Endothelial β1 Integrin-Mediated Adaptation to Myocardial Ischemia

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Keywords
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► cardiac vascular growth
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► cardioprotection

Abstract
Background Short episodes of myocardial ischemia can protect from myocardial infarction. However, the role of endothelial β1 integrin in these cardioprotective ischemic events is largely unknown.

Objective In this study we investigated whether endothelial β1 integrin is required for cardiac adaptation to ischemia and protection from myocardial infarction.

Methods Here we introduced transient and permanent left anterior descending artery (LAD) occlusions in mice. We inhibited β1 integrin by intravenous injection of function-blocking antibodies and tamoxifen-induced endothelial cell (EC)-specific deletion of Itgb1. Furthermore, human ITGB1 was silenced in primary human coronary artery ECs using small interfering RNA. We analyzed the numbers of proliferating ECs and arterioles by immunohistochemistry, determined infarct size by magnetic resonance imaging (MRI) and triphenyltetrazolium chloride staining, and analyzed cardiac function by MRI and echocardiography.

Results Transient LAD occlusions were found to increase EC proliferation and arteriole formation in the entire myocardium. These effects required β1 integrin, except for arteriole formation in the ischemic part of the myocardium. Furthermore, this integrin subunit was also relevant for basal and mechanically induced proliferation of human coronary artery ECs. Notably, β1 integrin was needed for cardioprotection induced by transient LAD occlusions, and the absence of endothelial β1 integrin resulted in impaired growth of blood vessels into the infarcted myocardium and reduced cardiac function after permanent LAD occlusion.

Conclusion We showed that endothelial β1 integrin is required for adaptation of the heart to cardiac ischemia and protection from myocardial infarction.

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Introduction

Coronary artery disease (CAD) is the leading cause of death around the globe. Due to diminished blood supply of the heart, it can result in myocardial infarction (MI), heart failure, and death. Current prevention strategies aim to reduce risk factors that negatively affect the cardiovascular system, such as hyperglycemia or diabetes mellitus, hyperlipidemia, smoking, hypertension, physical inactivity, and unhealthy dietary patterns. Notably, cardiac vascular growth was also observed as an important factor to protect from MI or to improve its outcome. Hereby, functional growth of coronary vessels creates additional and alternative routes to provide the ischemic myocardium with fresh blood, thus reducing MI size or preserving cardiac function after MI.

Hypoxic and mechanical stimuli are thought to be involved in vascular growth. Hypoxia activates hypoxia-inducible factor-1α to induce signals, such as vascular endothelial growth factor-A (VEGF-A) that preferentially triggers vascular growth and left ventricular (LV) remodeling and interest, since they bind collagen type I, which is involved in reperfusion.

Permanent LAD occlusion was performed to mimic the experiments on the adult mouse heart. Finally, we studied whether endothelial β1 integrin had a general function to protect the heart and preserve cardiac function upon MI, independent of short episodes of ischemia.

Our study uncovers an important role of endothelial β1 integrin in cardiac adaptation to LV ischemia as well as preservation of cardiac function after an MI.

Methods

Mice

For wild-type studies with antibody treatment, 10- to 15-week-old male C57BL/6J mice from Janvier were used. β1 integrin blocking antibody (BD Bioscience, 555002) and control antibody (BD Bioscience, 553957) were intravenously injected (1 mg/mL, 100 µL) 1 day before each 15-minute ischemia and before and after permanent occlusion. For genetic deletion experiments, global NOS3 (eNOS KO) mice were used. For endothelial Itgb1 deletion (Itgb1flEXKO), Cdhs5-CreERT2, homozygous Itgb1-loxp mice were crossed with homozygous Itgb1-loxp mice. As controls for eNOS KO mice, the corresponding C57BL/6J mice from Jackson Laboratory were used. As controls for Itgb1flEXKO mice, Cdhs5-CreERT2 mice were used, which received the same tamoxifen injections. Both mouse lines were treated with 100 µL tamoxifen (75 mg/kg body weight) for 5 consecutive days. All experiments were performed according to the German animal protection laws (Animal Ethics Committee of the Landesamt für Natur, Umwelt und Verbraucherschutz, North Rhine-Westphalia).

