutically, exosomes appear to mediate many beneficial properties of stem cells administered to the heart (5,6). These studies have mainly focused on the role of exogenous, not endogenous, exosomes,

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ABBREVIATIONS AND ACRONYMS

HSP = heat shock protein

miRNA = microribonucleic acid

RIC = remote ischemic pre-conditioning

TEM = transmission electron microscopy

TLR = toll-like receptor

which are present in the blood of humans and rodents in the order of 10^{10} exosomes/ml (7). These striking numbers raise crucial questions as to their role.

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Coronary artery obstruction produces myocardial ischemia, which is usually treated with myocardial reperfusion, although this paradoxically causes further lethal injury that currently lacks an effective clinical therapy (8). Heat shock proteins (HSPs) are powerfully cardioprotective (9–11), but clinical translation faltered without practical techniques for their induction or delivery. After myocardial infarction, large elevations in serum HSP70 can stimulate inflammatory cytokine release via toll-like receptor (TLR) 4 and the innate immune response (12,13). However, a more mild stimulation of innate immunity via TLR ligands is necessary for myocardial pre-conditioning 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, because recent evidence suggests that microvesicles can transfer protection by remote ischemic preconditioning (RIC) (18), we hypothesized that RIC would augment exosome production, thereby stimulating cardioprotection. We purified, characterized, and quantified exosomes from the plasma of rats and humans, demonstrating protection against ischemia and reperfusion both in vitro and in vivo. This cardioprotection was mediated by HSP70 on exosomes, which activated a pathway downstream of TLR4 involving extracellular signal-regulated protein kinases (ERK) 1 and 2 and p38 mitogen-activated protein kinase (MAPK), leading to phosphorylation of HSP27.

METHODS

These studies used male Sprague Dawley rats treated in accordance with the Animals (Scientific Procedures) Act of 1986, published by the United Kingdom Home Office, and the Guide for the Care and Use of Laboratory Animals from the U.S. 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. Exosomes were isolated by centrifugation from healthy, 30- to 45-year-old male volunteers after written consent and overnight fasting.

STATISTICAL ANALYSIS. Data are shown as mean ± SEM. Pairwise comparisons were made with the Student *t* test. One-way analysis of variance was followed by post-test analysis using the Tukey test for multiple comparisons. Two-way analysis of variance was carried out followed by Bonferroni correction to test for significance when performing multiple comparisons between different groups. A p value <0.05 was considered significant.

Additional details of the materials and methods are provided in the Online Appendix.

RESULTS

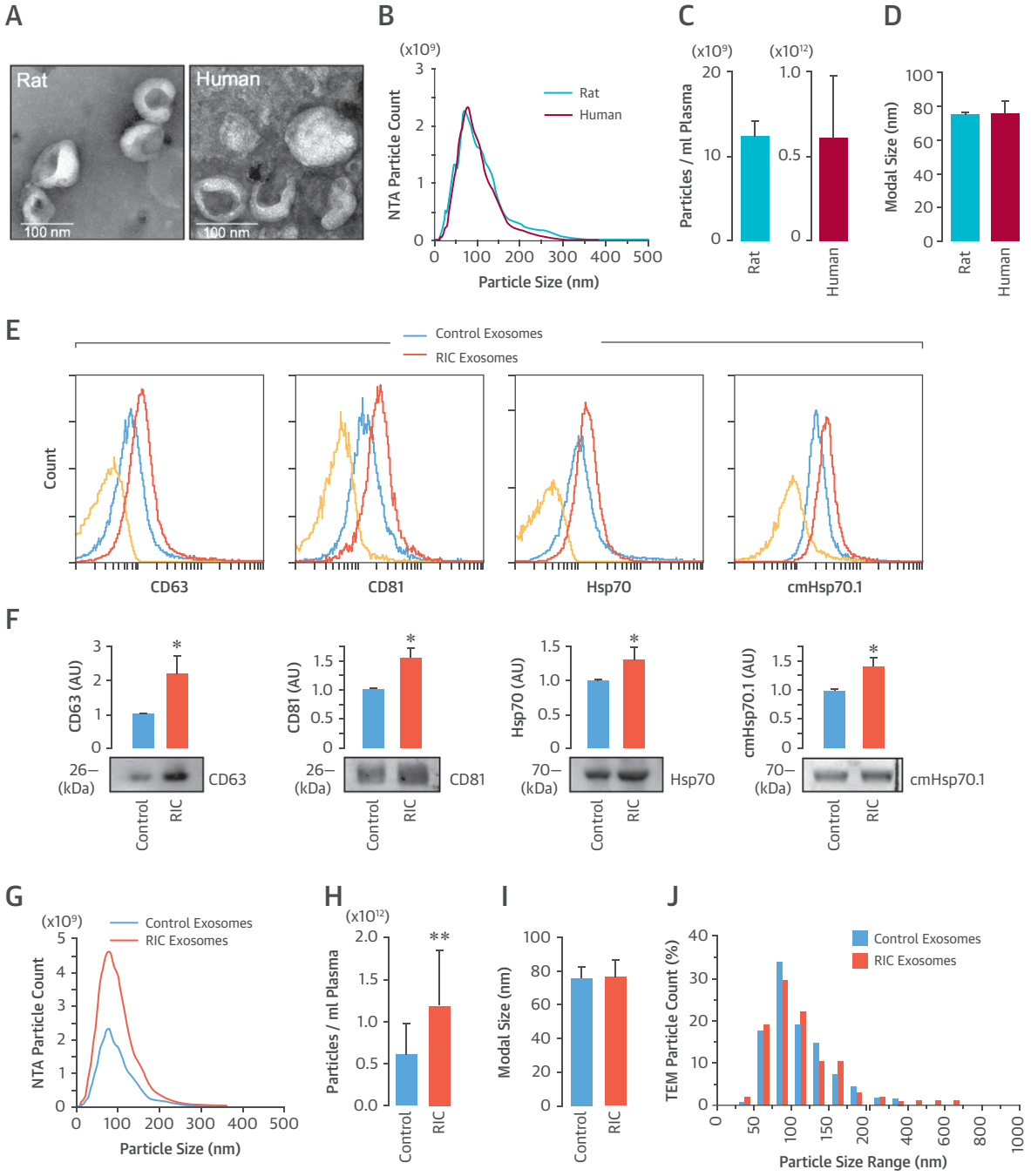
The exosome-rich fraction was purified from the blood of adult male rats and healthy human male volunteers using a standard protocol of serial, differential centrifugation, and ultracentrifugation steps. Using transmission electron microscopy (TEM), we observed the typical "cup-shaped" vesicles of exosomes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

