Microparticle release in remote ischemic conditioning mechanism

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MYOCARDIAL INFARCTION (MI) is a major cause of mortality worldwide (22). Although highly beneficial, myocardial reperfusion is paradoxically associated with cellular injuries including cardiomyocyte death (25). Therefore, new treatment strategies are required to protect the heart during myocardial reperfusion. Remote ischemic postconditioning (RCond), defined as brief intermittent episodes of ischemia and reperfusion in an organ or tissue remote to the heart, offers a novel approach to myocardial protection (10). First described by Kerendi et al. in 2005 (13), RCond is clinically applicable and has been successful in reducing infarct size in the human heart (5). However, the signaling pathways linking the remote organ or tissue to the heart remain unclear (11). A humoral factor appears to be involved (15), although its identity is currently unknown.

Microparticles (MPs) are small membrane vesicles that are released from cells in response to activation or apoptosis. MPs are present in the blood of healthy individuals. The number and cellular source of MPs are altered in various pathological states, including cardiovascular diseases (20). Endothelial microparticles (EMPs), reflecting endothelial injury, increase in patients with acute coronary syndrome and MI (4). MPs may play a role by interacting with circulating cells or components of the vessel wall. Indeed, MPs can be considered vectors of biological messages, such as induction of endothelial and vascular dysfunctions (7, 20). MPs may also have beneficial biological effects, promoting nitric oxide production (3) or vasculogenesis (18).

In this investigation, we sought to evaluate the role of circulating MPs in the messages relayed to the heart during RCond. Our aim was to characterize circulating MPs after RCond according to their cellular origins and procoagulant properties and to determine whether these MPs could be a biological vector of RCond.

MATERIALS AND METHODS

Study 1: MP isolation and characterization of MP phenotype after RCond in rats. Male Wistar rats weighing 260 ± 7 g were assigned to two groups of 10 rats: 1) control (anesthesia and blood sampling only) and 2) RCond (blood sampling 10 min after the end of limb ischemia). All animals were treated in accordance with 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 the study was approved the Animal Care and Use Committee. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg) and orotracheally intubated with a 16-gauge tube. The animals were ventilated using a small animal ventilator at a rate of 50–60 breaths per minute (Series Small animal Ventilator, SAR-830/P; CWE, Ardmore, PA). Body core temperature was monitored during the surgical procedure with a rectal thermometer and maintained at 37 ± 1°C by using a homeothermic blanket linked to a temperature control unit (HB101/2 RS; Bioseb, France). RCond was performed by thin elastic placed around the upper third of the hind extremity in tight position, as previously described (23). Limb ischemia was performed for 10 min followed by 10 min of reperfusion. The underskin temperature was measured in an interdigital space of the leg using an electrothermometer. Limb ischemia was confirmed by the pale color of the limb and the decrease in temperature (>3°C in all).

Subtotal blood (8 ml) from rats was collected in sodium citrate tubes after inferior vena cava puncture. Blood samples were centrifuged for 20 min at 240 g, and plasma was then isolated and centrifuged for 20 min at 1,500 g to obtain platelet-free plasma (PFP). As previously described (12), three hundred microliters of PFP was frozen and stored at −80°C for quantitative and qualitative analyses. Remaining PFP was subjected to a centrifugation at 21,000 × g for 45 min to pellet MPs for experiments. Supernatant was replaced by 100 μl 0.9% sodium chloride, and pellets were mixed together in a 2-ml
tube. A further centrifugation at 21,000 g for 45 min was performed, and the pellet was resuspended in 300 µl of 0.9% sodium chloride kept at 4°C. At this stage MPs were sufficiently washed to ensure that there were not contaminated by circulating plasma products. As previously described (2), membrane MP subpopulations were discriminated in both PFP and pellet MPs according to the expression of membrane-specific antigens. Numeration of platelet, leukocyte, endothelial, and erythrocyte MPs was performed using anti-CD61, anti-CD45, anti-CD45 (BioLegend, San Diego, CA), and anti-erythroid cell (BD Biosciences, San Jose, CA) labeling, respectively. Annexin V binding was used to count phosphatidylserine-expressing circulating MPs (3 µl of Annexin V/5 µl PFP; Beckman Coulter, Villepinte, France). An equal volume of sample and Flowcount beads were then added, and samples were analyzed in a flow cytometer 500 MPL System (Beckman Coulter). The size of MPs was determined with a Zetasizer ZS (Malvern, Worcestershire, UK). Particle sizing was based on photon correlation spectroscopy, and the results were expressed in nanometers. MP size was 740 ± 58 nm (627–821 nm).

