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Extracellular vesicles (EVs) in ischemic conditioning and angiogenesis: Focus on endothelial derived EVs

Edoardo Alfì [a], Cecilia Thairi [a], Saveria Femminò [b], Giuseppe Alloatti [c], Francesco Moccia [d], Maria F. Brizzi [b], Pasquale Pagliaro [a,*], Claudia Penna [a]

[a] Department of Clinical and Biological Sciences, University of Turin, Regione Gonzole 10, 10043 Orbassano, TO, Italy
[b] Department of Medical Sciences, University of Turin, Corso Dogliotti 14, 10126 Turin, Italy
[c] Uni-Astiss, Polo Universitario Rita Levi Montalcini, 14100 Asti, Italy
[d] Department of Biology and Biotechnology, University of Pavia, Laboratory of General Physiology, 27100 Pavia, Italy

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ABSTRACT

During myocardial ischemia, timely reperfusion is critical to limit infarct area and the overall loss of cardiac contractile function. However, reperfusion further exacerbates the damage of the ischemic heart. This type of injury is known as ischemia-reperfusion injury (IRI). Ischemic conditioning is a procedure which consists of brief cycles of ischemia and reperfusion in order to protect the myocardium against IRI. Remote ischemic conditioning (RIC), namely transient brief episodes of ischemia at a remote site before a subsequent damaging ischemia/reperfusion procedure of the target organ (e.g., the heart), protects against IRI. However, how the stimulus of RIC is transduced from the remote organ to the ischemic heart is still unknown. Recently, extracellular vesicles (EVs) have been proposed to have a role in the RIC procedure. The endothelium releases EVs and is also one of the tissues mostly exposed to EVs during their journey to the target organ. Moreover, EVs may have important roles in angiogenesis and, therefore, in the remodeling of post-ischemic organs. Here we analyze how EVs may contribute to the overall cardioprotective effect and the implication of the endothelium and its EVs in RIC mediated acute cardioprotection as well as in angiogenesis.

1. Introduction

The paradigm of cardioprotection induced by ischemic preconditioning (IPC) was established by Murry and colleagues [1]. These authors demonstrated that brief cycles of 5 min coronary occlusion interspersed with 5 min of reperfusion immediately prior to 40 min of coronary occlusion were able to significantly reduce infarct size (IS). Subsequently remote ischemic conditioning (RIC) was described [2]. Unlike IPC, which protects only those districts directly subjected to initial ischemia by coronary occlusion, in RIC the ischemic insult is applied to distant areas within the heart or distant organs. Among distant organs limbs [3] are included. RIC gained popularity for this intriguing feature and it is believed a more applicable type of conditioning for cardioprotection in humans. RIC can be applied before ischemia, that is remote preconditioning (RIPC), during organ ischemia, namely remote per-conditioning (RIPerC) and/or after organ ischemia, that is remote post-conditioning (RIPostC). All these procedures, collectively known as RIC, showed similar cardioprotective effects [4].

Several evidence suggest that many organs can undergo RIC in order to protect the heart from ischemia/reperfusion (I/R) injury (IRI). Indeed, cardioprotection can be evoked by ischemic episodes in distant organs, such as liver [5], small intestine [6], and kidney [7]. Different interesting mechanisms have been proposed to explain the cardioprotective effect of RIC [8] including: *i)* the RIC as a trigger of specific mediators in the preconditioned remote organ/limb, *ii)* the transfer of such mediators from remote organ/limb to the heart, and finally *iii)* the

Abbreviations: AMI, Acute myocardial infarction; EC, Endothelial cells; ECFCs, Endothelial colony forming cells; EEs, Early endosomes; EPC, Endothelial progenitor cells; eNOS, Endothelial nitric oxide synthase; ESCRT, Endosomal sorting complex required for transport; EVs, Extracellular vesicles; HUVECs, Human umbilical vein endothelial cells; ILVs, Intraluminal vesicles; I/R, Ischemia reperfusion; IRI, Ischemia reperfusion injury; IPC, Ischemic preconditioning; LEs, Late endosomes; MMP, Matrix metalloprotease; MS, Metabolic syndrome; MSCs, Mesenchymal stem cells; MVBs, Multivesicular bodies; PI3K, Phosphoinositide-3-kinase; RIC, Remote ischemic conditioning; RIPC, Remote ischemic preconditioning; RIPerC, Remote ischemic perconditioning; RIPostC, Remote ischemic post conditioning.

