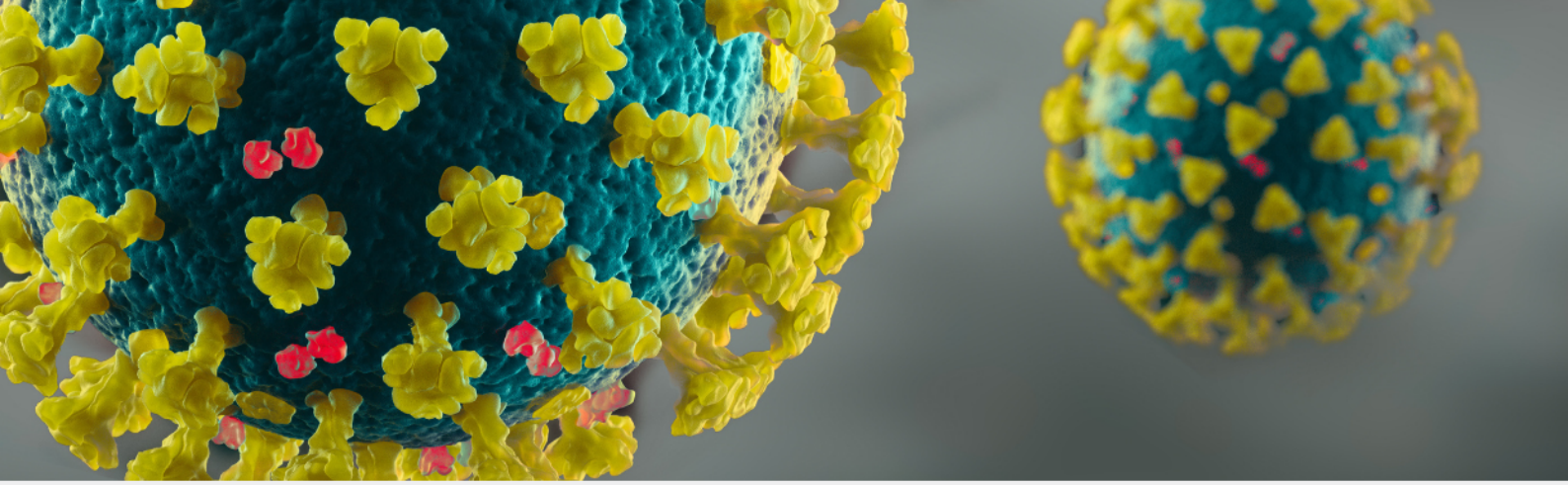

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RESEARCH ARTICLE

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Normoxic induction of HIF-1α by adenosine-A_{2B}R signaling in epicardial stromal cells formed after myocardial infarction

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Abstract

Myocardial infarction (MI) activates the epicardium to form epicardial stromal cells (EpiSC) that reside in the epicardial hypoxic microenvironment. Paracrine factors secreted by EpiSC were shown to modulate the injury response of the post-MI heart and improve cardiac function. We have previously reported that the expression of the angiogenic cytokines vascular endothelial growth factor A (VEGFA) and IL-6 is strongly upregulated in EpiSC by adenosine acting via the A_{2B} receptor (A_{2B}R). Since tissue hypoxia is well known to be a potent stimulus for the generation of extracellular adenosine, the present study explored the crosstalk of A_{2B}R activation and hypoxia-hypoxia-inducible factor 1 alpha (HIF-1α) signaling in cultured EpiSC, isolated from rat hearts 5 days after MI. We found substantial nuclear accumulation of HIF-1α after A_{2B}R activation even in the absence of hypoxia. This normoxic HIF-1α induction was PKC-dependent and involved upregulation of HIF-1α mRNA expression. While the influence of hypoxia on adenosine generation and A_{2B}R signaling was only minor, hypoxia and A_{2B}R activation cumulatively increased VEGFA expression. Normoxic A_{2B}R activation triggered an HIF-1α-associated cell-protective metabolic switch and reduced oxygen consumption. HIF-1α targets and negative regulators PHD2 and PHD3 were only weakly induced by A_{2B}R signaling, which may result in a sustained HIF-1α activity. The A_{2B}R-mediated normoxic HIF-1α induction was also observed in cardiac fibroblasts from healthy mouse hearts, suggesting that this mechanism is also functional in other A_{2B}R-expressing cell types. Altogether, we identified A_{2B}R-mediated HIF-1α induction as novel aspect in the HIF-1α-adenosine crosstalk, which modulates EpiSC activity and can amplify HIF-1α-mediated cardioprotection.

KEYWORDS

A_{2B} receptor, cardioprotection, HIF-1α, hypoxia, myocardial infarction

Abbreviations: A_{2B}R, adenosine A_{2B} receptor; CAIX, carbonic anhydrase IX; COL3A1, collagen, type III, alpha1; EPDC, epicardium-derived cells; EpiSC, epicardial stromal cells; HIF-1α, hypoxia-inducible factor 1 alpha; HRE, hypoxia response element; LDHA, lactate dehydrogenase A; MI, myocardial infarction; PDK1, pyruvate dehydrogenase kinase 1; PFKL, phosphofructokinase, liver type; PHD, prolyl hydroxylase; ROS, reactive oxygen species; VEGFA, vascular endothelial growth factor A.

1 | INTRODUCTION

The epicardium plays a pivotal role in heart development by providing instructive signals[1] and by giving rise to mesenchymal progenitor cells (epicardium-derived cells, EPDC), which differentiate into various cardiac lineages, such as smooth muscle cells, endothelial cells, pericytes, and cardiac fibroblasts.[2] In the healthy adult heart, the epicardium resides in a quiescent state and has been proposed as hypoxic progenitor cell niche.[3] Myocardial infarction (MI) activates epicardial cells to re-express fetal epicardial genes such as *Wt1* and again form mesenchymal cells, resulting in a thickening of the epicardial layer to about 100 μm.[4] While the regenerative capacity of the adult epicardial stromal cells (EpiSC) is not sufficient to restore myocardial tissue in mammals,[5] murine EpiSC have been shown to promote angiogenesis and favorably modulate post-MI wound healing by secreting paracrine factors such as vascular endothelial growth factor A (VEGFA) and IL-6.[4]

We have recently shown that the secretion of IL-6 and VEGFA by EpiSC is regulated by adenosine signaling via the A_{2B} receptor ($A_{2B}R$), which is highly expressed in EpiSC.[6] $A_{2B}R$ is one of four adenosine receptor subtypes (A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R) which belong to the G-protein-coupled receptor family. In EpiSC, only the $A_{2A}R$ is expressed at a comparable level as the $A_{2B}R$, however, the effects of selective $A_{2A}R$ activation on the cytokine response were negligible.[6] $A_{2B}R$ is coupled to Gs and Gq proteins[7] and, in contrast to the other adenosine receptors, requires micromolar adenosine concentrations to become activated.[8] Under conditions of inflammatory hypoxia, the transcription of $A_{2B}R$ is upregulated and extracellular adenosine is elevated to levels sufficient for $A_{2B}R$ activation.[9] $A_{2B}R$ signaling in the post-MI heart has been reported to exert both profibrotic and antifibrotic effects and to significantly impact infarct size.[10]

