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Modulation of Cellular Stress Response via the Erythropoietin/CD131 Heteroreceptor Complex in Mouse Mesenchymal-Derived Cells

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Abstract

Tissue protective properties of erythropoietin (EPO) have led to the discovery of an alternative EPO-signaling via an EPO-R/CD131 receptor complex which can now be specifically targeted through pharmaceutically designed short sequence peptides such as ARA290. However, little is still known about specific functions of alternative EPO-signaling in defined cell populations. In this study we investigated effects of signaling through EPO-R/CD131 complex on cellular stress responses and pro-inflammatory activation in different mesenchymal-derived phenotypes. We show that anti-apoptotic, anti-inflammatory effects of ARA290 and EPO coincide with the externalization of CD131 receptor component as an immediate response to cellular stress. In addition, alternative EPO-signaling strongly modulated transcriptional, translational or metabolic responses after stressor removal. Specifically, we saw that ARA290 was able to overcome a TNF α -mediated inhibition of transcription factor activation related to cell stress responses, most notably of serum response factor (SRF), heat shock transcription factor protein 1 (HSF1) and activator protein 1 (AP1). We conclude that alternative EPO-signaling acts as a modulator of pro-inflammatory signaling pathways and likely plays a role in restoring tissue homeostasis.

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Keywords

ARA290; oxidative stress; heat shock; apoptosis; TNF α ; homeostasis; mesenchymal stem cells

Introduction

At a local, non-systemic level, various tissues have demonstrated the ability to secrete erythropoietin (EPO) and express the EPO receptor (EPO-R) under conditions of stress, likely as part of a para-/autocrine local tissue-protective mechanism [1]. When systemically administered at supra-physiological levels, EPO is tissue-protective in various injury models [2]. Immuno-precipitation experiments revealed a co-localization of EPO-R with the common-beta-chain-receptor-subunit-beta 2 (CSF2RB) [3], also referred to as CD131. A growing body of evidence suggests that EPO-R/CD131 complex mediated signaling is responsible for observed tissue-protective effects of various EPO derivatives [4]. Notably, in a CD131 knock-out mouse EPO is devoid of tissue protective properties but demonstrates normal hematopoiesis [5].

In order to study the specific effects of signaling via the EPO-R/CD131 complex on cellular stress responses, we used the pharmaceutically designed 11 amino-acid *helix beta surface peptide*, referred to as ARA290 [4]. This peptide selectively binds, with high affinity, to the EPO-R/CD131 receptor heterodimer complex but not to classic EPO-R/EPO-R homodimer. Thus, clinically relevant side effects related to high dose EPO administration can be avoided [6]. *In vivo*, we and others have already shown the effectiveness of ARA290 in the nanomolar range to reduce tissue damage [7]. In the present study, we investigated CD131 expression profiles as well as the effects of EPO-R/CD131-mediated signaling on cellular stress responses *in vitro*. Our findings show that EPO-R/CD131-mediated signaling dampens pro-inflammatory and pro-apoptotic pathways. This was observed in different cell lines of mesenchymal origin, suggesting that this mechanism plays an important role in restoring homeostasis following tissue injury.

Materials and Methods

Animals

Primary cell lines were isolated from male C57BL/6 adult wild type mice (>6 weeks, > 20 g) purchased from The Jackson Laboratory (ME; www.jax.org). All animal procedures were performed in accordance with National Research Council guidelines and were approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital, Boston, MA.

Cell isolations and culture

Primary mouse fibroblastic cell lines were isolated as previously described [8]. Phenotypic uniform cultures between passages 3–10 from skin/adipose (mSFB's) or aortic (mAFB's) tissue of male C57BL/6 wild type mice as well as the mouse macrophage cell line J774A.1 (mJ774's) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco™, USA) supplemented with 10% heat inactivated fetal bovine serum (hiFBS), L-glutamine

[200 mM] and penicillin [10,000 U/l]/streptomycin [10 mg/ml] (P/S). Human microvascular endothelial cells (hMEC's) (Lot#:7F3109, Lonza™, CH) were maintained using MCDB 131 medium (Caisson Laboratories™, USA) supplemented with 10% hiFBS, P/S, hydrocortisone [1 µg/ml], dibutyl cAMP [80 µM], heparin [2 U/ml], endothelial growth cell supplement [25 µg/ml] (EGCS, Biomedical Technologies™, USA), ascorbic acid [0.2 mM]. Mouse bone marrow-derived mesenchymal stem cells (mBMSC's, GIBCO® Mouse C57BL/6 mesenchymal stem cells, Invitrogen™, USA) were maintained in MEM- α basal media supplemented with fibroblast growth factor 2 (both Invitrogen™, USA) and 10% hiFBS. Unless mentioned otherwise, cultures were switched to DMEM supplemented with 2% hiFBS, L-glutamine and 1 µM sodium pyruvate 24 hours prior to and following heat shock exposure. For heat shock experiments, cell lines were grown to confluence on fibronectin coated 10 mm Ø glass cover slips (Fisherbrand™, USA). Heat shock was administered via immersion in a temperature controlled 0.9% saline bath followed by transfer to a 12 well plate format at 37°C/5% CO₂ during recovery. If not specified otherwise components were purchased from Sigma Chemical USA.

Flow cytometry

Cells were fluorescently labeled in suspension with 1:250 dilutions of anti-mouse phycoerythrin-CD131 or isotype-control antibody (both BD Biosciences™, USA) in phosphate buffered saline (PBS) supplemented with 0.5 % hiFBS at 4°C for 40 min followed by fixation in 1% PBS-buffered paraformaldehyde. Intracellular labeling for phosphotyrosine/-serine was performed with the 4G10® Platinum/4A4 Phosphopack (Millipore™, USA) and anti-mouse Alexa Fluor® 488 (Invitrogen™, USA) secondary antibody using a BD Cytofix/Cytoperm® kit (BD Biosciences™, USA) buffer. Sorting of labeled isolates were performed using either a BD FACSCalibur® with CellQuestPro® or a BD SORP FACS Aria IIs® platform with BD Diva® operating software (all BD Biosciences™, USA) and data was further analyzed using FlowJo® software ([//www.flowjo.com](http://www.flowjo.com)).