We used nanoparticle tracking analysis to measure the number and size distribution of particles in purified, exosome-rich preparations; the modal size of particles purified from control rat plasma was 75 ± 2 nm (**Figures 1B and 1D**), corresponding to the exosomes' expected size, and the concentration was $0.1 \pm 0.02 \times 10^{11}$ ml^{-1} plasma (n = 5 rats) (**Figure 1C**). In human plasma, particle concentration was $6 \pm 3 \times 10^{11}$ ml^{-1} (n = 6) (**Figure 1C**), and average modal size was 75 ± 7 nm (**Figure 1D**). For simplicity, we refer hereafter to the isolated particles as "exosomes," although the isolated fraction also included some particles outside of the expected exosome size range.

Flow cytometry confirmed the expression of marker proteins for exosomes in the human samples. The tetraspanin molecules CD63 and CD81 (found in many exosomes), as well as HSP70, were all detectable at high levels (**Figure 1E**). Isotype control antibodies were negative (Online 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**).

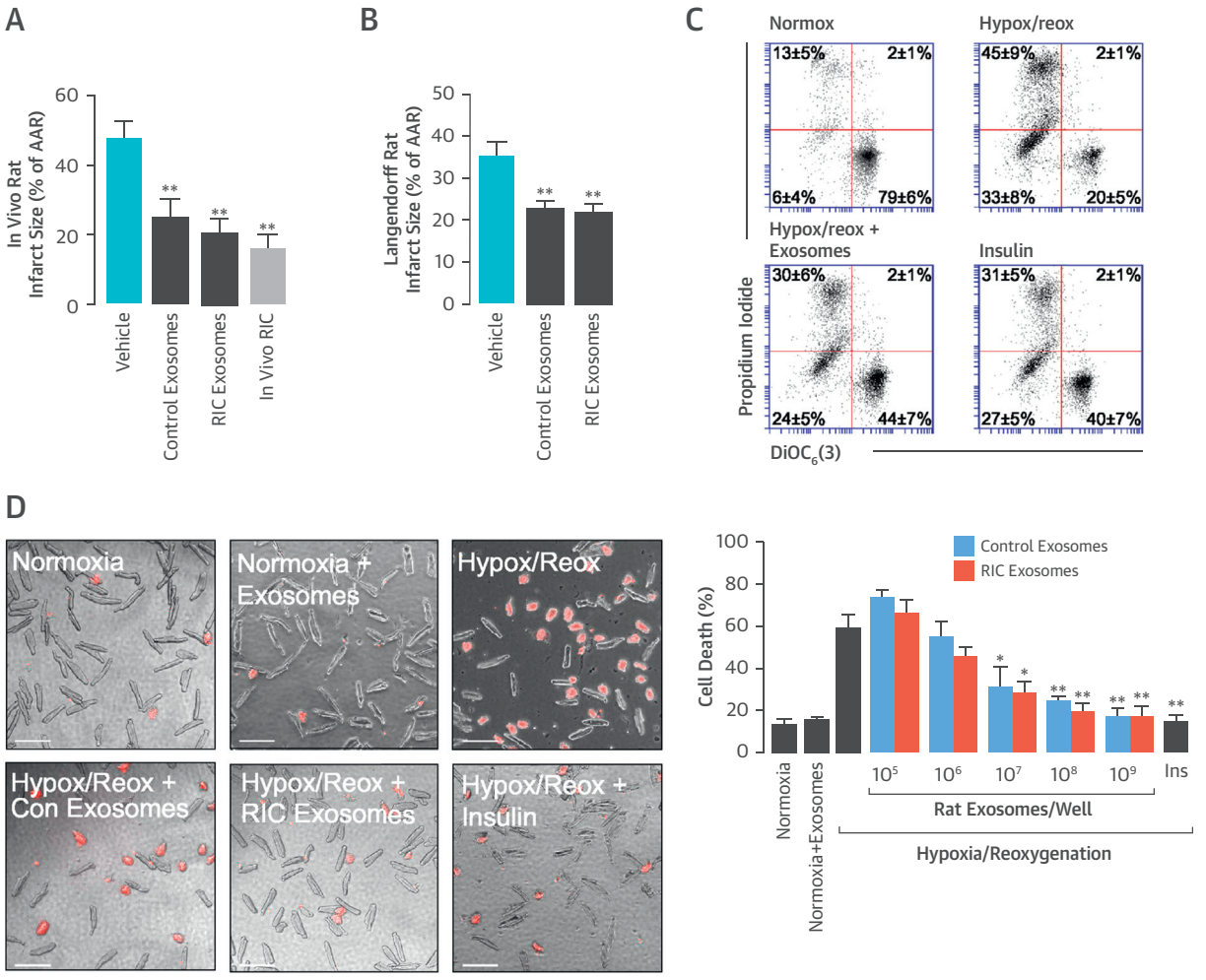
We also performed sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot

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 and 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 identified the presence of exosomal markers CD63, CD81, and heat shock protein (HSP) 70, both at baseline (control, **blue**), and after remote ischemic preconditioning (RIC) **(salmon)**. Strong staining was also observed using antibody cmHsp70.1. **Yellow curve** indicates exosome-coated microspheres stained with secondary antibodies (control). **(F)** Western blots confirmed the presence of exosomal marker proteins CD63, CD81, and HSP70 in human samples. Levels of all proteins were increased after RIC (*$p < 0.05$; n = 3). **(G)** NTA demonstrates size distribution of control and RIC exosomes. **(H to J)** The size distribution and concentration of human exosomes as determined by transmission electron microscopy (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 Provide Cardioprotection



(A) Exosomes purified from a control donor rat or a donor rat subject to RIC decreased infarct size (as percentage of area at risk [AAR]) in a naïve rat in an in vivo model of reperfusion injury. Protection was similar when RIC was applied to the rat hind limb in vivo (in vivo RIC) (**$p < 0.01$; $n = 6$). **(B)** Exosomes purified from a control rat, or 1 subject to RIC, decreased infarct size in an isolated, Langendorff-perfused rat heart model of reperfusion injury (**$p < 0.01$; $n = 6$ to 9). **(C)** Flow cytometry analysis shows that HL-1 cardiomyocytes were protected against hypoxia (Hypox) and reoxygenation (Reox) by pre-incubation with rat plasma exosomes, as determined by $DiOC_6$ (3) and propidium iodide (PI) staining ($n = 3$). Insulin indicates positive control. **(D)** Pre-incubation with rat exosomes decreased death in primary adult rat cardiomyocytes subject to hypoxia and reoxygenation. Representative images are shown from cells in conditions of normoxia, Hypox/Reox, with the addition of 10^9 exosomes/well, or insulin as a positive control, as indicated; **red** indicates PI. A dose-response curve was performed with 10^5 to 10^9 control or RIC exosomes/well and compared with insulin (Ins). (*$p < 0.05$; **$p < 0.01$ vs. Hypox/Reox alone; $n = 6$). **Bar**: 100 μm. Abbreviations as in Figure 1.

analyses to biochemically characterize the human samples and were able to detect CD63, CD81, and HSP70 (**Figure 1F**). Similar results were obtained using rat exosomes (Online Figure 2).

To investigate whether plasma exosomes are cardioprotective, we used 4 different experimental models. In an in vivo rat model, the entire exosome fraction isolated from a donor rat was administered to a naïve rat by intravenous tail vein injection 15 min before occlusion of the left anterior descending artery. The area at risk was the same in each group (Online 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**). Second, to exclude the influence of blood, Langendorff-perfused rat hearts were exposed to all of the exosomes isolated from donor rat blood during 15-min perfusion, before being subjected to 35-min ischemia followed by 2-h reperfusion. The area at risk was the same in each group (Online 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 2 in vitro 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 with 6 ± 4% in normoxia. The population of PI-detected dead HL-1 cells was 45 ± 9% compared with 13 ± 5% in normoxia (**Figure 2C**). Pre-treatment 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 reoxygenation increased cardiomyocyte cell death from 13 ± 3% to 59 ± 7% ($p < 0.001$; n = 5) (**Figure 2D**). The maximal tested dose of 10^9 exosomes/well was highly cardioprotective, reducing cell death to 17 ± 3%, which is similar to the effect of insulin (14 ± 3%), and this protection remained down to a dose of 10^7 exosomes/well, at which exosomes decreased death to 32 ± 8% (**Figure 2D**).

If exosomes can activate cardioprotective pathways, this raises the possibility that manipulating their levels in the blood might affect cardioprotection. No specific pharmacological inhibitors have yet been developed for in vivo use 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). Such RIC also renders the heart in a protected state (21,22).