Extracellular adenosine is generated by the purinergic ectoenzyme cascade starting from ATP and NAD,[11] which is highly active at the EpiSC surface.[6] Nucleotides are released into the extracellular space from dying cells after tissue injury, but they can also be actively released via nonlytic mechanisms, for example, vesicular exocytosis, ion channels, and transporters.[12] An adenosine-$A_{2B}R$-triggered release of ATP and NAD has been reported for EpiSC.[6] Signaling by ATP via P2X and P2Y receptors is generally considered to trigger pro-inflammatory responses.[13] These include NLRP3 inflammasome activation by ATP $P2X_7$ receptor ($P2X_7R$) signaling, which has also been identified in EpiSC.[6]

The balance between ATP and adenosine signaling is towards adenosine under hypoxic conditions,[14] and this switch has been implicated in the crosstalk of hypoxia and inflammation.[15,16] While hypoxia upregulates the expression of CD39 (ATP → AMP), CD73 (AMP → adenosine), and $A_{2B}R$,[17] it also triggers ATP release[18,19] and reduces expression of equilibrative nucleoside transporters mediating adenosine uptake.[20] These changes together tend to increase extracellular adenosine levels, thereby favoring adenosine-$A_{2B}R$ signaling. In line with this, $A_{2B}R$ activation has been shown to be critically involved in cardioprotection by ischemic preconditioning in the setting of MI.[21] Pretreatment with the $A_{2B}R$-selective agonist BAY 60-6583 mimicked preconditioning effects and preconditioning-mediated cardioprotection was abolished in $A_{2B}R$-deficient mice.[22] Details of the crosstalk between hypoxia and $A_{2B}R$ signaling in EpiSC have not been explored.

CD73 and $A_{2B}R$ have been identified as direct targets of hypoxia signaling via hypoxia-inducible factor (HIF)-1.[23,24] The stability and activity of the HIF-1 alpha subunit (HIF-1α) are tightly controlled by a multicomponent regulatory network.[14,25] Under normoxic conditions, prolyl hydroxylases (PHD) continually target HIF-1α for proteasomal degradation. Under hypoxic conditions, HIF-1α escapes this oxygen-dependent PHD targeting, translocates into the nucleus, dimerizes with the uniformly expressed HIF-1 beta subunit (HIF-1β), and binds to hypoxia response elements (HRE) in target gene promotors to activate transcription. Adenosine signaling via all four adenosine receptor subtypes has been reported to amplify this hypoxia-mediated HIF-1α induction in human foam cells.[26]

Classical HIF-1α targets include glycolytic enzymes to mediate a switch of energy metabolism from oxidative phosphorylation towards glycolysis. In addition, HIF-1α induces pyruvate dehydrogenase kinase 1 (PDK1), which prevents pyruvate usage for the TCA cycle and thus actively represses mitochondrial function.[27] By these steps, HIF-1α reduces mitochondrial oxygen consumption and allows cellular adaption to low oxygen levels. Adenosine-$A_{2B}R$ signaling has been shown to promote the hypoxia-triggered HIF-1α-mediated cellular adaption during myocardial ischemia.[28]

HIF-1α protein accumulation can also be regulated by hypoxia-independent pathways,[29] including the transcriptional control of HIF-1α by, for example, reactive oxygen species (ROS), TLR4 activation, and inflammatory cytokines.[30] Gq-PKC signaling has been shown to induce HIF-1α protein under normoxic conditions: In addition to increasing HIF-1α gene expression, angiotensin II enhances HIF-1α translation via a ROS-PI3K-mediated mechanism,[31] while endothelin-1 inhibits prolyl hydroxylation by reducing PHD2 mRNA and protein levels.[32] Whether adenosine can likewise induce normoxic HIF-1α induction via $A_{2B}R$-coupled Gq-PKC signaling has not been investigated so far. However, a direct and functionally important impact of $A_{2B}R$ signaling on HIF-1α induction seems to be likely, since it has been reported that ischemia- and hypoxia-mediated HIF-1α stabilization was diminished in whole hearts and isolated cardiomyocytes, respectively, from $A_{2B}R$-deficient mice.[28]

In this study, we analyzed the effects of adenosine-$A_{2B}R$ signaling on HIF-1α in cultured EpiSC from infarcted rat

hearts under normoxic and hypoxic conditions and explored the crosstalk of $A_{2B}R$ and hypoxia signaling. We found that $A_{2B}R$ signaling under normoxic conditions (20% O_2) strongly induced HIF-1α, which was of the same magnitude as under hypoxia (1% O_2). $A_{2B}R$ activation under normoxia triggered the metabolic switch towards reduced oxygen consumption, while only weakly inducing the HIF-1α negative regulators PHD2 and PHD3. The $A_{2B}R$-mediated normoxic HIF-1α induction was also observed in cardiac fibroblasts from noninjured hearts, suggesting that this mechanism might be relevant in other $A_{2B}R$-expressing cell types as well.

2 | METHODS

2.1 | MI animal model

Animal experiments were performed in accordance with the institutional and national guidelines for animal care in conformity to directive 2010/63/EU and were approved by the Landesamt für Natur-, Umwelt- und Verbraucherschutz. Induction of MI in male rats (Wistar, 250-300 g body weight, 12-16 weeks of age) by 60 minutes ligation of the left coronary descending artery followed by reperfusion was performed as previously described.[33] Animals were bred at the Zentrale Einrichtung für Tierforschung und Tierschutzaufgaben of the Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany.

2.2 | Isolation, culture and in vitro treatment of rat EpiSC and mouse cardiac fibroblasts

EpiSC were isolated from the surface of rat hearts 5 days post-MI using collagenase CLS II (Biochrom, Berlin, Germany) as described previously.[6] EpiSC were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 30% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine for up to 3 weeks and passaged every 3–4 days or cryopreserved for later use.

Cardiac fibroblasts were isolated from uninjured hearts of wild-type mice and $A_{2B}R^{-/-}$ mice on C57BL/6N background[34] (20–25 g body weight, 8–12 weeks of age) by retrograde coronary perfusion with collagenase CLS II using a Langendorff setup as described previously.[35] Cardiac fibroblasts were cultivated in DMEM supplemented with 20% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine.