Dye-based viability, cytotoxicity, metabolic assays

We used a fluorescence-based assay (LIVE/DEAD®, Invitrogen™, USA) in order to distinguish between viable (calcein positive) and non-viable (ethidium positive) cells following treatments. Images were acquired using an inverted microscope equipped with an incubation chamber and fluorescence imaging capabilities (Axiovert, Zeiss™, GER) and analyzed using ImageJ 1.43u (<http://rsb.info.nih.gov/ij/>). To quantify the amount of lactate dehydrogenase (LDH) released into supernates in response to cell stress, we used a colorimetric LDH activity assay kit (Sigma™, USA). Metabolic reducing activity of resazurin dye (AlamarBlue®, Invitrogen™, USA) was measured as previously described [9] using a Synergy HT® plate reader (BioTek™, USA) at 530 nm/590 nm (ex/em) filter settings.

ELISA-based assays

Protein concentrations of cell culture supernates or nuclear extracts were measured using enzyme-linked immunosorbent assay (ELISA) formats: mouse TNF α ; mouse IL6 (both BD

Pharmingen™, NJ); NFκB p50 (Panomics/Affymetrix™ Nuclear Extraction & TF ELISA Kit, CA)

Transcription Factor Activation Profiling Assay

Transcription factors (TF's) bound to genomic DNA were isolated using a nuclear extraction kit (Signosis®, USA). Using an oxidative stress transcription factor activation profiling plate array (Signosis®, USA), isolated TF's were then allowed to bind to promoter site specific, biotin-labeled probes, followed by column a purification step, hybridization to complementary probes sequences and colometric development in a plate format.

Gene expression analysis

RNA was extracted from cell cultures using AllPrep DNA/RNA/Protein Mini Kit® (Qiagen™). Total RNA quality was assessed by spectroscopy (NanoDrop® ND-1000, ThermoScientific™, USA) and micro fluidic gel analysis (Agilent™ RNA 6000 Nano Chip/Agilent™ 2100 Bioanalyzer, GER) followed by cDNA conversion using the SuperScript™ III Platinum® Two-Step qRT-PCR Kit (Invitrogen™, USA) or a RT² First Stand Kit® C-03 (SABiosciences™, USA) together with a Perkin Etus Thermal Cycler 480 (PerkinElmer™, USA). The gene expression patterns were then assessed by quantitative PCR (qPCR) with a RT² SYBR® Green/Rox qPCR Master Mix (SABiosciences™, USA) using a Stratagene™ mx3005P instrument (Stratagene™, USA) with. A melting curve was used to confirm the specificity of each primer pair. Each sample was run in triplicate to exclude outliers. Gene expression was analyzed using the commonly used CT-method (RT²qPCR Primer Assay User Manual, Version2.17, SABiosciences™) with GAPDH as the normalizer gene. The average gene expression was computed for each experimental condition (n = 3) relative to the control (n = 3). Forward (F) and reverse (R) sequence primers (see supplementary Table 1) were designed using Primer3 v. 0.4.0 (<http://frodo.wi.mit.edu/primer3/>) software based on coding DNA sequences (<http://www.ncbi.nlm.nih.gov/nucore>).

Statistics

The results are presented as mean ± SD and/or SEM. Pairwise comparisons were performed by a 2-tailed Student's t test. Statistical analysis was performed using Prism®4.0 (GraphPad, La Jolla, CA) software. In all tests, P<0.05 was considered statistically significant.

Results

Profiling of CD131 cell surface expression

First we used flow cytometry to evaluate CD131 cell surface expression in several cell lines of mesenchymal origin and found CD131 most consistently expressed in primary mouse aortic fibroblasts (mAFB's), mouse bone marrow derived mesenchymal stem cells (mBMSC's) [10] or the mouse macrophage cell line J774A.1 (mJ774's) [11]. When cultured under standard conditions, mAFB's exhibited stable CD131+ cell fractions of ~20% for up to 10 passages. When stimulated with bacterial lipopolysaccharide (LPS) or exposed to heat shock, CD131+ populations (without observed cell detachment) increased by 2 fold within 2 hours (Figure 1A). Adding the selective EPO-R/CD131 ligand ARA290 did not affect CD131 cell surface expression. Similarly, as shown in Figure 1B, mBMSC's under standard

culture conditions showed stable CD131+ cell fractions of ~40%, and stimulation with LPS or TNF α significantly increased this fraction. Also, induction of oxidative stress using tert-butylhydroperoxide (BHP) caused a corresponding dose-dependent increase in the number of CD131+ cells, suggesting a balanced response to cellular stress (Figure 1B). However, co-stimulation with ARA290, LPS or TNF α did not appear to alter this response. Conversely to what we saw in these fibroblastic cell lineages, mJ774's, which also robustly express CD131 along with IL3 and GM-CSF receptor [12], showed decreased CD131+ cell fractions following stimulation with ARA290, LPS or BHP (Figure 1C). Thus, CD131 expression increased in fibroblasts but decreased in macrophage-like cells in response to cellular stress and/or a pro-inflammatory environment. This in turn indicates an altered phenotype-specific responsiveness to EPO-R/CD131 complex mediated signaling under these conditions. Also, the observed changes of CD131 cell surface expression occurred almost immediately following stimulation and thus cannot be explained by de novo protein synthesis alone. We therefore assumed that in order to induce responsiveness to EPO *via* EPO-R/CD131 receptor complex shortly following cellular stress, preformed cytosolic CD131 would have to translocate to the cell membrane surface.

