The importance of hypoxia and EPHOSS for collection and processing of stem and progenitor cells to understand true physiology/pathology of these cells ex-vivo

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Abstract

Purpose of Review—Hematopoietic stem (HSCs), and progenitor (HPCs) cells reside in a hypoxic (lowered oxygen (O_2) tension) environment, *in vivo*. We review literature on growth of HSCs and HPCs under hypoxic and normoxic (ambient air) conditions with a focus on our recent work demonstrating the detrimental effects of collecting and processing cells in ambient air through a phenomenon termed Extra Physiologic Oxygen Shock/Stress (EPHOSS), and we describe means to counteract EPHOSS for enhanced collection of HSCs.

Recent Findings—Collection and processing of bone marrow (BM) and cord blood (CB) cells in ambient air causes rapid differentiation, and loss of HSCs, with increases in HPCs. This apparently irreversible EPHOSS phenomenon results from increased mitochondrial ROS, mediated by a *p53-Cyclophilin D* – mitochondrial permeability transition pore (MPTP) axis, and involves *hif-1α* and *miR210*. EPHOSS can be mitigated by collecting and processing cells in lowered (3%) O_2, or in ambient air in the presence of, cyclosporine A (CSA) which effects the MPTP, resulting in increased HSC collections.

Summary—Our recent findings may be advantageous for HSC collection for hematopoietic cell transplantation, and likely for enhanced collection of other stem cells types. EPHOSS should be considered when *ex-vivo* cell analysis is utilized for personalized medicine, since metabolism of cells and their response to targeted drug treatment *ex-vivo* may not mimic what occurs *in vivo*.

Keywords

Hematopoietic stem and progenitor cells; microenvironment; O_2 tension; EPHOSS and its mitigation

Introduction

Complex multicellular organisms began to evolve as the atmospheric oxygen (O_2) started increasing to reach its present levels of approximately 21% [1,2]. However, and perhaps

ironically, the production of blood cells *in vivo* in neonates and adults occurs in a microenvironment that is hypoxic [3*,4*,5, 6, 7**,8]. Blood cell production is dependent on critical cell-cell and cytokine-cell interactions between hematopoietic stem (HSCs) and progenitor (HPCs) cells, and their precursors and more mature cell offspring [9–11] and occurs mainly in the bone marrow (BM) microenvironment where HSCs and HPCs are near or in contact with stromal cells, osteoblasts and endothelial cells in a low O_2 environment that ranges from 1–4% with perhaps some slightly lower or higher O_2 levels [5,6,7**,8]. While HSCs and HPCs can be grown *ex-vivo* in atmospheric O_2, these rare life-saving cells proliferate better *in vitro* in hypoxia (usually   5% O_2), compared to normoxia (defined as atmospheric O_2), [12–17]. Colony assays of BM HPCs from mice or humans, or cord blood (CB) cells from humans demonstrate increased numbers and cell cycling of granulocyte macrophage (CFU-GM), granulocyte (CFU-G), macrophage (CFU-M), erythroid (BFU-E), megakaryocytic (CFU/BFU-Meg), and multipotential (CFU-GEMM; CFU-Mix) HPCs when *in vitro* culture conditions are hypoxic. Expansion of HPCs and HSCs *ex-vivo* are superior under hypoxic culture conditions [15,17].

Studies have evaluated the distribution of HSCs and HPCs in relationship to BM microenvironmental cells in context of regional O_2 levels. HSCs and cells within BM that support HSCs are mainly present in a niche predominately located at a lower region of the O_2 gradient, suggesting that regional hypoxia plays an important role in regulating HSC function [5]. More recent studies have refined concepts of HSC localization. One study, defined HSC phenotype within endosteal BM regions as being superior for homing and proliferative capacity, compared to these same phenotyped cells isolated from the central BM [18]. Another group performed *in vivo* measurements of local O_2 tension in live mice [7**] using two-photon phosphorescence lifetime microscopy to determine that absolute local O_2 tension of the BM was low (<32mm Hg) even though there was a very high vascular density. Although the BM as a whole was hypoxic, they found heterogeneity in local O_2 levels with the lowest (about 9.9mm Hg, or 1.3% O_2) present in deeper peri-sinusoidal regions. Under conditions of post-chemotherapy stress, HSCs and HPCs did not seek out specific niches defined by low O_2 for their preferential homing. Another group used 5-color imaging cytometric analysis to quantitate the distribution of HSCs and HPCs in femoral BM cavities [6]. HSCs and HPCs localized preferentially in endosteal zones, where they interacted closely with sinusoidal and non-sinusoidal BM microvessels. HSCs/HPCs exhibited a hypoxic metabolic profile defined by strong retention of pimonidazole and expression of *hypoxia inducing factor* (hif)-1α, regardless of: location in the BM, position next to vascular structures, or cell cycle state. Thus, the hypoxic phenotype of HSCs and HPCs in BM was cell, rather than location, specific. Endosteal BM areas did not contain the most hypoxic HSCs/HPCs, and *hif-1*α stabilization in these cells occurred independent of differences in O_2 levels at different anatomical sites.