Selective activation of $A_{2B}R$ was performed with 25 µM of $A_{2B}R$ agonists BAY 60-6583 (Bio-Techne, Minneapolis, MN, USA) or VCP 746 for 24 hours. VCP 746 was synthesized according to Valant et al.[36] from 2-amino-3-benzoyl-4-(3-(trifluoromethyl)phenyl)thiophene[37] and N^6-(6-aminohexyl)-adenosine.[38] Identity and purity were assessed by spectroscopic analysis (NMR, ESI-MS). For incubation under hypoxic conditions (1% O_2, 5% CO_2), an Heracell 150i CO_2 incubator with oxygen control (Thermo Fisher Scientific, Waltham, MA, USA) was used. To block $A_{2B}R$-mediated Gs-PKA-cAMP signaling or Gq-PKC signaling, EpiSC were pretreated with 1 µM cAMP response element-binding protein (CREB) inhibitor 666-15 (Bio-Techne) or 50 µM PKC inhibitor Go 6983 (Bio-Techne), respectively, for 1 hours followed by incubation with 25 µM BAY 60-6583 in presence of the inhibitors for 24 hours.

2.3 | Immunofluorescence

For analysis of nuclear HIF-1α accumulation, cells were seeded on cover slips and treated as described above. After fixation with ice-cold methanol for 5 minutes at 4°C, cells were stained for HIF-1α using primary rabbit-anti-HIF-1α antibody (1:100, ab179483; Abcam, Cambridge, UK) and Alexa Fluor (AF)594-conjugated anti-rabbit IgG secondary antibody (Thermo Fisher Scientific) diluted in PBS/0.2% saponin/5% normal goat serum. Cells were mounted with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Fluorescence signals were recorded with a BX61 fluorescence microscope (Olympus, Hamburg, Germany). Images were processed with ImageJ/Fiji.[39] For quantification of HIF-1α-positive nuclei, five independent views at 10× magnification were analyzed for each sample. In the DAPI image, nuclei were identified using the ImageJ "threshold" function, and an overlay mask was generated using the "analyze particles" function. The mask was transferred onto the HIF-1α/AF594 image to measure only the nucleus-associated fluorescence intensity. The threshold to discriminate HIF-1α-negative/positive nuclei was set by comparing untreated control cells with hypoxic cells. On average, 940 EpiSC and 360 cardiac fibroblasts per sample were analyzed.

2.4 | Western blot

To determine total protein levels of HIF-1α, HIF-2α, and PHD2, EpiSC were lysed by incubation in Pierce RIPA buffer supplemented with 1x Halt protease inhibitor cocktail and 5 mM EDTA (Thermo Fisher Scientific) for 20–30 min at 4°C. Protein content of cell lysates was determined using Pierce BCA protein assay (Thermo Fisher Scientific) according to the manufacturer's protocol. Polyacrylamide gel electrophoresis with 25-30 µg protein per lane and protein transfer on PVDF membranes was performed using the Bolt

Bis-Tris Mini Gel system and the iBlot2 Western Blotting system (Thermo Fisher Scientific) according to the manufacturer's instructions. Membranes were incubated with rabbit-anti-HIF-1α (1:300–1:1000, ab179483, Abcam), rabbit-anti-HIF-2α (1:500, NB100-122, Novus Biologicals, Littleton, CO, USA), rabbit-anti-PHD2 (1:1,000, #4835, Cell Signaling Technology, Danvers, MA, USA), and mouse-anti-actin (1:2000, MA1-744, Thermo Fisher Scientific) primary antibodies and AF Plus 555-anti-rabbit IgG, AF Plus 800-anti-mouse IgG or horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibodies (Thermo Fisher Scientific). As HRP substrates, the Pierce ECL Plus Western Blotting Substrate or the SuperSignal West Femto Chemiluminescence Substrate (Thermo Fisher Scientific) was used. Signals were detected and quantified with an iBright FL1000 Imaging System (Thermo Fisher Scientific). HIF-1α, HIF-2α, and PHD2 protein levels were normalized to actin as loading control by calculating ratios of the signal intensities.

2.5 | Quantitative real-time PCR

Total RNA of EpiSC was isolated with the RNeasy Micro Kit and transcribed into cDNA with the QuantiTect Reverse Transcription Kit (Qiagen Hilden, Germany) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) using TaqMan assays and Takyon ROX Probe Mastermix dTTP blue (Eurogentec, Seraing, Belgium) was performed at an ABI StepOnePlus System (Thermo Fisher Scientific) according to the manufacturer's protocol. Primer/probe sequences and predesigned TaqMan Gene Expression Assays (Thermo Fisher Scientific) are listed in supporting information Tables S1 and S2. Gene expression was normalized to housekeeper *Rplp0*, which was selected because of its stable gene expression in mesenchymal progenitor/stem cells[40] and upon hypoxic conditions,[41] by the ΔC_t method.

2.6 | High-pressure liquid chromatography

For analysis of purinergic ectoenzyme activities, EpiSC were incubated with 20 μM ATP, NAD or AMP in HBSS at 37°C and metabolites were determined after 2, 4, 12, 30, and 60 minutes by high-pressure liquid chromatography (HPLC). To this end, cell supernatants were applied to an ACQUITY UPLC H-Class System equipped with a Cortecs C18 UPLC column (3.0 × 150 mm, particle size 1.6 μm) (Waters, Milford, MA, USA). Purine separation was performed as previously described,[42] using a liner gradient of buffer A (200 mM KH_2PO_4/200 mM KCl, pH 6) and buffer B (200 mM KH_2PO_4/200 mM KCl/7.5% acetonitrile, pH 6). Absorbance was measured at 254 nm.

2.7 | Respirometry

The basal cell respiration rate was assessed with an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) by measuring oxygen consumption of EpiSC in cell suspensions (1×10^6 cells/ml in culture medium), generated after 24 hours incubation without or with 25 μM $A_{2B}R$ agonist BAY 60-6583. The O_2 slope was quantified after a short equilibration period in the linear phase of oxygen consumption.

2.8 | Glycolytic rate assay

The glycolytic rate of EpiSC was analyzed using the Seahorse XF Glycolytic Rate Assay Kit (Agilent Technologies, Santa Clara, CA, USA), which quantifies glycolysis by measuring medium acidification in relation to oxygen consumption. The assay was performed according to the manufacturer's protocol. After incubating EpiSC with BAY 60-6583 (25 μM) for 24 hours, the medium was changed to Seahorse XF DMEM medium without BAY 60-6583 for measurements in the extracellular flux analyzer Seahorse XFe96 (Agilent) to exclude perturbations. Subsequently to the assay, nuclei were stained with Hoechst 33342 to normalize oxygen consumption and proton efflux data to cell count. Data analysis was performed via the Seahorse XF Glycolytic Rate Assay Report Generator (Agilent).

2.9 | Lactate assay

Lactate levels in cell supernatants of EpiSC were quantified by the bioluminescent Lactate-Glo Assay (Promega, Madison, WI, USA) after incubation with BAY 60-6583 (25 μM) for 24 hours according to manufacturer's instructions.

2.10 | Statistical analysis

Experiments were performed with biological replicates in terms of EpiSC preparations from different animals. Values are presented as means ± SD. Statistical analyses were performed with GraphPad Prism. To compare multiple samples, one-way ANOVA with Tukey's multiple comparisons test, two-way ANOVA with Tukey's multiple comparisons test (comparison of rows within each column) or two-way ANOVA with Sidak's multiple comparisons test (comparison of cell means within rows) were applied as stated in the figure legends. For comparison of two samples, ratio paired *t* test was used. The threshold for statistical significance was set at $P < .05$.

