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Research Paper

Short-Term Hypoxia Dampens Inflammation *in vivo via* Enhanced Adenosine Release and Adenosine 2B Receptor Stimulation

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ABSTRACT

Hypoxia and inflammation are closely intertwined phenomena. Critically ill patients often suffer from systemic inflammatory conditions and concurrently experience short-lived hypoxia. We evaluated the effects of short-term hypoxia on systemic inflammation, and show that it potently attenuates pro-inflammatory cytokine responses during murine endotoxemia. These effects are independent of hypoxia-inducible factors (HIFs), but involve augmented adenosine levels, in turn resulting in an adenosine 2B receptor-mediated post-transcriptional increase of interleukin (IL)-10 production. We translated our findings to humans using the experimental endotoxemia model, where short-term hypoxia resulted in enhanced plasma concentrations of adenosine, augmentation of endotoxin-induced circulating IL-10 levels, and concurrent attenuation of the pro-inflammatory cytokine response. Again, HIFs were shown not to be involved. Taken together, we demonstrate that short-term hypoxia dampens the systemic pro-inflammatory cytokine response through enhanced purinergic signaling in mice and men. These effects may contribute to outcome and provide leads for immunomodulatory treatment strategies for critically ill patients.

1. Introduction

Hypoxia and inflammation are two closely linked phenomena that are encountered in many pathological processes; particularly in critical illness such as sepsis and trauma (reviewed in [12, 41]). Inflammation can lead to tissue hypoxia due to both enhanced demand and decreased availability of oxygen, the latter resulting from edema, microthrombi and changes in microcirculation (reviewed in [12]). Conversely, *in vitro* studies have demonstrated that hypoxia influences the immune response as well, with either pro- or anti-inflammatory effects, depending on the cell type (reviewed in [25]). *In vivo* data on immunomodulatory effects of hypoxia or hypoxia mimetics in animal models are conflicting. For instance, chronic hypoxia (11–27 days) in mice was reported to result in enhanced TNFα levels upon challenge with endotoxin [2], whereas the hypoxia mimetic DMOG was shown to exert distinct anti-inflammatory effects in endotoxemic mice [19]. A recent study in mice revealed that long-term hypoxia (>12 h) does not relevantly affect the immune response, but increases morbidity and mortality from skin and pulmonary infections [47]. Importantly however, hypoxia is often very short-lived in critically ill patients, as it is quickly corrected by oxygen supplementation and/or mechanical ventilation [31], but the immunologic effects of short-term hypoxia are unknown. Furthermore, no human data on immunomodulatory effects of hypoxia

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are available. These are nevertheless of clinical relevance, because the majority of critically ill patients suffer from inflammation-related conditions, and putative immunomodulatory effects of short-term hypoxia might therefore contribute to the outcome of these patients.

Hypoxia may modulate the immune response through a group of transcription factors called hypoxia-inducible factors (HIFs) [33]. There are several HIF isoforms, of which primarily HIF-1α has been implicated in regulation of the inflammatory response [33], although HIF-2α was also found to have immunomodulatory properties [23]. Moreover, hypoxia also induces enhanced signaling of the purine nucleoside adenosine [9], which has been shown to exert anti-inflammatory and tissue-protective effects [14]. In addition, adenosine signaling is also augmented by inflammation, through enhanced plasma levels of adenosine and increased expression of adenosine receptors. Therefore, hypoxia and inflammation might have additive or even synergistic effects on adenosine signaling [38].

In the present work, we evaluated the immunologic effects of short-term hypoxia *in vivo* in mice. Furthermore, we investigated the involvement of HIFs and adenosine signaling in these effects, and translated our findings to the human setting using an *in vivo* model of systemic inflammation in healthy volunteers.

2. Materials and Methods

2.1. Murine Studies

2.1.1. Animals and Ethics

Experiments were performed at the Experimental Centre at the University of Technology Dresden (Medical Faculty, University Hospital Carl-Gustav Carus), Germany, and the Radboud university in Nijmegen, the Netherlands. Experiments were in accordance with the facility guidelines at the University of Technology Dresden and were approved by the Landesdirektion Dresden, or with the requirements of the Dutch Experiments on Animals Act and the EC Directive 86/609, and approved by the Animal Ethics Committee of the Radboud university. Experiments were performed on male C57BL/6 mice (Charles River Laboratories International, Inc., L'Arbresle Cedex, France). For the experiments depicted in Fig. 3, Vav:cre [44] HIF1f/f [40] and HIF2f/f [18] transgenic mouse lines were obtained from the Jackson Laboratories (Bar Harbor, ME) and crossed in our facility. The obtained mouse lines are respectively: Vav:cre-HIF1αf/f (hematopoietic HIF1f/f) and Vav:cre-HIF2αf/f (hematopoietic HIF2f/f). The degree of HIF1α deficiency in hematopoietic cells of Vav:cre-HIF1α$^{f/f}$ mice *versus* WT littermates was defined by qPCR on CD45+ bone marrow cells, which revealed >90% reduction of WT HIF1α mRNA compared to WT littermates (Fig. S1). Since HIF2α messenger was scarcely detectable in hematopoietic cells of WT mice, we performed genomic PCRs on DNA isolated from mature white blood cells. In samples displaying Cre-recombinase, HIF2α PCRs revealed virtually full recombination of the floxed-HIF2α (Fig. S1). For the experiments depicted in Figs. 4 and 5, adenosine 2B receptor$^{f/f}$ (provided by Prof. Eltzschig), and B6.129P2-Il10^{tm1Cgn}/J mice ((MGI Cat# 5470153, RRID:MGI:5470153) (IL-10$^{-/-}$, The Jackson Laboratory, Bar Harbor, ME) and genetically matched controls were used (all C57BL/6 background). Due to the nature of the studies, blinding of the animal researchers was not possible. The laboratory analyses were performed by blinded personnel.