Myocardial Ischemia

For the induction of transient LAD occlusions, a closed-chest model was used, as previously described. Briefly, in an initial operation an occluder was implanted for temporary occlusion of the LAD, therefore a 7–0 prolene suture (Ethicon, Johnson and Johnson) was placed around the proximal LAD, ends were threaded through a polyethylene tube, the thorax was closed, and the suture ends were placed into a subcutaneous pocket. For permanent LAD occlusion, mice were treated the same way but the LAD was closed with a 7–0 silk suture (Seraflex, Serag-Wiessner) and myocardial ischemia was induced directly. In general, 7 days after the initial operation, the first ischemia for transient LAD occlusions was induced. However, during the proliferation analysis in blocking AB-treated mice, and when Repl/R and MI were combined for magnetic resonance imaging (MRI), the first ischemia was induced 2 days after the initial operation, so as to shorten the experimental procedure. Mice were anesthetized in an inhalation anesthetic set with oxygen-enriched air (O2 40%) and isoflurane (3% vol., Piramal
Healthcare). After anesthesia, the mice were placed in a supine position on a 37°C preheated surgery table, followed by respiration with oxygen-enriched air (O₂ 40%) and isoflurane (2% vol., Piramal Healthcare). After skin disinfection, electrocardiogram (ECG) electrodes were fixed to the fore- and hindfeet and the skin was reopened to pull out the suture ends. Afterwards, 15-minute or 60-minute ischemia was induced by sticking the suture ends to two magnets and setting them under tension to ligate the LAD. Induction of ischemia was controlled in ECG by ST-segment elevation followed by reperfusion of the LAD. Suture ends were placed back into the subcutaneous pocket or cut off after the last ischemia. The 15-minute ischemia was repeated three times every other day. Additional 60-minute ischemia was followed only if the infarct size (IS) and cardiac function were quantified. All mice received an occluder implantation and afterward were randomized to sham or ischemia/reperfusion (I/R) procedure. Mice were intraperitoneally (IP) injected with ketamine (100 mg/kg bodyweight, Ketanest S., Pfizer Pharma GmbH) and xylazine (10 mg/kg bodyweight, Rompun TM, Bayer Healthcare) for anesthesia and treated with Temgesic (0.05–0.1 mg/kg bodyweight) for analgesia.

Cardiac Function
For analysis of IS and cardiac function, ¹H MRI measurements were performed at 400.13 MHz using a Bruker AVANCE III 9.4 T Wide Bore NMR spectrometer (Bruker) driven by ParaVision 5.1, as described previously. In the case of late gadolinium enhancement (LGE) measurements, mice received an IP injection of 0.2 mmol Gd-DTPA per kg bodyweight immediately prior to the measurement. To calculate the LV ejection fraction (EF), defined as the difference between end-diastolic volume (EDV) and end-systolic volume (ESV) divided by EDV, the ventricular borders were selected manually by means of the ParaVision region-of-interest tool (Bruker). For quantification of IS, LGE-positive areas were selected manually and related to the LV volume. Transthoracic echocardiography was also used to analyze LV cardiac function, as previously described. Therefore, measurements were performed with a MS-400 scan head (Vevo 2100, VisualSonics, FUJIFILM). EDV, ESV, and EF were calculated from long-axis B mode in the Vevo laboratory software 1.7.1 (VisualSonics). Only mice that survived until the end of the experiment were used for analysis.

Osmotic Pump Implantation
Osmotic pumps (Alzet, model 1002) were filled with 20 mg/mL 5-bromo-2′-deoxyuridine (BrdU, Sigma) in phosphate buffer saline (PBS) and were implanted IP during the first 15-minute ischemia. The pumps remained in the mice until heart isolation.

Pimonidazole Treatment
To determine hypoxic regions in the myocardium during ischemia, mice were treated with pimonidazole (Hypoxyrrobe Green Kit, Hypoxyrrobe, Inc., Burlington, Massachusetts, United States). More specifically, after anesthesia, pimonidazole (60 mg/kg bodyweight) dissolved in PBS was injected IP and the LAD was occluded as described before, without reperfusion. After ischemia induction, hearts were isolated, perfused with ice-cold PBS, deep-frozen in nitrogen, and embedded in Tissue-Tek O.C.T. (Sakura Finetek GmbH) for cryosectioning.

