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Characterization of extracellular vesicles derived from cardiac cells in an *in vitro* model of preconditioning

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ABSTRACT

Preconditioning is a promising technique to protect the heart from ischaemia-reperfusion injury. In this context, the crosstalk between different cardiac cell types and especially the exchange of cardioprotective mediators has come into the focus of current research. Recently, extracellular vesicles (EVs), nano-sized structures, emerged as possible communication mediators. They are taken up by recipient cells and can alter gene expression or activate intracellular signal cascades. It has been shown that all cardiac cell types are able to secrete EVs, but so far the influence of an *in vitro* preconditioning stimulus on EV concentration and composition has not been investigated. Therefore, we stimulated primary cardiac myocytes and fibroblasts from neonatal rats, as well as H9c2 cells, with two known *in vitro* preconditioning stimuli: hypoxia or isoflurane. EVs were isolated from cell culture supernatants 48 h after stimulation by differential centrifugation and size exclusion chromatography. They were characterized by transmission electron microscopy, tunable resistive pulse sensing, miRNA array and Western blot analysis. The detected EVs had the typical cup-shaped morphology and a size of about 150 nm. No significant differences in EV concentration were observed between the different groups. The protein and miRNA load was affected by *in vitro* preconditioning with isoflurane or hypoxia. EV markers like Alix, CD63, flotillin-1 and especially heat shock protein 70 were significantly up-regulated by the treatments. Several miRNAs like miR-92b-3p, miR-761 and miR-101a-5p were also significantly affected. A migration assay confirmed the physiological benefit of these EVs. Taken together, our findings show that a model of *in vitro* preconditioning of cardiac cells does not influence EV concentration but strongly regulates the EV cargo and affects migration. This might indicate a role for EV-mediated communication in isoflurane- and hypoxia-induced *in vitro* preconditioning.

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Introduction

According to the World Health Organization, cardiovascular diseases (CVD) are still the leading cause of death in Western countries, yet about one of every three deaths in the United States is related to CVD, indicating that treatment possibilities have to be improved. The best option to limit the lethal effects of myocardial infarction is to restore the blood flow and supply the ischaemic region with oxygenized blood. Unfortunately, this reperfusion is also known to cause deleterious effects by inducing inflammatory and oxidative stress resulting in cardiomyocyte death and cardiac damage [1]. One possible intervention to reduce ischaemia/reperfusion (I/R) injury is pre- or post-conditioning. In 1986, Murry et al. demonstrated that brief cycles of hypoxia, a process later known as ischaemic preconditioning (IPC), protected the myocardium from a subsequent longer period of reduced perfusion [2]. In a comparable manner, volatile anaesthetics like isoflurane were shown to mimic this process and also protect the heart from I/R injury. This phenomenon is now termed anaesthetic induced preconditioning (AIP) [3]. The exact underlying mechanism of preconditioning still remains only partly understood. It involves the activation of the reperfusion injury salvage kinase (RISK) pathway, transcription factors like hypoxia-inducible factor 1α (HIF-1α) and also downstream mediators like heat shock 70 kDa protein (HSP70) and inducible nitric oxide synthase (iNOS) [4].

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Supplemental data for this article can be accessed here

One of these downstream mediators involved in preconditioning is HSP70, which can also be found in extracellular vesicles (EVs) [5]. EVs are nano-sized particles, which are secreted by almost every cell type. Subtypes of EVs are distinguished by their size and origin. Exosomes are the smallest vesicles (30–150 nm), are released from multivesicular bodies (MVBs) and their cargo consists of proteins [6], DNA [7,8] and RNA/miRNA [9,10]. The two other subpopulations of EVs are microvesicles (100–1000 nm) which directly bud from the cell membrane [11,12] and apoptotic bodies (1–5 μm) [13]. Recently, the benefits of IPC were also attributed to extracellular vesicles. Coronary perfusates from donor hearts, which were subjected to IPC, were able to attenuate infarct size in non-preconditioned recipient hearts. A depletion of EVs in these perfusates resulted in diminished cardioprotection [14]. In different models of cardiac ischaemia reperfusion, exosome-rich fractions were shown to reduce I/R injury by a pathway involving toll-like receptor 4 (TLR4) and cardioprotective HSPs [5]. In general, EVs can protect the myocardium by enhancing angiogenesis, reducing oxidative stress, diminishing cardiomyocyte death and attenuating the inflammatory response [15].

The majority of studies involving EVs in cardioprotection were performed with basally secreted EVs from unstimulated cells. To date, the influence of different cardioprotective stimuli on EV biogenesis, composition and function has not been investigated. Therefore, we focused on the question whether an *in vitro* preconditioning stimulus like hypoxia or the volatile anaesthetic isoflurane has an impact on EV formation and cargo composition. Additionally, we investigated whether the isolated EVs have any physiological benefit for cardiac cells. We showed that an *in vitro* model of preconditioning does not influence the secretion of EVs and their morphology but has an effect on EV size and especially their cargo. Fibroblast-derived EVs enhanced cell migration in an *in vitro* scratch assay and the effect was further enhanced by *in vitro* preconditioning.

Materials and methods

All experiments were executed in compliance with the local institution's Ethics Review Committee. An animal protection representative at the Institute of Animal Research of the Uniklinik RWTH Aachen approved all animal studies in accordance with the German Animal Protection Law §4, Section 3. The Animal Care and Use Committee of the local authorities approved the conducted experimental procedures (AZ 50.203.2 AC, LANUV NRW, Essen, Germany).

Cell culture

Cell culture medium, supplements and chemicals were obtained from Thermo Fisher Scientific (Waltham, MA, USA), Sigma-Aldrich (St. Louis, MO, USA) or Santa Cruz Biotechnology (Dallas, TX, USA) if not stated otherwise. Cells were cultivated in a humidified atmosphere at 37°C and 5% CO_2. H9c2 cells were obtained from America Tissue Type Collection (ATCC) and cultivated like the primary fibroblasts, in Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine and 1% antibiotic-antimycotic (AA). Primary cardiomyocytes were cultivated in DMEM supplemented with 10% horse serum instead of FBS. The medium was changed every 48–72 h.

Isolation of neonatal cardiomyocytes and fibroblasts

Cardiac fibroblasts and ventricular myocytes (further stated, primary cardiomyocytes) were isolated from 1–5 days-old Wistar rats as described previously [16,17]. Briefly, neonates were decapitated and the ventricles were collected in chilled calcium and bicarbonate free Hanks buffer (CBFHH) with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4: 140 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 5.6 mM glucose. The hearts were washed twice with CBFHH buffer and cut into pieces of approximately 1 mm^3. The extracellular matrix was digested with 5 ml of 91 U Collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 0.89 mg/ml pancreatin at 37°C and gentle shaking for 5 min. The supernatant was discarded and 6 ml of fresh enzyme solution were added. After 20 min at 37°C, the supernatant was removed, mixed with 5 ml FBS and stored at 4°C until further use. This digestion step was repeated 5–6 times. Isolated cells were centrifuged at 600 *g* for 10 min at 4°C (Rotina 420R, Heraeus, Hanau, Germany). The pellet was resuspended in medium and passed through a 100 μm cell strainer. To separate fibroblasts from cardiomyocytes, cells were plated at a density of 0.5×10^6/cm^2 for 2 h at 37°C and 5% CO_2. Subsequently, the supernatant was removed and adherent fibroblasts were cultivated in fresh medium until further use. The supernatant containing cardiomyocytes was centrifuged at 600 *g* for 10 min at 4°C. Cardiomyocytes were resuspended in medium and seeded at a density of 0.85×10^6 cells/cm^2 on culture plates coated with 5 μg/ml fibronectin in 0.02% gelatin. Cells were cultured for at least 7 d before use to ensure an adult phenotype.

