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Reduced calcium influx in the hypoxia-tolerant *Spalax*: The role of the erythropoietin receptor

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ABSTRACT

Tissue hypoxia is a condition that induces calcium influx into living cells. Calcium is a major player in maintaining cell signaling and homeostasis, and mediates the regulation of gene transcription and cell proliferation; however, acute and aggressive calcium influx induced by hypoxia eventually leads to programmed cell death.

The blind mole rat, *Spalax*, is a wild-spread burrowing mammal adapted to hypoxic environments. A tyrosine -to- phenylalanine (F481 in *Spalax* corresponding to Y485 in human full-length receptor; Y460 in human mature form) substitution is found in the erythropoietin receptor of *Spalax* and other species, which was previously shown to be strongly involved in the calcium channels activation and subsequent calcium influx.

The current work aimed to explore the dynamics of calcium transport across *Spalax* nonhematopoietic cells' membrane compared to above ground rat and mouse, and the role of the erythropoietin receptor of *Spalax* in the regulation of calcium influx under hypoxia.

We show here that Epo-induced calcium influx in HEK293 cells transfected with *Spalax* EpoR is significantly lower than that of mouse; in hypoxia this difference was even more pronounced. Western blots confirmed a significant increase of Erk phosphorylation after stimulation with erythropoietin under hypoxia in cells transfected with mouse full length erythropoietin receptor compared to *Spalax*. Native primary fibroblasts showed lower cytosolic calcium concentrations in *Spalax* cells when compared to those of rats under normoxic and hypoxic conditions. *Spalax* EpoR appears to play an important role in preventing deleterious consequences of hypoxia and maintaining cellular homeostasis under stress.

1. Introduction

The mole rat, (genus *Spalax)* belongs to a group of burrowing wild rodents of the Eastern Mediterranean region. *Spalax* inhabits sealed underground tunnels that provide protection from climatic changes, pathogens and predation; however, surviving in such environment requires efficient adaptive mechanisms to cope with extreme conditions, such as severe hypoxia and hypercapnia [1,2].

Erythropoietin (Epo) is one of the factors that were shown to be greatly stimulated in *Spalax* under both moderate and severe hypoxia [3]. Epo is a member of an extensive cytokine family that includes growth hormone, prolactin, interleukins 2 through 7, and others [4]. It is a 34 KDa glycoprotein, playing the role of a hormone, cytokine and a growth factor, expressed in fetal liver and kidney cells after birth [5,6]. Hypoxia and anemia are the main events that induce Epo gene expression. Once produced, erythropoietin is released into the blood flow towards the cells expressing erythropoietin receptor (EpoR). Epo physically interacts with EpoR homodimers that are expressed on the erythroid cell surface to stimulate erythropoiesis by generating a complex network of molecular signals involved in the control of cell proliferation, differentiation and death (reviewed in Ref. [7]). However, Epo circulates also in several non-hematopoietic tissues (like neurons and pancreatic cells), where it plays a role in the protection from apoptosis, ischemia and inflammation due to hypoxia, toxicity or injury [7]. EpoR is a member of the superfamily of cytokine receptors [8,9] with no kinase or other enzymatic activity identified [reviewed in Refs. [7–10]]. Human cytoplasmic EpoR region has eight tyrosines; upon Epo binding the receptor undergoes homodimerization followed by a conformation change leading to multiple tyrosine phosphorylation and activation of more than 40 different binding domains [reviewed in Ref. [10]]. JAK2 is the first signaling molecule that binds to the EpoR [11], allowing the phosphorylation of STAT5, calcium/PKC pathway and MAPK/ERK signaling cascade, phosphatidylinositol 3-kinase (PI3K) and the activation of serine/threonine protein kinase B (Akt), thereby

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triggering the signal transduction cascade necessary for Epo's biological activities [4,7,11–13].

Importantly, Epo induces a rapid rise in cytosolic free calcium concentration as well as activation of calcium-dependent protein kinase [14,15]. The increase in erythroblast Ca^{2+} is mediated by voltage-independent calcium channels [16,17] – a process later characterized as a complex interaction involving the transient receptor potential channels TRPC2 and TRPC6, PLCγ, and inositol 1,4,5-trisphosphate receptor [18,19]. Other members of TRPC family are also involved in Ca^{2+} regulation in different tissues and cell types [20]. Ca^{2+} is a second messenger molecule in the cell that is involved in cell signaling processes crucial for the regulation of healthy functions of neurons, muscles and other tissues [21]. Tuning of cytosolic calcium levels can either promote cell survival or cell demise and apoptosis [21–23]. Whilst in the resting cells, calcium concentration in the cytosol is maintained at low levels, typically 100 nM [22,24], the concentration can increase dramatically in response to a variety of environmental factors such as stressors and hormones. The increase of calcium concentration can be derived from the extracellular space or from the mitochondria, and the ER [24]. Moreover, under hypoxia, the elevation of the cytosolic Ca^{2+} stimulates the translation of HIF1-α, the protein needed for adequate cellular response despite the protein synthesis repressing conditions [25].

In a research conducted in 1999, researchers tested the impact of the eight cytosolic tyrosines of the EpoR on calcium influx upon EPO stimulation, and revealed that the Y460 has a crucial role in the increase of the cytosolic calcium levels [26]. Interestingly, a multiple alignment of EpoR sequences revealed that amino acid corresponding to Y460 in the human receptor is substituted by phenylalanine in the wild type *Spalax* EpoR [27]. Among all mammalian species for which EpoR sequence was published, only nine mammalian species harbor a phenylalanine substitution in position 460. Notably, six species out of the nine are *aquatic/subterranean* mammals. While *Spalax* is the only rodent, the rest of the species belong to carnivores (Table1).