Immunohistochemistry
Heart isolation was performed 7 days after the last 15-minute ischemia, except for the proliferation analysis in AB-treated mice, which was 2 days after the last 15-minute ischemia. For proliferation analysis, sections were pretreated with 2 M HCl for 30 minutes at 37°C and neutralized with 0.1 M sodium tetraborate, followed by primary and secondary antibody incubation. The following primary antibodies were used in this study: rat anti-BrdU (Abcam, ab6326), goat anti-PECAM-1 (R&D Systems, AF3628), mouse anti-α-SMA-Cy3 (Sigma, C6198), mouse anti-α-SMA (Sigma, A5228), and rat anti-β1 integrin (Millipore, MAB1997), and incubated overnight at 4°C. Secondary antibodies conjugated with fluorophores AF488 (Invitrogen Molecular probes), Cy3 (Jackson ImmunoResearch), and Cy5 (Jackson ImmunoResearch) were incubated in the dark for 1 or 3 hours at room temperature. Cell nuclei were stained with DAPI (Sigma). For hypoxia analysis, sections were stained with fluorophore-coupled antibodies for HP-FITC-MAb (detects pimonidazole adducts) and mouse anti-α-SMA-Cy3 (Sigma, C6198) overnight at 4°C. Cell nuclei were stained with DAPI (Sigma).

Hearts with considerable tissue damage and areas of cryosections containing part of the occlusion system were excluded from quantification.

Triphenyl Tetrazolium Chloride Staining
The triphenyl tetrazolium chloride (TTC) staining was performed to determine the IS and area at risk (AAR), after 60-minute I/R. After anesthesia, hearts were isolated, cannulated over the aorta and washed with NaCl solution to remove remaining blood clots. Afterwards, the LAD was permanently closed with a silk suture and the hearts were perfused with 1% Evans blue solution (in NaCl). Hearts were frozen at –20°C for approximately 1 hour, transversally cut, transferred into TTC solution containing 8 parts 0.1 M Na₂HPO₄, 2 parts 0.1 M NaH₂PO₄ (pH 7.4), and 10 mg/mL TTC and incubated at 37°C for 5 to 10 minutes under shaking. Images were taken with a stereomicroscope (Leica M26 with Leica KL 1500 LCD), and infarcted area and AAR in the LV were analyzed with the software DISKUS (Vers. 4.30.7, Carl H. Hilgers, DISKUS mikroskopische Diskussion).

Microinfusion
To image the entire coronary vasculature, hearts were washed and retrograde-perfused via the thoracic aorta with Microinfusion (Flow Tech Inc.), previously described but modified. For optical clearing, hearts were incubated in methyl salicylate (Sigma Aldrich) and afterward imaged under a stereomicroscope (Leica M26 with Leica KL 1500 LCD).
Primary Human Cell Culture, Transfection, and Mechanical Stretching

Primary HCAECs (male, 21 years old), purchased from PELO-Biotech, were cultured in a humidified atmosphere at 5% CO₂ and 37 °C. HCAECs were grown in a microvascular EC growth medium kit enhanced (PELOBiotech), plated on stretch chambers (STEX) or on 12-well plates, and used up to passage 6. To selectively knock down the expression of β1 integrin, HCAECs were transfected with one of the following ITGB1-siRNAs (β1-siRNA) (Invitrogen) via electroporation (4D-Nucleofector System, LONZA) and incubated for 48 hours:

- β1-siRNA1: 5′-CCUAAGUCAGCAGUAAGCAAUU-3′
- β1-siRNA2: 5′-UGCGAGUGGGUGCUGUAGUA-3′
- β1-siRNA3: 5′-GGGAGCCACAGCAUUACAUUAA-3′

As control, a non-targeting small interfering RNA (siRNA; Invitrogen) with a similar GC content was used. Additionally, HCAECs were mechanically stretched for 30 minutes in the case of proliferation studies and for 15 minutes to analyze integrin activation by means of a manual cell-stretching case of proliferation studies and for 15 minutes to analyze integrin activation by means of a manual cell-stretching system (STREX, STB-10). Knockdown efficiencies were analyzed by quantitative real-time polymerase chain reaction (PCR) and Western blotting.

In Vitro Proliferation Analysis and β1 Integrin Activation

For analysis of in vitro proliferation, 10 μM BrdU (Sigma) was added to the transfected HCAECs and incubated for 2 hours. In the case of stretching experiments, BrdU was added at the beginning of the stretching. After pretreatment of fixed cells with 2 M HCl and subsequent neutralization with 0.1 M sodium tetraborate, immunofluorescence staining was performed using mouse anti-BrdU antibody (BD Bioscience, 555627) as primary and anti-mouse antibody conjugated with AF488 (Invitrogen Molecular probes) as secondary antibodies. For β1 integrin activation analysis, HCAECs were fixed in 4% PFA after mechanical stretching, and the immunostaining was performed using mouse anti-human activated β1 integrin antibody (Merck Millipore, MAB2079Z) as primary and anti-mouse antibody conjugated with Cy5 (Jackson Immunoresearch) as secondary antibodies.