In vitro preconditioning of cardiac cells

A model of *in vitro* preconditioning of cardiomyocytes was performed at d 7–8 after isolation. Due to the limited availability of primary cardiomyocytes and their lack of proliferation, all cells from a single preparation were split in one control and only one treatment group (hypoxia or isoflurane). At least 15×10^6 cells were used per group. Fibroblasts as well as H9c2 cells are able to proliferate, so treatments with hypoxia and isoflurane were possible from every preparation. Fibroblasts were preconditioned in passage 1–2 at 95% confluence. H9c2 cells were treated at 95% confluence. Both cell types were preconditioned in 10-cm dishes and five dishes per group were used. Cells were washed with PBS and preconditioned in regular medium, supplemented with 1% EV-depleted FBS. Treatment with hypoxia or isoflurane was performed in a modular incubator chamber (Billups-Rothenberg Inc., San Diego, CA, USA) at 37°C. A model of hypoxic *in vitro* preconditioning was initiated by flooding the chamber with 95% N_2 and 5% CO_2 for 5 min at a flow rate of 3 l/min and maintained for 1 h at 0.5 l/min as described previously [18]. A model of anaesthetic *in vitro* preconditioning was achieved by isoflurane treatment of the cells, using the same flow rates (3 l/min for 5 min, 0.5 l/min for 4 h) and a mixture of 20% O_2, 75% N_2, 5% CO_2 enriched with 1.5 Vol% isoflurane (Forene®, AbbVie, North Chicago, IL, USA) as described previously [17]. After the treatment, all cells were kept in the same medium at regular cell culture conditions for 48 h.

EV purification from cell culture supernatant

At 48 h after the *in vitro* preconditioning stimuli [19], EVs were isolated as illustrated in Scheme 1. The cell culture supernatant was pooled for each group and differentially centrifuged at 4°C for 10 min at 300 *g*, then 15 min at 2000 *g* and finally 45 min at 12,000 *g* followed by steady filtration through a 0.22 µm filter (VWR, Radnor, PA, USA). Vivaspin 20 concentrators with a 100,000 Dalton cut-off (Sartorius AG, Goettingen, Germany) were washed with 5 ml PBS at 6000 *g* for 1 min. The cell culture supernatant was then concentrated to a final volume of 1 ml. EVs were purified using Exo-spin™ Midi columns (Cell Guidance Systems, Cambridge, UK) for size exclusion chromatography (SEC). The columns were washed with 20 ml PBS, then 1 ml of the concentrated cell culture supernatant was applied to the column and 500 µl fractions were collected. Fractions 7 and 8 were EV enriched (Supplementary Figure 1). Fractions were collected in protein low bind tubes (Eppendorf, Hamburg, Germany), pooled and stored at –80°C until further analysis.

Tunable resistive pulse sensing (TRPS)

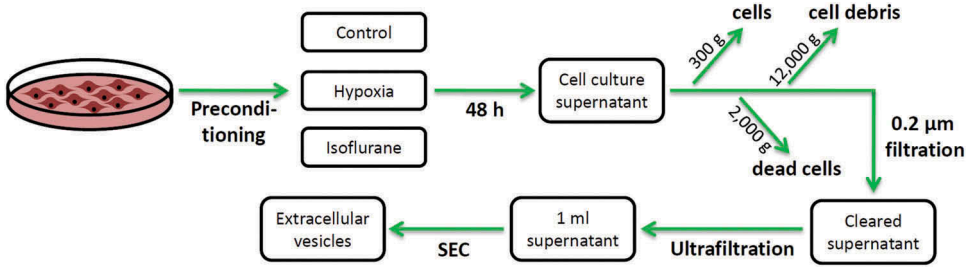
Size and concentration of isolated EVs were quantified by TRPS using the qNano (Izon Science, Oxford, UK). All samples were diluted at least two-fold with 0.03% Tween® 20 in PBS (v/v) prior to the measurement. NP200 (Izon Science, Oxford, UK) pores and a pressure of 7 or 8 mbar were used. Calibration runs were performed with 210 nm diameter carboxylated polystyrene beads (Izon Science, Oxford, UK) before and after each sample. All samples were measured two times. The system was cleaned after each measurement with PBS for 3 min at 20 mbar. Concentration and size were determined using the provided software (Izon Control Suite, version 3.2, Izon Science, Oxford, UK).

Protein measurement

Protein concentration of the EV-rich fractions was determined using the micro bicinchoninic acid assay (BCA assay, Thermo Fisher Scientific) according to the manufacturer's protocol. Samples were diluted 1:10 with ddH_2O prior to the measurement.

MiRNA isolation

miRNA was isolated according to the manufacturer's protocol (miRCURY RNA isolation kit, Exiqon,



Scheme 1. **Isolation procedure of cardiac cell-derived EVs.** Cardiac cells in EV-depleted medium were treated with hypoxia (<1% O_2) for 1 h or with 1.5% (v/v) isoflurane for 4 h at 37°C. Control cells were maintained at cell culture conditions. After 48 h the supernatant was pre-centrifuged, filtered and concentrated. EVs were isolated by size exclusion chromatography (SEC).

Denmark); 800 µl of purified EVs were used for miRNA extraction. The miRNA was eluted with 20 µl RNase free H_2O and the eluate was again loaded on the column for another elution step. The extracted miRNA was stored in DNA/RNA LoBind tubes (Eppendorf, Hamburg, Germany) at –80°C until further analysis.

Western blot

Expression of EV markers was analysed by Western blotting. Immediately after the isolation, EVs were lysed in lithium dodecyl sulphate buffer (LDS buffer) either containing 100 mM dithiothreitol (DTT) for reducing conditions or the appropriate amount of water for non-reducing conditions and boiled at 95°C for 5 min. Equal amounts of proteins were separated on 10% TGX™ FastCast™ gels (Bio-Rad, Hercules, CA, USA) and stain-free imaging was performed to ensure equal loading of proteins [20]. Afterwards, proteins were transferred to a PVDF (polyvinylidene difluoride) membrane (Bio-Rad, Hercules, CA, USA) and blocked with 5% (w/v) milk in TBS-T (tris buffered saline containing 0.05% Tween (v/v)). For detection, the membranes were probed with the primary antibodies overnight at 4°C and gentle shaking (Supplementary Table 1). Blots were developed with the Clarity Western ECL Blotting substrate (Bio-Rad, Hercules, CA, USA) using a peroxidase conjugated secondary anti-mouse antibody (Abcam, Cambridge, GB, Ab6820, 1:5000). Chemiluminescence was detected with the ChemiDoc MP imager (Bio-Rad, Hercules, CA, USA). Band intensities were quantified by using the Image Lab software (Bio-Rad, Hercules, CA, USA).

Ultracentrifugation

Due to the slightly lower amounts of EVs derived from primary cardiomyocytes we concentrated them by ultracentrifugation for better visualization by electron microscopy; 800 µl of SEC-purified EVs were centrifuged at 118,000 *g* for 3 h at 4°C (Beckman Coulter Optima MAX-XP, TLA-55 fixed angle rotor, Krefeld, Germany). The pellet was then resuspended in 50 µl PBS and immediately used for negative staining as described in the transmission electron microscopy section.