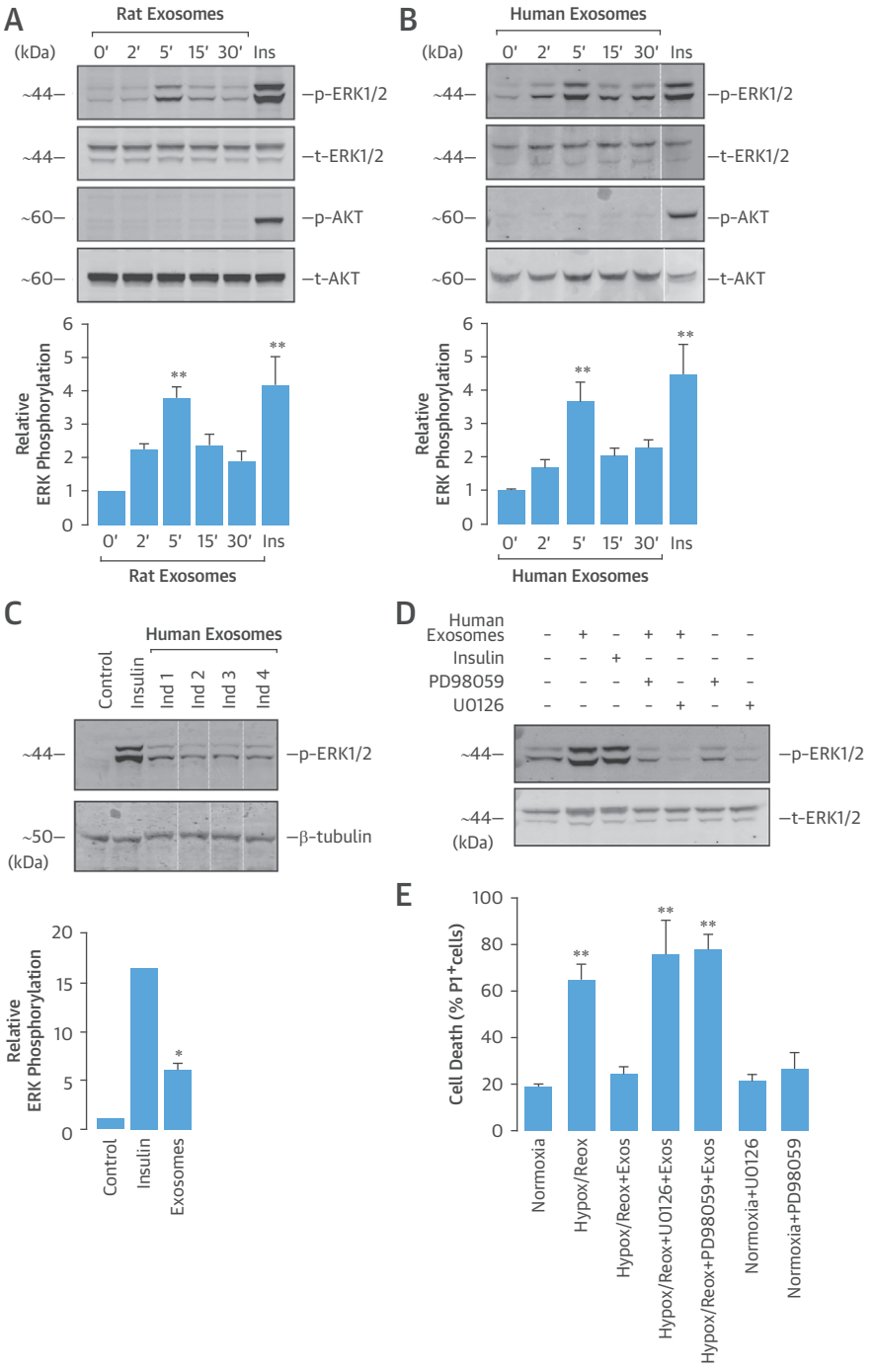
We first looked for any effect on plasma exosomes in humans subject to RIC. Exosomes were isolated from healthy volunteers both before and after a standard RIC procedure and were characterized by nanoparticle tracking analysis and TEM. RIC exosomes exhibited the same size and shape as control exosomes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

The ability of exosomes from RIC-treated rats to protect the heart was then determined. 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. Although RIC exosomes slightly increased protection, the difference was nonsignificant (**Figure 2D**). This suggested that although exosomes after RIC remain cardioprotective and their numbers increase, this increase is insufficient to substantially affect cardioprotection in this model.

We then studied the intracellular signaling pathways activated by exosomes on cardiac cells using Western blot analysis. Adding 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 of exosome exposure, decreasing by 15 min; however, there was no increase in protein kinase B (AKT) phosphorylation after exosome treatment (**Figure 3A**), despite its phosphorylation by insulin. We saw a very similar time course of ERK1/2 phosphorylation with human exosomes, with maximal phosphorylation after 5 min; again, no AKT phosphorylation was detected in response to exosomes at any time point despite the insulin-caused dual activation of ERK1/2 and AKT (**Figure 3B**). These results suggest that exosomes do not activate insulin-receptor signaling. For further validation, we used human exosomes to stimulate HL-1 cardiomyocytes and again observed phosphorylation of ERK1/2 after 5 min without any AKT effect (Online Figure 5). Activation of ERK1/2 was tested in response to exosomes purified from 4 different human volunteers; 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 (MAPK/ERK kinase). Both drugs completely inhibited ERK1/2 phosphorylation in response to human exosomes (**Figure 3D**) as well as eliminated cardioprotection in adult rat cardiomyocytes treated with human exosomes (**Figure 3E**). In control experiments, the inhibitors alone had no effect (Online Figure 6).