Microscopy and Analysis

All immunofluorescence stainings were imaged using a confocal laser scanning microscope (LSM 710 or LSM 880, Zeiss). Proliferating ECs and arterioles were counted manually and related to the myocardial area in mm². Proliferating HCAECs were quantified either by manual or a semiautomated counting method in FIJI (Image, NIH). The myocardial area was determined applying the freehand selection tool in FIJI, area or intensity of PECAM-1, DAPI or β1 integrin in heart sections and in HCAECs after mechanical stretching was analyzed with FIJI as well.

Magnetic-Activated Cell Sorting of ECs

For collection of cardiac ECs from Itgb1iECKO and control mice, hearts were isolated and dissociated with a gentleMACS dissociator (Miltenyi Biotec). All further steps were performed according to the customer protocol (Miltenyi Biotec; “Preparation of single-cell suspensions from mouse heart,” “CD45 MicroBeads,” and “CD31 MicroBeads”). EC pellets were used either for quantitative real-time PCR and therefore homogenized with peqGold TriFast (Peqlab) or lysed in RIPA buffer for Western blot analysis.

Quantitative Real-Time PCR

Total RNA from cultivated HCAECs was extracted with an RNasey Mini kit (Qiagen), according to the manufacturer’s instructions. Total RNA isolation from magnetic-activated cell sorting (MACS)-sorted murine ECs was performed by using the phenol/chloroform extraction. Quantitative real-time PCR was performed with Brilliant III Ultra-Fast SYBR Green QPCR Master Mix and the thermal cycler Stratagene Mx3000P (Agilent Technologies). All samples were analyzed in triplicates. The following primers were used:

- human ITGB1 forward: 5′-CATCTGCGAGTGTGGGTCT-3′
- human ITGB1 reverse: 5′-GGGTAATTGTTCGCCGACTT-3′
- human RPLP0 forward: 5′-CAGCAGTCTACAACCTCTAGAG-3′
- human RPLP0 reverse: 5′-CAGTGGCAAATCCGAGAC-3′
- human HPRT forward: 5′-TGACACTGCGAAGAACATGCA-3′
- human HPRT reverse: 5′-GGCTTCCTTTTCCACGCAAGCT-3′
- human B2M forward: 5′-TTTTCATCCATCGACATGTA-3′
- human B2M reverse: 5′-CCTCCATGATGCTGCTACA-3′
- mouse Itgb1 forward: 5′-AATGCGGAGGACACCGG-3′
- mouse Itgb1 reverse: 5′-TGACTAAAGTGGCTCTGCTGAG-3′
- mouse Rplp0 forward: 5′-GATGCCGCGGAAACAGCG-3′
- mouse Rplp0 reverse: 5′-ACAAGTAGCAATTTTGGATAATCA-3′
- mouse Hprt: forward: 5′-CAGGACTAAAGACCCTGCT-3′
- mouse Hprt reverse: 5′-GCTGTGAAAAGGACCTCT-3′
- mouse B2m forward: 5′-GGGCGGAGACGCTACTG-3′
- mouse B2m reverse: 5′-GCTATTATTCTGCTGACT-3′

The efficiencies of β1 integrin knockout (in vivo) or knockdown (in vitro) were determined by means of the ΔΔCT method, where RPLP0/Rplp0, HPRT/Hprt, and B2M/β2m were used as housekeeping genes for normalization.

Western Blotting

Cultivated human or MACS-sorted murine ECs were lysed in RIPA buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% IGEPAL, 0.25% Na-deoxycholate, 1 mM EDTA) containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail, Roche). Total protein concentrations were determined by means of the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein were used for the SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). For protein separation, 4 to 15% SDS-gels (Bio-Rad) and Mini-PROTEAN Tetra Cell (Bio-Rad) were used. Western blotting was performed by using the Trans-Blot Turbo Transfer System (Bio-Rad) and rabbit anti-GAPDH (Abcam, ab9485), rat anti-β1 integrin (Millipore, MAB1997) for MACS-sorted murine ECs or goat anti-β1 integrin (Santa Cruz, sc-6622) for human ECs as primary antibodies and HRP-conjugated donkey anti-rabbit (Cell Signaling Technologies, 7074), donkey anti-rat (Jackson Immunoresearch, 712-035-150) and donkey anti-goat (Jackson Immunoresearch, 712-036-150) as secondary antibodies.
ImmuNoResearch, 705–035–147) as secondary antibodies. To determine the efficiencies of β1 integrin-knockout (in vivo) or knockdown (in vitro), a semiquantitative band density analysis was performed (Fjll, ImageJ, NIH), where GAPDH was used as a housekeeping protein for normalization.