* Corresponding author.
E-mail address: pasquale.pagliaro@unito.it (P. Pagliaro).

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modulation end effectors within the heart enhancing its resistance to sustained IRI.

Recently, extracellular vesicles (EVs) have been proposed as potential mediators of cardioprotective signals of RIC [9] (Fig. 1) and potential carriers of protective signals in general [10]. In particular, in the context of chronic ischemic myocardial disease, intramyocardial treatment with human EVs may increase the perfusion of post-ischemic myocardial tissue by inducing arteriolar and capillary growth. It seems that the EVs are able to activate the Akt/eNOS and mitogen-activated protein kinase signaling pathways resulting in increasing both the stroke volume and cardiac performance. However, contrasting results are reported about the ability to induce changes in vascular density and to improve cardiac function under conditions of chronically ischemic myocardium and/or diet-induced metabolic syndrome (MS) [11–14].

In accordance with these studies, the main goal of this review is to recap the most recent knowledge implicating EVs in RIC mediated-cardioprotection, focusing on the role of endothelium-derived EVs and their involvement in angiogenesis. Indeed, in addition to producing most of the circulating EVs, the endothelium is constantly exposed to circulating EVs not only at the interfaces with remote organs and the target tissue in general, but also throughout the vasculature of the whole body. These features make the endothelium a fundamental mediator of the cross-talk between the remote organ and the heart and so a potential mediator of the RIC and subsequent angiogenesis.

2. Extracellular vesicles: Classification, content and biogenesis

EVs are lipid bilayer-coated particles secreted by different cell types into the extracellular space and thereafter into the circulation. However, EVs can be also found in several other body fluids, such as saliva, urine, breast milk, and cerebrospinal [15–18]. EVs can be mainly classified based on their size in small (<100 nm or < 200 nm) and medium/large EVs (>200 nm) and based on their origin in exosomes and micro-vesicles [19] (Fig. 2). Although a third category of EVs has been suggested, named apoptotic bodies, their genesis and function deeply differ from those of the two former categories and they are not thought to act as humoral factors in intercellular communication [20]. EVs (micro-vesicles and exosomes) are broadly enriched with several bioactive molecules, e.g., lipids, proteins, and nucleic acids, such as messenger RNAs (mRNAs) and non-coding RNAs [21,22]. However, several EV features depend on the microenvironment from which they are secreted [23]. For this and other reasons, besides being involved in both cardiovascular physiology and pathophysiology [24,25], EVs have been proposed as potential diagnostic and/or prognostic biomarkers, in different clinical settings [26,27].

2.1. Micro-vesicles (medium/large EVs)

Micro-vesicles, equally known as micro-particles or ectosomes, have a diameter ranging from 100 to 1000 nm [19]. They are released upon calcium-dependent enzyme recruitment that modify the asymmetry of the plasma membrane and elicit cytoskeleton changes. In particular, the protrusion of phosphatidylserine from the inner to the outer side of the membrane via the aminophospholipid translocases (mainly flippases and floppases) is triggered by the increase in intracellular calcium levels. Moreover, the calcium signal engages calpain to further destabilize the plasma membrane actin-cytoskeleton anchorage, thereby inducing these specialized plasma membrane regions to bud outwards and originate EVs [22].

2.2. Exosomes (small EVs)

Exosomes are the smallest class of EVs, ranging from 30 to 150 nm, which are produced in the endosomal compartment [21]. Briefly, newly formed endocytic vesicles fuse with early endosomes (EEs), which, in turn, gradually mature into late endosomes (LEs). Inward budding of LE membrane results in the formation of intraluminal vesicles (ILVs) and LEs are, therefore, also known as multivesicular bodies (MVBs). MVBs can then be targeted to lysosomes for degradation or move to and fuse with the plasma membrane, thereby releasing ILVs as exosomes (Fig. 2). The formation of ILVs occurs in specific regions of the LE membrane