FIGURE 3 Activating the ERK1/2 Signaling Pathway



(A) Western blot analysis of adult rat cardiomyocytes shows robust and rapid activation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) after stimulation with rat plasma exosomes. Protein kinase B (AKT) was not activated (insulin indicates 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. **(D)** Pre-incubation (15 min) with the inhibitors of ERK1/2 phosphorylation, U0126 and PD98059, prevented ERK1/2 phosphorylation in adult rat cardiomyocytes stimulated with human exosomes for 5 min (n = 3 independent experiments). **(E)** In adult rat cardiomyocytes subjected to hypoxia/reoxygenation, U0126 and PD98059 blocked protection by human exosomes (n = 3); *p < 0.05 and **p < 0.01 versus 0′ time point **(A and B)**, versus control **(C)**, or versus normoxia **(E)**. Abbreviations as in Figure 2.

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 phosphoserine 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}; we subsequently used this phosphorylation site to define the signaling pathway leading to HSP27 phosphorylation.

We found that, in contrast to endothelial cells, exosomes were not internalized by primary cardiomyocytes (Online Figure 7), suggesting that protection was mediated by a receptor-ligand interaction. Extracellular HSP70 is considered to be a danger-associated molecular pattern, which can activate immune responses by binding to TLR4 (13,23). Our flow cytometry experiments suggested that HSP70 is present on exosome surfaces. 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, which 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 reoxygenation, and by itself, the neutralizing antibody did not affect cell survival (**Figure 4D**).

Upstream of HSP27 phosphorylation, 1 of the most well-established activators is p38MAPK. Using SB203580, an inhibitor of p38MAPKα/β, we observed that HSP27 phosphorylation in response to exosomes was blocked, whereas upstream ERK1/2 phosphorylation remained unaffected (**Figure 5A**). As discussed earlier, 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.

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 HSP27 phosphorylation (**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 to be extracellular debris, exosomes are now understood to be vesicles communicating between cells. Evidence suggests that exosomes from cultured stem cells are cardioprotective (5,6); now, the present study has focused on endogenous plasma exosomes. We found in vitro and in vivo evidence that plasma exosomes are cardioprotective in an acute setting, mediated by exosomal HSP70 that stimulated TLR4 signaling, and leading to the activation of ERK1/2, p38MAPK, and subsequent HSP27 phosphorylation in cardiomyocytes (**Central Illustration**).

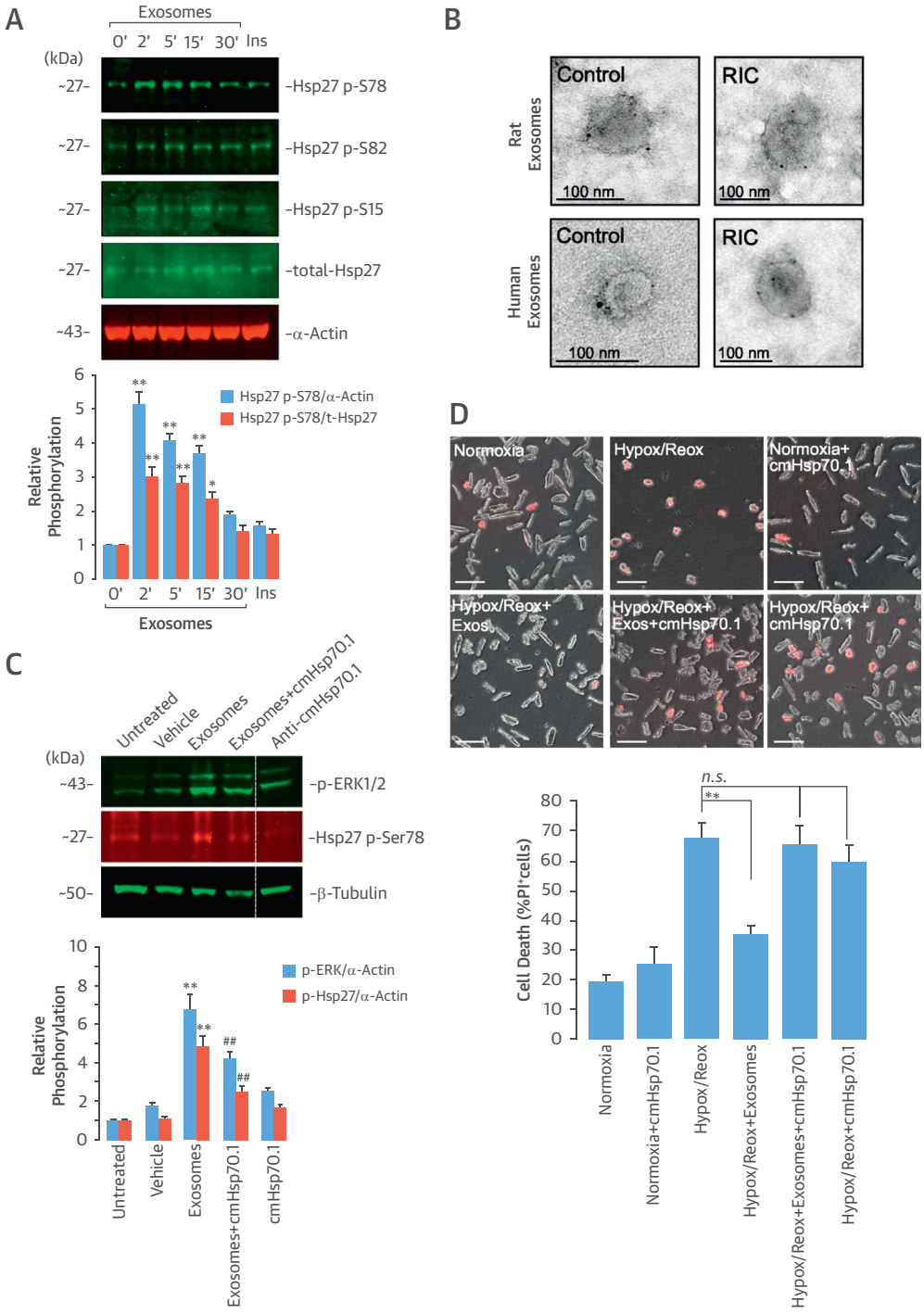
The increase in exosome number stimulated by RIC is interesting, but to date there are no tools available to prevent exosome release in vivo; therefore, rigorous exclusion of the role of exosomes in RIC awaits the development of specific chemical inhibitors or knockout models. Alternatively, several other candidate proteins that may mediate RIC have been proposed, including stromal-derived factor-1α (24) and interleukin-10 (25).