Extra Physiologic Oxygen Shock/Stress (EPHOSS)

While the biology of HSCs/HPCs and other stem cells (embryonic, mesenchymal, and neural) are now considered in context of anatomical site positioning *in vivo*, and in context of O_2 tension for growth differences *ex-vivo* [8,19], no attempts have been made to assess initial effects of even brief exposure of HSCs and HPCs to ambient atmospheric O_2

regardless of whether or not the cells collected in ambient air are subsequently processed, cultured or injected into animals under normoxia or hypoxia. Our most recent studies [20**] now demonstrate that even very brief exposure to ambient air has a rapid and apparently irreversible effect that changes the metabolism of HSCs and HPCs. Through a phenomenon that we termed EPHOSS, this results in rapid loss of HSC numbers with concomitant increases in HPCs, due to rapid differentiation of HSCs. Mechanisms of EPHOSS encompass ambient air-induced production of mitochondrial reactive oxygen species (ROS), and induction of the mitochondrial permeability transition pore (MPTP) opening. This occurs with BM and also human CB cells, which is consistent with reports that human CB cells are also in a hypoxic environment [21]. EPHOSS is mediated by interactions with the MPTP and *cyclophilin D* (*CypD*) and *p53*, with links to expression of *hif-1α*, and the hypoxamir, *miR210*. This information is important for hematopoietic cell transplantation (HCT), especially for CB HCT where numbers of cells from single collections are low. While efforts have focused on enhancing the current clinical efficacy of these cells for HCT via *ex-vivo* expansion of these cells, or by increasing their homing capabilities [22,23], to compensate low collection numbers, being able to collect more HSCs in a CB collection could greatly enhance the efficacy of CB for HCT. In fact, EPHOSS, and means to prevent its action, will likely extend to many other stem cell types, including embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), adipose stem cells (HSCs), and other tissue specific stem cells that normally reside in a hypoxic environment *in vivo*.

ROS can be toxic, but also has differentiation inducing activity [24*]. We reasoned that collecting mouse BM cells and doing all processing and eventual culture of the cells or their injection into mice under low O_2 tension might mitigate production of mitochondrial ROS, and subsequent ROS-induced differentiation of the mouse BM HSCs [20**]. For this to be successful, it was necessary for all procedures to be done in a hypoxic chamber in which everything used (media, plastic ware, glassware, syringes, etc.) was pre-equilibrated to 3% O_2 in the hypoxic chamber for 18 hrs prior to collection, processing, and eventual culture or injection into mice under 3% O_2. This resulted in ~3–5 fold enhanced collection of mouse BM long-term repopulating (LT)-HSCs, rigorously determined by phenotype and functional engraftment of competitive repopulating units (CRU) as defined by donor cell chimerism and limiting dilution analysis, with concomitant decreases in HPCs, defined by phenotype for short-term repopulating (ST)-HSCs and multi-potential progenitor (MPPs) cells, and by function using colony assays for CFU-GM, BFU-E, and CFU-GEMM.

Collection of cells in 3% O_2 and then placing them in ambient air for as short as 20–30 minutes (the shortest time in which we were able to process the cells) resulted in greatly reduced numbers of HSCs and increased numbers of HPCs. Additionally, collection and processing of cells in air, or collecting of cells at 3% O_2 and placement in ambient air greatly increased production of mitochondrial ROS, mitochondrial mass/activity, and high mitochondrial membrane potential. EPHOSS did not link to apoptosis, nor did it influence the homing efficiency of the collected cells. Collection and processing of human CB CD34^{+} cells in 3% O_2 also resulted in about a 3 fold increase in rigorously defined human HSCs [25], demonstrating that effects of EPHOSS were not limited to BM [20**].

