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Resveratrol regulates paracrine function of cardiac microvascular endothelial cells under hypoxia/reoxygenation condition

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The secreted factors from cardiac microvascular endothelial cells (CMECs) regulate the physiological activity of adjacent tissues and could be modulated by myocardial ischemia/reperfusion injury (MIRI). How this paracrine function of CMECs is regulated by MIRI and resveratrol remains to be elucidated. CMECs pretreated with/without resveratrol were subjected to hypoxia/reoxygenation (H/R). Apoptosis was measured by flowcytometry. Protein antibody arrays were performed to find the alteration of cytokine secreted by CMECs. The Gene Ontology analysis was applied to interpret the function of modulated factors. We revealed resveratrol inhibited apoptosis of CMECs dose-dependently after H/R and reached its peak effect at the concentration of 100 μM. 29 factors were significantly changed by H/R, and resveratrol at 100 μM changed 98 types of factors compared with the H/R group. Among these factors, eight were increased by H/R and then were decreased by resveratrol. Eleven were attenuated by H/R and further decreased by resveratrol. Insulin-like growth factor binding protein-1 was upregulated by H/R and it was further increased by resveratrol. The altered factors were involved in cell proliferation, cell growth, cell motility, chemotaxis, angiogenesis and vasculogenesis. The study suggests that resveratrol inhibits the apoptosis and modulates the paracrine function of CMECs under ischemia/reperfusion condition.

1. Introduction

Despite re-establishing complete epicardial patency by timely coronary revascularization, the residual mortality and the rate of complications of acute myocardial infarction remain high, which primarily results from myocardial ischemia/reperfusion injury (MIRI) (Gunata and Parlakpinar 2020). Microvascular endothelial injury was recently considered playing an initial role in MIRI. The microvascular endothelium serves as an interface between restored blood flow and cardiomyocytes, so it suffers from reperfusion injury earlier than cardiomyocytes. Besides, compared to ischemia alone, ischemia followed by reperfusion was demonstrated to induce massive microvascular injury (Hollander et al. 2016). Booming evidence demonstrated that microvascular injury increased infarct size, induced systolic dysfunction and worsen clinical outcome, which made microvascular protection a novel research focus in MIRI (Reinstadler et al. 2016; Sezer et al. 2018). The paracrine or autocrine effects of cardiac microvascular endothelial cells (CMEC) on functions of various adjacent tissues were recognized broadly (Dal Lin et al. 2015; Jiang et al. 2019; Pellowe et al. 2019). Ischemia/reperfusion jeopardize not only the barrier function of microvasculature, but also the paracrine function. Different factors secreted by CMECs communicating with cardiomyocytes and blood cells were modulated by MIRI, for example, neuregulin-1β (NRG-1β) (Lin 2020), angiopoietin-like 4 (ANGPTL4) (Qi et al. 2020), Interleukin (IL) (Verrier 1996). Until now, there is still lack of investigation about how the paracrine function is modulated by MIRI and the endothelium-protective medicine.

Resveratrol, a natural polyphenolic compound, has been demonstrated a cardioprotective function especially the infarct-limiting effect against MIRI by diverse studies (Mao et al. 2019). More importantly, endothelial preservation *via* various pathways such as Akt, silent information regulator, or oxidative stress attenuation might be some of important mechanisms (Repossi 2020; Zhang et al. 2019). However-it remains unclear whether resveratrol regulate the paracrine function of CMECs under MIRI condition and how the cytokine profile was altered. Therefore, we adopted an in vitro model of CMECs suffering ischemia/reperfusion to determine the modulation of paracrine function and its regulation by the endothelial protector resveratrol.

2. Investigations and results

2.1. Resveratrol reduced cellular apoptosis after hypoxia/reoxygenation (H/R) exposure

The previously described *in vitro* model of endothelial reperfusion injury (2 hours of hypoxia and afterwards 12 hours of reoxygenation) significantly induced the apoptosis of CMECs. Flow cytometry (FCM) analysis revealed that H/R upregulated the apoptotic rate compared to the control (27.27±1.70% vs. 8.05±0.55%, $p = 0.0004$, Fig. 1). To define the optimal working solution, CMECs were treated with different concentrations of resveratrol for 30 minutes before H/R. Resveratrol attenuated H/R induced apoptosis dose-dependently at below 200 μM. The concentration at 100 μM reached the peak effect of anti-apoptosis, which reduced apoptotic rate to 15.01±0.79% after H/R exposure. ($p = 0.0028$, Fig. 1).