Cytoprotective, anti-apoptotic effects of selective EPO-R/CD131 stimulation

Sub-lethal cellular damage is more likely to trigger a controlled process of self-induced cell death/apoptosis if repair mechanisms fail within a short time period post injury. We first studied aspects of EPO-R/CD131 mediated signaling that more likely reflect an immediate modulation of cellular stress responses rather than more delayed changes in transcriptional profiles. A Live/Dead[®] assay was used to evaluate the effect of ARA290 on recovery of mAFB's after immersion heat shock. We saw a significant reduction in cell death if ARA290 was provided within the first hour of an eight hour recovery phase (Figure 2A). In addition, we developed an assay based on confluent human microvascular endothelial cell (hMEC's) cultures in order to measure the influence of ARA290 on cytoskeletal-driven cell contraction in response to cell stress. In one experiment, eight hours post heat shock, ARA290 and human recombinant EPO (hrEPO) supplementation maintained significantly higher surface coverage of the cultured endothelium compared to controls. Interestingly, a higher surface coverage with viable endothelial cells was also observed with addition of TNF α , with or without ARA290 (Figure 2B). Again, we used mAFB's to evaluate the dose dependence of BHP-induced oxidative stress. Following a recovery phase, we measured lactate dehydrogenase (LDH) activity released into supernates as an index of cytotoxicity. We saw a decreased cytotoxicity if ARA290 was supplemented during recovery, most significantly at a 10 μ M BHP concentration (Figure 2C). We also investigated the effect of EPO-R/CD131-signaling on the ability of cells to maintain a reducing environment within their cytoplasm in the context of oxidative stress and a pro-inflammatory environment using an assay based on the redox indicator dye resazurin in BMSC's. ARA290 supplemented during a six hour recovery phase following BHP-induced oxidative stress did not appear to significantly affect mitochondrial oxidation capacity but abrogated the increase induced by LPS and TNF α (Figure 2D). Thus, ARA290 appears to prevent a further deterioration of the redox capacity of cells promoted by LPS and TNF α in conditions of oxidative stress.

Modulation of inflammatory secretion profiles in response to cell stress

Cells can sense and respond to deleterious changes in their environment in several distinctive ways. Most notably, mesenchymal stem cells used in this study besides their potential to differentiate into various mesenchymal-derived phenotypes [13], have demonstrated an outstanding capacity to respond to and modulate inflammatory environments *via* various secretion products [14] with a net effect of accelerated recovery and improved functional repair of tissues [15]. One characteristic feature of MSC's in cell culture is constitutively high secretion levels of IL6. We found that both LPS and TNF α inhibited IL6 secretion by mBMSC's, which was further diminished by subsequent ARA290 exposure. Furthermore, ARA290 mitigated IL6 secretion stimulated by BHP induced oxidative stress. Interestingly, IL6 secretion by mBMSC's was unaffected by ARA290 alone (Figure 3A). IL6 secretion by primary mAFB cultures was strongly induced by LPS (~100 fold), TNF α (~100 fold) and BHP (~5 fold), while ARA290 strongly inhibited this induction (Figure 3B). We also evaluated effects of ARA290 on activated mJ774's, and found that following a pre-stimulation with LPS- or BHP for two hours, ARA290 significantly mitigated TNF α secretion (Figure 3C). We conclude that EPO-R/CD131 mediated signaling inhibits the secretion of cytokines with a pro-inflammatory net-effect such as IL6 and TNF α .

Modulatory effects on transcriptional stress responses

Our findings so far indicated a robust, functional expression of CD131 by cells of mesenchymal background. As already mentioned, mBMSC's, which showed highest CD131 positive fractions in cell culture, possess an extraordinary capacity to modulate inflammatory environments e.g. by various secretion products. We assumed that a more long-standing effect of CD131-mediated signaling following cellular stress and/or within a pro-inflammatory environment would also effect the transcriptional response e.g. of mBMSC's. Thus, we performed gene expression analysis of pathways related to apoptosis, inflammation, oxidative stress, hypoxia, cell cycling and proliferation. Experimental conditions included a two hour period of BHP-induced [1 μ M] cell stress followed by a recovery phase of six hours. A stress response was modulated by supplementing recovery media with the following components, alone or in combination (including non BHP-induced controls): AR290 [2 μ M], recombinant mouse EPO (rmEPO) [2 μ M], LPS [10 ng/ml], mouse TNF α [2 ng/ml]. In the following we elaborate on the most significant changes in cell stress responses mediated through alternative EPO-signaling (for details see Figure 4). First, in the absence of cell stress, expression of EPO-R/CD131 receptor complex appeared not to be influenced in a relevant way by ARA290 or rmEPO stimulation alone. As a general trend, LPS, TNF α and BHP predominately induced EPO-R (~5–10 fold) over CD131. Evaluation of pathways related to apoptosis revealed Birc5 (survivin) and Bag1, both inhibitors of caspase activation, to be upregulated (~10 fold) by ARA290 or mrEPO added during recovery following BHP-induced oxidative stress. Also with BHP induction, TNFRsf1a and FasR (TNFRsf6) receptors were strongly induced. However, co-stimulation of LPS/TNF α with ARA290/mrEPO appeared to selectively mitigate FasR induction during recovery. Various regulators of cell cycle progression, most notably P53 and P73 were strongly induced by LPS or TNF α stimulation alone. Conversely, BHP induced oxidative