Transmission electron microscopy (TEM)

Native EVs were incubated for 30 min on formvar carbon coated grids (Nickel Grid 200 mesh, Electron Microscopy Sciences, Hatfield, PA, USA) and fixed with 3% glutaraldehyde in H_2O (Sigma-Aldrich, St. Louis, MO, USA). Grids were washed five times for 5 min with H_2O. Negative staining was performed with 4% uranyl acetate in 2% methyl cellulose (ratio of 1:9, v/v) for 10 min in the dark and on ice. Excess liquid was removed using a filter paper. The grids were air dried for 10 min. Samples were imaged using a LEO 906 E transmission electron microscope (Zeiss, Oberkochen, Germany), operated at an acceleration voltage of 60 kV.

Microarray analysis

Arrays were performed as described elsewhere [21,22]. For microarray analysis, three independent experiments were performed. Isolated miRNAs were labelled using the Flashtag RNA labelling kit according to the manufacturer's protocol (Genisphere, Hatfield, PA, USA). miRNAs of each sample were subjected to a tailing reaction for 15 min at 37°C (2.5 mM $MnCl_2$, ATP, Poly A Polymerase). Afterwards, the ligation of the biotinylated signal molecule to the target miRNA sample was performed for 30 min at RT (1 x Flash Tag ligation mix biotin, T4 DNA ligase) and stopped with the appropriate stop-solution. Each sample was hybridized to a GeneChip® miRNA 4.0 Array for 18 h at 48°C and 60 rpm (Affymetrix, Santa Clara, CA, USA). Afterwards, the chips were washed and stained on Fluidics Station 450 (Fluidics script FS450-0002) and scanned on a GeneChip® Scanner 3000 7G (both, Affymetrix, Santa Clara, CA, USA). Data were analyzed by using expression console software 1.4 (Affymetrix, Santa Clara, CA, USA). Summarization and normalization of the expression values were performed with robust multi-array average (RMA) [23,24]. Detected miRNAs whose expression levels were significantly different ($p < 0.05$) and showed a linear-fold change of at least 1.3 compared to the control group, were considered as differentially expressed.

Cell migration assay

Cardiac fibroblasts were seeded at a density of 12,000 cells/well in a 96-well plate, maintained at 37°C and 5% CO_2 overnight and starved the next day with 1% EV-depleted FBS medium for 24 h. The monolayer was scratched with a 200 µl pipette tip as described previously [25,26]. Cells were washed with PBS and incubated with 5×10^8 EVs/ml in 1% EV-depleted FBS medium for 24 h. PBS was used as negative control. For evaluation of the scratch, cells were fixed with 4% (v/v) formaldehyde, washed with PBS containing 0.05% Tween 20, permeabilized with 0.1% (v/v) Triton X-100 and stained with Fluoromount-G containing 4',6-diamidino-2-phenylindole (DAPI, SouthernBiotech Birmingham, Al, USA). Images of stained cells were taken with the EVOS cell

imaging system (Thermo Fisher Scientific, Waltham, MA, USA). Scratches were quantified by using ImageJ and the MRI_Wound_Healing_Tool (NIH, Bethesda, MD, USA).

Statistical analysis

miRNA calculations and statistical significances between two groups (paired Student's *t*-test) were performed with AltAnalyze (Version 2.0.9.4, Cincinnati, OH, USA) [27]. Data analysis (except miRNA calculations) and graph design were performed using GraphPad Prism, version 6.07 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significances for the Western blot results were assessed by two-way ANOVA with appropriate adjustments for multiple measurements. Sizes and concentrations of EVs derived from fibroblasts and H9c2 cells as well as cell migration assays were analyzed by one-way ANOVA. Tukey and Dunnett post-hoc tests were performed. Student's unpaired *t*-tests were conducted to analyze size and concentration of primary cardiomyocytes derived EVs. If not indicated differently, data are expressed as mean ± SEM. A level of $p < 0.05$ was considered statistically significant.

Results

The heart consists of different cell types with cardiomyocytes and cardiac fibroblasts as the main constituents [28]. Therefore, to investigate the impact of different *in vitro* preconditioning stimuli on EV production and secretion in a cardiac system, two cell systems were used: primary cardiac fibroblasts and cardiomyocytes. These cells were isolated from neonatal rat hearts and showed purity larger than 90% (Supplementary Figure 2). Even though we utilised a high purity preparation, some other contaminating cells might have still been present and, additionally, variations in different preparations could not be excluded. To prevent these preparation specific variations in cell purity, yield and viability, the cardiomyocytic cell line H9c2 was also investigated. We could further demonstrate that our treatments were not cell toxic (Supplementary Figure 3).

Primary cardiac cells and H9c2 cells secrete extracellular vesicles

To investigate whether these cell types were able to secrete EVs, the cells were cultured in 1% EV-depleted FBS medium for 48 h. Subsequently, EVs were isolated according to a protocol combining sequential centrifugation, ultrafiltration and SEC (Scheme 1). Characterization of these secreted EVs was performed by TEM. All three cell types secreted EVs which showed the typical collapsed cup-shaped morphology that is a result of drying the sample (Figure 1(a-c)). The size range was 100–170 nm.

Treatment with isoflurane or hypoxia does not influence EV size in primary cardiac cells

With TEM, only few EVs can be analyzed at a time and it is difficult to detect a shift in EV size distribution after an *in vitro* model of preconditioning. Therefore, the EVs were further analyzed via TRPS using the qNano (Izon Science, Oxford, UK). For each measurement, at least 500 EVs were analyzed. Primary cardiomyocytes, fibroblasts and H9c2 cells secreted EVs with a modal size of about 150 nm (Figure 2(a-c)). For the primary cells, an *in vitro* model of preconditioning stimuli had no impact on the EV size. However, H9c2 cells tend to secrete larger EVs, especially after isoflurane treatment (Figure 2(c), con: 140.3 nm, Iso: 166.5 nm, $p = 0.015$).

EV secretion is not affected by in vitro preconditioning

Since TEM is not a quantitative method to measure size and concentration of EVs, EVs were further analyzed using additional methods. Total protein concentration was used to compare EV concentrations but total numbers could not be obtained with this method. For the exact EV number we used the qNano (Izon Science, Oxford, UK) and compared the results obtained from micro BCA assay with those from TRPS.

Protein concentrations ranged from 32.91 µg/ml up to 51.32 µg/ml in cardiomyocytes, 63.05 µg/ml up to 80.76 µg/ml in primary fibroblasts and from 94.58 µg/ml up to 119.2 µg/ml in H9c2 cells (Figure 3(a–c)). No significant differences were detected between the different treatments in each cell type. In general, the differences in total protein concentrations between the cell types were due to the different amounts of primary cardiac cells and fibroblasts/H9c2 cells used for preconditioning. When particle concentrations were quantified by TRPS, a similar trend was observed. Generally, primary cardiomyocytes secreted EVs in a range of 2.1×10^9–4.1×10^9 particles/ml (Figure 3(a)) and fibroblasts in a range of 3.1×10^9–3.8×10^9 particles/ml (Figure 3(b)). Fibroblasts and cardiomyocytes did not actively secrete significantly more or less EVs after an *in vitro* model of preconditioning compared to untreated cells. The size distribution also indicated that the amount of small EVs is not enhanced or reduced by our treatments in these cells (Figure 3(a,b)) Surprisingly, the EV concentration of H9c2 cells (5.36×10^9 particles/ml for control) decreased due to