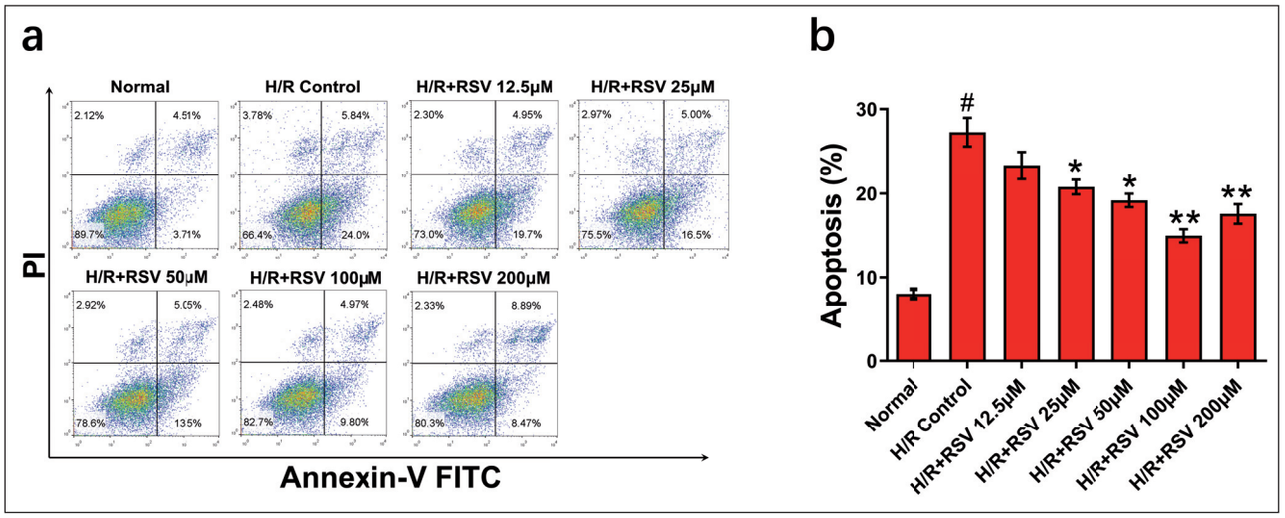

Fig. 1: Resveratrol inhibited the H/R induced CMECs apoptosis dose-dependently. (a). Representative flowcytometric pictures of apoptotic quadrants in different groups. (b). Histogram of apoptotic rates in different CMECs groups. Data were expressed as the means±SEM (n = 3 in each group). # $p < 0.05$ versus the normal group. * $p < 0.05$ versus the H/R group. H/R, hypoxia/reoxygenation. RSV, Resveratrol.

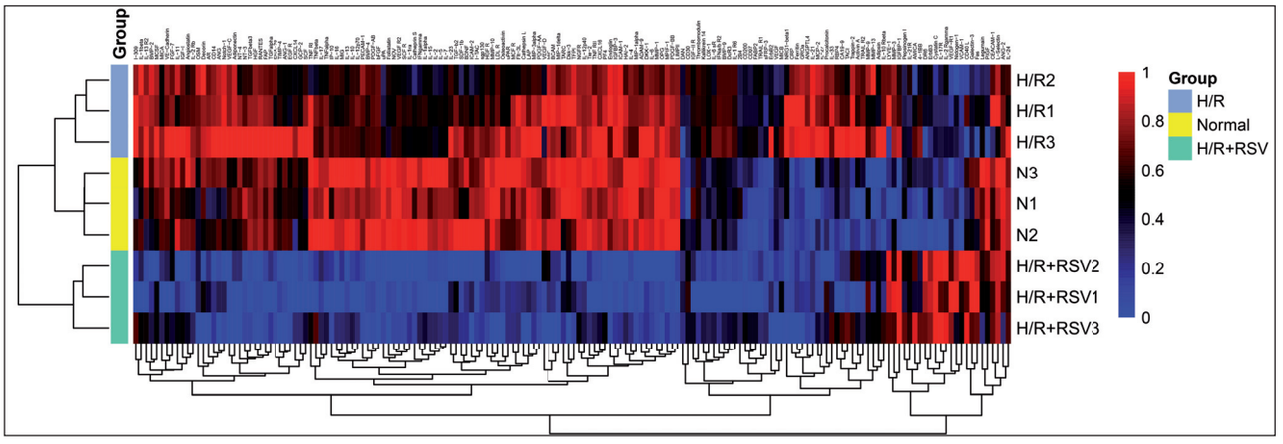

Fig. 2: Cluster analysis heat-map based on significantly altered factors and samples showed the nearest neighbor correlations of factors. Color gradient represent different concentration range of secreted factors. Each group included biologically repeated three samples (absolute fold-change >1.5 and p<0.05) H/R, hypoxia/reoxygenation. RSV, Resveratrol.