2.2. Experimental Protocol

After randomization using the sealed envelope method, mice were placed in an air-tight cage that was continuously flushed with either medical air (normoxia) or an hypoxic nitrogen/medical air mixture (fraction of inspired oxygen [FiO_2] of 9%) at the same airflow rate. Endotoxin (*E. coli*, serotype 0111:B4, Sigma-Aldrich, St Louis, MO, USA) dissolved in normal saline was administered intraperitoneally at a dose of 5 mg/kg. Mice were sacrificed by exsanguination through orbita extraction under deep isoflurane anesthesia. Blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes, centrifuged (14,000 *g*, 5 min, room temperature), was and plasma was stored at −80 °C until analysis. Splenic tissue was snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

2.3. Cytokine Analysis

Plasma concentrations of TNF, IL-1β, IL-6, KC, and IL-10 were measured using a Luminex assay (Milliplex, Merck Millipore, Billerica, MA, USA) or ELISA (Duoset or Quantikine, R&D systems, Minneapolis, MN, USA).

2.4. mRNA Expression Analysis

Spleen tissue was homogenized using a Tissuelyzer LT instrument (Qiagen, Venlo, the Netherlands) and RNA was isolated with the RNeasy kit (Qiagen). Up to 1 μg of RNA was used for cDNA synthesis with iScript (Bio-Rad, Veenendaal, the Netherlands).

qPCR was performed on a CFX96 (Bio-Rad, Hercules, CA, USA). The following primer-probe sets were used (all from Life Technologies, Carlsbad, CA, USA): TNF: Mm00443258_m1, IL-1β: Mm00434228_m1, IL-6: Mm00446190_m1, KC: Mm04207460_m1, IL-10: Mm00439614_m1, VEGF (Mm00437306_m1), B2M (Mm00437762_m1) and HRPT (Mm01545399_m1). ΔCt values were calculated as the difference between the Ct value of the target gene and the geometric mean of the Ct values of two housekeeping genes (B2M and HRPT). Fold regulation ($2^{|\Delta\Delta Ct|}$) was determined by normalizing ΔCt to the reference group (normoxia-saline).

2.5. Tissue Adenosine Measurements

Nucleosides were extracted from spleen tissue by sonification in 850 μL of ice cold 0.4 N perchloric acid. The mixture was vortexed and 10 μL of this solution was used for protein assay. The remaining volume was centrifuged at 14000 rpm at 4 °C for 10 min and 710 μL of the supernatant was vortexed with 40 μL phenol red (0.1 mg/mL in water) and 356ul 0.6 N $KHCO_3$/KOH. After addition of 111 μL ammonium phosphate and 50 μL of 0.18 N H_3PO_4 the sample was vortexed, and centrifuged at 14000 rpm for 5 min. 1000 uL of the supernatant was used for adenosine measurements by HPLC [26].

2.6. Human Studies

2.6.1. Subjects and Study Design

The human experiments were carried out in two phases (ClinicalTrials.gov identifiers NCT01889823 and NCT01978158) for safety reasons. All experiments were in accordance with the declaration of Helsinki. After approval from the local ethics committee of the Radboud University Medical Center, thirty healthy, male volunteers gave written informed consent to participate in the experiments. Subjects were screened and had a normal physical examination, electrocardiography, and routine laboratory. Exclusion criteria were febrile illness during the 2 weeks before the experiment, high altitude exposure in the three months prior to the experiment, use of prescription drugs, history of spontaneous vagal collapse, and participation in a previous trial with endotoxin administration.

In the first phase, effects of short-term hypoxia in the absence of systemic inflammation were studied. Ten subjects were exposed to hypoxia for 3.5 h by titration of FiO_2 to a peripheral saturation (SaO_2) of 80–85%, using an nitrogen/medical air mixture and an air-tight respiratory helmet (CaStar, StarMed, Italy). The airflow was adjusted to prevent carbon dioxide rebreating and prevent hypercapnia. In the second phase, 20 subjects participated in endotoxemia experiments. These subjects were randomized using the sealed envelope method to hypoxia ($n = 10$) as described above, or normoxia (medical air, FiO_2

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