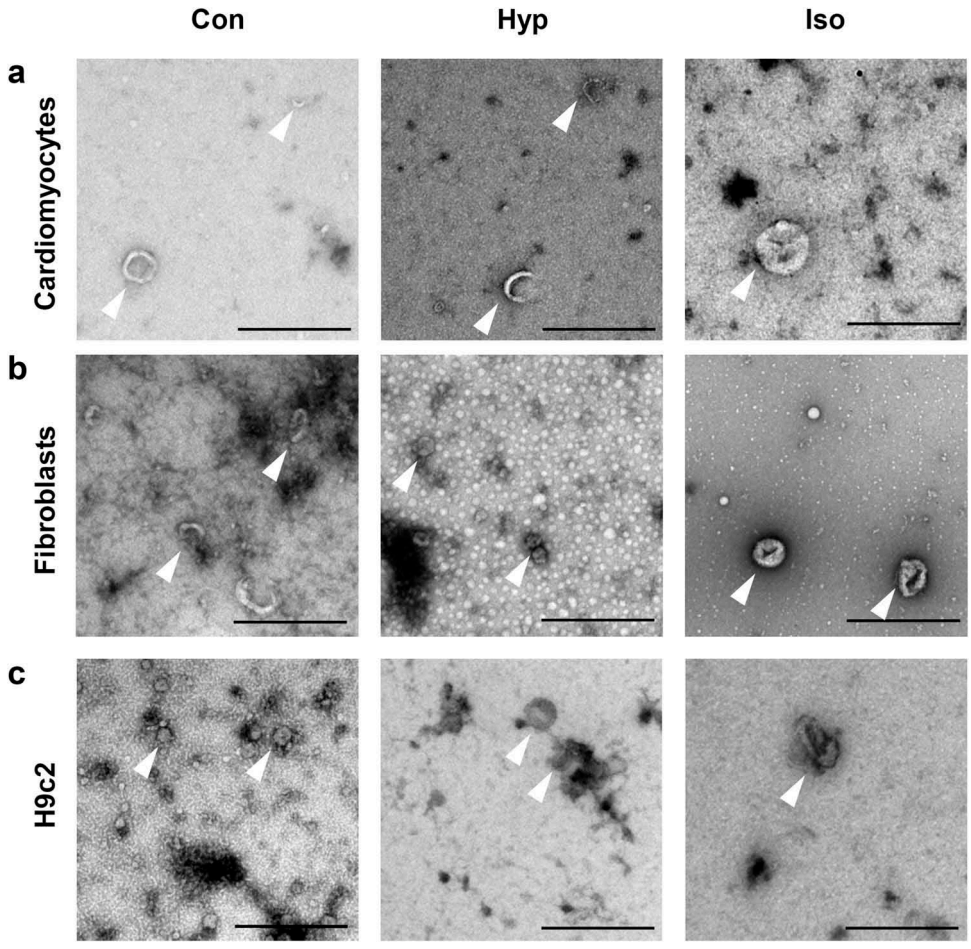


Figure 1. **Transmission electron microscopy (TEM) of cardiac cell-derived EVs after *in vitro* preconditioning**. Cells were treated with hypoxia (Hyp), isoflurane (Iso) or left untreated as control (Con). After 48 h EVs were isolated by SEC and incubated on formvar-coated grids. Negative staining with uranyl acetate in methyl cellulose was performed. Representative images of EVs derived from primary cardiomyocytes (a), cardiac fibroblasts (b) and H9c2 cells (c). Scale bar represents 500 nm.

the hypoxic (3.73×10^9 particles/ml) and especially isoflurane (Iso: 2.39×10^9 particles/ml) treatment. The amount of small EVs also decreased due to those treatments, whereas the number of EVs larger than 150 µm stayed the same (Figure 3(c)). However, these drops in EV concentration were not significant.

Protein composition of EVs changes after in vitro preconditioning

While the total protein concentration and EV number did not significantly change after *in vitro* preconditioning, we next investigated whether the EV composition was affected. Equal amounts of EV lysates were subjected to SDS-PAGE and Western blotting and the common EV markers CD63, Alix, flotillin-1 as well as the small EV specific marker CD81 were detected [6]. In addition, HSP70, an EV marker that has also been described to be a cardioprotective mediator, was analyzed. To verify equal protein loading, total protein content per lane was quantified using stain-free technology [20]. No differences were observed for all groups independent of treatments (Figure 4(a)), therefore equal loading was assumed. GP96 could not be detected, which is a marker of large EVs and should not be present in small EV preparations (Supplementary Figure 4) [6].

Surprisingly, EV protein composition was affected by *in vitro* preconditioning. The effect was cell type and stimulus dependent and not all investigated markers were regulated to the same extent (Figure 4(b)). In primary cardiomyocytes, EV composition was strongly influenced by hypoxia but not by isoflurane treatment. *In vitro* preconditioning with hypoxia significantly increased the level of CD63 (2.3-fold, $p < 0.005$) compared to control in EVs. Alix (1.7-fold, $p = 0.232$) as well as the HSP70 (1.8-fold, $p = 0.222$) content were also slightly increased after treatment with hypoxia in those vesicles. Isoflurane treatment affected Alix (1.6-fold, $p = 0.247$), CD63 (1.4-fold, $p = 0.477$) and CD81 levels in EVs (1.4-fold, $p = 0.587$) but no significant differences

