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Rapid communication

Cardioprotection by remote ischemic preconditioning of the rat heart is mediated by extracellular vesicles ☆

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ABSTRACT

Remote ischemic preconditioning (RIPC) of the heart is exerted by brief ischemic insults affected on a remote organ or a remote area of the heart before a sustained cardiac ischemia. To date, little is known about the inter-organ transfer mechanisms of cardioprotection by RIPC. Exosomes and microvesicles/microparticles are vesicles of 30–100 nm and 100–1000 nm in diameter, respectively (collectively termed extracellular vesicles [EVs]). Their content of proteins, mRNAs and microRNAs, renders EV ideal conveyors of inter-organ communication. However, whether EVs are involved in RIPC, is unknown. Therefore, here we investigated whether (1) IPC induces release of EVs from the heart, and (2) EVs are necessary for cardioprotection by RIPC. Hearts of male Wistar rats were isolated and perfused in Langendorff mode. A group of donor hearts was exposed to 3 × 5-5 min global ischemia and reperfusion (IPC) or 30 min aerobic perfusion, while coronary perfusates were collected. Coronary perfusates of these hearts were given to another set of recipient isolated hearts. A group of recipient hearts received IPC effluent depleted of EVs by differential ultracentrifugation. Infarct size was determined after 30 min global ischemia and 120 min reperfusion. The presence or absence of EVs in perfusates was confirmed by dynamic light scattering, the EV marker HSP60 Western blot, and electron microscopy. IPC markedly increased EV release from the heart as assessed by HSP60. Administration of coronary perfusate from IPC donor hearts attenuated infarct size in non-preconditioned recipient hearts (12.9 ± 1.6% vs. 25.0 ± 2.7%), similarly to cardioprotection afforded by IPC (7.3 ± 2.7% vs. 22.1 ± 2.9%) on the donor hearts. Perfusates of IPC hearts depleted of EVs failed to exert cardioprotection in recipient hearts (22.0 ± 2.3%). This is the first demonstration that EVs released from the heart after IPC are necessary for cardioprotection by RIPC, evidencing the importance of vesicular transfer mechanisms in remote cardioprotection.

1. Introduction

Remote ischemic conditioning (RIPC), where a remote area of the heart or another organ is submitted to brief cycles of ischemia–reperfusion, protects the heart against a lethal ischemic insult with efficiency comparable to that of classic in-situ ischemic protocols [1,2]. Although effector pathways of RIPC have been well described, it is currently unclear how cardioprotective signals are propagated between organs [3]. Humoral and neuronal aspects have been hypothesized, but vesicular transfer mechanisms have not been evidenced in inter- or intra-organ communication of RIPC signals.

Exosomes and microvesicles/microparticles (collectively termed extracellular vesicles, EVs) are membrane-bound structures secreted by a wide range of mammalian cell types via distinct mechanisms [4,5]. Since EVs contain a high concentration of RNAs and proteins, and since EVs can be secreted and specifically taken up by other cells, they are prime medium for intercellular signal transfer mechanisms [5]. Thus, it is not surprising that EVs have been shown to modulate several essential cellular functions, including cell survival mechanisms [6,7]. However, to date, it is not known whether EVs are involved in the transmission of cardioprotective signals in ischemic conditioning maneuvers, particularly, their role in the propagation of RIPC has never been studied.

Therefore, here we aimed to investigate whether the release of EVs from the heart is induced by preconditioning stimuli; and to test if EVs are necessary for RIPC-induced cardioprotection by assessing that RIPC can be exerted in the presence and absence of EVs.

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2. Materials and methods

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996) and was approved by the animal ethics committee of the Semmelweis University, Budapest, Hungary.

2.1. Experimental setup, heart perfusion protocol, and assessment of infarct size

Male Wistar rats (250–350 g) were anesthetized by 85 mg/kg ketamine and 10 mg/kg xylazine and heparinized. Hearts were isolated and perfused in Langendorff mode with 37 °C Krebs–Henseleit solution for 20 min for stabilization; then hearts were randomized to the following groups. Perfusate donor hearts either received aerobic perfusion for an additional 30 min (CON) or were exposed to 3 × 5-5 min ischemia and reperfusion (PRE). Perfusate recipient hearts were perfused with collected perfusate from either CON or PRE hearts (CON PERF and PRE PERF, respectively). Another group of hearts received perfusate of PRE hearts which had been previously depleted of EVs (DEPL PERF). All hearts were then exposed to a 30 min global ischemia and 2 h reperfusion (Fig. 1).