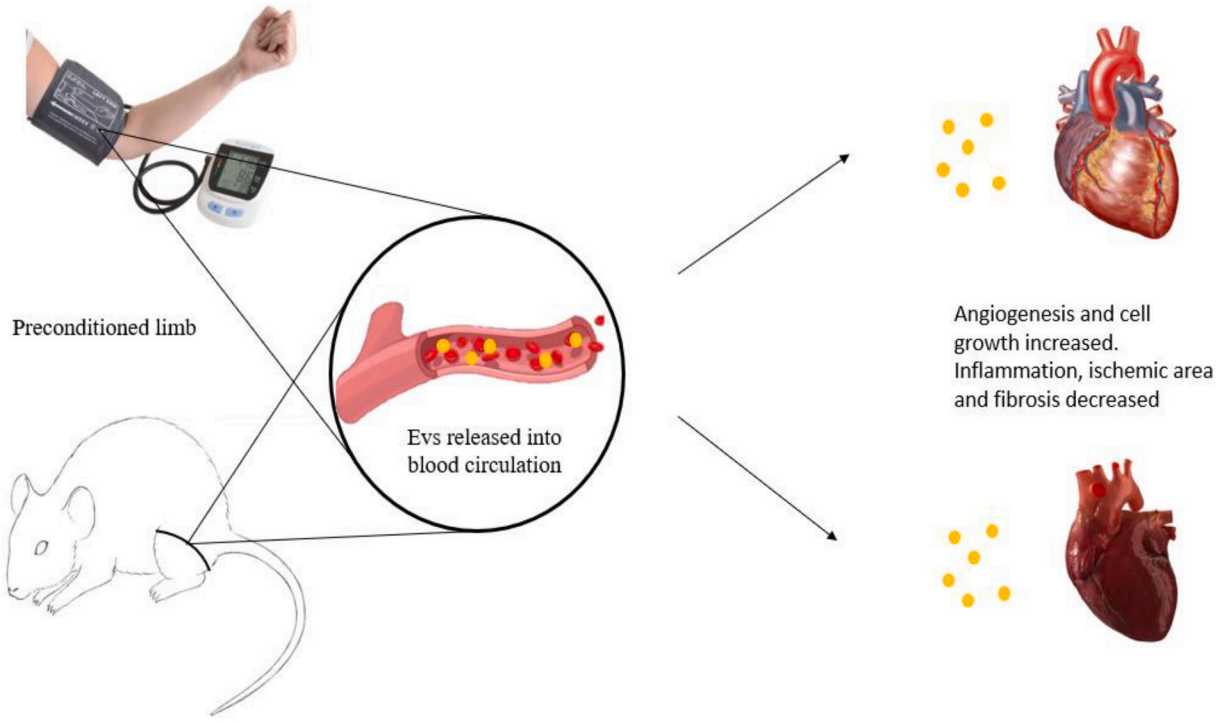

Fig. 1. Schematic model representing the putative cardioprotective mechanism of Remote Ischemic Conditioning (RIC). EVs (Extracellular vesicles) are released into the circulation upon RIC and home to the heart where they exert their protective role.