stress suppressed this induction. In this context, co-stimulation during cell stress recovery with ARA290 and mrEPO appeared to mitigate (>10 fold) the induction of P73. We saw the same trend with several known effectors of P53/P73 including CyclinD3 (CcnD3), Cyclin-dependent kinase inhibitors 1c/2a (Cdkn1c/2a), Cyclin dependent kinase 5 (Cdk5), Cullin3 (Cul3) [16], E2F transcription factors 4 (E2F4) [17] and Growth arrest and DNA-damage-inducible beta protein (Gadd45b) [18]. Similarly, ARA290 and mrEPO also decreased LPS- and TNF α -induced upregulation of genes related to a more general activation of cell growth and reorganization, most notably Mitogen-activated protein kinase 1/14 (MAPK1/14) [19]. Among marker genes of increased oxidative stress such as Glutathione S-transferase M3 (Gstm3), Glutathione peroxidase 1 (Gpx1) or Superoxide dismutase 1 (Sod1) or Peroxiredoxin 1 (Prdx1), Gstm3 was most responsive to BHP induced oxidative stress in that it was suppressed (~5 fold) by co-stimulation of ARA290/mrEPO with LPS/TNF α . Among genes related to protein stabilization or degradation such as heat shock transcription factor protein 1 (HSF1), Heat shock proteins 27/90ab (HSP27/90ab), Prefoldin subunit 1 (Pfdn1), Chaperonin containing TCP1, subunit 2 (Cct2), Ubiquitin-conjugating enzyme E2 E1 (Ube2E1) or Autophagy related protein 2a (Atg2a), HSP27 and Atg2a were most notably upregulated (~5–100 fold) by LPS, TNF α and BHP. Here, ARA290/mrEPO co-stimulation appeared to act synergistically. However, ARA290/mrEPO stimulation during recovery from BHP induced oxidative stress mitigated HSF1 induction by LPS/TNF α . Thus, as expected, our findings show that effects of ARA290 are largely superimposable with those of native erythropoietin (mrEPO), confirming the relevance of alternative EPO signaling via EPO-R/CD131 complex. Also, the effects appeared to be mostly limited to a modulation of cellular stress responses with or without a pro-inflammatory stimulus induced by LPS and/or TNF α but not under conditions where cellular stress was absent. If added during the recovery phase following BHP induced oxidative stress, ARA290/mrEPO mitigated LPS/TNF α induced gene expression. Thus, EPO-R/CD131 mediated signaling appears to act as a negative co-modulator of LPS or TNF α mediated signaling in the context of cell stress responses.

Modulatory effects on intracellular phosphotyrosine/-serine levels

Phosphorylation of functional residues of various proteins is a highly dynamic event occurring during signal transduction, transcriptional modulation or the regulation of enzymatic activity and is controlled by kinase and phosphatase activity. Again, we chose mBMSC's based on characteristic high levels of tyrosine phosphorylation and low levels of serine phosphorylation. Using an anti-phosphotyrosine/-serine based assay stimulation with LPS or TNF α resulted in decreased levels of phosphorylation in mBMSC's within 20–40 minutes of exposure. Subsequently we saw that with tyrosine-phosphorylation, ARA290 acted synergistically, with serine-phosphorylation antagonistically. This effect was most notable under conditions where LPS/TNF α was removed, followed by a recovery phase of 20 minutes with or without ARA290 added (Figure 5A, B). Effects of ARA290 related to BHP induced oxidative stress were less consistent, varying greatly with dose and exposure time to BHP. Thus, again we concluded that an important aspect of EPO-R/CD131 complex mediated signaling is a likely to counteract cellular responses induced by a pro-inflammatory environment.

Modulatory effects on transcriptional control

The distinctive changes we saw in the global phosphorylation state of mBMSC's in response to oxidative stress and/or pro-inflammatory activation suggested a strong, kinase-driven effect on transcriptional regulation. Therefore we went on to evaluate effects of ARA290 on relative phosphorylation levels of P50 protein (NFκB p50), a major component of *Nuclear Factor kappa-light-chain-enhancer of activated B cells* (NFκB) protein complex, using an ELISA based assay. The NFκB-dependent gene activation is involved in most responses related to cellular stress and inflammation. Well known activators are LPS and TNFα, but also reactive oxygen species [20]. We exposed BMSC's to LPS, TNFα or BHP, followed by a recovery phase with or without ARA290. As expected, we saw a significant increase in NFκB p50 nuclear translocation with all stressors. Although data was inconclusive, ARA290 supplemented to recovery media appeared to inhibit phosphorylation and thus activation of LPS or TNFα induced NFκB p50 with a seemingly opposite effect related to BHP induced oxidative stress. Similarly, in mJ774's LPS-induced NFκB p50 phosphorylation appeared to be mitigated by ARA290 (suppl. Figure 1). In order to further confirm a modulation of various pathways already demonstrated on a transcriptional level (see Figure 4) we continued to profile nuclear translocation of various transcription factors (TF's) related to oxidative stress responses using a DNA hybridization-based assay. A comprehensive summary of those results is given in Figure 5C. Apart from *Serum Response Factor* (SRF), which was upregulated over 2 fold, ARA290 did not alter transcription under non-cell stress/non-inflammatory conditions. This changed in the case of BHP induced oxidative stress followed by a recovery phase with treatment with either ARA290 and or TNFα. ARA290 treatment post BHP- induced cell stress induced *Nuclear Factor of Activated T-cells* (NFAT) 3 fold, along with a ~1.5 – 1.8 fold increase in *Activating Transcription Factor 4* (ATF4) and *Hypoxia Inducible Factor* (HIF). Conversely, nuclear translocation of *Nuclear Translocation of Activator Protein 1* (AP1), *Core Binding Factor* (CBF/NFY), *Early Growth Response Protein 1* (EGR1), *E-Twenty Six* TF family (ETS), *Forkhead Box Protein O1* (FOXO1), *Heat Shock Factor* (HSF), *Interferon Regulatory Factor* TF family (IRF), NFκB, *Nuclear Factor (erythroid-derived 2)-like* (2NRF2/ARE), P53, *Small Mothers Against Decapentaplegic* TF family (SMAD) was significantly inhibited. In contrast to ARA290, TNFα essentially inhibited the activation of all evaluated TF's if added during recovery from BHP induced oxidative stress. Outstanding, however, were effects seen by co-stimulation with both ARA290 and TNFα post BHP-induced cell stress, showing increased activation of almost all marker TF's, most significantly of SRF (>5 fold), HSF (>2 fold) and AP1 (>2 fold). Intriguingly, *Signal Transducer and Activator of Transcription 3* (STAT3), which has been associated with classical EPO-R/EPO-R complex mediated signaling [21] was least affected. Taken together, our findings suggest that alternative EPO signaling predominately acts as modulator of cellular stress responses with little to no effect during cellular homeostasis.