Study 2: MP isolation and characterization of MP phenotype after RCond in humans. Eight healthy donors were recruited. The study was approved by the ethics committee at the University Hospital of Angers (France), and donors gave written informed consent. Donors were Caucasian men who did not have any accepted cardiovascular risk factors. The mean age of donors was 31 ± 2 years. They did not carry out any strenuous physical activity during the last 48 h. As described by Hoole et al. (12), RCond was performed by using a blood pressure cuff placed around the arm. The cuff was inflated to 200 mmHg by Hoole et al. (12), RCond was performed by using a blood pressure cuff placed around the arm. The cuff was inflated to 200 mmHg pressure for 5 min, followed by 5 min of deflation, to permit reperfusion. This sequence was repeated two more times.

Peripheral blood (25 ml) from donors was collected in sodium citrate tubes. Venous puncture was performed in the contralateral arm before and 5 min after RCond. Blood samples were centrifuged by using the same protocol as for rats. A total of 200 µl PFP was frozen and stored at −80°C for quantitative and qualitative analyses using flow cytometry. Numeration of platelet, leukocyte, endothelial, and erythrocyte MPs was performed by using anti-CD41, anti-CD45, anti-CD146, and anti-CD235a (Beckman Coulter) labeling, respectively. Additionally, annexin V binding was used to numerate phosphatidylserine-expressing circulating MPs. As described above for rats, an equal volume of sample and Flowcount beads were added, and samples were analyzed in a flow cytometer.

Study 3: infarct size measurement after RCond-derived rat MPs injection. RCond-derived rat MPs were injected intravenously into rats undergoing myocardial ischemia. Infarct size was measured and then compared with rats receiving RCond during similar myocardial ischemia. Thus male Wistar rats weighing 288 ± 9 g were randomly assigned to one of the following groups (Fig. 1): 1) MI alone (n = 6); 2) MI + RCond started 20 min after coronary ligation (n = 6); or 3) MI + injection of RCond-derived rat MPs (MI + MPs) (n = 5).

All rats underwent a total of 40 min coronary ligation followed by a 2-h reperfusion by using an open chest surgical approach. A median thoracotomy was performed. After removal of the pericardium so as to expose the heart, a 7-0 polypropylene suture (Premio 7/0; Peters surgical) was placed around the proximal portion of the left anterior descending coronary artery (LAD). The ligature ends were passed through a small length of plastic tube to form a snare. For reversible coronary artery occlusion, the snare was pressed onto the surface of the heart directly above the coronary artery. Ischemia was confirmed by blanching of the myocardium below the suture and dyskinesis of the ischemic region. After 40 min of occlusion, reperfusion was achieved by loosening the snare and confirmed by a marked hyperemic response at reperfusion. Two hours after reperfusion, the heart was harvested. The LAD was re-occluded using the 7-0 monofilament suture kept in place. The ascending aorta was retrogradely perfused ex vivo by using Evans Blue. The left ventricle was sliced transversely from apex to base into five to six 1-mm slices. The slices were incubated in 1% triphenyltetrazolium chloride (TTC; Sigma) in phosphate buffer solution, pH 7.4, at 37°C for 20 min and then fixed in 10% formalin. For each section, the area at risk (nonblue) and infarct size (white area) were quantified with computerized planimetry using ImageJ software (National Institutes of Health, Bethesda, MD). Infarct size was expressed as a percentage of total left ventricular area at risk (INF%AAR), and risk area was expressed as a percentage of left ventricular area (AAR%LV).

The estimated plasma volume of each rat was calculated as previously described (17). Briefly, total rat blood volume was estimated using the formula [0.06×body wt (g) + 0.77] and assuming a 43% hematocrit. The number of RCond-derived rat MPs to be injected was then estimated from the mean circulating level of MPs detected in the rat plasma following RCond. For example, estimated total blood volume of a rat of 250 g body wt was 15.77 ml and with the assumption of a 43% hematocrit, estimated plasma volume was 8.99 ml. Because mean concentration of MPs after RCond was 3,310 events/µl of plasma, 29.77×10⁶ RCond-derived rat MPs were injected (8.99×3,310×10⁶). The profile of circulating RCond-derived MPs as well as injected RCond-derived MPs was analyzed by flow cytometry. The injection was performed in the femoral vein 2 min before myocardial reperfusion.

Statistical analysis. Statistical tests were performed using SPSS 14 software (SPSS, Chicago, IL). Data were expressed as means ± SE,
and \( n \) represents the number of experiences. Between-group differences were compared using \( \chi^2 \)-squared tests for categorical variables and one-way ANOVA, followed by post hoc Fisher’s least significant difference-corrected multiple comparison test for continuous variables. Circulating levels of MPs before and after RCond in humans were compared by paired \( t \)-test.