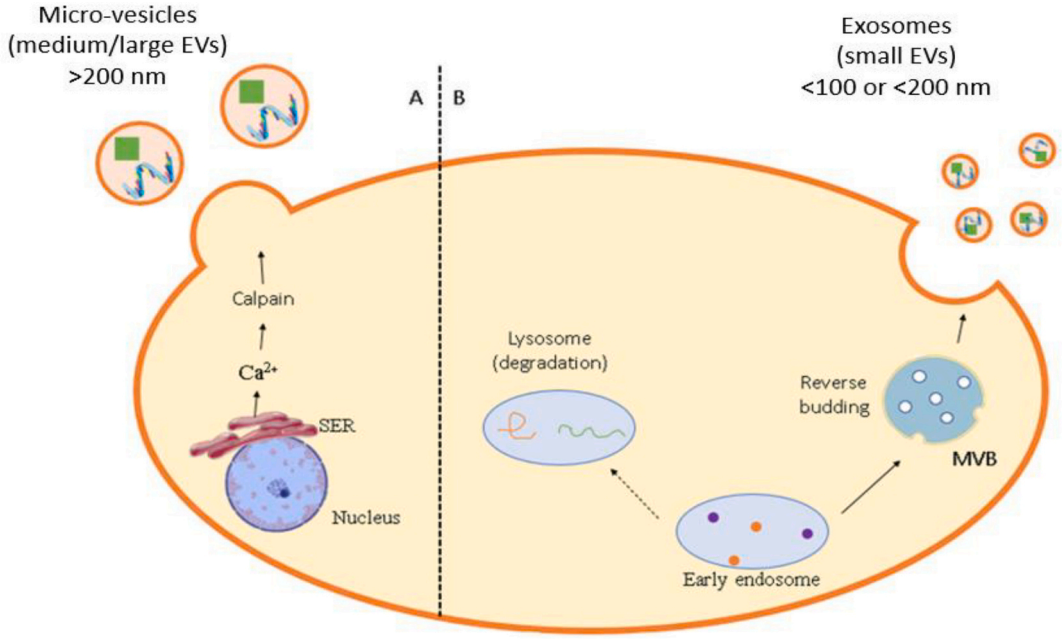

Fig. 2. Schematic representation of EVs biogenesis. A) Micro-vesicles biogenesis. B) Exosomes biogenesis. SER (Smooth endoplasmic reticulum); MVB (Multi-vesicular body).

enriched with tetraspanins and requiring the Endosomal Sorting Complex Required for Transport (ESCRT), which acts in concert with accessory proteins, such as Alix and the tumor susceptibility gene 101 (TSG101). Yet, exosomes are enriched with heat shock proteins (HSP70 and HSP90) and tetraspanins (CD9, CD63 and CD81) and express markers of their cell of origin [20,21].

3. EVs and endothelium in cardioprotection

Although their role is still controversial, several studies have suggested endothelial-derived EVs (ECs-EVs) as a novel tool for the treatment of cardiovascular diseases [28] (Table 1). One of the first studies suggesting the potential role of ECs-EVs in RIC mediated cardioprotection was performed by Davidson and colleagues [29]. Using human umbilical vein endothelial cells (HUVECs)-derived EVs, they demonstrated that the exposure of primary cardiomyocytes to EVs reduces IRI-induced cellular damage paving the way to identify ECs-EVs as crucial mediators of cardioprotection in response to RIC. In fact, in an ex vivo model, Giricz et al. [9] demonstrated that the heart subjected to IPC releases EVs able to protect the naïve heart, thus simulating RIC. These two studies provide the proof-of-concept that conditioning procedures almost double the release of EVs both in vitro and ex vivo (isolated perfused rat heart). Intriguingly, EVs obtained by preconditioned HUVECs only exert their protective effect when given in a dose-dependent manner, reflecting the trend observed after preconditioning. On the other hand, Jeanneteau et al. [30] report that EV number after RIC does not increase either in rats or in humans. However, the relative ECs-EV number ($CD54^+$ and $CD146^+$) and the pro-coagulant Annexin V^+ EVs increased in rats, after 10 min of limb ischemia followed by 10 min of reperfusion, and in humans after three cycles of 5 min ischemia followed by 5 min reperfusion. Treatment with preconditioned rat derived EVs did not show any significant improvement in the IRI model with an ischemic area pretty similar to the control group [30].

The paucity of studies and their heterogeneity does not allow to draw clear-cut conclusions on the role of ECs-EVs as mediators of RIC. In addition, whether the endothelium should be considered a RIC target, a mediator, or both, is even harder to assert. For instance, Chen and collaborators [31] demonstrated that rats treated with RIC-derived EVs improved cell remodeling, heart function related parameters, and angiogenesis after myocardial infarction. RIC treatment consisted in 10 cycles of 2 min reperfusion and 2 min bilateral hindlimb ischemia using tourniquets, at 20-s intervals and the animals were injected with RIC EVs every three days up to 28 days. The investigators also demonstrated that HSP70 enriched in EVs is a crucial mediator. On the other hand, no experiment assessing the cellular origins of these circulating EVs was performed, making it difficult to establish which and how much different tissues contribute to circulating EVs and, therefore, to RIC. Indeed, several cell types produce EVs. ECs, macrophages, fibroblasts and cardiomyocytes, are the primary sources of EVs locally in the heart [25–32]. However platelets [33], mesenchymal stem cells (MSCs) [34] and various cell types produce their own EVs. In this regard, a very recent study reported that upon pretreatment with a Chinese medicine, Tongxinluo, cardiomyocytes release EVs which protect cardiac microvascular ECs after 18 h hypoxia followed by 2 h reoxygenation by activating eNOS, thus, unveiling a cardioprotective mechanism which involves the cross-talk between cardiomyocytes and ECs. The mechanism was also established in vivo. The authors suggested that cardioprotection relies on changes in the EVs content upon Tongxinluo treatment. In particular, EVs were found enriched in specific long non-coding RNAs which downregulates miR-145-5p and leads to eNOS activation [35].