Discussion

Tissue protective properties of EPO via the alternative EPO-R/CD131 heteromer receptor complex can be specifically targeted by engineered EPO-derivatives such as ARA290 [4]. In this study we characterized the impact of alternative EPO signaling on stress/inflammatory

responses of different cell lines of mesenchymal origin in order to better understand tissue protective effects previously described in various *in vivo* models of injury [2]. Also, the particular cell lines were chosen based on their responsiveness to stimulations with the ARA290 peptide which coincided with a high degree of CD131 positive fractions in cell culture. Initially we confirmed that CD131, which is involved in intracellular signal transduction of IL3/5 and GM-CSF receptors [22], is also expressed on fibroblastic, non-hematopoietic phenotypes. Whereas IL3/5 receptor expression has not been associated with fibroblastic phenotypes, the expression of GM-CSF and its receptor by fibroblasts under co-culture conditions has been reported [22]. However, fibroblasts predominately express fibroblast growth factor (FGF) receptors which share certain characteristics with GM-CSF receptor signaling, including the involvement of heparin/proteoglycans in ligand-receptor binding [23]. Thus, in non-hematopoietic tissues, CD131 expression is likely to be part of a unique profile of related surface receptors, including EPO-R. A characterization of EPO-R expression in various cell types has proven problematic in part due to unspecific epitope binding sites [24]. However, it has been convincingly demonstrated that co-localization of EPO-R/CD131 with *Vascular Endothelial Growth Factor Receptor 2* (VEGF-R2) in CD34+ endothelial progenitor cells is mandatory for a non-calcium dependent induction of *endothelial NO-Synthase* (eNOS) following the stimulation with *Vascular Endothelial Growth Factor* (VEGF) or EPO [25]. These findings suggest that alternative EPO-signaling via EPO-R/CD131 is likely to participate in the activation of various other signaling pathways. Here we show that an increased CD131 cell surface expression occurs almost instantly in primary mAFB's or mBMSC's following cell stress by heat shock, BHP or pro-inflammatory activation *via* LPS and TNF α . Thus, the most plausible mechanism of this CD131-upregulation would be the externalization of pre-formed receptor units opposed to a more protracted transcriptional upregulation. Conversely, 'constitutively activated' mJ774 macrophages showed highest CD131+ cell fractions under non-stress, non-inflammatory culture conditions with apparent internalization or shedding in response to LPS or BHP-induced cell stress. This may be related to a previously described [26] significant co-expression of IL3 and GM-CSF receptors in these cells, as opposed to fibroblastic cell lineages. We conclude that changes in CD131 surface expression appear to be a specific response to cell stress and inflammation that affects the responsiveness to EPO, at least in the selected cell lines.

We also show that ARA290 stimulation following the induction of cell stress decreases apoptosis, mitigates LDH release due to cellular damage, and preserves cytoskeletal-dependent cell contractility. ARA290 also abrogated a state of metabolic hyperactivity triggered by LPS and TNF α ; this may in turn decrease energy expenditure and preserve resources towards recovery and repair processes. In these experiments, ARA290 was most effective if given within the first hour following the induction of cell stress. Therefore, cytoprotective effects became apparent in a time interval where transcriptional activation of genes was unlikely to play a crucial role in overcoming cellular damage. This understanding concurred with our finding that ARA290 was also able to significantly alter intracellular phosphorylation levels of tyrosine or serine residues as functional groups of various enzymes and regulatory proteins early. At least in mBMSC's, ARA290 showed both

synergistic and antagonist effects on LPS- and TNF α -mediated signaling in terms of overall phosphorylation levels.

In addition, we found that another key function of alternative EPO signaling appears to be the modulation of phenotype-dependent secretion profiles. Following cellular damage or pro-inflammatory activation, most cells will not only passively respond to but also actively interact with their environment on a local and systemic level e.g. by secreting various cytokines or chemokines. Concurrent to a previously reported mitigation of inflammatory responses in a burn injury model, we saw that ARA290 treatment suppressed LPS or BHP-induced TNF α secretion in mJ774's macrophages. In fibroblastic phenotypes, mRNA levels of TNF α can also be induced [27] but this did not translate into a tangible secretion of TNF α in cell lineages used in this study. However, primary mAFB's showed a robust induction of IL6 secretion that was significantly mitigated following the exposure to stressors such as LPS, TNF α or BHP. In mBMSC's, expression of IL6 was also significantly mitigated by ARA290 stimulation that followed induction with LPS, TNF α or BHP.