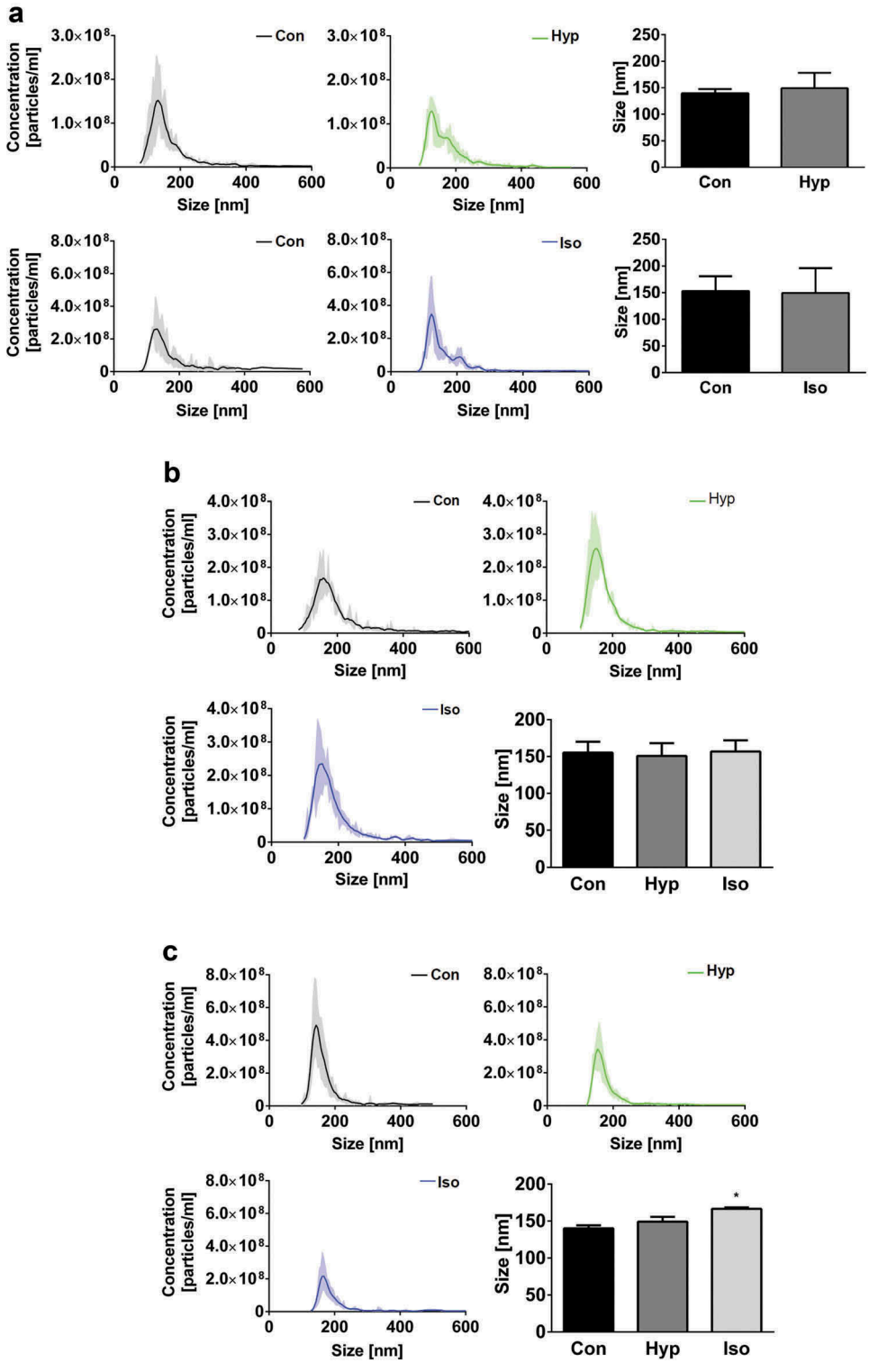


Figure 2. **Size characterization of EVs released after *in vitro* preconditioning with hypoxia or isoflurane**. EV size was quantified by TRPS using a NP200 pore. Modal size distribution of 2–3 representative experiments ± SEM from primary cardiomyocytes (a), cardiac fibroblasts (b) and H9c2 cells (c). Bar diagrams represent the modal size ± SEM of pooled EV-rich fractions of 3 independent experiments. * = $p < 0.05$ vs. control.

could be detected, whereas flotillin-1 was not affected. Interestingly, the HSP70 content in these EVs was even decreased after isoflurane treatment (Figure 4(c)). In contrast, isoflurane treatment of primary fibroblasts resulted in significantly increased levels of Alix (2.7-fold, $p < 0.0001$), CD63 (2.2-fold, $p < 0.0005$) and HSP70 in EVS (4.0-fold, $p < 0.0001$) compared to untreated cells, whereas flotillin-1 and CD81 levels were

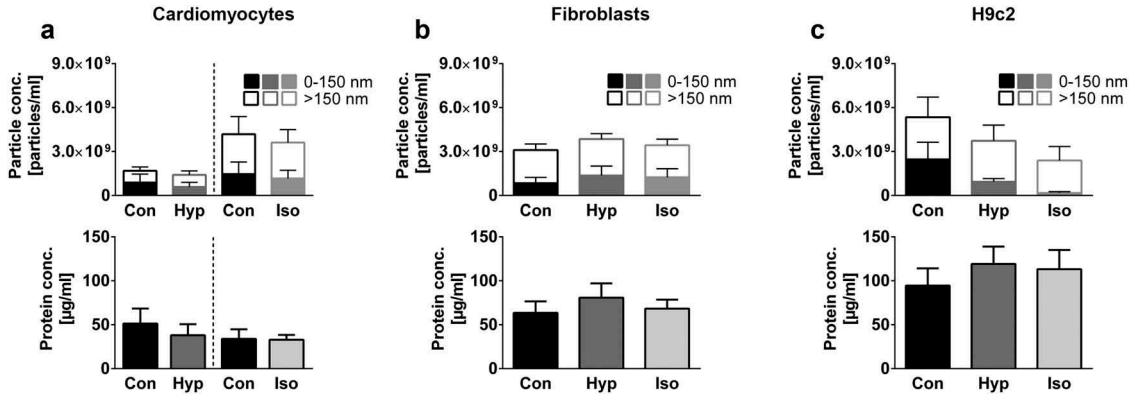


Figure 3. EV **and protein concentration after *in vitro* preconditioning with hypoxia or isoflurane**. EV concentration was quantified by TRPS and further subdivided in two different fractions 0–150 nm and >150 nm. Protein concentration was determined by micro BCA assay. Primary cardiomyocytes (a), fibroblasts (b) and H9c2 cells (c). Data represent means ± SEM of 3–9 independent experiments.

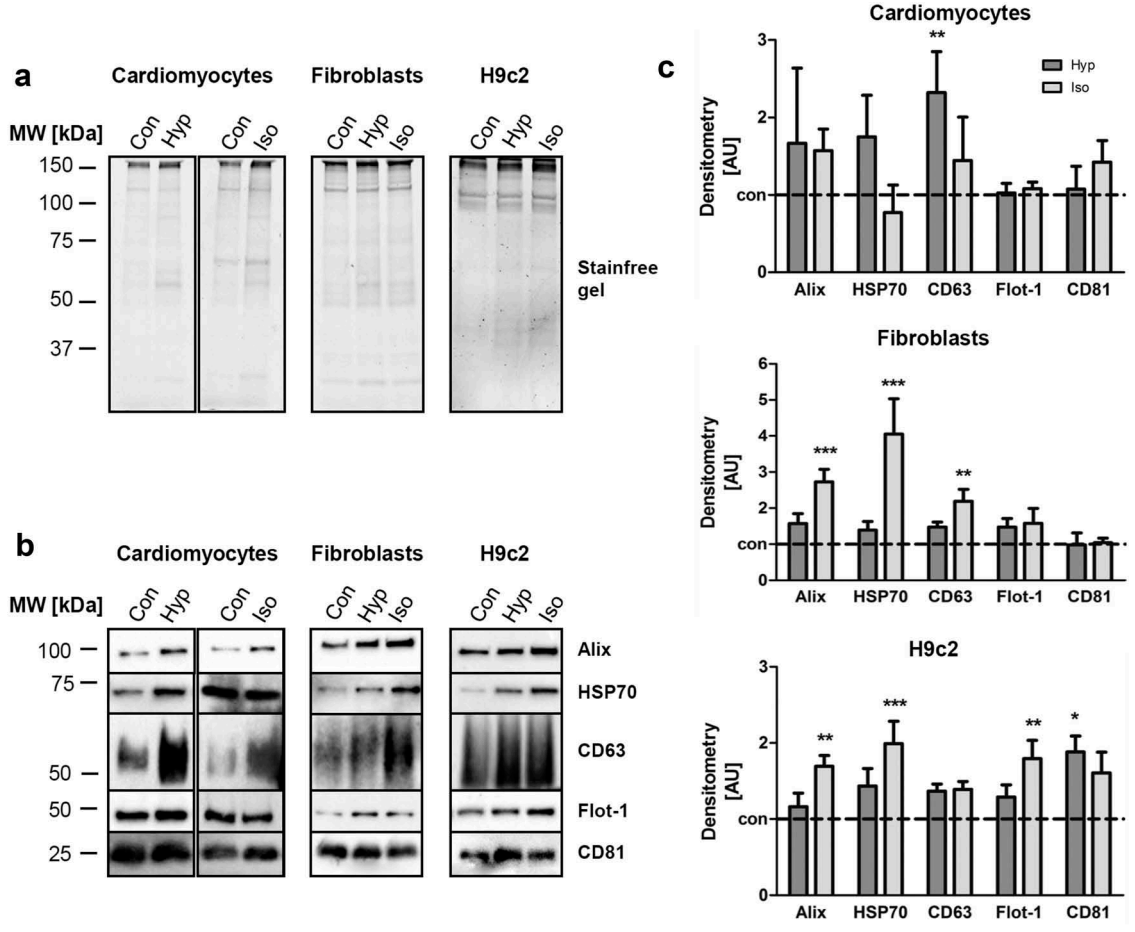


Figure 4. **EV marker expression in isolated EVs after *in vitro* preconditioning**. EVs were subjected to SDS-Page and western blot. Stain-free images confirmed equal protein loading (a). The commonly used EV markers Alix, HSP70, CD63, flotillin-1 and CD81 were detected (b). To quantify the EV marker expression, western blot bands were quantified and normalized to control (c). Depending on the cell type, 1–3 µg were loaded on each gel. Data represent means ± SEM of 4–12 independent experiments depending on the cell type and marker and displayed in arbitrary units (AU, * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0001$).