3.1. Microenvironment impacts on EVs

Several groups reported that pharmacological modulation of the microenvironment can dictate EVs' features. For example, cardiac progenitor cells release anti-hypoxic EVs upon Ticagrelor administration [36], whereas the efficacy of MSCs-derived EVs in the treatment of acute myocardial infarction is enhanced by Atorvastatin, likely favoring endothelial function through the upregulation of the long non-coding RNA H19 [37]. Intriguingly, an inflammatory environment could also affect the production of protective EVs. For instance, we demonstrated that the interaction between endothelial-derived EVs and the coronary endothelial layer is necessary to trigger cardioprotection via the activation of the MEK1/2/eNOS/GC pathways. Yet, ECs pre-exposed to interleukin 3 (IL-3) release EVs enriched in the eNOS-antagonist caveolin-1 that may hamper EV cardioprotective properties [38]. Of note,

Table 1
Mechanisms of modulation of cardioprotection and angiogenesis by EVs.

References	Model		EV Origin	EV Content	Main results
Main topic: cardioprotection					
[9]	Rat Hearts	Ex vivo I/R 30′ + 2 h	EVs from ex vivo rat hearts subjected to IPC	NC	EVs reduce infarct size
[29]	Primary Rat Cardiomyocytes	H/R 2 h30′ + 30’	EVs from HUVECs subjected to 30′ hypoxia	NC	EVs protect against hypoxia via ERK1/2
[30]	Rats	In vivo IR 40′ + 2 h	EVs from rats subjected to RIC	NC	EVs do not reduce infarct size
[31]	Rats	In vivo IR	EVs from rats subjected to RIC	NC	EVs decrease collagen deposition and infarct ratio, increase angiogenesis and increase the expression of eNOS, HIF1α, Angiopoietin-1, VEGF and Hsp70
[31]	CMVECs	H/R 12 h + 24 h	EVs from rats subjected to RIC	NC	EVs increase cell proliferation and migration, increase ratio of G1 cells, inhibit apoptosis, increase tube formation and increase the expression of Hsp70
[35]	CMECs	H/R 18 h + 2 h	EVs from Tongxinluo-pretreated cardiomyocytes subjected to H/R (18 h/2 h)	Linc-ROR➔ downregulation miR-145-5p ➔ activation of p70s6k1 ➔ eNOS activation	EVs reduce cell death, increase eNOS phosphorylation and NO production via p70s6k1 phosphorylation
[36]	HL-1 cardiomyocytes	Hypoxia 24 h	EVs from hCPCs pretreated with ticagrelor (72 h)	NC	EVs decrease cell death, attenuate intracellular HIF1α, increase pERK42/44 (measured 5 min after the end of EV treatment)
[37]	HUVECs	Hypoxia 12 h	EVs from Atorvastatin-treated MSCs	LncRNA H19 ➔ regulates miR-675 expression ➔ activation of VEGF and ICAM-1	EVs increase tube length and migration rate, decrease apoptosis
[37]	Rats	In vivo I/R	EVs from Atorvastatin-treated MSCs	LncRNA H19 ➔ regulates miR-675 expression ➔ activation of VEGF and ICAM-1	EVs increase cardiac functional recovery, increase angiogenesis, decrease inflammation (lower IL-6 and TNFα levels)
[38]	H9C2	H/R 2 h + 1 h	EVs from IL-3 treated-HUVECs or EV from HUVECs	Caveolin-1	IL-3-EVs do not improve cell viability, while EVs do
[38]	Rat Hearts	Ex vivo I/R 30′ + 60′	EVs from IL-3 treated-HUVECs or EV from HUVECs	Caveolin-1	IL-3-EVs do not reduce infarct size, while EVs do
[40]	H9c2	Normoxia	EVs from HUVECs subjected to H/R (12 h/4 h)	NC	EVs reduce viability, increase ROS formation and increase p38 and JNK1/2 phosphorylation