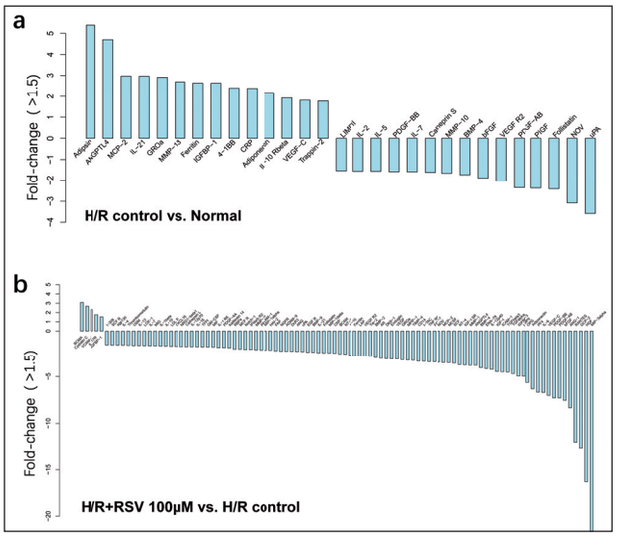

Fig. 3: Differentially secreted factors in pairwise comparison (absolute fold-change >1.5, p<0.05). (a). Histogram of changed factors in the H/R group compared with the normal group (absolute fold-change >1.5 and p<0.05). (b). Histogram of changed factors in the RSV group compared with the H/R group (absolute fold-change >1.5 and p<0.05). H/R, hypoxia/reoxygenation. RSV, Resveratrol.

2.2. Cytokine secretion from CMECs was altered by H/R and resveratrol

To reveal the cellular secretion after H/R and its modulation by resveratrol, human cytokine antibody array was used to evaluate the levels of factors in the supernatant of the cultures. A cluster analysis of the cytokine profile is shown in Fig. 2. The altered factors of significance were defined as the factors with absolute fold-change >1.5 and p value <0.05 in comparing the H/R group with the control group and/or in comparing the resveratrol group with the H/R group. 29 types of factors were significantly modulated by H/R compared with the control, including 15 downregulated ones and 14 upregulated ones. Among these factors, adipsin was increased most (5.39 fold-p=0.026, Fig. 3A), and urokinase-type plasminogen activator (uPA) was decreased most (3.59^{-1} fold-p=5.97×10^{-4}, Fig. 3a). Resveratrol disposure at the concentration of 100 μM regulated the secretion of 98 factors compared with the H/R group, including five upregulated ones and 93 downregulated ones. B-cell maturation antigen (BCMA) was the most upregulated factor (3.09 fold-p=0.046, Fig. 3B) and macrophage inflammatory protein-3α (MIP-3α) was the most downregulated factor (21.73^{-1} fold-p=0.003 , Fig. 3b).

Twenty factors were significantly changed both in comparing the H/R group with the control group and in comparing the resveratrol group with the H/R group (Fig. 4a), Including eight factors that were increased by H/R and were decreased by resveratrol,

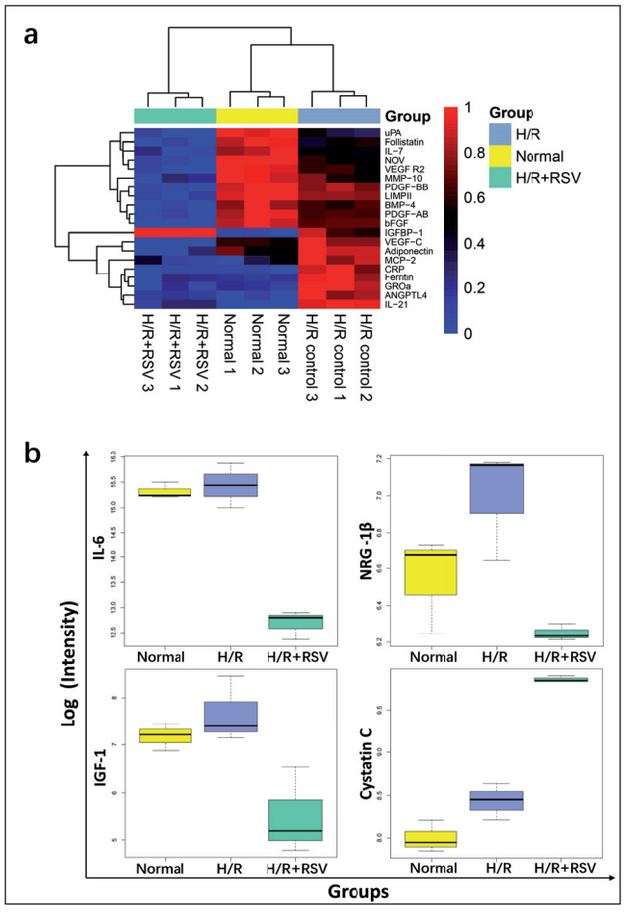

Fig. 4: Notably changed factors revealed by pairwise comparisons or of significant meanings. (a). Heat-map of twenty significantly changed factors in comparisons of the H/R group versus the control group as well as the RSV group versus the H/R group. (b). Boxplots of IL-6, IGF-1, NRG-1β and cystatin C among different groups. H/R: hypoxia/reoxygenation. RSV, Resveratrol. IL-6, interleukin-6. IGF-1, insulin-like growth factor-1. NRG-1β, neuregulin-1β.