Hearts then were cut into 6–8 slices, slices were weighed, and infarct size was assessed by TTC-staining. Infarct size was expressed as a percentage of the total heart weight.

2.2. Isolation of EVs and EV depletion

EVs were isolated from collected coronary perfusates by filtration and differential centrifugation. Briefly, perfusates were dialyzed against 0.45% saline containing 5 mM EDTA for 4 h at room temperature then vacuum-distilled to 40 mL. Concentrated perfusates were filtered through 800 nm filter (Merck, Darmstadt, Germany) and centrifuged at 12,200 ×*g* for 20 min at 4 °C. Pellets were saved as microvesicle/microparticle fraction. Then supernatants were filtered through 200 nm filter (Merck, Darmstadt, Germany) and centrifuged at 100,000 ×*g* for 90 min at 4 °C. Pellets were saved as exosome-rich pellet and the supernatant was saved as EV-depleted perfusate. EV-depleted perfusates were then reconstituted to their original volume with Krebs–Henseleit solution and used in heart perfusion experiments.

2.3. Characterization and assessment of quantity and size distribution of EVs

Isolated vesicles were visualized by transmission electron microscopy. Vesicle pellets were fixed with 4% formaldehyde, postfixed in 1% OsO_4. EVs were block-stained with 1% uranyl acetate in 50% ethanol, then dehydrated in graded ethanol, and embedded in Taab 812 (Taab Laboratories, Aldermaston, UK). Ultrathin sections were cut and then analyzed with a Hitachi 7100 electron microscope.

Hydrodynamical average particle size of EVs in perfusates was measured by Dynamic Light Scattering (DLS) apparatus Zetasizer Nano ZS (Malvern Instruments, Malvern Hills, UK) (n = 3–4).

The presence and amount of EVs were assessed by HSP60 immunoblots from vesicular pellets and EV-depleted perfusates.

For a detailed Methods section please see Supplementary data online.

2.4. Statistical analysis

Values are expressed as mean ± SEM. One way analysis of variance (ANOVA) followed by Fisher LSD post-hoc test was used to determine differences in infarct size.

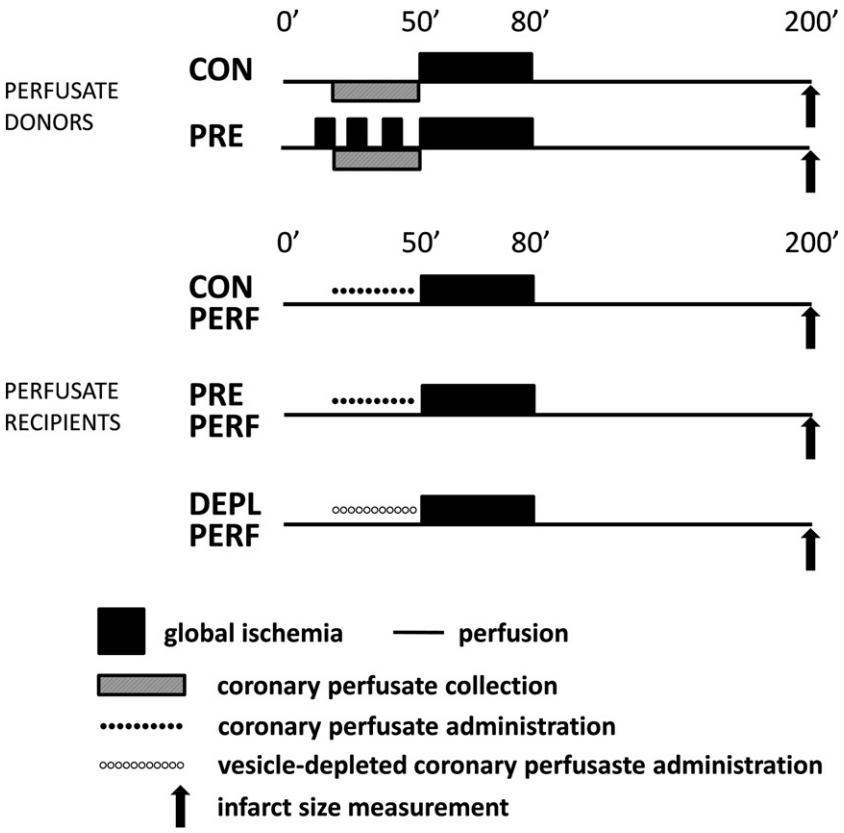

Fig. 1. Experimental protocol of Langendorff-perfused rat hearts. CON: control; PRE: preconditioned; PERF: perfused; DEPL: depleted.