**RESULTS**

**Study 1: circulating levels of MPs and their cellular origins after RCond in rats.** The total number of circulating MPs was not significantly increased in the RCond group compared with the control group (3,310 ± 650 vs. 1,994 ± 257 events/μl of plasma, respectively; \( P = 0.08 \); Fig. 2A). Phenotypical characterization of the cellular origin of MPs showed a significant increase in endothelium-derived (CD54\(^+\)) and Annexin V\(^+\) MPs in the RCond group versus the control group (Fig. 2B and C). MPs from other cellular origins did not significantly differ between these two groups of rats, including those from platelet-(CD61\(^+\)), leukocyte- (CD45\(^+\)), and erythrocyte-derived cells (Fig. 2D–F). It should be noted that only 0.98 ± 0.37% of leukocyte MPs were CD54\(^+\), indicating that CD54\(^+\) MPs reflected endothelial cell-derived MPs.

**Study 2: circulating levels of MPs and their cellular origins after RCond in humans.** Comparison between before and after RCond showed no significant difference for the total number of circulating MPs (13,385 ± 2,338 vs. 14,908 ± 2,653 events/μl of plasma, respectively; \( P = 0.67 \); Fig. 3A). As for rats, phenotypical characterization of the cellular origin of MPs showed a significant increase in endothelium-derived (CD146\(^+\)) and Annexin V\(^+\) MPs in the RCond group versus the control group (Fig. 3, B and C, and Fig. 4). MPs from other cellular origins did not significantly differ between the two groups (Fig. 3, D–F).

**Study 3: infarct size measurement after rat RCond-derived MPs injection.** Mortality rate was similar in all MI groups (MI = 23.1%, MI + RCond = 18.2%, MI + MPs = 18.2%, not significantly different). The ischemic area induced by LAD ligation (AAR%LV) did not differ among the three groups (MI = 39.4 ± 1.9%, MI + RCond = 39.2 ± 5.5%, MI + MPs = 44.2 ± 3.2%, not significantly different; Fig. 5).

As shown in Fig. 5, RCond induced infarct size reduction (INF%AAR = 24.4 ± 5.9% in MI + RCond group vs. 54.6 ± 4.7% in the MI alone group, respectively; \( P < 0.01 \)). Contrary to MI + RCond, infarct size was not decreased in MI + MPs, compared with MI alone (INF%AAR = 50.2 ± 6.4% vs. 54.6 ± 4.7%, respectively, not significantly different).

Phenotypical characterization of cellular origins of MPs showed no differences between injected MPs and those of circulating MPs (Fig. 6). These results suggest that the populations of MPs used for injection remain similar to those of circulating MPs.

![Histograms show total circulating MP levels (A) and endothelium-derived MPs (CD54\(^+\); B), procoagulant MPs (Annexin V\(^+\); C), platelet-(CD61\(^+\); D), leukocyte- (CD45\(^+\); E), and erythrocyte-derived MPs (F) from control group (\( n = 10 \)) and RCond group (\( n = 10 \)). Results are expressed as events per microliter of plasma and given as means ± SE. *\( P < 0.05 \) vs. control group.](http://ajpheart.physiology.org/)

*Fig. 2. Circulating MP levels in 2 groups of rats. Histograms show total circulating MP levels (A) and endothelium-derived MPs (CD54\(^+\); B), procoagulant MPs (Annexin V\(^+\); C), platelet-(CD61\(^+\); D), leukocyte- (CD45\(^+\); E), and erythrocyte-derived MPs (F) from control group (\( n = 10 \)) and RCond group (\( n = 10 \)). Results are expressed as events per microliter of plasma and given as means ± SE. *\( P < 0.05 \) vs. control group.*
DISCUSSION

RCond is a new noninvasive procedure with high potential for clinical application in patients presenting an acute MI. However, the mediator of RCond is still unknown. The present study demonstrated increased levels of EMPs and procoagulant MPs during RCond in both rats and humans. However, injection of rat RCond-derived MPs to rats did not mimic RCond-derived cardioprotection.

The actual mechanism of RCond and the messages relayed to the heart are still unknown. We, and others, have demonstrated that RCond activates the reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE) pathways in the heart, both signal transduction cascades initially described in local ischemic pre- or postconditioning (11, 29, 30). Numerous studies sought to determine the vector between the remote organ and the heart. Several pathways, which may interact with each other, have been proposed (11): a neurogenic pathway, a systemic response, and/or a humoral pathway. In the neural hypothesis, one or various endogenous substances, released at the reperfusion, activate afferent neurofibers, resulting in a cardioprotective signal on the myocardium (9). In the systemic response, ischemia and reperfusion in the remote organ could...
change gene transcription, thereby suppressing proinflammatory gene expression (14). There are strong arguments for the existence of a humoral factor, released by the remote organ after ischemia, which is then transported to the heart (15). Shimizu et al. (28) showed that plasma or dialysate of plasma obtained from donor rabbits or humans subjected to RCond, using four cycles of 5 min limb ischemia and 5 min reperfusion, decreased ischemia-reperfusion injury in isolated hearts and cardiomyocytes models. The identity of this potential humoral factor is still unknown, even if several endogenous substances such as adenosine (24) and opioids (32) could be involved at some point. Thus, the link between the remote organ and the heart is still unclear, and a proteomic study failed to clarify the connexion (16). MP characteristics are consistent with the low molecular mass and hydrophobic nature of the circulating factor that has been shown in Langendorff-perfused rabbit heart (28). Despite clear increased levels of EMPs and procoagulant MPs after RCond in both humans and rats, injection of rat RCond-derived MPs to rats before reperfusion did not induce cardioprotection in our model.