A number of well recognized factors in the process of MIRI were also identified by the antibody arrays, however, the significant alteration of these factors was found only in one-time comparison rather than the pairwise comparisons. For example, IL-6 as a pro-inflammatory factor was downregulated by resveratrol in a large extent (6.66^{-1} fold, p=0.0001). However, some well-acknowledged protective factors were unexpectedly changed. NRG-1β as an endothelial protector which was not significantly upregulated by H/R (1.37 fold, p=0.170) was surprisingly decreased by the resveratrol treatment (1.69^{-1} fold, p=0.024). Similarly, the level of insulin-like growth factor-1 (IGF-1) was not significantly elevated in the H/R group compared with control (1.40 fold, p=1.00), while it was reduced by resveratrol (4.55^{-1} fold, p=0.025). Some novel factors identified by recent studies which play pivotal role in MIRI were also sifted out by our study, for example, although cystatin C was not significantly changed by H/R (1.35 fold, p=0.05), it was dramatically increased by resveratrol (2.68 fold, p=0.0001). (Figure 4b).

2.3. Gene ontology (GO) annotation of the function of significant altered factors.

Some growth factors, cytokine and chemokine participate in the pathophysiological mechanism in MIRI and in the protective mechanism of resveratrol. The GO analysis was used to annotate the functions of significantly altered factors sifted out according to above standard. With the controlled vocabulary, the GO project described the gene and gene product attribute in any organism. 'Molecular function' and 'biological process' of selected factors were analyzed and the related terms were identified, including positive and negative regulation of cell proliferation, cell growth, cell motility, chemotaxis, positive and negative regulation of angiogenesis, and vasculogenesis. These factors were sorted by different function categories and were listed in the Table.

3. Discussion

This study adopted an *in vitro* ischemia/reperfusion model of CMECs to illuminate the modulation of paracrine function by H/R and by resveratrol treatment. These results revealed that large amounts of secreted factors from CMECs were significantly eleven that were decreased by H/R and were further attenuated by resveratrol. Besides, insulin-like growth factor binding protein-1 (IGFBP-1) was upregulated by H/R and was further increased by resveratrol (Fig. 4a). altered by H/R and were further sharply changed by resveratrol. The function of altered factors mainly focused on cell proliferation, cell growth, chemotaxis, cell motility and angiogenesis. As far as we know, there is bare of study investigating how resveratrol

Table: Gene ontology annotation of differentially modulated factors in conditioned media which related mainly with cell proliferation, cell growth, cell motility, chemotaxis, angiogenesis and vasculo-genesis

GO TERM / GO ID	H/R vs. Normal	H/R vs. Normal	Resveratrol vs. H/R	Resveratrol vs. H/R
	Upregulated	Downregulated	Upregulated	Downregulated
Regulation of cell growth/ GO:0001558	ErbB2; IGFBP-1	bFGF; IL-2; NOV	IGFBP-1;IL-17 R;	bFGF; VEGF; BMP-9; IGFBP-3; NOV; CXCL16;
Positive regulation of cell proliferation / GO:0008284	IL-21; VEGF-C	uPA; VEGF R2; PDGF-AB; bFGF; IL-7; IL-2; PDGF-BB		bFGF; PDGF-BB; IL-21; HGF; VEGF-C; PDGF-AB; VEGF R2; IL-6; SCF R; PDGF-AA; Notch-1; NT-3; IGF-I SR; IL-7; OSM; uPA; sgp130; ENA-78; VEGF; IP-10; EGF R; AR; IGF-I; FGF-7; GM-CSF; MCSF
Negative regulation of cell proliferation / GO:0008285	GROa; VEGF-C; 4-1BB	BMP-4		IGFBP-3; MPIF-1; HGF; GROa; VEGF-C; IL-6; BMP-4; TGFbeta3; BMP-2; Notch-1; IGF-I SR; OSM; sgp130; IL-1beta; IL-1 R6; AR; IGF-I
Chemotaxis/ GO:0006935	GROa; MCP-2	uPA; bFGF		bFGF; MPIF-1; GROa; RANTES; MIP-1alpha; IL-4; MIP-3alpha; MIG; CXCL16; uPA; I-TAC; ENA-78; MCP-2; IP-10; GCP-2; I-309
cell motility /GO:0048870				Endoglin
Vasculogenesis /GO:0001570		VEGF R2		VEGF R2; Endoglin; FAP; BMP-9; OSM; VEGF
Positive regulation of angiogenesis / GO:0045766	ANGPTL4; VEGF-C	VEGF R2; bFGF		bFGF; HGF; VEGF-C; RANTES; VEGF R2; ANGPTL4; Tie-2; BMP-9; VEGF; IL-1beta; IL-1 R6
Negative regulation of angiogenesis / GO:0016525				Tie-2; BMP-9; PF4; IP-10
Angiogenesis /GO:0001525	ANGPTL4; VEGF-C; MCP-2	NOV; uPA; VEGF R2		NOV; VEGF-C; VEGF R2; ANGPTL4; FAP; Tie-2; PDGF-AA; ANG; BMP-9; uPA; VEGF; MCP-2

regulate the paracrine or autocrine function of CMECs under ischemia/reperfusion condition.