3. Results

3.1. Ischemic conditioning increases the amount of EVs released into coronary perfusate

Coronary perfusates from preconditioned hearts (PRE) contained more EVs than perfusates isolated from control (CON) hearts as evidenced by Western blot against HSP60, a well-accepted marker of EVs (Fig. 2a). Electron micrographs revealed that these EVs can be classified as microvesicle/microparticles as defined by a diameter of >100 nm and light vesicular structure (Fig. 2b upper panels), and exosomes of <100 nm in diameter (Fig. 2b lower panel).

To further evidence that size of the particulate matter in coronary perfusate conforms to the range of microvesicles and exosomes, we assessed their size distribution by DLS in perfusates from PRE hearts. As the representative diagram (Fig. 2c) shows, three populations of particles could be distinguished in our samples. Beside a small fraction of particles with hypothetical diameter of approximately 10 nm, the main particulate constituents fell into the size range of exosomes (<100 nm) and microvesicles (100–1000 nm).

3.2. Perfusates depleted of EVs did not decrease infarct size

Isolated hearts that received 3 × 5-5 min ischemia and reperfusion (PRE) before 30 min global ischemia demonstrated a significantly reduced infarct size as compared to aerobically perfused hearts (CON). In hearts perfused with coronary perfusates collected from PRE hearts (PRE PERF) infarct size was significantly lower than in hearts perfused with coronary perfusate from CON hearts (CON PERF). Perfusates of PERF hearts which had been depleted of EVs were also given to recipient hearts (DEPL PERF). Infarct size after 30 min ischemia and 2 h reperfusion in DEPL PERF hearts did not differ significantly from the extent of infarction observed in CON PERF hearts (Fig. 2d).

4. Discussion

We have shown here for the first time in the literature that the release of EVs from the heart after preconditioning stimuli is increased and that EVs are responsible for the transmission of remote conditioning signals for cardioprotection.

Previously several humoral and neuronal transmitter mechanisms have been hypothesized to play a role in the propagation of remote ischemic conditioning, however, to date none of them is generally accepted. Dickson et al. have proposed first the involvement of humoral transmission pathways showing that transfusion of blood from preconditioned rabbits confers cardioprotection in a naïve non-preconditioned animal against ischemia/reperfusion injury [8]. Later, Breivik et al. showed that the soluble factor is likely to be hydrophobic [9]. The role of neuronal pathways has also been studied, but results are also still controversial [10,11].

Here we evidence a novel, vesicular mechanism for the transmission of cardioprotective signals from a preconditioned heart to another heart subjected to coronary occlusion and reperfusion, which might explain how the suspected humoral and/or released neuronal factors of remote conditioning are transmitted. Ischemia-induced release of EVs from cultured cardiomyocytes was reported by Malik et al. [12] recently, which