Despite great interest in the potential for exosomes to deliver cargo inside of cells, we were unable to detect any uptake of fluorescently-labeled exosomes into primary cardiomyocytes even after 24-h incubation, despite their rapid uptake into endothelial cells. Combined with the rapidity of the signaling response and its dependence upon HSP70 and TLR4 signaling, this strongly suggests that cardioprotection is mediated via direct ligand-receptor interaction on the cell surface. Because exosomes also contain other cargo such as miRNA, it will be interesting to determine whether they are capable of delivering longer-term benefit via known cardioprotective miRNAs (26).

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; this was required to induce cardioprotection. Potential downstream targets of ERK1/2 include p38MAPK, which phosphorylates HSP27 (27). 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 2 isoforms that are protective when transiently activated before ischemia (28).

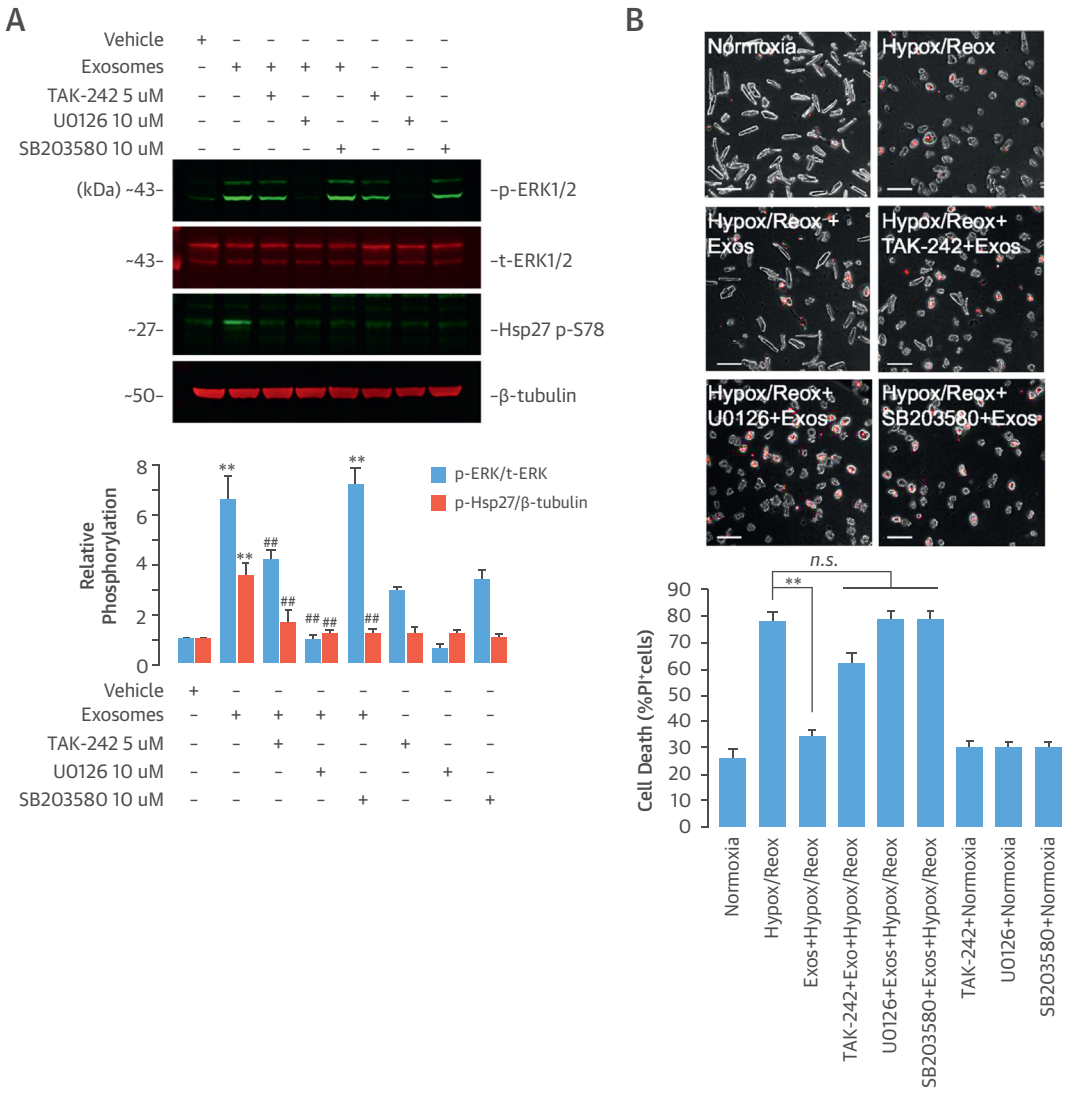
One of the most well-characterized members of the cardioprotective, small HSP family, HSP27 is highly expressed in the myocardium (16). In addition to controlling protein folding, small HSPs exert