As cells of the largest number in the myocardial tissue, CMECs exceed approximately two folds of cardiomyocytes in quantity (Singhal et al. 2010). The cardiac microvasculature network serves as the pathway of microcirculation supplying nutrition and oxygen, defends against inflammatory cells or toxics as a barrier. Moreover, a vital role of CMECs is secreting factors to communicate with cardiomyocytes, hemocytes as well as adjacent CMECs, which is called paracrine or autocrine function. Complex interactions among different cell types are involved in MIRI pathophysiological process since restored blood flow contacting myocardium. Cytokine, chemokine and growth factors serve as mediator or messenger in the interaction among different types of cells, in the cellular response to environment, and in the maintenance of homeostasis. On one hand, pro-inflammatory factors including IL, tumor necrosis factor-α (TNF-α), monocyte chemotactic protein (MCP) and angiotensin-II (Ang-II) aggravate myocardial inflammatory responses (Rodrigues and Granger 2010; Singhal 2010; Yellon and Hausenloy 2007). On the other hand, factors are secreted to protect the myocardium such as nitric oxide (NO) and NRG-1β (Chandrasekar et al. 2004; Leucker et al. 2011). We previously investigated the paracrine alteration of CMECs by a traditional Chinese medicine Tongxinluo (Cui 2016). However, since Tongxinluo is a complex mixture contained thousands of chemicals rather than a monomer, the altered factors profile during MIRI seemed confusing, which might reflect synergetic effects of diverse contents. Resveratrol was demonstrated to decrease levels of some well-known proinflammatory cytokines. Using a Sprague Dawley rats MIRI model, a recent study revealed resveratrol significantly reduced the levels of serum IL-1β, TNF-α, and IL-6 (Xing et al. 2021). Resveratrol downregulated the levels of IL-22, IL-17A and granulocyte-macrophage colony-stimulating factor (GM-CSF) after irradiation (Khalil et al. 2020). Besides, it inhibited the protein and/or mRNA production of p-NF-κB, IL-1β, IL-6 and IGF-1 at 7 and 28 days after myocardial infarction in male C57/BL mice (He et al. 2021). Based on these previous reports, our study provided a more complete description of altered paracrine function as well as a more specific focus on cardiac microvasculature.

There were some novel findings through our mid-throughput study. MIP-3α-alternatively named as CCL20, serves as the primary ligand for its only receptor CC chemokine receptor 6 (CCR6), a property shared with the antimicrobial defensins (Schutyser et al. 2003). The CCL20-CCR6 interaction participates in the chemoattraction of immature dendritic cells, memory T-cells and B-cells that produce many of the inflammatory molecules. MIP-3α was found significantly elevated under inflammatory or some pathophysiological conditions including cancer (Gordy et al. 2020), rheumatoid arthritis and nonalcoholic fatty liver disease (Chu et al. 2018; Schutyser et al. 2003). The MIP-3α level after cerebral ischemia reperfusion injury was elevated at beyond 2000-fold, which indicates cerebral MIP-3α production deteriorated exacerbation of ischemic injury via further production of inflammatory mediators. It might be a novel therapeutic target for cerebral ischemia (Terao et al. 2009). Another study demonstrated β-caryophyllene improved neurological deficits of cerebral ischemia-reperfusion injury by reducing the inflammation through dramatically inhibited the expression of MIP-3α (Liu et al. 2021). Although there is still no systematic investigation about how MIP-3α impact MIRI, Fu Qiang et al. recently reported that microRNA-19α eliminated the H/R-induced injury in cardiomyocytes through directly targeting CCL20 and attenuating the activity of mitogen-activated protein kinase (MAPK) signaling pathway. In our study MIP-3α secretion was sharply suppressed by resveratrol to a level of less than one twentieth, which might be one of the anti-oxidant and anti-inflammatory mode of actions of resveratrol against MIRI.