3 | RESULTS

3.1 | Induction of HIF-1α under normoxic conditions by $A_{2B}R$ activation

To explore the effects of adenosine-$A_{2B}R$ signaling on HIF-1α induction in EpiSC, cells were isolated from rat hearts 5 days post-MI as described previously.[33] EpiSC were stimulated with the $A_{2B}R$ agonist BAY 60-6583, and immunofluorescence analysis revealed that $A_{2B}R$ activation with BAY 60-6583 under normoxic conditions (20% O_2) for 24 hours substantially induced nuclear HIF-1α protein accumulation which reached similar values as under hypoxia (1% O_2) (Figure 1A,B). This normoxic HIF-1α induction was also observed after stimulation with another $A_{2B}R$ agonist, VCP 746,[43,44] confirming the specificity of the effect (Figure 1B). Western Blot analysis of EpiSC cell lysates showed that nuclear HIF-1α accumulation was accompanied by an increase in total HIF-1α protein levels (Figure 1C). HIF-2α protein levels in EpiSC were not significantly altered, neither in response to $A_{2B}R$ activation nor under hypoxic conditions (supporting information Figure S1A).

To test, whether HIF-1α induction under normoxic conditions extends to other cardiac stromal cells, we isolated cardiac fibroblasts from uninjured hearts of wild-type mice. As shown in Figure 1D, both BAY 60-6583 and VCP 746 induced nuclear HIF-1α protein accumulation to the same extent as hypoxia (Figure 1D). This stimulatory effect was $A_{2B}R$-specific, since it was greatly attenuated in cardiac fibroblasts isolated from $A_{2B}R$-deficient mice (Figure 1D). The hypoxia-mediated HIF-1α induction on the other hand was comparable to that in wild-type cardiac fibroblasts (Figure 1D).

Since $A_{2B}R$ is coupled to both Gs and Gq proteins,[7] we analyzed which of these signal transduction pathways are involved in $A_{2B}R$-mediated HIF-1α induction using the CREB inhibitor 666-15 to target Gs-PKA-cAMP signaling and the PKC inhibitor Go 6983 to target Gq-PKC signaling. As shown in Figure 1E, 666-15 did not influence $A_{2B}R$-mediated nuclear accumulation of HIF-1α, while Go 6983 significantly attenuated this effect. This suggests that $A_{2B}R$-mediated HIF-1α induction is mediated by Gq signaling.

3.2 | Effects of $A_{2B}R$ activation on expression of HIF subunits and regulatory network components

Next we analyzed, whether $A_{2B}R$ mediates HIF-1α protein accumulation by modulating gene expression of HIF subunits or components of the HIF regulatory protein network[14,25,45,46] by qRT-PCR. As summarized in Figure 2A,B, $A_{2B}R$ activation significantly enhanced HIF-1α mRNA expression in EpiSC both under normoxic and hypoxic conditions, and this might have contributed to the observed HIF-1α protein induction (Figure 2A). Gene expression of HIF-2α (*Epas1*) remained unaltered (Figure 2A). Hypoxia alone did not influence HIF-1α gene expression (Figure 2A) but strongly increased expression of PHD2 (*Egln1*) and PHD3 (*Egln3*) (Figure 2B), which are known HIF-1α targets and are part of a negative regulatory mechanism that limits HIF-1α accumulation in prolonged hypoxia.[47] In contrast, $A_{2B}R$ activation only weakly stimulated the expression of both of these enzymes (Figure 2B), suggesting that the HIF-1α-limiting negative feedback was less pronounced than under hypoxia.

Since a downregulation of PHD2 protein was reported to be involved in endothelin-1-mediated HIF-1α induction,[32] we also measured PHD2 protein levels in our system. As shown in Figure 2C, $A_{2B}R$ activation did not alter PHD2 protein levels, neither under normoxic conditions nor under hypoxia with profound PHD2 induction. Similar to the findings at the mRNA level, this points towards a notable difference in the induction of HIF-1α-limiting negative feedback between $A_{2B}R$ and hypoxia signaling.

3.3 | Crosstalk of adenosine-$A_{2B}R$- and hypoxia-HIF-1α-mediated signaling

Since adenosine is generated from extracellular ATP by an ectoenzyme cascade (Figure 3A), whose components CD39 and CD73, like $A_{2B}R$, have been reported to be upregulated in hypoxia,[23] we analyzed the effects of $A_{2B}R$ activation and hypoxia on the expression of central purinergic ectoenzymes together with pro-inflammatory ATP receptor $P2X_7R$ and $A_{2B}R$ in EpiSC. As shown in Figure 3B, both $A_{2B}R$ activation and hypoxia reduced expression of $P2X_7R$ (*P2rx7*) and the reduction by hypoxia seemed to be stronger but was not increased in combination with $A_{2B}R$ activation (Figure 3B). $A_{2B}R$ activation significantly reduced CD39 (*Entpd1*) and CD38 expression, while CD73 (*Nt5e*) and $A_{2B}R$ (*Adora2b*) were enhanced (Figure 3B). In contrast, hypoxia unexpectedly did not augment the expression of CD39, CD73, or $A_{2B}R$ in EpiSC neither did it alter expression of NAD-degrading CD38 or ATP/NAD-degrading CD203a (*Enpp1*) (Figure 3B). Consistent with this finding, the kinetics of extracellular ATP, NAD, and AMP degradation as measured by HPLC was not different in normoxia and hypoxia (Figure 3C). However, despite having no detectable effects on its own, hypoxia amplified $A_{2B}R$-mediated induction of CD73 and $A_{2B}R$ (Figure 3B). Together, these data suggest that in EpiSC the impact of hypoxia on extracellular purinergic metabolism and adenosine generation is rather limited.

To more directly explore the crosstalk of adenosine-$A_{2B}R$ and hypoxia-HIF signaling pathways, we analyzed the induction of selected HIF-1α and $A_{2B}R$ target genes in EpiSC. As was expected, hypoxia markedly increased HIF-1α targets