FIGURE 4 Surface HSP70 Mediates ERK1/2 and HSP27 Phosphorylation



(A) Treatment of adult rat cardiomyocytes with human exosomes activates HSP27 phosphorylation at different serine epitopes (n = 3); *p < 0.05, **p < 0.01 versus control conditions. **(B)** Electron microscopy of rat exosomes immunostained with cmHsp70.1 demonstrates the presence of HSP70 on exosomal surface both before and after RIC (**black dots** = 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. **Bar**: 100 μm. Experiments are shown as mean ± SEM, n = 3; **p < 0.01. Abbreviations as in Figures 1 to 3.

FIGURE 5 ERK- and p38MAPK-Dependent Induction of HSP27

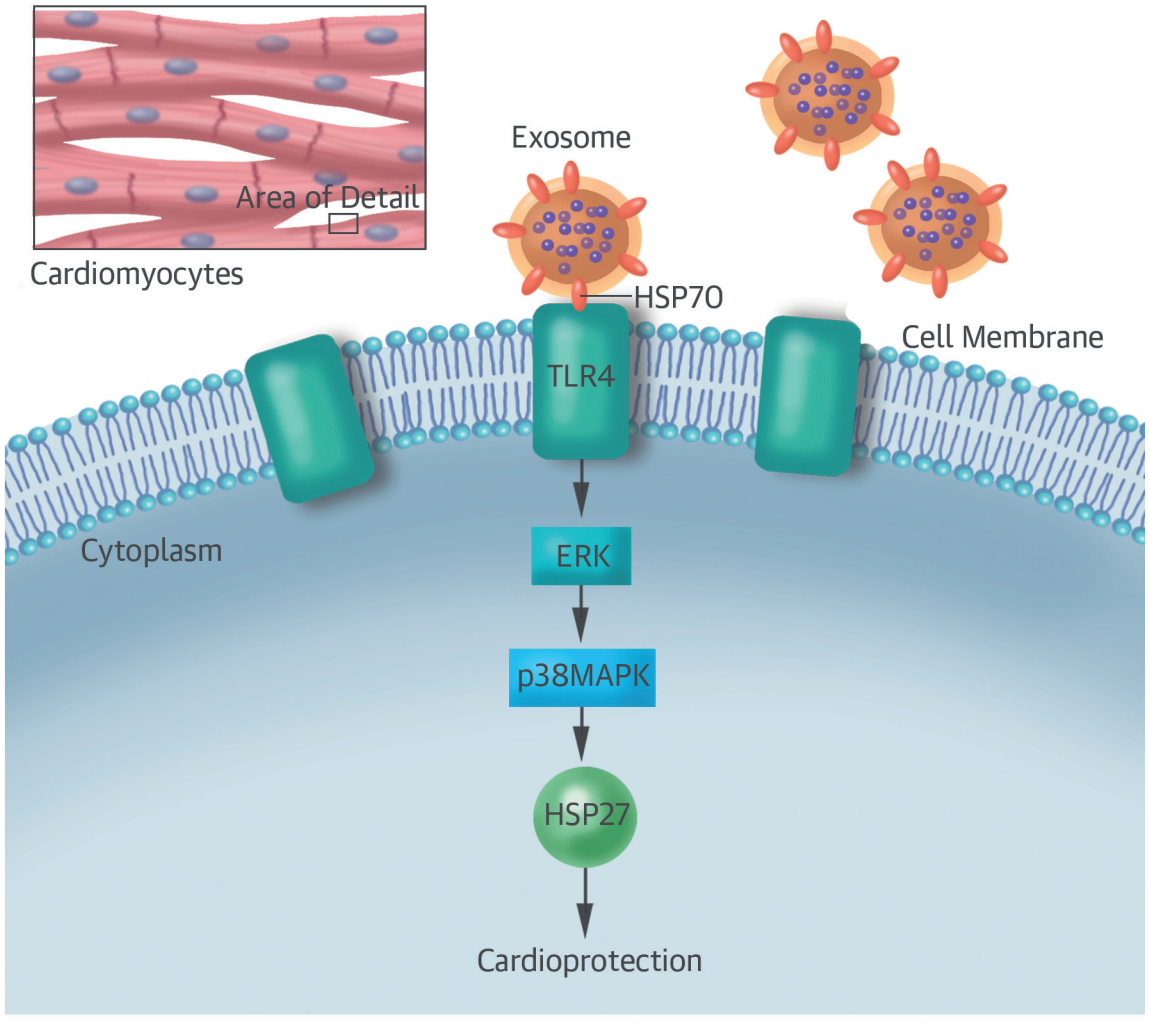


(A) Inhibitors of toll-like receptor 4 (TAK-242), ERK1/2 (U0126), or p38 mitogen-activated protein kinase (p38MAPK) (SB203580) block the phosphorylation of both ERK1/2 and HSP27-Ser^{78} in response to exosomes. On their own, TAK-242 and SB203580 slightly increased ERK1/2 phosphorylation, but without activation of HSP27 ($n = 3$); **$p < 0.01$ versus vehicle; $^{\#\#}p < 0.01$ versus exosomes. **(B)** The inhibitors in the previous text blocked protection by exosomes on adult rat cardiomyocytes subjected to hypoxia/reoxygenation, ($n = 3$); **$p < 0.01$. **Bar**: 100 μm. Abbreviations as in Figures 1 to 3.

powerful protection against oxidative stress and apoptosis and maintain sarcomeric structure (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 to our knowledge that endogenous plasma exosomes can activate this HSP.

We previously developed a neutralizing antibody against the highly-conserved, exposed C-terminal of HSP70 (19), demonstrating 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. Heat shock proteins, including HSP27 and HSP70, are intracellular chaperones important for

CENTRAL ILLUSTRATION **Exosome-Stimulated Cardioprotection**



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The proposed mechanism of action by which exosomes communicate cardioprotective signals to the heart is mediated by heat shock protein (HSP) 70 on exosomes. This activates a pathway downstream of toll-like receptor 4 (TLR) involving extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and p38 mitogen-activated protein kinase (p38MAPK), leading to phosphorylation of HSP27.