This family of structurally related chemotactic cytokines like MIP-3α, also called chemokine, induce the migration of leukocytes throughout the body under physiological as well as inflammatory circumstances. Chemokine plays roles in various procedures like autoimmunity, morphogenesis, angiogenesis, tumor growth and metastasis by activating 7-transmembrane-domain G protein-coupled receptors on their target cells (Laudanski et al. 2014). Like MIP-3α, growth related oncogene α (GROα), IL-21, and MCP-2 related to inflammation and apoptosis were all found upregulated by H/R and downregulated by resveratrol in our study. GO term analysis indicated chemotaxis was a common function of some altered cytokines, implying that dysfunctional endothelial cells in MIRI directed various leukocytes infiltration. GROα is also called chemokine (C-X-C motif) ligand 1 (CXCL1), the chemo-attractant secreted by ischemic tissue that could active the early inflammatory reaction such as neutrophil infiltration (Kobayashi 2008; Olson and Ley 2002; Sager et al. 1991). Al-Amran and Shahkolahi (2013) and Mersmann (2010) revealed that some therapeutics protecting myocardium against reperfusion injury shared common mechanisms of inhibiting CXCL1 and subsequently suppressing leukocyte infiltration. IL-21 is the cytokine which possesses broad immunomodulatory function through forming a heterodimeric receptor complex with its unique receptor and the common cytokine receptor γ chain (Spolski and Leonard 2014; Wang et al. 2018; Weir et al. 2012). IL-21 is involved in anti-tumor and antiviral responses, in the development of autoimmune diseases and certain inflammatory disorders (Spolski and Leonard 2014). Wang et al. (2018b) found that IL-21 was elevated during the acute MIRI in mice and in vitro, and exogenous IL-21 disposure markedly exacerbated the myocardial injury related to an increase in CXCL-1 and macrophage inflammatory protein-2 (MIP-2) expression and infiltration of neutrophils through downstream Akt/ NF-κB signaling and p38 MAPK/ NF-κB pathways. Treatment with the anti-IL-21 monoclonal antibody induced the opposite effect. MCP-2 is a pleiotropic chemo-attractant protein that attracts lymphocytes, monocytes, eosinophils and basophils, and under infectious or inflammatory conditions it could be secreted by microvascular endothelial cells (Struyf et al. 2009). Myocardial inflammation and cardiomyocyte apoptosis are well-acknowledged key mechanisms in MIRI (Gunata and Parlakpinar 2020). The secretion of these chemotactic factors was inhibited by resveratrol, meaning that resveratrol enhanced microvascular endothelial barrier against leukocytes infiltration. This might explain that infiltration of inflammatory cells outside the microvasculature was alleviated by resveratrol as a previous histopathological study showed (Xing 2021). Similarly-IL-7, produced by the endothelium under pathological conditions (Schroten et al. 2012), was recently found playing an intensifying role in MIRI by promoting cardiomyocyte apoptosis through the regulation of macrophage infiltration and polarization (Yan et al. 2021). IL-7 knockout or anti-IL-7 antibody treatment contributed to reduction in myocardial infarction area, decrease of serum troponin T level and improvement in cardiac function (Yan 2021). hence, IL-7 suppression by resveratrol revealed in our study might also constitute the protective mechanisms.

In our study, uPA (also called PLAU) was downregulated most by H/R and was further decreased after resveratrol disposure. There is scarce of study investigating the function of uPA in MIRI. UPA, a serine proteinase catalyzing the generation of plasmin on the cell surface and activating downstream signaling pathways, is revealed to promote remodeling and repair, and it is considered to be a protective molecular according to recent studies. Using a septic human umbilical vein endothelial model, Long et al. (2019) demonstrated uPA promoted cell viability and after silencing uPA expression cell apoptosis was enhanced. UPA promotes wound healing through a plasminogen-independent mechanism of inducing the detachment of β-catenin from the cytoplasmic tail of N-cadherin (NCAD) *via* triggering its phosphorylation at Tyr654 (Diaz et al. 2021). Intravenous treatment with recombinant uPA three hours after the onset of cerebral ischemia induces NCAD-mediated repair of synaptic contacts in the area surrounding the necrotic core (Diaz et al. 2021). In our study, H/R reduced the secretion of uPA, which meant the protection of CMECs was jeopardized by H/R. However, resveratrol cannot reverse this trend, and further studies are needed to clarify the issue.

Some protective factors were found dramatically elevated by resveratrol in our study, and this trend was previously unreported. For instance, cystatin C, a potent endogenous inhibitor of lysosomal cysteine proteinases, was upregulated by resveratrol. Cystatin C is abundant in the central nervous system, and a previous study illuminated that Cystatin C exerts neuro-preservative function by preventing oxidative injury (Tizon et al. 2010) and consequently reduced the infarct volume in a rat ischemic stroke model (Fang et al. 2017). The protective mechanisms include maintaining lysosomal integrity and promoting autophagic flux by elevating light chain 3-II and beclin-1 (Fang et al. 2019). More importantly, cystatin C was lately updated as a novel cardioprotective factor to reduce the oxidative stress and the apoptosis of cardiomyocytes by inactivating the NF-κB signaling pathway in cardiomyocytes (Su et al. 2020). Thereby, resveratrol enhancing the secretion of cystatin C might also be one of its mechanisms in treating MIRI.