**Statistical Analysis**

Statistical significance was determined by using Excel (Microsoft) or Prism (GraphPad Inc.). To compare two groups, an unpaired two-tailed Student’s t-test was used, whereas for multiple comparisons a one-way analysis of variance with subsequent Dunnett’s post hoc test was performed. To compare survival rates, in a Kaplan–Meier curve a log-rank (Mantel-Cox) test was used. p-Values less or equal to 0.05 are shown either in the figures or mentioned in the figure legend. All values are shown as means ± standard error of the mean.

**Results**

**Transient Episodes of LV Cardiac Ischemia Lead to β1 Integrin-Dependent Cardiac EC Proliferation and Arteriole Formation in the Entire Myocardium**

We used a closed-chest mouse model to introduce transient LAD occlusions, leading to repetitive episodes of ischemia followed by reperfusion (Rep/R) in the LV myocardium (+Fig. 1), similar to a previously published mouse model. 

The procedure employed an occluder to block the blood flow through the LAD three times every other day for 15 minutes each (+Fig. 1A–G). The LAD occlusions resulted in a transient transmural ischemia. This was indicated by ST-segment elevation on ECG that resolved when the LAD was opened again (+Fig. 1D). Hypoxia staining revealed that only the LV (“ischemic myocardium”), but not the RV and septum (“non-ischemic myocardium”), turned hypoxic during the LAD occlusions (+Supplementary Fig. S1A–D, available in the online version and +Fig. 1E, F). As a control (RepSham), an occluder was implanted around the LAD, and the surgery was performed three times as described above (+Fig. 1A–D), but without occluding the LAD.

First, we studied the proliferation of cardiac ECs in response to the repeated LAD occlusions and the role of β1 integrin in this proliferative response by intravenously injected β1 integrin blocking antibodies (β1-B-AB) versus isotype-matched control antibodies (ctrl-AB) into mice (+Fig. 1H–O). When mice were treated with ctrl-AB, Rep/R increased cardiac EC proliferation in both the ischemic (+Fig. 1R) and non-ischemic myocardium (+Fig. 1H–K, P). In contrast, β1-B-AB abolished the Rep/R-induced cardiac EC proliferation in both, the ischemic and non-ischemic myocardium (+Fig. 1L–O, Q, S).

Next, we counted the number of arterioles in the myocardium of the hearts undergoing either Rep/R or RepSham treatments (+Fig. 2). Notably, the arterioles increased in number, both in the ischemic and non-ischemic myocardia at the level of papillary muscles upon Rep/R (+Fig. 2A–D, I, K). In contrast, no increase in the number of arterioles was observed upon Rep/R when mice were treated with β1-B-AB (+Fig. 2E–H, J, L).

**Transient Cardiac Ischemia Protects from Myocardial Infarction and Subsequent Cardiac Dysfunction via β1 Integrin**

We then investigated whether the Rep/R treatment reduced the IS of an MI induced by an additional 60-minute ischemia (+Fig. 3A). Consistent with previous results, transient cardiac ischemia reduced the IS as compared with the RepSham treatment, analyzed in MRI and TTC staining (+Fig. 3B–E, J; +Supplementary Fig. S2A–E; and +Video 1, available in the online version). Rep/R also improved cardiac function after MI, as determined by preserved LV EF (+Fig. 3L).