correct protein homeostasis. HSP70 is secreted from cells by exosomes (20) and is present in the serum of healthy individuals (29). Beyond HSP70's well-established intracellular protective role, an intriguing new, *extra*cellular role is emerging for HSP70 (30). For instance, supporting cells in the inner ear can secrete HSP70 to protect hair cells from cell death in response to ototoxic drugs (31). Plasma HSP70 is believed to play a role in activating the adaptive and innate immune response (32). Although activating the innate immune system is generally considered to be detrimental to the heart, a small degree of activation is protective (15); this is highly reminiscent of the manner in which sublethal episodes of ischemia are protective during ischemic pre-conditioning. Importantly, mice lacking TLR4 appear resistant to the beneficial effects of pre-conditioning (14,15). Our data suggest that exosomal HSP70 can activate protective pathways in cardiomyocytes via TLR4, part of the ancient, innate immune system response.

STUDY LIMITATIONS. 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 we cannot exclude that nonexosomal HSP70 may be involved, or that other known inducers of TLRs are present. Because TAK-242 did not entirely block ERK1/2 phosphorylation by exosomes, it is possible that either TLR4 was incompletely inhibited by TAK-242 or ERK1/2 can be partially activated by an alternative, parallel route such as TLR2. Furthermore, because exosome isolation from biological fluids is challenging, accurate direct measurement of plasma exosome concentration before and after RIC awaits improved technology. We did not quantify the increase in exosome concentration in animals after exosome administration to confirm a mass-action effect of exosomes. Because 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, we could not directly compare rat and human exosomes. All samples were compared to vehicle, which could potentially have influenced results.

CONCLUSIONS

Given that plasma exosomes are cardioprotective, these nanoparticles are worthy of further investigation in our search for ways to protect the myocardium at risk of severe injury.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Exosomes are nanometer-sized vesicles released from cells into the blood. Exosome-rich extracts from plasma activate signaling pathways that can protect against experimental ischemia-induced injury in cardiac myocytes.

TRANSLATIONAL OUTLOOK: Further work is required to develop simpler methods to isolate and purify exosomes, to understand the pathways regulating their release and distribution, and to explore the potential therapeutic value of the endogenous protective mechanisms they activate.

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APPENDIX For additional methods and supplemental figures, please see the online version of this article.