Unexpectedly, some protective factors were downregulated by resveratrol, including ANGPTL-4, NRG-1β, vascular endothelial growth factor (VEGF), IGF-1, platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). The secretion of ANGPTL-4 and VEGF-C by CMECs was significantly promoted under H/R circumstance, while the levels of IGF-1 and NRG-1β had increasing trend without statistical significance. A previous study demonstrated that ANGPTL-4 inhibited no-reflow phenomenon and kept vascular integrity during myocardial reperfusion (Galaup et al. 2012). VEGF was well-recognized as a factor against MIRI either (Chen et al. 2016; Zhou et al. 2019). Thus, the elevation of ANGPTL-4 and VEGF in response to MIRI might be an endogenous activated protective mechanism in CMECs against MIRI. In contrast, some protective factors against MIRI such as PDGF, bFGF were downregulated by H/R, which indicated a few intrinsic protective factors from CMECs were depressed by H/R. Hausenloy and Yellon (2004) put forward the concept of reperfusion injury salvage kinase (RISK) protective mechanism. It means that some growth factors preserve myocardium against ischemia/reperfusion injury by activating the pro-survival kinase cascades including PI3K/Akt and p42/p44 extra-cellular signal-regulated kinases (ERK1/2) pathways. GO term analysis showed that above mentioned factors, such as VEGF, IGF-1, PDGF, bFGF and uPA have effects on cell proliferation and growth, and ANGPTL4 belonged to a category of factors related to angiogenesis. Previous studies have already proved that ANGPTL-4 (Qiu et al. 2021), VEGF (Chen 2016; Zhou 2019), IGF-1(Liao et al. 2019), NRG-1β (Wang et al. 2018), PDGF (Edelberg et al. 2002) and bFGF (Wang et al. 2015) exert their myocardial preservation more or less through activating RISK pathway. Although resveratrol downregulated these factors, however, resveratrol was demonstrated to activate both the PI3K/Akt (Yu et al. 2021; Zhang 2019) and the ERK1/2 (Das et al. 2006) pathways. These clues implied that resveratrol activated the RISK mechanism in a direct manner or *via* some other nonprotein factors such as NO, rather than in an indirect manner of promoting growth factors to activate the RISK cascades.

In addition, our study indicated that resveratrol reduced the level of bone morphogenetic protein-4 (BMP-4). BMP-4 enhanced apoptosis by promoting the Jun N-terminal kinase/mitogen-activated protein kinase pathway, and treatment of attenuating BMP downstream pathway relieved reperfusion injury (Pachori et al. 2010). Thus, it is hypothesized that reducing BMP-4 by resveratrol is also contributing to its protection against MIRI.

There are some limitations in this current study. Firstly, protein array is a mid-throughput method investigating various kinds of protein factors at the meanwhile, and this method could only illuminate the alteration of already recognized protein factors. Thus, it remains unclear whether there are any undiscovered protein factors or non-protein factors participated in MIRI changed. Secondly, our study was limited to demonstrate the change of previous mentioned injurious factors such as TNF-α, endothelin and angiotensin-II due to limited sample size. Finally, because we adopted a simple model of MIRI at only one time point, the continuous trends of altered factors cannot be revealed and still need further investigations.

In conclusion, resveratrol protect CMECs against H/R promoted apoptosis in a dose-dependent manner, and resveratrol achieved its optimal effect at 100 μM. H/R significantly altered the secreted factors of CMECs, and some factors was further modulated by resveratrol. Resveratrol decreased inflammatory factors inducing leukocytes infiltration such as MIP-3α, GROα, and IL-21, while it attenuated some growth factors activating RISK cascades such as ANGPTL-4, VEGF and NRG-1β. GO annotation identified the function of altered factors included cell proliferation, cell growth, cell motility, chemotaxis, angiogenesis and vasculogenesis.