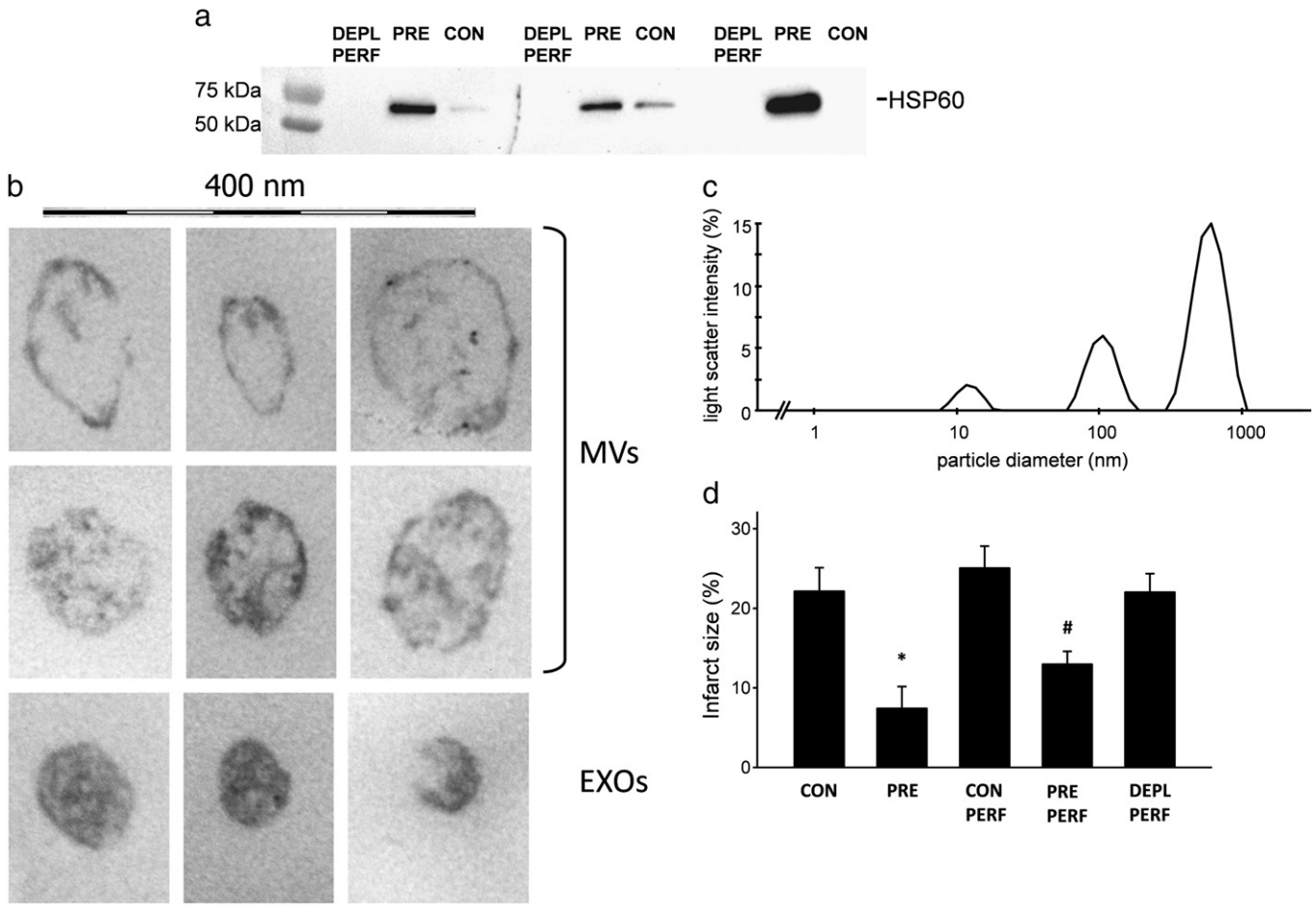

Fig. 2. a: HSP60 immunoblots of depleted perfusates (DEPL PERF), EV pellets of preconditioned (PRE) and control perfusates (CON). b: Representative electron micrographs of EVs in the size ranges of microvesicles and exosomes isolated from coronary perfusate of preconditioned hearts. Scale bar represents 400 nm. c: DLS analysis of perfusates showing distribution of different vesicle populations. d: Infarct size indicated as percent of the total heart volume. Results are expressed as mean ± SEM; n = 5–8. *p < 0.05 vs. CON, #p < 0.05 vs. CON PERF.

is in agreement with our current findings that EV-release of isolated hearts increases after brief ischemic episodes. Elsewhere, exosomes derived from mesenchymal stem cell cultures have been shown to exert cardioprotection in mice [13], and microvesicles isolated from cell culture medium of GATA-4-overexpressing bone marrow stem cells protected neonatal cardiomyocytes from ischemic injuries [14]. Since in the latter two reports EVs from untreated cells induced pro-survival signals, based on our current findings, we cannot exclude the possibility that EVs released from the heart under basal conditions might be also cardioprotective, would their amount be as high as after preconditioning stimuli. Seemingly controversial to our findings, microvesicles derived from blood of animals underwent hind limb ischemia/reperfusion failed to decrease infarct size in rats [15], which might suggest that exosomes rather than microvesicles are responsible for the propagation of cardioprotective signals. Nonetheless, despite our present findings clearly show that an increased release of EVs is indispensable for RIPC-induced cardioprotection ex-vivo, further experiments are warranted to investigate this phenomenon using a clinically relevant in-vivo model for remote conditioning by hindlimb ischemia.

In summary, this is the first demonstration that EVs are the carrier mechanism of the cardioprotective effect of RIPC of the heart, although, further molecular and in-vivo experimentation is warranted to decipher the nature of actual effector factors carried by these vesicles.

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Conflict of interest statement

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2014.01.004.

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