[50]	Primary Rat Cardiomyocytes	H/R 3 h + 1 h	EVs from hyperglycemic HUVECs, hyperglycemic rats and diabetic patients	CD81 and HSP70	EVs from hyperglycemic conditions lose the ability to protect (the viability of cardiomyocytes is not rescued)
[51]	HL-1 Cardiomyocytes	H/R 2 h30’ + 20 h	EVs from normoglycemic and diabetic rats subjected to RIC	NC	Only EVs from normoglycemic rats protect against H/R
Main Topic: Angiogenesis					
[12]	Pigs	In vivo I/R with an ameroid constrictor	EVs from MSCs	NC	EVs increase capillary and arteriolar density, increase CO and SV, increase phosphorylation of MAPK and eNOS
[13]	Pigs with High Fat Diet	In vivo I/R with an ameroid constrictor	EVs from MSCs	NC	EVs increased arteriolar but not capillary density, increased CO and SV
[32]	HMECs	Normoxia (in vitro) and Matrigel plug (in vivo)	EVs from HMECs	MiR-214 ➔ stimulates angiogenic program	EVs increase tube length, increase migration and angiogenic sprouts in vitro; stimulate neovessel formation in vivo
[39]	HUVECs	Normoxia	EVs from ASCs preconditioned with endothelial differentiation medium	MiR-31 ➔ targets factor-inhibiting HIF1	EVs increase migration and tube formation
[58]	HUVECs	Normoxia	EVs from MSCs subjected to 72 h hypoxia	NC	EVs increase proliferation, migration and tube formation
[58]	Rats	In vivo I/R 30’	EVs from MSCs subjected to 72 h hypoxia	NC	EVs reduce infarct size, improve cardiac function, increase angiogenesis
[61]	HUVECs	Normoxia	EVs from HUVECs	MMP-2 and MMP-9	EVs improve migration and tubulogenesis
[62]	HUVECs	Normoxia	EVs from HUVECs and HDMECs	Downregulation of miR-106b + upregulation of lncRNA HOTAIR and MALAT1	EVs increase vascularization bioactivity
[66]	HMECs, HUVECs	Normoxia	EVs from MSCs	mRNA for PI3K ➔ Akt and NO	EVs improve proliferation and migration via Akt/NO pathway
[67]	RMECs	Normoxia	EVs from ECFCs	>20 miRNAs ➔ stimulate angiogenesis	EVs increase cell migration
[68]	Mice	In vivo I/R 30’	EVs from MSCs subjected to hypoxia for 24 h	MiR-486-5p ➔ targets MMP19	EVs improve cardiac function, enhance vascular density, decrease infarct size

NC = not considered; Other acronyms and abbreviations as in the text.

Kang and colleagues [39] have found that adipose derived-stem cells, when cultured in endothelial differentiation medium, release miR-31 enriched EVs that enhance angiogenesis by targeting and down-regulating the factor-inhibiting hypoxia-inducible factor-1 (HIF-1). However, a different study reported that, upon hypoxia/reoxygenation treatment (12 h in hypoxic buffer into a hypoxic chamber and then reoxygenated for 4 h), HUVECs produce apoptotic and hypoxic EVs, promoting apoptosis and oxidative stress in H9c2 cells in vitro [40]. Thus, RIC could positively modulate the microenvironment, leading not only to induce the release of protective EVs [29] in situ, but also to exert direct effect at the site of the maneuver application [41]. Nevertheless, we must consider that, besides the remote conditioning effects

[9,42,43], the presence, content, and EV characteristics may be influenced by several different conditions [33,44], including acute coronary syndrome (in patients/animal-models) and mechanical stimulation [45,46], which could hamper their cardioprotective action [10,33].