**Requirement of Endothelial β1 Integrin for Cardiac EC Proliferation and Arteriole Formation in the Myocardium**

To investigate whether β1 integrin in the endothelium was required for Rep/R-induced cardiac EC proliferation, we selectively deleted the Itgb1 gene in the ECs of adult mice (Itgb1EcKO) using tamoxifen-inducible Cdh5-CreER T2 expression (+Fig. 4). These adult mice expressed Cre recombinase only in the endothelium, but not in myeloid cells, and were compared with tamoxifen-injected Cre control mice (+Fig. 4A). An up to 70% knockdown efficiency of β1 integrin in ECs sorted from tamoxifen-treated mouse hearts was observed on both mRNA and protein levels (+Supplementary Fig. S3; for uncropped Western blots, see +Supplementary Fig. S6, available in the online version). In the controls, transient ischemia induced a twofold increase in cardiac EC proliferation in the non-ischemic myocardium as well as a three- to fourfold increase in EC proliferation in the ischemic myocardium (+Fig. 4B–E, J, L). In contrast, tamoxifen-induced EC-selective gene depletion of β1 integrin strongly reduced the proliferative response of cardiac EC to the short periods of cardiac ischemia (+Fig. 4F–I, K, M).
Fig. 1  Transient episodes of left ventricular (LV) cardiac ischemia require β1 integrin to induce cardiac EC proliferation in the entire myocardium. (A–C) Illustrations of (A) an implanted occluder at the LAD to introduce (B, C) 3 × 15-minute myocardial ischemia treatment with reperfusion (RepI/R) in a closed-chest mouse model. (D) ECG recording before (baseline; black line), during (ischemia; orange line), and after the LAD occlusion (reperfusion; green line). (E) Illustration of an adult mouse heart, including the LAD with an occluder installed. The location of cross-section through the mouse heart (as illustrated in panel F and used for immunohistochemical analyses) is also indicated. (F) Schematic illustration of a cross-section through the ischemic and non-ischemic myocardium. (G) Study design to inhibit β1 integrin by antibody injection. A shortened experimental protocol is shown in brackets. (H–O) Representative immunofluorescence images of sections through the non-ischemic myocardium; mice received either (H–K) ctrl-AB or (L–O) β1-B-AB before each 15-minute ischemia or sham treatment. Sections were stained for BrdU (red), platelet endothelial cell adhesion molecule-1 (PECAM-1; CD31; green), and DAPI (blue). (P–S) Quantification of BrdU-positive ECs per mm² in (P, Q) the non-ischemic and (R, S) the ischemic myocardium upon ctrl-AB or β1-B-AB treatment. Reported values are means ± SEM. Ctrl-AB with RepSham: n = 5; ctrl-AB with RepI/R: n = 6; β1-B-AB with RepSham: n = 5; β1-B-AB with RepI/R: n = 4. Statistical significance was determined using unpaired two-tailed Student’s t-test. EC, endothelial cell; LAD, left anterior descending artery; LCX, left circumflex artery; RCA, right coronary artery; SEM, standard error of mean.
Next, we analyzed the number of arterioles within the ischemic and non-ischemic myocardia in the Itgb1<sup>IECKO</sup> mice (►Fig. 5). Consistent with the previous experiments, more arterioles were found in both parts of the myocardium upon RepI/R (►Fig. 5A–D, I, K). In contrast to the situation in the ischemic myocardium (►Fig. 5K, L), endothelial β1 integrin was required for a statistically significant RepI/R-induced arteriole formation in the non-ischemic myocardium (►Fig. 5E–H, I, J).

**Contribution of Endothelial β1 Integrin to Ischemia-Induced Cardioprotection**

We next analyzed the role of endothelial β1 integrin in cardioprotection conveyed by transient cardiac ischemia. To this end, an MI was induced after RepI/R treatments (►Fig. 5O), and the hearts were analyzed over the following days for IS and LV EF. Notably, the IS in adult Itgb1<sup>IECKO</sup> mice was found to be substantially larger compared with that of tamoxifen-induced controls (►Fig. 5M–R), and the LV EF was reduced on the first day after the MI (►Fig. 5S).

**Growth of Blood Vessels into the Infarcted Myocardium and Maintenance of Cardiac Function upon MI Require Endothelial β1 Integrin**

Next, we occluded the LAD permanently to induce a severe MI (without reperfusion), with the goal to study the role of endothelial β1 integrin in vascularization and function of the infarcted myocardium in the absence of RepI/R (►Fig. 6). Growth of blood vessels into the infarcted myocardium was analyzed by Microfil compound injection (►Supplementary Fig. S1E–H, available in the online version). Here, β1-B-AB was found to reduce vessel density in the infarcted area and to numerically reduce heart function (►Fig. 6A–G). Tamoxifen-induced EC-specific depletion of β1 integrin prevented the growth of blood vessels into the infarcted area (►Fig. 6H–K), and worsened heart function (►Fig. 6L–N). Notably, 3 of 8 mice (37.5%) without endothelial β1 integrin died in the first 4 days after the MI induction, while 2 of 10 control animals (20%) died only after 7 days (►Fig. 6S). Histology of the hearts revealed myocardial rupture due to thinning of the LV myocardium as the likely reason for cardiac death (►Fig. 6O–R, exemplified for Itgb1<sup>IECKO</sup> mice).
Identification of eNOS-Regulated β1 Integrin Expression in Cardiac Endothelium