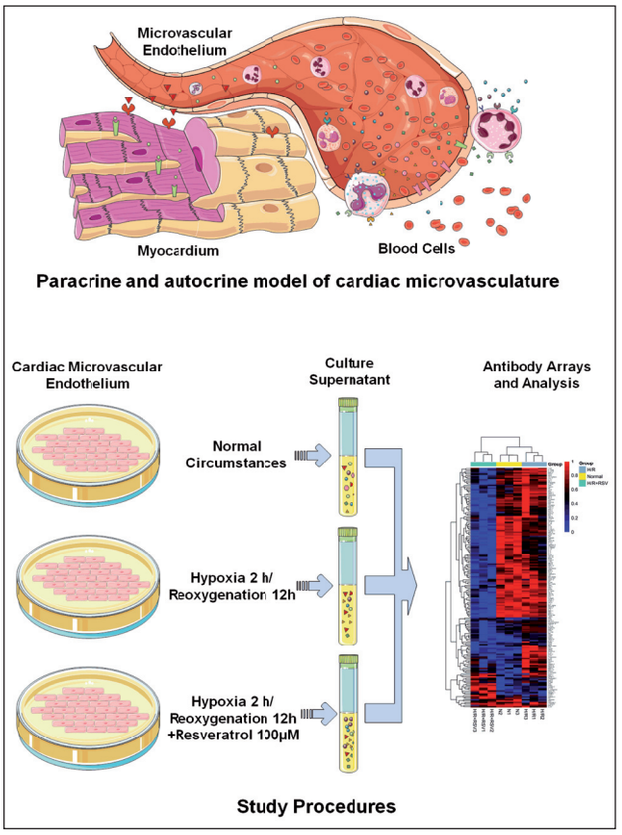

Fig. 5: Study overview

4. Experimental

4.1. Cell culture and disposure

Human CMECs in the logarithmic phase were bought from ScienCell (San Diego, CA, USA) and were cultured according to the instructions. To be concise, a complete endothelial cell medium which contained 5% fetal bovine serum, 1% penicillin/streptomycin and 1% endothelial cell growth supplement were used in cell culture. CMECs were subcultured at the ratio of 1:3 reaching 90% confluence. Resveratrol was purchased from Sigma-Aldrich (St. Louis, MO, USA). CMECs of the third passage were treated with serum-free DMEM or resveratrol DMEM solution at gradually changed concentrations (12.5, 25, 50, 100, 200 μM) for 30 min before hypoxia exposure. A H/R procedure was performed as previously described (Chen et al. 2013; Cui et al. 2016), that is to say, CMECs were incubated in a sealed GENbox jar with a catalyst (Bio-Me´rieux) inside to scavenge oxygen for 12 h, followed by transferring cells to normoxic circumstance for 2 h.

4.2. Cellular apoptosis evaluation

FCM with FACS Calibur System (Becton-Dickinson, FranklinLakes, NJ, USA) was adopted to evaluate cellular apoptosis. The Annexin V-fluorescein isothiocyanate (FITC) /propidium iodide (PI) Kit (Biosea Biotechnology, Beijing) was used as the manufacturer instructed. To be brief, CMECs after disposure were harvested and resuspended in 500 μL of buffer. The cells were stained with Annexin V solution and propidium iodide (PI) sequentially in the dark for 15 min at room temperature, afterwards they were collected and analyzed with FCM. FCM results were further quantified by Flowjo (Version 7.6.1). Four quadrants were divided according to whether the cells were stained by Annexin V or PI. Apoptotic rate = cell number of early apoptotic quadrant (Annexin V+/PI-) and late apoptotic quadrant (Annexin V+/PI+) / total cell number × 100%. The concentration at which resveratrol reduced cellular apoptosis in largest extent was defined as the best working concentration.

4.3. Cytokine antibody chip analysis

Secretion of cytokines, growth factors and chemokines were assayed using a median-throughput scanning method, Quantibody human cytokine antibody array 7000 kit (RayBiotech, Catalog No. QAH-CAA-7000-Norcross, GA, USA). Culture medium after disposure from the normal group, the H/R group and the resveratrol group at optimal working concentration were obtained and centrifugated, thereafter the cytokine antibody chips were used to detect the concentration of factors in the supernatant using the enzyme-linked immune-absorbent assay (ELISA) method. 320 kinds of multiplexed antibodies spotted onto eight slide chips consist the median-throughput antibody arrays. The fluorescent signal intensity of microarray was visualized using InnoScan 300 microarray scanner (Innopsys, France), and relative values were acquired and normalized. Three independent samples in each group were used to avoid bias. The experiment processes were carried out by CapitalBio Technology (Beijing, China).

4.4. Gene ontology analysis

GO as a bioinformatic web-based tools (http://geneontology. org) was conducted to analyze the proteins of interest (absolute fold change > 1.5). GO is originally proposed to unify the representation of genes and gene products of many species, which covers three main domains including cellular component, molecular function and biological process. Fisher's exact test was used to find if there was more overlap between the differentially expressed factors list and the GO annotation list than would be expected by chance. GO terms, which are updated based on the evolvement of biological science, were used as powerful tools to summarize the specific roles of genes and their products. The p value denoted the significance of GO terms enrichment in the differentially expressed factors. The lower the p value, the more significant the GO Term (p value ≤ 0.05 was recommended).

4.5. Statistical analysis

All the data were obtained from least three independent experiments and were expressed as mean values±SEM. One-way analysis of variance was performed to compare more than two groups, and paired Student's t-test was performed to compare two groups (IBM SPSS statistics 25.0). A p value of <0.05 (two sided) was considered statistically significant. IQR method was used to standardize the original data and the pooled analysis was performed using R method.

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