4. EVs, metabolic syndrome and remote conditioning

Along with microenvironmental cues, sex [47] and comorbidities, such as metabolic syndrome (MS), dictates EV features mainly impacting on their cargo. Given the role played by diabetes in hampering cardioprotection and angiogenesis, here we focus on type 1 and 2 diabetes mellitus (T1DM and T2DM, respectively) [48]. The impact of other comorbidities on EVs are discussed in a recent review [47]. It seems that EVs lose their protective properties in diabetes. Indeed, it has been reported that small EVs from adipocytes of T2DM mice may have a role in the transduction of pathological signals resulting in the exacerbation of myocardial IRI. The same results were obtained with EVs derived from human T2DM and reversed by downregulating miR-130b-3p, suggesting a new standpoint in the treatment of T2DM [49]. Davidson and colleagues demonstrated that EC-EVs lose their cardioprotective activity when taken from T2DM rats/patients [50]. This may explain, at least in part, the lack of protection of RIC in diabetic settings [51]. Surprisingly, while EVs from diabetic animals were found ineffective in transducing protection, their removal worsens hypoxia/reoxygenation-induced damage. As in T2DM, the role of EVs in T1DM is controversial. Various studies suggest that EVs may contribute to the autoimmune process by carrying autoantigens resulting in β-cell dysfunction [52], whereas others support the anti-inflammatory effect of EVs occurring in TD1M [53]. Interestingly, it has been reported that the EV number is increased in T1DM compared to T2DM and healthy subjects [54].

5. Endothelium, EVs and angiogenesis

Angiogenesis is a well-regulated process that plays a fundamental role in the embryo development, and in vascular homeostasis of mature organisms, during tissue regeneration and repair. The angiogenic process occurs in various pathological conditions including inflammation and the post-ischemic phases of vascularization [55]. Indeed, post-ischemic vascularization, which is mainly driven by angiogenesis, is essential to rescue organ function upon an ischemic event and is the final target of a vast array of strategies, collectively denoted as therapeutic angiogenesis [56]. EVs deriving from different MSC sources can stimulate endothelial cell proliferation and tube formation in vitro and increase capillary density and local blood perfusion in small and large animal models of acute and chronic myocardial ischemia [12,57,58] (Table 1). However, the route of EV delivery (intramyocardial vs. intravenous) is crucial to boost in vivo angiogenesis under conditions of chronic myocardial ischemia [12]. Furthermore, in a setting of diet-induced metabolic syndrome, intramyocardial delivery of EVs resulted in an increased arteriolar, but not capillary density [13]. Since different EC subpopulations may exist within the same vascular network [59], it is likely that MSCs-derived EV content can be sensed or not as a pro-angiogenic input depending on the vascular position of recipient EC (arteriolar or capillary). The above evidence, however, suggests that vascular ECs undergo angiogenesis by EVs. Furthermore, endothelial-derived EVs could directly contribute to transduce RIC into a pro-angiogenic output [60]. Early work demonstrated that cultured HUVECs shed EVs, ranging from 300 to 600 nm and containing matrix metalloprotease-2 (MMP-2) and MMP-9, which autocrinally stimulate endothelial migration [61]. Further studies unveiled that ECs-EVs promoted EC migration and tube formation by the horizontal transfer of miR-214, which was also required to promote EV-dependent neovascularization in vivo [32]. The proof-of-concept that vascular ECs may release pro-angiogenic EVs has been recently described in [62]. This report demonstrated that HUVECs and human dermal microvascular endothelial cells (HMECs) pre-treated with ethanol (0–200 mM) release EVs that are, in turn, able to promote endothelial migration in vitro and neovascularization in vivo. This response involves two pro-angiogenic long non-coding RNAs, i.e., MALAT1 and HOTAIR, in ECs-EVs [62] and is consistent with the emerging notion that moderate alcohol consumption could exert a beneficial effect on endothelial function [63]. Paradoxically, the circulating pro-angiogenic EVs detected after intense physical exercise are mainly secreted by pro-coagulant rather than vascular ECs [64]. However, a powerful source of EVs in the cardiovascular system is represented by endothelial colony forming cells (ECFCs), which represent endothelial progenitor cells (EPC) truly belonging to the endothelial lineage [65]. ECFCs are mobilized in peripheral blood and redirected towards ischemic tissues to stimulate local angiogenesis and physically engraft within neovessels, according to the process known as vasculogenesis [65]. An early study demonstrated that the horizontal transfer of a precise subset of mRNAs enabled ECFCs to activate an angiogenic program in HUVECs and HMECs [66]. ECFCs-derived EVs stimulate in vitro proliferation and tube formation in both EC types and promote neovascularization in vivo [66]. The pro-angiogenic effect of ECFCs-derived EVs was mediated by the transfer of mRNAs encoding for PI3K in recipient ECs, which led to Akt activation and eNOS phosphorylation [66]. A recent report confirmed that ECFCs-derived EVs, whose size ranges from 60 to 1500 nm, convey ≈20 mRNAs, including miR-486-5p and miR-26A, which stimulate retinal microvascular EC migration in vitro [67]. Furthermore, the intravitreal injection of these ECFCs-derived EVs reduced the avascular area in a mouse model of oxygen-induced retinopathy [67]. Recently, treatment with either miR-486-5p overexpressing EVs or EVs derived from hypoxia-preconditioned MSCs promoted angiogenesis and cardiac recovery in a non-human primate acute myocardial infarction (AMI) model. In this model MMP19-VEGFA fibroblast cleavage signaling contributed to angiogenesis [68].