Previously others and we provided evidence for eNOS as a possible downstream effector of β1 integrin in vitro. To analyze the relevance in vivo, we checked β1 integrin expression in eNOS KO mice. Here, the intensity of endothelial β1 integrin expression was found to be increased in eNOS KO mice when their myocardium was compared with that of control mice (Supplementary Fig. S4A–C, available in the online version), whereas the EC area was not substantially different (Supplementary Fig. S4D, available in the online version).

Requirement of β1 Integrin for Basal and Mechanically Induced Proliferation of Primary HCAECs

To investigate whether our in vivo results on endothelial β1 integrin in adult mouse hearts were (in principle) applicable to the human situation, ITGB1, the gene for human β1 integrin, was silenced in primary HCAECs. Using three different siRNAs, we obtained a more than 90% knockdown efficiency on the mRNA level, even when the ITGB1 gene expression was normalized to three different housekeeping genes (Supplementary Fig. S5A–C, available in the online version). The siRNAs also reduced the human β1 integrin protein to a substantial extent (Supplementary Fig. S5D, E).
for uncropped Western blots, see ►Supplementary Fig. S6, available in the online version). Notably, knockdown of ITGB1 reduced the number of proliferating HCAECs by more than 20% (►Supplementary Fig. S7A–E), even the total number of ITGB1-silenced HCAECs was numerically reduced compared with control-silenced cells within 2 days of cultivation (►Supplementary Fig. S7F, available in the online version). We also found that mechanical stretching (mimicking the effect of vasodilation on ECs 18) activated β1 integrin in HCAECs (►Supplementary Fig. S8A–E, available in the online version) and that the integrin was strictly required for mechanically induced proliferation of HCAECs (►Supplementary Fig. S8F–O, available in the online version).

Discussion
The β1 integrin is part of the largest group of integrins and needed for assembly of BMs, cardiomyocyte function and differentiation, mechanosensing, EC reorientation, cross-activation of VEGF receptors, flow-mediated vasodilation, release of angiocrine signals, and other processes. 10,14,23,41,44,45 Studies could
Fig. 5 Transient episodes of cardiac ischemia require endothelial Itgb1 expression to induce arteriole formation in the non-ischemic myocardium and reduce infarct size (IS) after myocardial infarction (MI). (A–H) Representative immunofluorescence images through the non-ischemic myocardium of (A–D) control and (E–H) Itgb1iECKO mice after RepSham or RepI/R treatments. Sections were stained for αSMA (red) and PECAM-1/CD31 (green). (I–L) Quantification of αSMA/PECAM-1-positive arterioles per mm² (I, J) non-ischemic and (K, L) ischemic myocardium of control or Itgb1iECKO mice. Reported values are means ± SEM. Control with RepSham: n = 6; control with RepI/R: n = 5; Itgb1iECKO with RepSham: n = 6; Itgb1iECKO with RepI/R: n = 7. (M, N, P, Q) Representative MRI of (M, N) control and (P, Q) Itgb1iECKO mouse hearts after RepI/R treatments and MI (60-minute ischemia) in short and long axis. IS or infarcted areas (as revealed by LGE) in the left myocardium are indicated by dashed lines. (O) Timeline of the experimental procedure. (R) Quantification of the IS 1 day after MI in control and Itgb1iECKO mice undergoing RepI/R treatment. (S) Quantification of the LV ejection fraction at 0, 1, 7, and 28 days after MI with reperfusion in control versus Itgb1iECKO mice. Reported values are means ± SEM. Control with RepI/R: n = 7; Itgb1iECKO with RepI/R: n = 6. Statistical significance was determined using unpaired two-tailed Student’s t-test. *p-Values: 1 d = 0.04. LGE, late gadolinium enhancement; LV, left ventricular; MRI, magnetic resonance imaging; SEM, standard error of mean.
show that a global heterozygous knockout of β1 integrin worsened the outcome after MI. Furthermore, it was shown that cardiomyocyte-specific deletion of β1 integrin causes fibrosis, cardiac failure, issues in mechanotransductive response, and bone mesenchymal stem cells overexpressing Itgb1 revealed a protective role after MI for cardiomyocytes. In addition, an EC-specific knockout of β1 integrin was used to study the role of this integrin in vascular leakage and endotoxemia in the mouse.
However, the role of endothelial β1 integrin in cardio-protective ischemic events, ischemia-induced cardiac vascular growth, and preservation of LV cardiac function after MI has, to our knowledge, not yet been investigated.