Although EPO-R/CD131 receptor complex mediated effects related to recovery appeared to be most relevant if ARA290 was given immediately following induced cell stress and/or sub lethal cell damage, we also aimed at characterizing a more prolonged modulation of the transcriptome/proteome related to cellular stress responses [28]. By profiling this response in BHP-induced mBMSC's with or without an additional LPS or TNF α mediated pro-inflammatory activation, we showed that both components of the EPO-R/CD131 receptor complex are induced, thus likely increasing responsiveness to alternative EPO-signaling over time. Stimulation of BMSC's with ARA290 or mrEPO alone did not noticeably affect expression profiles. Conversely, following BHP-induced stress, the effect of ARA290 or mrEPO was a synergistic upregulation of most screened pathway genes. However, the most prominent effects of ARA290 or mrEPO were seen in the context of a co-stimulation with LPS or TNF α . Here, alternative EPO-mediated signaling showed mitigating effects on genes that were strongly upregulated by LPS or TNF α following cell stress induction via BHP, such as FasR, known to mediate pro-apoptotic actions of TNF α or P73, involved in cell cycle control. Overall, mitigating effects on LPS/TNF α induced transcription were apparent in pathways related to apoptosis, inflammation and cell cycle control. However, we also saw synergistic effects of ARA290 or mrEPO with LPS/TNF α co-stimulation with gene transcription related to protein stabilization or degradation. Nevertheless, BHP-induced cells remained highly responsive to LPS/TNF α stimulation which, is more likely to be detrimental to cell survival during a phase of recovery from cellular damage such as already demonstrated under conditions of ischemia [29]. In this respect, alternative EPO-signaling might act as a functional antagonist of a pro-inflammatory activation of cells, instead channeling cellular resources into repair mechanisms which will promote cell survival. Finally, alternative EPO-signaling strongly affected the transcriptional activation of various nuclear promoter sites related to cell stress responses and cell cycle control. However, our evaluation of a possible direct control of alternative EPO-signaling on LPS/TNF α induced NF κ B-mediated transcription remained inconclusive.

One insightful conclusion from our findings is that alternative EPO-signaling via the EPO-R/CD131 receptor complex is unlikely to play a significant modulatory role under normal

circumstances. This changes dramatically under conditions of cellular stress and a pro-inflammatory environment. However, a distinction has to be made between an isolated induction of cellular damage using e.g. heat shock or BHP-induced oxidative stress vs. solely a stimulation using LPS or TNF α . Regarding the latter, ARA290 overall appears to mostly mitigate a pro-inflammatory activation of responsive cell lines whereas with isolated cellular damage, ARA290 appears to help maintain a functional cellular apparatus. Both effects results in a net effect of reduced cell death and functional recovery with a return to homeostasis. Since tissue injury in vivo is usually closely related to inflammation and conditions of microbial invasion, we speculate that a potential function of alternative EPO-signaling might be to promote cell survival at the expense of other functions. In the context of cellular stress, other investigators have previously proposed that cellular protective heat shock responses and inflammatory pathway activation via interleukin 1[30], LPS and TNF α [31–33] or activation of NF-kappaB [34] may be mutually exclusive. Thus, our observation of an anti-apoptotic effect along with the blocked increased metabolic activity induced by LPS or TNF α could be intended at diverting intra-cellular resources into self-repair, as opposed to an energy-consuming activation of pro-inflammatory pathways. In summary this study confirms the relevance of cytoprotective actions of alternative EPO-signaling as a net effect resulting from modulatory effects both on immediate and delayed cellular stress responses. Our findings support the existence of an innate mechanism to resolve states of cell stress and pro-inflammatory activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Erythropoietin (EPO) triggers an alternative pathway via heteroreceptor EPO/CD131.
- ARA290 peptide specifically binds EPO/CD131 but not the canonical EPO/EPO receptor.
- Oxidative stress and inflammation promote cell surface expression of CD131.
- ARA290 prevents tumor necrosis factor-mediated inhibition of stress-related genes.
- Alternative EPO signaling modulates inflammation and promotes tissue homeostasis.

Figure 1. Changes in CD131 cell surface expression reflect a cellular stress response
FACS analysis of PE-CD131 positive (+) cell fractions following exposure to LPS, TNF α , heat shock and BHP with/without ARA290 (% $\pm$ SD, n = 6, *P<0.05 vs. control condition): (A) mAFB's, (B) mBMSC's, (C) mJ774's. (abbrev.: PE=fluorophore/R-Phycoerythrin; SSC-H=Side Scatter-High granularity)

Figure 2. Characterization of cytoprotective effects of alternative EPO-signaling on mesenchymal lineage cells

(A) Heat shock (65°C/20 sec) of mAFB's with ARA290 or ARA297 (inactive control) supplemented during recovery followed by Live/Dead® dye staining at 8 h (n=12). (B) Heat shock (55°C, 10 sec) of hMEC's followed by cell contraction analysis at 8 h (ratio of cell covered fluorescent to non-fluorescent surface area) (n=12). (C) Dose response toxicity of BHP (0.1–100 μM/2 h; 6 h recovery) on mAFB's (n=6). (D) Resazurin-based assay: modulation of metabolic reducing activity of mBMSC's recovering from 1 h of BHP-induced cell stress (n=24). (all: mean ± SD, *P<0.05 vs. control condition)

Figure 3. Alternative EPO-signaling modulates cytokine secretion profiles

Effects of ARA290 on cytokine secretion measured in supernates compared to standard media culture conditions (=control) with a defined cell stress induction period followed by a recovery period: (A) IL6 secretion of mBMSC's, (B) IL6 secretion of mAFB's, (C) TNF α secretion of mJ774's (all: n = 6, mean $\pm$ SD, *P<0.05 vs. control condition)