not affected. Hypoxia resulted in a slight but not significant increase of Alix, CD63, HSP70 and flotillin-1 (Figure 4(c)) in EVs. The myoblast cell line H9c2 showed a similar expression profile as the primary fibroblasts. The CD81 level was significantly up-regulated in EVs after hypoxic stimulation (1.9-fold, $p < 0.05$) and also

affected by isoflurane treatment (1.6-fold, $p = 0.133$). Isoflurane treatment likewise resulted in a significant increase of Alix (1.7-fold, $p < 0.005$), HSP70 (2.0-fold, $p < 0.0001$) and also flotillin-1 (1.7-fold, $p < 0.005$). On the other hand, only a slight trend in CD63 overexpression was observed in EVs after *in vitro* preconditioning with hypoxia or isoflurane (both 1.4-fold, Hyp: $p = 0.135$, Iso: $p = 0.097$) (Figure 4(c)).

Our Western blot results indicated that even though protein loading was equal for all treated groups, *in vitro* preconditioning resulted in modified EV-protein composition. The commonly used EV markers were differentially affected by hypoxia or isoflurane treatment.

In vitro preconditioning affects the miRNA composition

Since Western blot analysis revealed that the strongest changes in vesicular protein composition after *in vitro* preconditioning were observed for fibroblasts, we decided to investigate the miRNA cargo of these EVs. Therefore, a GeneChip® miRNA 4.0 array was performed.

To identify miRNAs that were affected by the different treatments, the expression levels of control and treated groups were compared and the fold change was calculated. All miRNAs with a fold change of at least log2: 0.37 (linear 1.3) and a p-value <0.05 were considered as significantly changed. By applying these criteria, 11 miRNAs that were significantly regulated by hypoxia (five up, six down) and 14 miRNAs that were significantly affected by isoflurane treatment (seven up, seven down) were identified (Supplementary Figure 5). Further investigation revealed that seven miRNAs from the hypoxia treated group were related to CVD: miR-541-5p, miR-761 and miR-155 were up-regulated whereas mir-290, miR-92b-3p, let-7a-1-3p and let-7c-2-3p were down-regulated (Figure 5(a)). For the isoflurane treated group nine miRNAs were related to CVD: miR-320-3p, miR-384-3p, miR-351, miR-352 and let-7f-2 were up-regulated whereas let-7f-1-3p, miR-101a-5p, let-7a-1-3p and let-7c-2-3p were down-regulated (Figure 5(b)).

These results indicated that the miRNA cargo of EVs is also affected by *in vitro* preconditioning with hypoxia or isoflurane.

EVs from preconditioned cells enhance cell migration

To test whether the isolated EVs have any physiological benefit, a migration assay with primary fibroblasts was performed. Since the most promising changes in EV cargo composition were detected in EVs derived from primary fibroblasts, they were transferred to primary fibroblasts to investigate autocrine effects. After 24 h, all fibroblast-derived EVs significantly reduced the scratch (Figure 6(a–e)) compared to the buffer control (PBS = 1.34 ± 0.08 mm^2, Con = 0.97 ± 0.06 mm^2, Hyp = 0.73 ± 0.08 mm^2 and Iso = 0.76 ± 0.07 mm^2). In addition, an incubation of scratched cells with EVs derived from hypoxia treated cells resulted in a

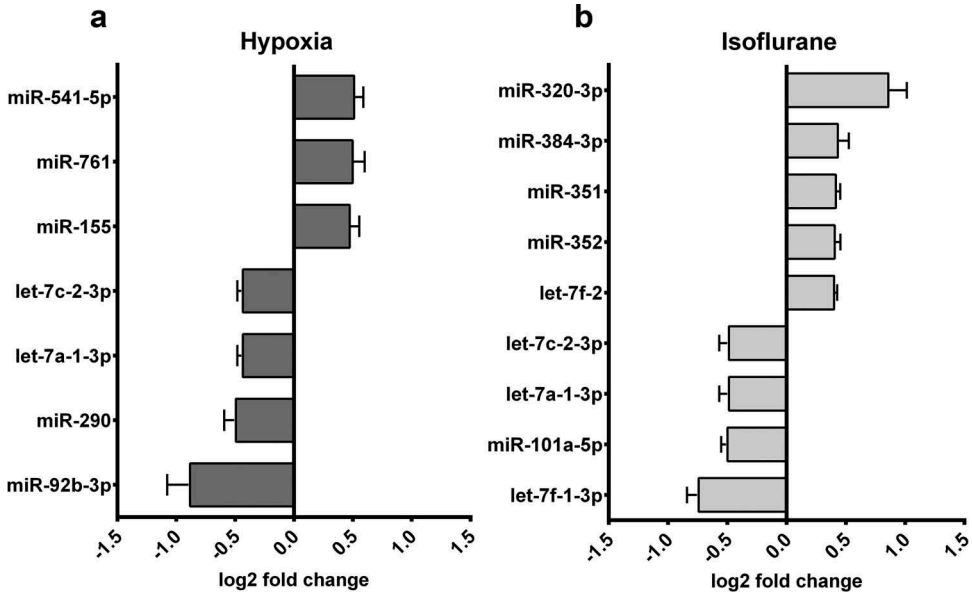


Figure 5. **miRNAs regulation by *in vitro* preconditioning with hypoxia or isoflurane**. miRNA was isolated from fibroblast-derived EVs and a microarray was performed. Significantly regulated cardiac related miRNAs after treatment with hypoxia (a) or isoflurane (b) are depicted. All displayed miRNAs were significantly regulated compared to the control group. Data represents means ± SEM of 3 independent experiments ($p < 0.05$).