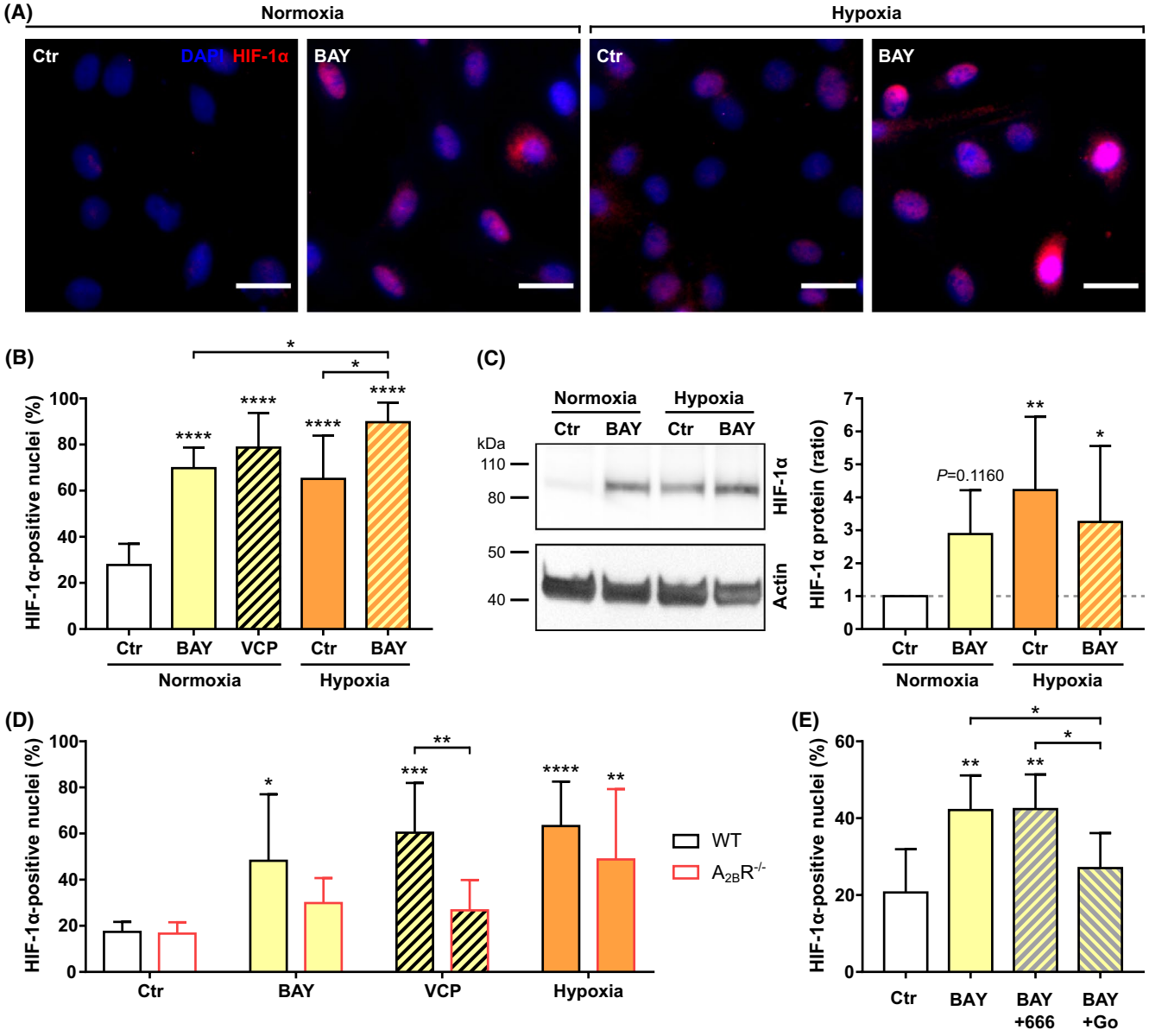

FIGURE 1 Induction of HIF-1α by $A_{2B}R$ activation. Epicardial stromal cells (EpiSC) isolated from rat hearts 5 days post-myocardial infarction (MI) were incubated in absence (Ctr) and presence of $A_{2B}R$ agonist BAY 60-6583 (BAY, 25 μM) under normoxic (20% O_2) or hypoxic (1% O_2) conditions for 24 hours and analyzed for HIF-1α protein accumulation by immunofluorescence and Western Blot. A, Representative images of immunofluorescence analysis of nuclear HIF-1α accumulation (red). Nuclei were stained with DAPI (blue). Scale bar 30 μm. B, Quantification of HIF-1α-positive nuclei from (A) using ImageJ. Additionally, EpiSC were incubated with the $A_{2B}R$ agonist VCP 746 (VCP, 25 μM). Means ± SD (n = 4-9 EpiSC preparations). One-Way ANOVA and Tukey's multiple comparisons test. C, Western blot analysis of HIF-1α in EpiSC lysates. Representative blot with actin as loading control (left panel) and quantification of HIF-1α protein levels normalized to actin (right panel). Means ± SD (n = 6 EpiSC preparations). One-Way ANOVA and Tukey's multiple comparisons test. D, Cardiac fibroblasts were isolated from healthy hearts of wild-type (WT) or $A_{2B}R$-deficient ($A_{2B}R^{-/-}$) mice. Nuclear HIF-1α accumulation was analyzed by immunofluorescence after incubation with $A_{2B}R$ agonists BAY and VCP under normoxic (20% O_2) or hypoxic (1% O_2) conditions for 24 hours. Means ± SD (n = 7-8 cardiac fibroblast preparations). Two-Way ANOVA and Tukey's multiple comparisons test (treatment vs Ctr) or Sidak's multiple comparisons test ($A_{2B}R^{-/-}$ vs WT). E, Rat EpiSC were stimulated with $A_{2B}R$ agonist BAY in absence and presence of CREB inhibitor 666-15 (666) and PKC inhibitor Go 6983 (Go) for 24 hours. Means ± SD (n = 7 EpiSC preparations). One-Way ANOVA and Tukey's multiple comparisons test. *P < .05, **P < .01, ***P < .001, ****P < .0001

carbonic anhydrase IX (CAIX, *Car9*) and VEGFA (Figure 3D). $A_{2B}R$ activation also stimulated VEGFA expression and $A_{2B}R$ activation together with hypoxia enhanced VEGFA even further (Figure 3D). In contrast, $A_{2B}R$ activation significantly reduced CAIX expression (Figure 3D), implying that $A_{2B}R$ signaling triggers only a specific subset of HIF-1α targets, differing from that induced by hypoxia. Expression of HIF-2α target genes *Abl2* and *Tgfa* was not affected by $A_{2B}R$