Fig. 5. Infarct size assessment in MPs-treated rats. A: infarct size is expressed as a percentage of area at risk (INF%AAR; black column), and area at risk expressed as a percentage of left ventricular area (AAR%LV; white column) in 3 groups of rats: 1) MI (n = 6); 2) MI + RCond (n = 6); 3) MI + MPs (n = 5). *P < 0.05 vs. MI group. B: representative pictures from each group of a mid-left ventricle slice after TTC staining.

Fig. 6. Flow cytometer dot plot [forward scatter (FSC) vs. fluorescence levels of fluorescently labeled microparticles] showing circulating (left) and injected (right) MP profile. MPs were stained with CD61-FITC antibody or CD54-biotin antibody plus streptavidin-FITC and then measured using fluorescence threshold triggering.
To the best of our knowledge, the present study is the first to examine the effect of RCond on circulating MP levels. Interestingly, we observed similar results in both rats and humans. We found an approximately sevenfold increase in rats and a ~10-fold increase in humans in circulating EMP levels after RCond. EMPs are a novel marker of endothelial injury (8). Various stimuli have been shown to provoke EMP release in such prothrombotic or proinflammatory states, or during oxidative stress (8). Nitric oxide synthase decoupling may also participate in the production of EMP (31). Moreover, low laminar shear stress on the vessel wall is associated with high EMP levels (6). Ex vivo studies have analyzed the effects of these EMPs; for example, human EMPs can induce severe endothelial dysfunction in rat aortic rings by altering the endothelial nitric oxide transduction pathway (7). Thus EMPs could be considered to be endothelial dysfunction markers. Furthermore, studies in humans have shown association between EMPs and clinical manifestations or risk factors. EMPs are released by ischemic tissues, in acute coronary syndromes (4) or prolonged muscle ischemia (18). In this study, we demonstrated that EMPs are also released during short-term peripheral ischemia. In addition, RCond induced an approximately sixfold increase in rats and a ~12-fold increase in humans in the circulating level of procoagulant MPs, which are characterized by expressing phosphatidylserine on their outer leaflet. During this short-term ischemia, in both rats and humans, the loss of endothelial integrity and the apoptosis of endothelial cells may have induced a procoagulant state, with the subsequent release of MPs. As for EMPs, significant increases in procoagulant MP levels have been shown in the circulating blood of patients with acute coronary syndrome (19) and prolonged muscle ischemia (18). In this study, procoagulant MPs originated mainly but not exclusively from endothelial cells. The nonsignificant upward trend of platelet- and leukocyte-derived MPs may be involved in this increase in procoagulant MPs after RCond. Furthermore, other cellular types could also play a role, such as smooth muscle cells. At last, we observed that the total number of circulating MPs did not differ after RCond, despite a strong upward trend in both rats and humans. In the present study, only the phenotype of MPs differed after RCond, as previously described in other pathological state (26).

Limitations. Because the circulating time course of the injected RCond-induced MPs is unknown in our model, the lack of response to MPs injection may be due to a rapid MP sequestration. However, the half-life of transfused platelet-derived MPs has been reported to exceed 5 h in humans (27). Furthermore, similar approaches using exogenous injection of MPs were previously tested in our group, inducing relevant responses (1, 21). Thus we showed that circulating levels of MPs and EMPs were increased in patients with septic shock. These MPs isolated from septic patients were then injected intravenously into mice, providing evidence that increased circulating MPs are protective against vascular hyperreactivity (21). Furthermore, circulating MPs isolated from patients with metabolic syndrome injected to mice induced vascular dysfunction (1). Finally, MPs collected after RCond massively expressed phosphatidylserine and were procoagulant, with a potential thrombogenic effect following injection. However, we observed an infarct size decrease in the MI + RCond group, although procoagulant MPs were released into the plasma of these rats.

In summary, this study demonstrated that RCond induces similar MP release in both rats and humans, especially EMPs. However, these MPs did not appear to be a biological vector of RCond between the remote organ and the heart in our experimental model.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