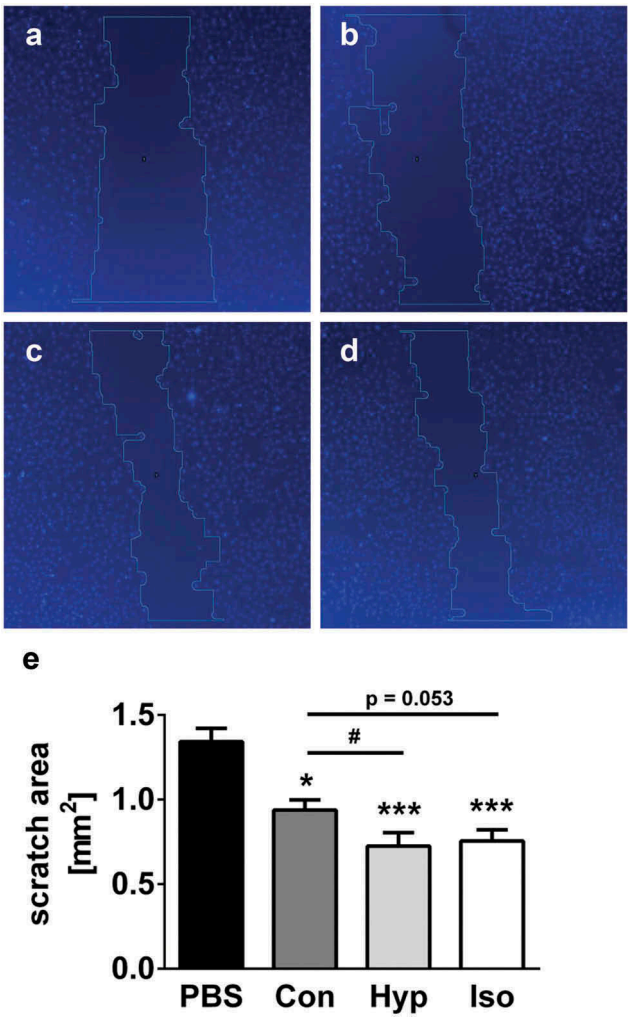


Figure 6. **Cell migration assay**. Fibroblasts were seeded in 96-well plates and scratched with a 200 µl pipette tip. Cells were treated with fibroblast-derived EVs with a concentration of $5*10^8$ EVs/ml for 24 h and afterwards stained with DAPI. The scratched area was quantified with ImageJ of PBS (a) or EVs derived from control (b), hypoxia- (c) and isoflurane-treated (d) cells. Data represent means ± SEM of 8 independent experiments (e) and is displayed in mm^2 (#* = $p < 0.05$, *** = $p < 0.0001$).

significantly reduced scratch area compared to EVs from control cells ($p < 0.05$). A similar effect with EVs derived from isoflurane preconditioned cells was observed ($p = 0.053$).

Discussion

Almost every cell type in the human body is capable of secreting EVs. Due to their lipid bilayer those EVs are able to withstand enzymatic digestion, e.g. in the blood, and therefore serve as transportation tools for proteins, lipids and nucleic acids in almost every body fluid. Recently, EVs came in focus as potential mediators of cardioprotection. Regarding this, researchers usually investigated EVs which were secreted from untreated cells (basal secretion) [29,30]. In contrast, we analyzed whether models of *in vitro* preconditioning with hypoxia or isoflurane had any influence on EV concentration, size or cargo. We hypothesized that the EV concentration and their cargo may be affected by these treatments. Surprisingly, significant changes were observed in the EV composition while the concentration was not significantly affected.

It has previously been shown that all cell types in our study are able to secrete EVs [31–33],but it remained to be elucidated whether these cell types secrete EVs after a certain stimulus and if these EVs might provide any benefit in cardioprotection. Numerous studies could show that EVs can act in an autocrine or paracrine fashion and mediate cardioprotection [34–36]. It has been demonstrated that cardiomyocytes can secrete EVs [31]. In another study, stimulation with hypoxia resulted in the release of EVs that were enriched in miR-30. These EVs were transferred between cardiomyocytes which counter-regulated autophagy [37]. The capability of cardiac fibroblasts to secrete EVs has also been demonstrated [33,38]. In contrast to the protective effects from cardiomyocytes derived EVs, it has been shown that EVs from fibroblasts provoke hypertrophy in cardiomyocytes [38]. In another study it was shown that fibroblast-derived EVs are less protective then endothelial progenitor derived EVs or EVs from cardiosphere-derived cells, specified by tube formation and proliferation assays [39,40]. Nevertheless, fibroblasts comprise the largest cell population in the heart and therefore it was necessary to investigate whether the applied stimuli had any effect on those cells in our study too [41]. Like cardiomyocytes and fibroblasts, H9c2 cells are also known to secrete EVs [32]. H9c2 cell-derived EVs can be taken up by endothelial cells and might induce proliferation and angiogenesis *in vitro* [32].

In our study, EVs were purified with a combination of ultrafiltration and SEC. We were able to isolate EVs in a size range of exosomes and small microvesicles. TEM images also showed the typical cup-shaped structure, which is expected from exosomes and microvesicles [11,42]. Since those vesicles cannot be distinguished by size, most likely a mixture of both EV subsets was isolated. Nevertheless, Kowal et al. recently demonstrated that different EV subsets might be distinguished by explicit EV markers. According to this publication, the presence of CD81 and the absence of GP96 in our study, indicated that the isolated vesicles belong to the group of small EVs [6]. Regarding the size, there was also no significant difference between the experimental groups in both primary cell types. Nevertheless, isoflurane treatment of H9c2 cells triggered the secretion of significantly larger EVs compared to the control group. The reason for isoflurane treatment to result in the release of larger EVs is still not clear and needs

further investigation. To date, there is no study which has investigated the impact of isoflurane on EVs, especially regarding size. Further analysis is also needed to investigate the underlying molecular mechanisms.

In our study, no significant differences in EV concentrations were detected across the experimental groups. In contrast, others have demonstrated that the EV concentration can be affected by certain stimuli [5]. Adult cardiac myocytes treated with physiological concentrations of ethanol, for example, secreted more EVs [43]. Others demonstrated that stimulation with hypoxia for 48 h resulted in an elevated EV concentration but also with a higher rate of apoptosis probably due to the abiding absence of oxygen [44]. In addition, starvation of H9c2 cells also increased the release of EVs [32]. In contrast, we saw a reduction of EV release from H9c2 cells upon hypoxia and especially isoflurane treatment. Our controversial results in this particular area are probably due to the different stimuli we applied. *In vitro* preconditioning probably affects EV generation and release differently from that in the literature described stimuli. Additionally, our experiments indicated that the myoblast cell line H9c2 reacted differently from our *in vitro* preconditioning model than the investigated primary cardiomyocytes and fibroblasts. One possible explanation for the discrepancy between primary cardiomyocytes and the H9c2 cells is the phenotype of the cell line. The cardiac phenotype of H9c2 cells can be enhanced by differentiation with starvation in combination with retinoic acid treatment [45]. Whether this has an influence on EV size and secretion is not known yet. Additionally, H9c2 cells are immortalized which will also result in a different phenotype and behaviour compared to primary cells.

Even though the EV number was not increased, *in vitro* preconditioning influenced EV biogenesis. Surprisingly, EVs secreted from both primary cardiac cell types as well as the cell line H9c2 differed in cargo composition, depending on the applied *in vitro* preconditioning stimulus. Whether alterations in the secreting cells or modifications in EV sorting mechanisms are responsible for these results needs to be further investigated. We could exclude that the differences we observed were a consequence of increased EV number or protein loading by TRPS, micro BCA and stain-free analysis. This discrepancy between the observed changes in EV cargo and unchanged EV concentration also demonstrated that EV marker expression or regulation in EVs should not be used as a standalone method for EV quantification, as was shown in another publication [14]. HSP70 is widely used as an exosomal marker but recent studies showed that this protein is also present in multiple other EV types like microvesicles [6]. Besides its function as an exosomal marker, HSP70 plays a key role in cardioprotection and was significantly up-regulated in our study after treatment with isoflurane in primary fibroblasts and H9c2 cells. Isoflurane also triggered the expression of HSP70 in several other studies, which did not investigate this protein in context of EVs and also displayed other organoprotective properties [46,47]. In a study of oxygen-glucose deprivation induced H9c2 cell injury, a treatment with isoflurane was able to suppress IL-6, IL-8 and TNF-α production, thereby preventing ischaemic heart disease [48]. These findings were supported by a study which showed that isoflurane mediated protection against oxidative stress through an up-regulation of miR-21 in cardiomyocytes [49]. In a recent study it was also shown that HSP70, displayed on the surface of EVs, activated HSP27 through TLR4 signalling and thereby provides cardioprotection [5]. Taken together, these findings support our hypothesis that *in vitro* preconditioning with isoflurane stimulates cardiac cells to secrete EVs with enhanced cardioprotective properties.