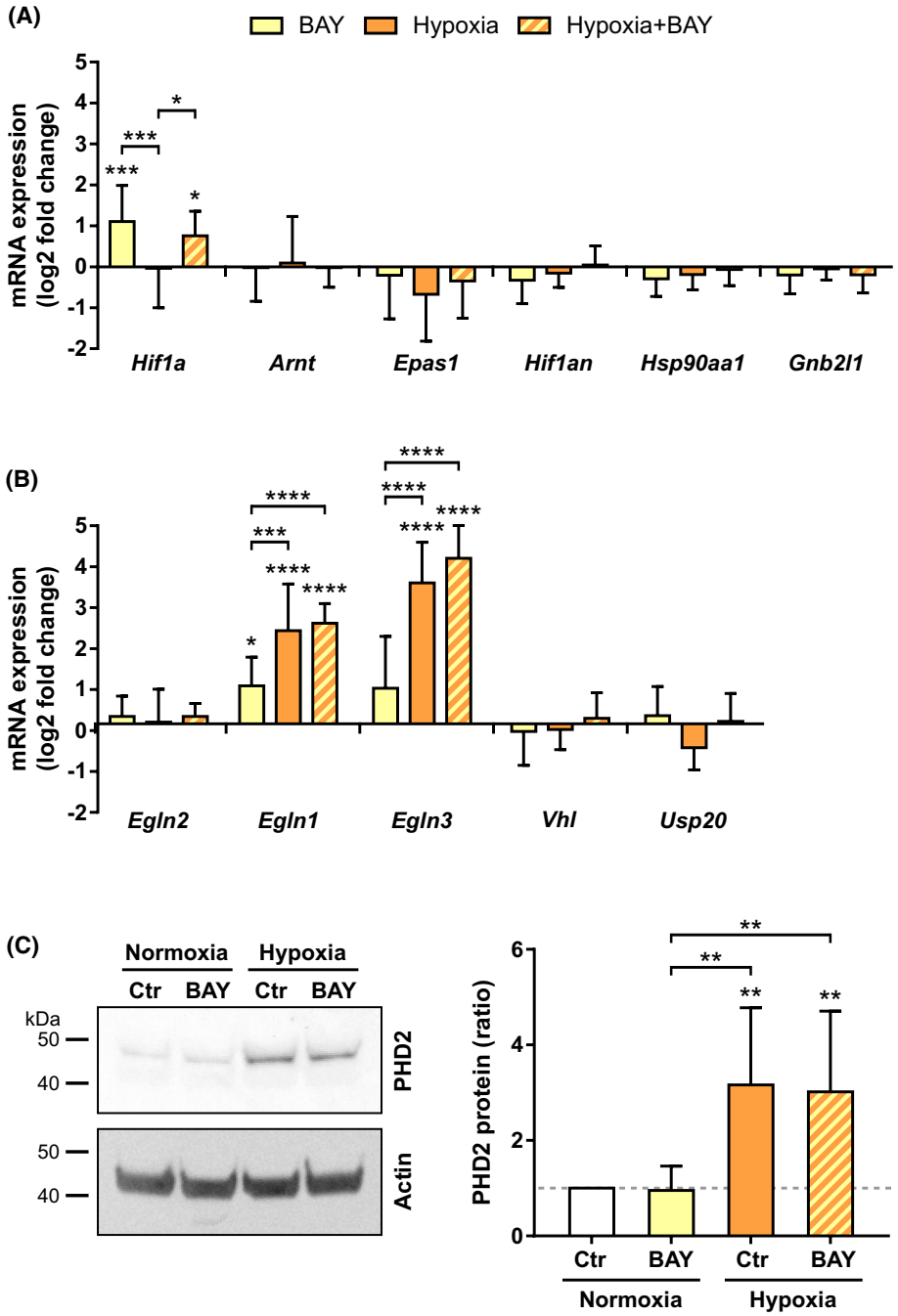

FIGURE 2 Expression of HIF subunits and regulatory network components after $A_{2B}R$ activation. EpiSC isolated from rat hearts 5 days post-myocardial infarction (MI) were analyzed in absence (Ctr) and presence of $A_{2B}R$ agonist BAY 60-6583 (BAY) under normoxic (20% O_2) or hypoxic (1% O_2) conditions after 24 hours. A, Expression analysis of HIF transcription factor subunit genes and genes of factors directly binding to HIF-1α by qRT-PCR: *Hif1a* (HIF-1α), *Arnt* (HIF-1β), *Epas1* (HIF-2α), *Hif1an* (FIH), *Hsp90aa1* (HSP90A), and *Gnb2l1* (RACK1). Data are presented as log2 fold changes in relation to Ctr. Means ± SD (n = 6-11 EpiSC preparations). B, Expression analysis of genes involved in HIF-1α ubiquitylation by qRT-PCR: *Egln2* (PHD1), *Egln1* (PHD2), *Egln3* (PHD3), *Vhl* (VHL), and *Usp20* (VDU2). Means ± SD (n = 6-11 EpiSC preparations). C, Western Blot analysis of PHD2 protein levels. Representative blot (left panel) with actin as loading control and quantification of protein levels normalized to actin (right panel). Means ± SD (n = 6 EpiSC preparations). One-Way ANOVA and Tukey's multiple comparisons test. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$

activation (supporting information Figure S1B). As to $A_{2B}R$ signaling targets, hypoxia did not alter the expression of IL-6 and alpha 1 chain of type III collagen (COL3A1) (Figure 3E). $A_{2B}R$ activation showed the expected upregulation of IL-6[6] and downregulation of COL3A1 expression in EpiSC, and there was no difference between normoxic and hypoxic conditions (Figure 3E). Thus, hypoxia does not seem to generally amplify adenosine-$A_{2B}R$ signaling in EpiSC.