		GENE OF INTEREST (abbreviations as: http://www.ncbi.nlm.nih.gov/nucleotide)															
pre (2 hours)	post (6 hours)	CD131	EpoR	Bag1	Birc5	Bax	Bcl2	TNFRsf1a	FasR	Perp	Triap1	p53	p73	Ccnd1			
none	Control	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
none	ARA290 [2uM]	10.92	1.18	1.42	1.07	1.37	0.67	3.810551992	0.88	2.828427125	2.639015822	0.24	0.94	3.917688119	1	1	
none	rmEpo [2uM]	3.10	0.88	1.13	1.73	1.63	0.37	0.73	0.97	1.94	3.14	0.71	1.61	1.72	1	1	
LPS [10ng/ml]	continued	11.58	96.47	7.48	2.33	1.68	0.62	16.56	573.64	6.54	84.39	4.89	726.09	94.09	1	1	
LPS	stopped	5.79	4.95	10.94	2.05	1.31	0.45	14.16	361.04	4.25	59.10	4.18	439.59	71.90	1	1	
LPS	ARA290	11.70	44.82	8.96	1.98	1.67	0.48	27.55	668.60	6.43	84.33	4.76	1158.47	100.36	1	1	
LPS	rmEpo	4.51	51.27	12.33	3.13	1.56	0.58	16.76	551.42	6.00	91.14	5.02	588.33	80.06	1	1	
TNFα [2ng/ml]	TNFα	10.88	76.06	6.16	2.70	1.44	0.58	15.34	844.53	8.15	89.26	3.46	1187.74	92.22	1	1	
TNFα	stopped	7.57	37.71	7.13	2.29	1.19	0.42	9.86	503.90	5.55	54.30	2.70	781.99	77.76	1	1	
TNFα	ARA290	8.94	63.12	10.09	2.32	0.93	0.35	11.61	404.78	16.74	71.56	9.86	1357.75	259.21	1	1	
TNFα	rmEpo	2.97	165.13	10.16	2.20	0.66	0.23	9.65	348.38	9.56	69.87	11.17	721.32	61.33	1	1	
BHP [1uM]	Control	0.84	11.24	2.33	1.77	1.17	0.35	34.77	14.52	1.42	8.22	0.72	4.68	0.35	1	1	
BHP	ARA290	0.87	14.52	4.96	9.58	5.74	6.28	29.18	42.81	14.11	8.92	13.45	3.29	0.16	1	1	
BHP	rmEpo	0.74	8.05	16	10	3	1.01	42	161	0.78	11.71	22.16	8	6.02	1	1	
BHP	LPS	6.06	3.24	3.94	1.38	8	0.99	21.11	422	1.20	6.23	5.46	1003	4.37	1	1	
BHP	LPS + ARA290	11.86	22.78	1.71	0.48	0.57	0.06	38.58	0.33	6.82	19.29	9.51	44.32	10.06	1	1	
BHP	LPS + rmEpo	5.46	18.37	1.75	0.99	0.62	0.11	20.25	6.91	4.72	16.79	30.70	21.86	28.91	1	1	
BHP	TNF-α	5.46	58.89	6.23	3.39	2.60	0.77	19.43	90.51	5.93	11.95	10.48	491.14	39.12	1	1	
BHP	TNF-α + ARA290	29.24	83.24	1.36	0.90	0.43	0.08	58.08	2.93	9.25	35.50	13.55	1.71	50.21	1	1	
BHP	TNF-α + rmEpo	51.63	23.24	1.75	1.30	0.95	0.20	28.48	4.10	11.63	38.32	12.64	2.27	30.91	1	1	
		GENE OF INTEREST (abbreviations as: http://www.ncbi.nlm.nih.gov/nucleotide)															
pre (2 hours)	post (6 hours)	Ccnd3	Cdkn1c	Cdkn2a	Cdk5	Cul3	E2f4	Gadd45b	MAPK1/Erk2	MAPK14/p38	Grb2	Rac1	cKiiu/CD117	FGF1			
none	Control	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
none	ARA290 [2uM]	1.45	1.68	0.73	0.77	0.67	0.69	0.85	1.54	0.37	0.94	0.66	0.88	0.51	1	1	
none	rmEpo [2uM]	1.92	1.83	0.33	0.62	0.48	0.39	0.38	1.89	0.92	0.49	0.59	0.77	0.83	1	1	
LPS [10ng/ml]	continued	13.04	37.27	19.74	5.15	1.21	2.68	6.58	6.50	49.94	13.99	7.13	3.38	0.68	1	1	
LPS	stopped	7.74	32.04	9.48	2.78	2.13	2.23	7.98	8.78	47.80	12.15	8.66	21.88	1.28	1	1	
LPS	ARA290	16.94	35.53	14.47	5.84	1.87	4.09	6.79	10.59	68.59	11.36	9.04	8.97	2.95	1	1	
LPS	rmEpo	9.27	35.41	12.24	4.64	2.39	3.08	8.32	9.53	51.98	11.88	11.38	7.06	2.95	1	1	
TNFα [2ng/ml]	TNFα	13.36	24.90	16.47	4.57	1.33	3.24	9.51	6.44	34.56	10.40	6.73	5.32	4.51	1	1	
TNFα	stopped	9.84	24.56	10.08	2.98	1.01	2.44	5.45	6.48	36.50	9.30	5.49	2.97	1.91	1	1	
TNFα	ARA290	6.63	22.39	4.91	2.19	1.95	2.10	4.76	10.51	49.69	8.49	8.09	13.01	2.93	1	1	
TNFα	rmEpo	4.43	11.45	3.09	1.54	1.82	1.82	4.77	8.68	49.61	8.48	7.25	8.44	2.63	1	1	
BHP [1uM]	Control	5.94	16.91	6.45	2.93	0.75	1.11	25.46	4.56	5.70	1.61	1.47	0.86	0.59	1	1	
BHP	ARA290	22.47	35.02	4.56	6.68	0.22	2.81	49.52	0.18	2.89	10.63	2.46	4.83	12.73	1	1	
BHP	rmEpo	14.12	9	43	8	1	2	57	38	58	1	4	3.27	1.85	1	1	
BHP	LPS	22.16	125	10	10	5	5	105	114	271	5	1	1.85	3.89	1	1	
BHP	LPS + ARA290	1.92	6.15	2.25	0.69	0.32	0.30	4.41	7.78	15.45	0.20	0.40	0.82	0.79	1	1	
BHP	LPS + rmEpo	2.71	9.51	4.23	1.06	0.27	0.51	6.59	8.69	33.36	0.63	0.50	4.86	1.23	1	1	
BHP	TNF-α	11.39	33.36	12.04	3.81	1.16	2.28	29.65	34.54	100.43	2.23	1.42	5.13	4.99	1	1	
BHP	TNF-α + ARA290	2.19	5.90	3.03	0.67	0.11	0.38	4.00	6.15	15.35	0.30	0.39	0.33	0.44	1	1	
BHP	TNF-α + rmEpo	2.36	11.16	6.45	1.20	0.36	0.52	7.21	3.92	7.35	0.61	0.71	0.55	0.11	1	1	
		GENE OF INTEREST (abbreviations as: http://www.ncbi.nlm.nih.gov/nucleotide)															
pre (2 hours)	post (6 hours)	Frn3	Gstm3	Gpx1	Sod1	Prdx1	HSP1	HSP27	HSP90ab	Pdcl1	Gclt2	Ube2E1	Atg2a				
none	Control	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
none	ARA290 [2uM]	11.24	1.04	0.62	0.74	3.89	0.93	2.33	0.71	0.66	0.60	3.76	3.34	1	1		
none	rmEpo [2uM]	5.20	1.16	0.84	0.48	1.52	0.68	3.21	1.14	1.99	0.45	1.92	0.99	1	1		
LPS [10ng/ml]	continued	42.52	23.10	6.43	5.03	9.46	2.04	78.95	5.47	0.85	0.51	4.87	6.85	1	1		
LPS	stopped	134.18	19.12	8.17	7.13	9.89	1.81	30.41	5.40	0.01	0.48	6.24	4.91	1	1		
LPS	ARA290	82.42	20.00	5.87	4.08	13.14	2.60	39.70	7.03	0.01	0.85	7.52	7.92	1	1		
LPS	rmEpo	57.28	23.26	9.77	8.60	14.42	2.34	54.46	8.17	0.03	0.87	10.70	6.36	1	1		
TNFα [2ng/ml]	TNFα	72.66	20.61	4.91	3.38	7.27	2.05	36.82	6.57	0.01	0.72	5.71	6.52	1	1		
TNFα	stopped	52.75	15.11	5.65	4.18	8.45	1.65	30.98	5.37	0.02	0.57	5.53	3.87	1	1		
TNFα	ARA290	63.91	15.67	7.85	6.22	11.17	1.93	22.89	4.43	0.04	0.63	7.84	5.55	1	1		
TNFα	rmEpo	39.14	14.68	6.98	3.88	10.17	1.81	0.99	4.72	0.02	0.56	7.55	6.67	1	1		
BHP [1uM]	Control	3.92	10.56	2.11	1.20	1.51	1.20	7.57	11.96	1.99	1.21	1.02	2.96	1	1		
BHP	ARA290	12.83	21.86	2.83	5.21	4.23	5.54	113.00	24.59	3.03	1.73	4.67	9.18	fold change < 0.1*	1		
BHP	rmEpo	17.15	26	6	4	2.39	3	437	47	6	3	1.28	7.88	0.2	1		
BHP	LPS	11.88	33	14	10	1.69	8	1596	115	13	5	1.06	3.13	0.2	1		
BHP	LPS + ARA290	60.97	2.16	0.71	0.47	2.73	0.25	146.02	6.63	0.61	0.27	1.16	11.87	0.5	1		
BHP	LPS + rmEpo	64.00	3.32	0.88	0.70	1.21	0.62	160.90	10.06	1.94	0.21	1.92	13.92	1	1		
BHP	TNF-α	65.34	11.79	2.27	2.08	1.02	2.64	155.42	6.68	6.32	1.28	0.95	4.41	2	1		
BHP	TNF-α + ARA290	103.97	1.96	0.58	0.34	1.59	0.38	319.57	8.22	2.48	0.25	2.83	42.81	5	1		
BHP	TNF-α + rmEpo	8.17	3.63	0.86	0.01	1.99	0.57	164.00	11.70	3.98	0.27	2.48	72.00	> 10*	1		