In our study, primary cardiomyocytes seemed to secrete different EVs regarding the investigated marker expression profile than H9c2 cells and fibroblasts. In agreement with our findings, Malik et al. had already shown by mass spectrometry analysis, that the EV composition of rat cardiomyocytes is different from that derived from other cell types [43]. In our study, primary cardiomyocytes seemed to be more sensitive to the hypoxic than the isoflurane treatment. It was shown that hypoxic preconditioning protects neonatal cardiomyocytes against hypoxia/reoxygenation injury [50]. In a recent study it was also shown that hypoxia can alter the miRNA cargo of cardiomyocyte derived EVs and thereby regulate autophagy [37]. We acknowledge that the precise mechanisms of hypoxia induced EV secretion from cardiomyocytes need to be investigated in future studies. Since it has been shown that hypoxia affects EV biogenesis in few studies, the influence of isoflurane in EV biogenesis has not been described so far. We are the first to describe the link between EV biogenesis and anaesthetic preconditioning.

Since Alix was strongly up-regulated after isoflurane treatment in fibroblasts and its involvement in miRNA enrichment in EVs has been described, we also investigated the miRNA cargo of fibroblast-derived EVs [51]. The microarray analysis identified several miRNAs that were regulated by our treatments. Of these 25 microRNAs, several have already been described in the context of CVD. miR-92b-3p was significantly down-regulated in our study and an inhibition of a closely related miRNA, miR-92a, has been described to be associated with attenuated apoptosis of cardiomyocytes [52]. miR-290, which was also down-regulated during hypoxic

treatment, regulates the visinin-like protein 1 (VSNL1) expression [53]. VSNL1 is associated with heart failure [54] and an overexpression of miR-290 would decrease protein expression and could therefore contribute to cardioprotection. Accordingly, an overexpression of miR-761 as observed in our study, could protect the heart due to its ability to inhibit mitochondrial fission and attenuate cardiomyocyte apoptosis [55]. The beneficial effects of miR-155, which was up-regulated in EVs after hypoxic treatment, are controversially discussed in the literature [56–58]. Even though some publications associate this miRNA with heart failure, one publication describes the anti-inflammatory properties of miR-155 [59]. Isoflurane treatment triggered the overexpression of miR-351, miR-384-3p and miR-352 in fibroblast derived EVs. These miRNAs are associated with cardioprotection or are regulated in different preconditioning models [60,61]. Especially an up-regulation of miR-351 has been described to be important for myogenic progenitor cell proliferation and differentiation, whereas a knockdown leads to apoptosis [62]. An up-regulation of miR-101 suppresses the Cystic fibrosis Transmembrane Conductance Regulator (CFTR), which can lead to increased mitochondrial levels of reactive oxygen species (ROS) [63–65]. Compatible with these findings and the potential cardioprotective benefit, miR-101a-5p was down-regulated in our study after isoflurane treatment. Surprisingly, miR-320-3p was up-regulated. An overexpression of miR-320 is related to increased sensitivity to I/R injury or inhibition of cell proliferation and migration [31,66]. However, this miRNA was also found in a model of hypoxic *in vitro* preconditioning with H9c2 cells, probably contributing to antiapoptotic effects [44]. The expression of several let-7 miRNAs in our purified EVs was affected by both preconditioning stimuli. First discovered in *C. clegans*, the let 7 family is also strongly associated with CVD [67,68]. In our study two let-7f miRNAs were regulated contrary after preconditioning with isoflurane, let-7f-2 was up-regulated whereas let-7f-1-3p was down-regulated. Let-7f is generally associated with myocardial infarction and angiogenesis [68,69]. The precise mechanisms and targets of our let-7f miRNAs and their potential cardioprotective effects need to be unravelled in future studies [70–72]. Additionally, both miRNAs let-7a-1-3p and let-7c-2-3p were down-regulated after an *in vitro* preconditioning with isoflurane, indicating similar sorting mechanisms for both miRNAs due to the treatments. An overexpression of this miRNA family is associated with heart hypertrophy, ischaemic cardiomyopathy and atherosclerosis [68,73–75]. It has also been shown that HSP70 is a direct target of let-7c. Jiang et al. demonstrated that HSP70 is negatively correlated with let-7c levels in fibroblasts, which supported our observations in protein and miRNA analysis after *in vitro* preconditioning [76]. Further investigations are needed for these particular miRNAs to unravel their potential beneficial effects. Furthermore, those miRNAs that were significantly regulated in our study but have so far not been associated with CVD, need to be further investigated to elucidate their potential cardioprotective properties.

The cell migration assay demonstrated the general physiological benefit of EVs, as has been shown in other studies [25,77]. The autocrine effect of fibroblast derived EVs was thereby confirmed. Additionally, we could show that EVs from hypoxia-treated cells significantly enhanced scratch area reduction compared to those derived from unstimulated cells. Since we also saw a strong tendency for EVs derived from isoflurane preconditioned cells to have the same effect, we conclude that EVs from *in vitro* preconditioned cells have physiological effects on cell migration. Since we saw in our study that different stimuli trigger the release of EVs with varying composition, we suggest that this might be the reason for the differences between the hypoxia and isoflurane group in our migration experiments. Our findings are similar to those from Zhang et al., who have demonstrated protective effects from EVs derived from hypoxic treated H9c2 cells, by investigating the miRNA content of those EVs and their targets. Several miRNAs with potential antiapoptotic effects like miR-152-3p and let-7i-5p were investigated [44].

The primary limitation of this study is that there is still no ideal purification method to isolate pure EVs. As a result, the isolated EV fractions may still be contaminated with proteins or lipids that are not necessarily associated with the isolated EVs. Nevertheless, we were able to separate the majority of protein contaminations from the EV-rich fractions. Because of the limited availability of primary cardiac myocytes it was not possible to perform experiments with both stimuli at the same time. Therefore, we had to split the cells of a single preparation in one control and one treatment group and thus interpret and illustrate the results from these cells separately. Additionally, the stimulation experiments were performed within an idealized setting. The EV release after preconditioning in a complex organism might therefore be different and has to be analyzed in the future. The explicit mechanisms which led to the differences in EV cargo composition in our study need to be further analyzed. To date, it is not clear whether this effect is a result of alterations in the cells or specific modifications in the EV sorting mechanism. The performed microarray should be considered as a first glimpse of which miRNAs might be affected and might provide cardioprotection. Further studies are needed to confirm their role in

preconditioning and cardioprotection in our *in vitro* setting. The migration assay gave first insight in the physiological relevance of EVs derived from treated cells, compared to control cells. Nevertheless, it has to be investigated if the observed effects were due to migration or proliferation by applying different inhibitors [78]. The effect on other cell types needs to be investigated in detail as well as the mediators which are responsible for the observed effects.

In conclusion, the isolated EVs showed the typical cup-shaped structure and showed no differences in concentration following an *in vitro* preconditioning stimulus, whereas the protein composition was affected. The effects differed from marker to marker and were stimulus dependent. Therefore, commonly used EV markers cannot be used to quantify EVs since they are themselves affected by different stimuli. Interestingly, the diverse cell types reacted differently to the applied stimuli. This might indicate that *in vitro* preconditioning triggered the release of tailored EVs that might be involved in intracellular communication. The cell migration assay gave a first indication that fibroblast-derived EVs after preconditioning alter physiological effects in recipient cells. In the next step, proteomic analysis and real-time polymerase chain reactions of the regulated miRNAs should be performed to elucidate which proteins or miRNAs are affected by these models of *in vitro* preconditioning. Also, the functional as well as biological relevance needs to be further demonstrated in future studies. This might also shed light on the potential cardioprotective properties of these EVs. Furthermore, the protective properties should also be investigated in animal models of IR to enable its translation into clinical practice.

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