Mechanisms of EPHOSS

We evaluated mechanisms of EPHOSS for obtaining insight into its biology, and also for potential alternative means of collecting HSCs in order to mimic the enhancing effects of HSC collection at low O_2 tension [20**]. Collection of BM or CB cells at low O_2 would present a logistical problem that even if solved would make collection of cells cumbersome and expensive. We focused on the MPTP as a potential key to EPHOSS [20**]. Although oxidative stress favors induction of the MPTP opening which can result in the swelling of mitochondria, and uncoupling of OXPHOS, that leads to apoptosis and necrosis [26,27], this MPTP opening can be transient and function in a regulatory capacity conducive to modulating differentiation of stem cells. A key regulatory component of the MPTP is CypD, which regulates induction of the MPTP [28,29]. Interestingly, cyclosporine A (CSA), a small molecule inhibitor of CypD which binds CypD and antagonizes induction of the MPTP [30,31], is FDA approved, and is used as an immunosuppressant to treat graft vs. host disease for HCT, as well as a treatment possibility for heart attack and stroke [32,33]. We reasoned that CSA might be useful to protect against effects of MPTP induction, and if this was successful it might be rapidly considered for collection of HSCs in ambient air by mimicking effects of low O_2 tension. We found that collection and processing of mouse BM or human CB in ambient air but in the immediate and continued presence of CSA resulted respectively in greatly enhanced numbers of phenotypically identified HSCs and functional CRUs for mouse BM, and SCID Repopulating Cells (SRCs) for human CB [20**]. To maintain HSC numbers in CB through the cryopreservation and thaw procedures necessary for CB banking, it is likely that CSA may have to be present throughout the freeze/thaw procedures.

To implicate the MPTP in EPHOSS further, we assessed whether or not *CypD* deletion (−/−), which is known to prevent induction of the MPTP [34–36], might protect against effects of EPHOSS for enhanced collection of HSCs from mouse BM. *CypD* −/− mouse BM cells collected and processed in air were greatly increased in phenotypically-defined and functional HSCs, with decreased numbers of HPC compared to *CypD* +/+ mouse BM. *CypD* −/− BM LT-HSC were also significantly reduced in production of mitochondrial ROS. Evaluating mouse *CypD* −/− spleen cells by the Seahorse XF96 flux analyzer, demonstrated that basal respiration and maximal respiratory capacity was higher in *CypD* −/− cells than in wild type control cells [20**].

We were also able to link *p53* −/− BM cells to a *p53-CypD*-MPTP axis in mechanisms of EPHOSS [20**]. Using *hif-1*α and *miR210* −/− mouse BM cells we also linked *hif-1*α and *miR210* to EPHOSS [20**], although exact mechanisms have not yet been worked out. *CypD* −/− and *p53* −/− had EPHOSS-protective effects, while *hif-1*α −/− and *miR210* −/− abrogated the protective effect seen under hypoxic harvesting and processing of the cells. Our studies highlight how interpretation of experimental results of mouse gene deletion models can be influenced once EPHOSS is considered.

Broad Implications for Role of EPHOSS in Interpretation of Studies of HSCs/HPCs and Other Stem/Progenitor Cell Types

Our studies on EPHOSS [20**] clearly link this phenomenon to HSCs, HPCs, and regulation of hematopoiesis. However, we believe that this phenomenon has much broader implications, not only for understanding the potential true *in vivo numbers*, characterization, and function of HSCs and HPCs, but also in the context of development and pathologic cell types. Many types of adult stem cells exist naturally in niches *in vivo* that are hypoxic [37], and ESCs, which are found in the inner mass of blastocytes, and cancer stem cells (CSCs), reside in hypoxic environments [38–40]. ROS is important in the growth, differentiation and the regulation of these cells [24*,41–43]. *Ex-vivo* growth in lowered O_2 tension favors the growth of ESCs, induced pluripotent stem cells (iPSCs), CSCs, as well as MSCs, ASCs, and other cells [24*,44–47]. While much has been written about the metabolism of HSCs, HPCs, ESCs, CSCs, and MSCs amongst a plethora of other stem/progenitor cell types [48*,49–53], critical consideration should now be given as to how accurate these measurements and analyses are with regards to the metabolism of these cells and their function *in vivo* in hypoxic environments. Thus, many studies on metabolism of stem/progenitor cells may have to be re-evaluated in context of EPHOSS. This is especially of relevance for future efforts of personalized medicine, since such treatments would be based on gene expression patterns and response of a person's tissue to *ex-vivo* treatment. However, metabolic profiling for the development of specific therapeutic strategies meant to target, for example, CSCs [54–56] may not accurately represent the metabolism of these cells as they exist in their microenvironment *in vivo*, since these cells are harvested and studied in atmospheric oxygen, and have already been subjected to consequences of EPHOSS.