Notably, RIC has been shown to stimulate EPC recruitment in the ischemic myocardium in rabbits, thereby increasing capillary density and coronary blood flow [69]. Likewise, RIC was proven effective in enhancing the levels of circulating EPCs in a rat model of hindlimb ischemia [70]. Although focused on EPC populations belonging to the myeloid lineage [65], these preliminary reports pave the way to future research on large animal (which exhibit higher ECFC levels) models of AMI with the aim to assess whether RIC may induce myocardial revascularization through ECFCs-derived EVs. To investigate whether RIC is able to stimulate vascular ECs, at local (i.e., at the site of conditioning) or distant (i.e., in the ischemic myocardium) sites, to release pro-angiogenic EVs would be also of particular interest.

6. Conclusion

RIC is a powerful tool to treat IRI, positively affecting the final infarcted area after myocardial ischemia and reperfusion. Despite the promising results on the potential cardioprotective effect of RIC, the mechanisms still remain elusive. Among proposed candidates, EVs caught the attention of many research groups. Once EVs are released in the circulation, they are conveyed from the preconditioned limb or organ to the heart where they exert their protective function. However, there is not enough clear-cut evidence to establish the role of endothelium in RIC. Besides a few number of studies and their discordant results, an important issue concerns the huge variability in the protocols of preconditioning. As reported in the previous paragraph, the timing used in RIC protocols are very different from each other not only in vivo but also in vitro, where the use of hypoxic buffers in tandem with the incubation of cells into hypoxic chambers adds further variability. The standardization of such methods should be among the future challenges. Another point is EV characterization. As aforementioned, almost every cell type produces EVs, an aspect that should be taken into consideration particularly when complex animal models are used. EVs characterization based on their physical parameters should be integrated with methodologies updating their cell of origin. On the other hand, the use of

HUVECs as an endothelial model is surely convenient but not so representative. Studies on other cell cultures such as primary endothelial cells isolated from the main vessels or adult vascular bed in general should be carried out. Moreover, the production and the release of EVs from these endothelial cell cultures may not completely resemble the in vivo condition in the presence of comorbidities which could affect endothelial function by modifying their circulating level and content. In this view, circulating ECFCs might represent an alternative, but reliable, model to investigate whether and how cardiovascular risk factors hamper the ability of endothelial cells to release cardioprotective EVs. Moreover, ECFCs could themselves represent the cellular vehicle recruited by RIC to deliver pro-angiogenic EVs to the ischemic organ.

Author contributions

E.A., C.P., F.M. and P.P. drafted manuscript; E.A and C.T. prepared table and figures and revised the paper contributing equally to this work; G.A., M.F.B., C.P. and P.P. edited and revised manuscript; all authors approved the final version of manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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