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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