Severe hypoxia and oxygen fluctuations challenges faced by *Spalax* in its natural habitat, and the importance of maintaining subtle changes in cytosolic calcium concentrations, lead us to the assumption that *Spalax* EpoR plays role in maintaining lower -cytosolic calcium levels protecting it from hypoxia and severe oxygen levels fluctuations.

In the present study we found that EpoR-mediated calcium influx is reduced in *Spalax*, likely evolved as an adaptation to protect cells from calcium-induced apoptosis in hypoxia.

2. Results

2.1. Cytosolic calcium concentrations in primary fibroblasts

Since *Spalax* is a hypoxia tolerant mammal, we hypothesized that it evolved strategies to minimize the deleterious effects of hypoxia. To test this, the overall cytosolic free calcium concentrations of *Spalax* native lung/skin primary fibroblasts were compared to these of a rat. The cells were cultured under normoxic or hypoxic conditions, and the concentrations were calculated according to the K_d obtained from the calibration curve (Appendix Figs. A1 and A2; Table A3). While rat cells demonstrated an expected significant increase in cytosolic calcium concentrations under hypoxic conditions, *Spalax* cells showed a milder calcium influx increase, with a stronger response to hypoxia in the lungs compared to the skin. Remarkably, in both tissue types the calcium concentrations under hypoxic conditions in *Spalax* cells did not exceed the values of rat cells under normoxic conditions (Fig. 1).

Table 1
Nine mammalian species harbor Y460F substitution in EpoR.

Species	EpoR fragment	Ecological niche	Order
Spalax	SSNGPFSHPYENSL	Subterranean	Rodents
Walrus	SS-GPFSNPYENSL	Aquatic	Carnivores
Sea otter	SSNGPFSNPYENSL	Aquatic	Carnivores
Hawaiian monk seal	SSNGPFSNPYENSL	Aquatic	Carnivores
Weddell seal	SSNGPFSNPYENSL	Aquatic	Carnivores
Polar bear	SSNGPFSNPYENSL	Semi aquatic	Carnivores
Panda	SSNGPFSNPYENSL	Others	Carnivores
European polecat	SSNGPFSNPYENSL		Carnivores
Dog	SLNSPFLNPYENSL		Carnivores

2.2. Calcium concentration dynamics mediated by Spalax and mouse full length EpoR expressed in HEK293 cells

In order to assess the elevation of the cytosolic EpoR-dependent calcium concentrations, we transfected HEK293 cells with *Spalax*, mouse full length EpoR, or a vehicle (empty vector). Immunofluorescent analysis was applied to confirm the expression of HA-tagged EpoR. Fig. 2 depicts EpoR expression in HEK293 cells. The quantification of the fluorescence signals demonstrated successful expression, with notably higher levels of *Spalax* EpoR than the mouse counterpart. Noteworthy is the observation that transfected *Spalax* EpoR demonstrates higher abundance on cell surface, shown as higher co-localization of the receptor with stained membrane structures (See Appendix Fig. A12).

Ca^{2+} response to Epo-stimulation under normoxia or hypoxia was then characterized in HEK293 cells stably expressing erythropoietin receptor. Fig. 3 shows the elevation of the cytosolic calcium concentrations 40 min after the stimulation with Epo under normoxic or hypoxic conditions (6 h in 1% O_2, 5% CO_2 and 37 °C). The measured fluorescence intensity demonstrates massive calcium influx into cells expressing mouse EpoR, with remarkably high levels in hypoxia (Fig. 3).

To quantify the calcium elevation under hypoxia after stimulation with Epo, each cell was marked and the initial and the maximal signal were taken to calculate the ratio of F_t/F_0 which shows the percentage of the calcium elevation after the stimulation. Then, the average ratios were calculated for each cell line type and divided by the average ratios of the control group (pcDNA- transfected cells) under the corresponding conditions.

$$Ratio = \frac{Fluorescent\ signal\ after\ stimulation}{Fluorescent\ signal\ before\ stimulation} \times 100\%$$

Fig. 3 shows the average ratios of calcium elevations in *Spalax* and mouse EpoR-transfected cells under normoxia and hypoxia as a relation to the corresponding control group of the pcDNA transfected cells (See Appendix Table A7 for the number of cells and positions of each group of interest, randomly chosen before the start of the time lapse experiment). This experiment demonstrates that the response to Epo stimulation is greater in mouse compared to *Spalax*, and is more prominent under hypoxic conditions (~2-fold in -mouse, compared to ~1.3 fold in *Spalax*).

2.3. A delay in calcium influx mediated by Spalax EpoR

To assess the difference in the *response time* to Epo stimulation between mouse- and *Spalax* EpoR- transfected cells, the cytosolic calcium concentrations were recorded by time-lapse fluorescence microscopy (see Appendix Videos A10 & A11). It was observed that under hypoxic conditions, the cytosolic calcium concentrations in mouse EpoR-expressing cells started to rise after 10 min following stimulation with Epo, whereas in the *Spalax* EpoR-transfected cells the cytosolic calcium concentrations started to mildly rise only 20 min following stimulation. This suggests that the response time may have an important role in the reaction to Epo stimulation under hypoxia (Fig. 4; refer to Appendix Videos A10 & A11 for time-laps imaging).