Here, we provide evidence for an important role of endothelial β1 integrin in vascular growth and cardioprotection (see ▶ Fig. 7). We found that short episodes of myocardial ischemia are sufficient to induce proliferation of cardiac ECs as well as formation of arterioles in the ischemic and non-ischemic myocardia in a β1 integrin-dependent manner (see ▶ Fig. 7). In contrast to EC proliferation, arteriole formation depends on endothelial β1 integrin in the non-ischemic, but not in the ischemic part of the adult mouse heart. This is particularly striking given that Repl/R treatment more strongly induces cardiac EC proliferation in the ischemic myocardium compared with the non-ischemic one. These regional differences in the adult heart might be due to additional responses taking place when the myocardium encounters hypoxia and subsequent inflammatory events.50,51 For example, whereas arteriole formation in the non-ischemic myocardium is likely to predominantly depend on mechanical stimulation (e.g., via increased shear stress/mechanical stretch) and thus β1 integrin as a mechanosensory protein on ECs, hypoxia-induced responses also take place in the ischemic myocardium. The latter responses might trigger arteriole formation independent of local EC proliferation and β1 integrin on ECs.

One possible scenario might be the recruitment of smooth muscle cells (SMCs) by migration, causing an arterialization of capillaries,52 which could be independent of EC proliferation. Further, it is noteworthy that Red-Horse and colleagues have recently shown that collaterals are also formed via “artery reassembly” in the neonatal heart,53 and that Adams and colleagues have shown that ECs from sprouting capillaries (that develop in hypoxic areas) can relocate to participate in

![Fig. 7 Visual Summary: Endothelial β1 integrin is required for myocardial ischemia-induced vascular growth, growth of blood vessels into the infarcted myocardium and preservation of cardiac function. (A) Adult mouse heart, with a coronary vasculature similar to the human situation, received (B) transient LAD occlusions, mimicking temporary ischemia observed in patients with CAD. (C) These transient occlusions trigger β1 integrin-dependent vascular growth and cardioprotection. (D) Permanent LAD occlusion, mimicking the situation in patients with MI, not receiving proper reperfusion. (E) Endothelial β1 integrin is required for growth of blood vessels into the infarcted myocardium and preservation of cardiac function.](image)
the formation of arteries, thus representing possible scenarios by which arterioles might form in the ischemic myocardium without increased cardiac EC proliferation.

The here-presented data show that pharmacological inhibition of β1 integrin abolished Repl/R-induced cardioprotection, shown in MRI measurements and TTC staining. Since a simultaneous decrease in cardiac function is observed in the RepSham and the Repl/R group, β1 integrin seems to have a general role in cardioprotection that extends beyond its role in Repl/R-induced cardioprotection.

To validate this point, the role of β1 integrin in a chronic ischemia model without Repl/R. In this model, we could generate a stronger ischemia, which induced growth of blood vessels into the infarcted myocardium, comparable to a previous study. Notably, our experimental results revealed that endothelial β1 integrin was required for proper vascular growth and maintenance of cardiac function independent of Repl/R. Further, we showed was required for proper vascular growth and maintenance of preconditioning, eNOS cyto

What is known about this topic?

- Short episodes of myocardial ischemia can protect the heart from myocardial infarction (MI).
- To date, β1 integrin has been targeted among others in cardiomyocytes, myofibroblasts, and pericytes, but not in endothelial cells (ECs) to uncover its endothelial role in myocardial ischemia.
- A role of endothelial β1 integrin has been identified in vascular leakage and endotoxemia of the mouse heart.

What does this paper add?

- Endothelial β1 integrin is shown to be required for cardiac EC proliferation during short episodes of left ventricular (LV) ischemia in mice in vivo and for proliferation of human coronary artery ECs in vitro.
- Endothelial β1 integrin is required for arteriolar formation induced by short episodes of LV ischemia in the non-ischemic myocardium.
- Endothelial β1 integrin has a general role in vascular growth into the infarct region and preservation of cardiac function after chronic myocardial infarction.

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Conflict of Interest

None declared.

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