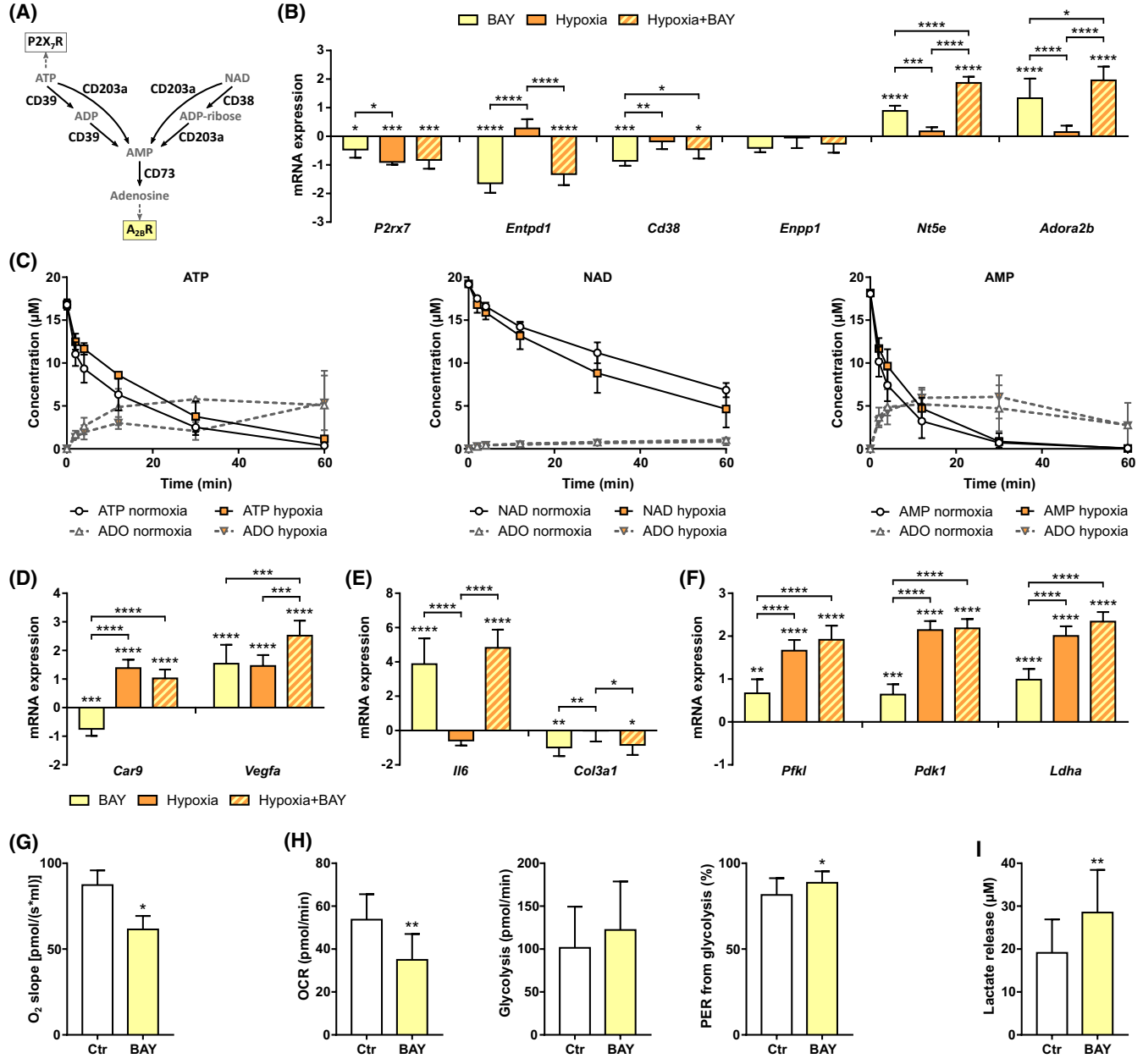

FIGURE 3 Crosstalk of adenosine-A_{2B}R- and hypoxia-HIF-1α-mediated signaling. EpiSC isolated from rat hearts 5 days post-myocardial infarction (MI) were incubated in absence (Ctr) and presence of A_{2B}R agonist BAY 60-6583 (BAY) under normoxic (20% O_2) or hypoxic (1% O_2) conditions for 24 hours. Gene expression was analyzed by qRT-PCR. A, Scheme of the ectoenzyme cascade mediating generation of adenosine from extracellular ATP and NAD. B, Gene expression of ATP receptor *P2rx7* ($P2X_7R$), ATP- or NAD-degrading enzymes *Entpd1* (CD39), *Cd38* (CD38), and *Enpp1* (CD203a), adenosine-generating enzyme *Nt5e* (CD73), and adenosine receptor *Adora2b* ($A_{2B}R$). Data are presented as log2 fold changes in relation to Ctr. Means ± SD (n = 4–6 EpiSC preparations). One-Way ANOVA and Tukey's multiple comparisons test. C, Degradation of extracellular ATP, NAD, and AMP by EpiSC after 24 hours incubation under normoxic and hypoxic conditions as measured by high-pressure liquid chromatography (HPLC). Two-Way ANOVA and Sidak's multiple comparisons test, $P \geq .05$. Means ± SD (n = 3 EpiSC preparations). D–F, Expression of (D) HIF-1α target genes *Car9* (CAIX) and *Vegfa* (VEGFA), E, A_{2B}R target genes *Il6* (IL-6) and *Col3a1* (collagen, type III, alpha1), and F, glycolytic enzyme genes *Pfkl* (phosphofructokinase, liver type), *Pdk1* (pyruvate dehydrogenase kinase 1) and *Ldha* (lactate dehydrogenase A). Means ± SD (n = 4-6 EpiSC preparations). One-Way ANOVA and Tukey's multiple comparisons test. G, Oxygen consumption of EpiSC after 24 hours incubation with BAY under normoxic conditions determined by respirometry. Means ± SD (n = 3 EpiSC preparations). Ratio paired t test. H, Extracellular flux analysis for glycolytic rate quantification in EpiSC after 24 hours incubation with BAY under normoxic conditions. OCR, oxygen consumption rate; PER, proton efflux rate. Means ± SD (n = 4 EpiSC preparations). Ratio paired t test. I, Lactate concentration in the supernatant of EpiSC after 24 hours incubation with BAY under normoxic conditions. Means ± SD (n = 6 EpiSC preparations). Ratio paired t test. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$

A major function of HIF-1α signaling is cellular adaption to low oxygen levels by reducing mitochondrial oxygen consumption.[27] $A_{2B}R$ signaling has been proposed to promote this metabolic switch for myocardial adaptation during ischemia by upregulating the expression of glycolytic enzymes.[28] Gene expression analysis of EpiSC showed that $A_{2B}R$ activation under normoxic conditions significantly increased the expression of phosphofructokinase, liver type (PFKL), PDK1, and lactate dehydrogenase A (LDHA) (Figure 3F). To directly explore the effects of $A_{2B}R$ signaling on the energy metabolism of EpiSC, we first performed a respirometric analysis after $A_{2B}R$ activation under normoxic conditions. As shown in Figure 3G, $A_{2B}R$ activation markedly reduced oxygen consumption, which is consistent with an $A_{2B}R$-HIF-1α-mediated metabolic reprogramming. The Agilent Seahorse XF glycolytic rate assay (based on the analysis of the extracellular flux of oxygen and protons which quantifies glycolysis by means of CO_2-independent medium acidification) confirmed the $A_{2B}R$-mediated reduction of oxygen consumption (Figure 3H). There was a trend for an $A_{2B}R$-mediated increase in the glycolytic rate, and the proportion of released protons that derive from glycolysis was significantly enhanced after $A_{2B}R$ activation (Figure 3H), altogether indicating an augmented glycolytic activity. Consistent with this finding, $A_{2B}R$ activation significantly enhanced the release of lactate by EpiSC (Figure 3I), again indicating a normoxic $A_{2B}R$-mediated metabolic switch towards oxygen-saving glycolysis.

4 | DISCUSSION

Modulation of extracellular adenosine generation and signaling by hypoxia is well established.[14] In the heart, adenosine has been shown to be critically involved in the attenuation of tissue inflammation as well as cardioprotection by ischemic preconditioning.[14,16,21] In this study we demonstrate, that even under normoxic conditions adenosine increases HIF-1α in EpiSC, which is mediated by $A_{2B}R$ signaling. Activation of $A_{2B}R$ with selective agonists resulted in increased HIF-1α mRNA expression and profound HIF-1α protein accumulation in the nucleus. This effect was PKC-dependent, similar to the Gq-mediated angiotensin II- and endothelin-1-triggered normoxic HIF-1α induction.[31,32] However, we did not observe $A_{2B}R$-mediated reduction of PHD2 at the gene and protein level, implying that the mechanism of HIF-1α-accumulation is different from endothelin-1. Since we found no changes in the expression of components of the canonical HIF-1α regulatory pathway, the $A_{2B}R$-mediated effect might involve, in addition to the observed upregulation of HIF-1α gene expression, one or more alternative post-transcriptional events directly acting on HIF-1α protein, such as enhanced translation,[31] phosphorylation/dephosphorylation,[48] enhanced binding to the chaperone HSP90,[45] or deubiquitylation.[46] On transcriptional level, RACK1 (*Gnb2l1*), which competes with HSP90 for HIF-α binding, and the deubiquitylating enzyme VDU2 (*Usp20*) were not regulated by $A_{2B}R$ activation (Figure 2A,B). Interestingly and in contrast to HIF-1α, HIF-2α expression was not changed by $A_{2B}R$ activation on mRNA or protein level. Hypoxia also did not induce HIF-2α (Figure 2A and supporting information Figure S1A) or its target genes *Abl2* and *Tgfa* (supporting information Figure S1B), suggesting that in our system an HIF-2α response is not relevant.