Another area to consider for effects of EPHOSS would be aging and senescence and its effects on the metabolism, and response of stem cells from aged animals or humans to cytokines/growth modulating factors. Aging has detrimental effects on HSCs and many other tissue-specific stem cells [57–65]. Since ROS has been linked as a driver in the aging process, it is possible that stem cells from aged animals and humans may be especially susceptible to EPHOSS-linked production of mitochondrial ROS after collection and processing of these cells in ambient air. The therapeutic potential of even aged HSCs may be enhanced if EPHOSS is mitigated during their collection/harvest.

Many factors influence the regulation of stem/progenitor cells *in vivo*. For example, the enzyme, Dipeptidylpeptidase (DPP)4 which can truncate and change the functional activity of a large number of cytokines/growth factors, and other growth modulating proteins [66–69]. DPP4 is found within cells and in the serum, and is also present on cell surfaces of HSCs, HPCs, mature hematopoietic, and other cells as CD26. How DPP4 works *in vivo* and in context of EPHOSS remains to be determined.

Conclusion

EPHOSS is a new, interesting, and likely important phenomenon which will have relevance to understanding the true physiology and pathology of stem and progenitor cells and how they will best be assessed for future therapeutic modalities [20**]. Information on HSCs,

HPCs, and other stem and progenitor cells and their interactions with microenvironmental niche cells, which have reached extremely high levels of sophistication [70–73], may now also have to be considered for re-evaluation in the context of present and future knowledge of the effects of EPHOSS on cellular processes. EPHOSS may also influence metabolism and differentiation of lymphocytes, monocytes and granulocytes, and other mature tissue cells. Figure 1 diagrams the potential impact of EPHOSS, and implications of EPHOSS for different stem, progenitor, and more mature cell types, whether normal or from patients or animals with malignant and non-malignant disorders, and should be considered as an important and worthy pursuit. More mechanistic insight into EPHOSS is warranted.

Acknowledgements

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Key points

- HSCs and HPCs reside *in vivo* in a microenvironment that is quite hypoxic compared to that of ambient atmospheric O_2.

- Collection and processing of mouse BM and human CB in ambient air underestimates numbers of HSCs in these tissues due to EPHOSS that is triggered by higher O_2 than that found *in vivo*. This results in increased production of mitochondrial ROS, and differentiation of HSCs.

- EPHOSS appears to be irreversible and mediated by a *p53-cyclophilin D-MPTP* axis, and is associated with expression of *hif-1α*, and *miR210*.

- Mitigation of EPHOSS by collection and processing of BM and CB in hypoxia, or alternatively in air in the continuous presence of CSA, results in higher numbers of HSCs.
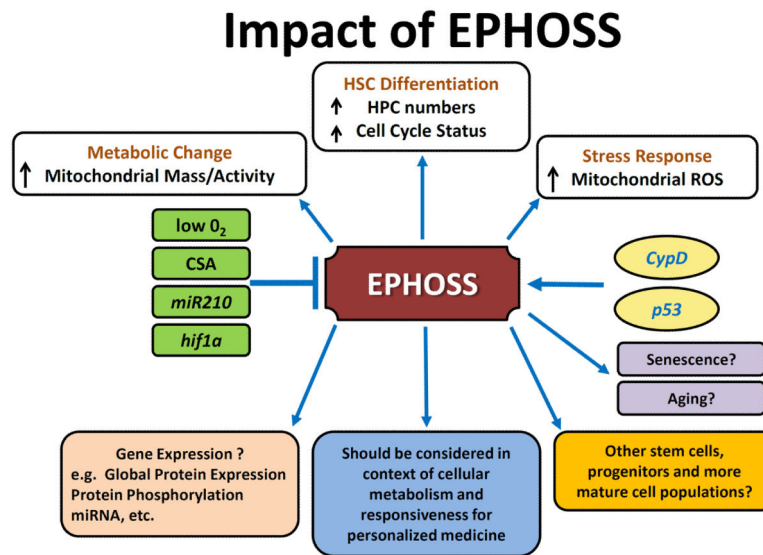
Figure 1.
A Diagrammatic representation of the impact of EPHOSS on cells and cellular processes. Abbreviations: EPHOSS, extra physiologic oxygen shock/stress; ROS, reactive oxygen species; miR, micro RNA; *hif1*α, *hypoxia inducing factor 1*α; O_2, oxygen; CSA, cyclosporine A; cypD; cyclophilin D; HSC, hematopoietic stem cell; HPC, hematopoietic progenitor cell.