Figure 4. Transcriptional profiling of cell stress responses in mBMSC's

Effects of ARA290 and rmEPO on pathway marker genes related to apoptosis, cell cycling, inflammation, hypoxia, oxidative stress or heat shock in mBMSC's following 2 h of induction with/without LPS, TNFα or BHP and 6 h recovery with/without ARA290, rmEPO, TNFα or LPS. (shown: qPCR/ Ct-method; n=4 pooled samples/condition; fold change color-coded: 0.1>x>10 for respective marker genes (columns) under various stressor-conditions (rows) compared to a non-stressed control=1).

Figure 5. EPO-R/CD131 dependent kinase activity and nuclear translocation of transcription factors in mBMSC's

FITC/anti- (A) phosphotyrosine, (B) phosphoserine FACS-based profiling (mBMSC's: cell population shift [%], mean $\pm$ SD, * P <0.05 vs. control condition), (C) nuclear TF-translocation profiling in cell stress induced mBMSC's (shown: Transcription Factor Activation following 2 h of induction with/without BHP and 6 h recovery with/without ARA290 and TNF α ; n=6 pooled samples, *fold change color-coded: 0.5>x>2 compared to a non-stressed control=1). (abbrev.: mAlexa488=fluorophor/mouse sec.antibody; SSC-H=Side Scatter-High granularity)