The impact of hypoxia-HIF-1α signaling on extracellular nucleotide degradation and $A_{2B}R$ signaling was only small. As reported previously by us, EpiSC strongly express $A_{2B}R$ and adenosine-generating CD73 under basal conditions and this was accompanied by a high activity of several purinergic ectoenzymes.[6] In the present study, hypoxia did not change gene expression of ATP- and NAD-degrading ectoenzymes CD39, CD38, and CD203a, nor of CD73 that was described to be a direct target of HIF-1α.[23] In line with these findings, degradation of extracellular ATP, NAD, and AMP at the EpiSC cell surface remained unaltered. Hypoxia did also not significantly upregulate gene expression of $A_{2B}R$ in EpiSC, but other classical HIF-1α targets (CAIX, VEGFA, PHD2, PHD3, PFKL, PDK1, and LDHA) were regularly induced. Only in combination with $A_{2B}R$ activation, there was a small hypoxia-mediated increase in CD73 and $A_{2B}R$ gene expression without any functional impact on $A_{2B}R$-mediated modulation of IL-6 and COL3A1. Collectively, these data provided evidence that the direct influence of hypoxia on adenosine generation and $A_{2B}R$ signaling in EpiSC is only minor. However, adenosine-$A_{2B}R$ signaling by itself increased CD73 and $A_{2B}R$ gene expression in EpiSC (Figure 3B) and triggers the release of ATP and NAD as substrates for adenosine generation in EpiSC,[6] likely forming an adenosine-sustaining loop as we have recently proposed.[6] Therefore, an initial burst of adenosine might be sufficient to trigger sustained adenosine generation and $A_{2B}R$ signaling in EpiSC. In the infarcted heart, an acute, hypoxia-triggered burst of adenosine in the epicardial vicinity could be initiated by various cell types within the injured tissue, including T cells whose CD73-mediated adenosine-generating ability has been shown to be of crucial importance in the post-MI wound healing.[49] Thus, despite a limited direct effect of hypoxia on EpiSC in vitro, tissue hypoxia might still be an important trigger of adenosine-$A_{2B}R$ signaling in EpiSC in the infarcted heart.

The adenosine-$A_{2B}R$-HIF-1α axis and hypoxia-HIF-1α signaling substantially differed in the induction of several classical HIF-1α targets in EpiSC. $A_{2B}R$ activation resulted in VEGFA induction comparable to that by hypoxia, associated with some induction of glycolytic enzymes but only weak induction of PHD2 and PHD3 and even a repression of CAIX. Such trigger-specific effects have also been

described for inflammation-related HIF-1α target gene expression after HIF-1α induction by the Gs-coupled adenosine receptor $A_{2A}R$ in macrophages,[50] suggesting that normoxic adenosine-HIF-1α can upregulate a cell-specific subset of HIF-1α targets that differs from hypoxia-HIF-1α, both via Gs- and Gq-coupled pathways. The limited induction of PHD as negative regulators of HIF-1α by adenosine-$A_{2B}R$ signaling may lead to a more sustained HIF-1α activity in comparison to the transient HIF-1α response to acute hypoxia, allowing prolonged cellular adaption and protection.

$A_{2B}R$ signaling reduced oxygen consumption and increased glycolytic activity of EpiSC, suggesting that $A_{2B}R$ activation not only promotes the hypoxia-HIF-1α-mediated switch in energy metabolism as was shown by Eckle et al.[28] but can also trigger this protective metabolic reprogramming in a hypoxia-independent fashion. In line with this interpretation, $A_{2B}R$ activation before MI can mimic ischemic preconditioning-mediated cardioprotection.[22] In this process, adenosine-$A_{2B}R$ signaling has been generally considered as downstream effector of hypoxia-HIF-1α.[21] It is tempting to speculate that the newly identified reciprocal mechanism of adenosine-$A_{2B}R$-mediated induction of HIF-1α may contribute to preconditioning as amplifying loop in such a way that the well-established HIF-1α tissue-protective activity is augmented by the ischemia-triggered increase of extracellular adenosine. Of note, we previously found that $A_{2B}R$ activation under normoxic conditions induces the release of ATP as substrate for adenosine generation,[6] and this might contribute to this amplification loop.

Already within the healthy heart, the epicardial layer constitutes a hypoxic microenvironment.[3] It is therefore reasonable to assume that EpiSC in the post-MI heart are subjected to significant hypoxia as well as increased adenosine concentrations. Here we found that both hypoxia and $A_{2B}R$ activation increased the expression of the angiogenic HIF-1α target VEGFA, individually or together cumulatively. VEGFA has been identified as one of the paracrine factors secreted by EpiSC, which reduce infarct size and improve cardiac function.[4] Interestingly, HIF stabilization by depletion of PHD2 and PHD3 in endothelial cells has been recently reported to reduce post-MI fibrotic scar formation and preserve cardiac function via paracrine factors.[51] Similar to these findings, an $A_{2B}R$-mediated sustained induction of HIF-1α by endogenous post-MI adenosine in EpiSC may be involved in their known beneficial paracrine effects on the infarcted myocardium[4] and thus may constitute a novel therapeutic option to improve cardiac healing.

Interestingly, the $A_{2B}R$-mediated normoxic HIF-1α induction was also observed in mouse cardiac fibroblasts from healthy hearts, indicating that the identified mechanism is not restricted to post-MI EpiSC but may apply to other $A_{2B}R$-expressing cell types. $A_{2B}R$ expression is widely distributed among numerous tissues[52] and can be induced by diverse cues in addition to hypoxia, such as proliferation, inflammation, and endogenous adenosine.[52,53] Therefore, it is likely that under conditions of increased extracellular adenosine, $A_{2B}R$-mediated HIF-1α induction may be operative in various other cell types as well, to promote sustained HIF-1α-mediated tissue-protective effects.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

J. Hesse designed and conducted experiments, analyzed data, and wrote the manuscript. W. Groterath conducted experiments and analyzed data. C. Alter and C. Owenier provided mouse cardiac fibroblasts. J. Steinhausen executed glycolytic rate analyses. A. Marzoq and Z. Ding performed surgery for MI induction. B. Steckel performed HPLC measurements. C. Czekelius synthesized VCP 746. J. Schrader supervised the experiments and edited the manuscript. All authors revised the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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