Molecular and Physiological Factors of Neuroprotection in Hypoxia-tolerant Models: Pharmacological Clues for the Treatment of Stroke

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ABSTRACT: The naked mole-rat possesses several unique physiological and molecular features that underlie their remarkably and exceptional resistance to tissue hypoxia. Elevated pattern of Epo, an erythropoietin (Epo) factor; c-fos; vascular endothelial growth factor (VEGF); and hypoxia-inducible factors (HIF-1α) contribute to the adaptive strategy to cope with hypoxic stress. Moreover, the naked mole-rat has a lower metabolic rate than any other eutherian mammal of comparable size that has been studied. The ability to actively reduce metabolic rate represents a strategy widely used in the face of decreased tissue oxygen availability. Understanding the different molecular and physiological factors that induce metabolic suppression could guide the development of pharmacological agents for the clinical management of stroke patient.

KEYWORDS: brain injury, hypoxia, naked mole-rats, stroke, metabolic suppression, neuroprotection

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Introduction

The naked mole-rat is a eusocial rodent that exhibits several unique biological features that underlie its ability to resist tissue hypoxia. Physiological hypercapnia that is combined with hypoxia in the burrowing environment of naked mole-rats is a synergistic natural neuroprotective mechanism. The naked mole-rat retains in the adult life the metabolic features of embryonic metabolism, such as deficient thermoregulation, fetal hemoglobin, and low thyroid hormones.[1] These features contribute to the healthy life duration and the observed hypoxia tolerance ability in the naked mole-rat. General features of resistance to tissue hypoxia include progressive dynamics of physiological functions.[1] Following the onset of a stroke, adaptation to tissue hypoxia is impaired,[2] resulting in the death of metabolically vulnerable neurons in stroke patients.

However, a hypoxia-tolerant mammal such as the naked mole-rat has the ability to resist tissue hypoxia by actively reducing brain oxygen demand to match the supply.[3–5] This is functionally related to different protective strategies, including modulation of immune response, thrombolysis, antioxidant defense, and activation or inactivation of pre-existing proteins.[3,6] These mechanisms reflect a cassette of events that work in synergy at the cellular and systemic levels to compensate for the discrepancy between oxygen supply and demand. The expansion of knowledge will now depend on understanding that under conditions where adaptive homeostatic mechanisms are properly initiated, damage to the brain during stroke can be considerably reduced or even prevented by adapting the strategies used by the naked mole-rat to maintain homeostasis of brain energy demand and supply. The general knowledge on neuronal defense mechanisms against hypoxia is still incomplete, and a cohesive analysis of this phenomenon in a hypoxia-tolerant species has been difficult to achieve. With the hope of contributing to the existing discourse on the defense mechanism against hypoxia, we focus on the physiological and molecular adaptations in the naked mole-rat. We highlight specific physiological and molecular characteristics of hypoxia tolerance in the naked mole-rat; the longest living rodent and a unique model of hypoxia tolerance. We describe how these adaptations might provide potential clues on strategies to adapt for the clinical management of tissue hypoxia during conditions such as stroke where oxygen demand fails to match the supply.

Physiological Mechanism of Hypoxia Neuroprotection in the Naked Mole-rat

Resistance to tissue hypoxia is thought to be generally controlled by metabolic suppression in the naked mole-rat.[7]

Metabolic suppression is a physiological adaptation that is associated with oxygen consumption being actively reduced without causing any harm to the brain and other vital organs when exposed to hypoxic insults.[8–10] Metabolic suppression is the altered state of metabolism adaptation that occurs in hibernators[3] and hypoxia-tolerant species,[11] such as the naked mole-rat.[12] Since the metabolic rate is at a declined level in a hypoxia-tolerant mole-rat, the supply of adenosine triphosphate (ATP) through glycolysis and oxidative phosphorylation pathways must match the cellular demand for ATP. This is a vital prerequisite that must be met for the long-term survival of the naked mole-rat during severe hypoxia. A fall in the metabolic rate may be associated with a decline in both brain energy (ATP) demand and supply, prompting the onset of a new lower level of metabolism for ATP turnover during the period of oxygen deficiency in the brain of the naked mole-rat.

There are two possible perspectives to support the idea of metabolic suppression strategy of neuroprotection in the naked mole-rat. (I) Metabolic regulation could act as the inherent mechanism for neurons to defend the cell against hypoxia. (II) A cellular stress response mechanism and synthesis of stress proteins may protect the cell. The mechanism may occur by posttranslational alteration of proteins or by expression of new proteins through a signal transduction system to the nucleus. These cascades of events may in turn reinforce the influence of survival factors or may even inhibit apoptosis. The cellular stress response and synthesis of stress proteins may lead to an increased capacity for a healthy environment inside the cell. These proteins work as cellular "chaperones" by unfolding misfolded cellular proteins and helping the cell to dispose of unneeded denatured proteins.[13] Recent experimental data have demonstrated the importance of the processing of unfolded proteins in cell survival and cell death[14] that could represent part of the salvage mechanism of metabolic programming in a hypoxia-tolerant mole-rat. The metabolic rescue process in the naked mole-rat is thought to facilitate multiple-fold systematic suppression of glucose and oxygen demand and supply pathways throughout the hypoxia period resulting in neuroprotection. In this context, the brain of the naked mole-rat may be protected from hypoxia by using multiple mechanisms that can actively induce metabolic suppression for cellular survival. If the brain metabolism of humans can be manipulated and accelerated by a drug treatment that is safe and effective enough, it could greatly improve the treatment of stroke. Successful elucidation of the mechanisms of metabolic regulation as shown by the naked mole-rat could represent a new and metabolic therapy for the clinical management of stroke.

In animals without the capability to regulate brain metabolism, the onset of hypoxia causes damage of brain and other vital organs, indicating that the ability to maintain oxygen homeostasis is necessary for the survival of all vertebrates. The evolution of specialized structures for oxygen delivery that includes a doorway (lungs), a transportation medium (red blood cells), the highway route (main arteries), local routes (vasculature), and a pumping machine (heart) provides the multifaceted physiological machineries necessary for optimal oxygenation in the cells. Such multifaceted physiological machineries are present in all mammals, including humans. The naked mole-rat (*Heterocephalus glaber*) has the ability to use the same physiological machineries to facilitate a unique physiological strategy of metabolic suppression to target cellular oxygen concentration and initiate protective responses. This ability is not common in humans.

In a series of experiments approved by the ETSU committee for animal care research, we determined whether the metabolic adaptive responses initiated at the cellular level during acute and chronic hypoxia adaptations in the naked mole-rat can be induced by hypoxia. As shown in Figure 1, hypoxia significantly decreased metabolic rates in the naked mole-rat. Although core body temperature (Tb) also decreased during hypoxia, the effect of hypoxia in suppressing core Tb was not significant, indicating that the naked mole-rat can actively suppress its metabolic rate when exposed to extreme hypoxia (3%) under constant ambient temperature. Collectively, these results suggest that suppression of brain metabolism could represent a neuroprotective strategy applicable in conditions where delivery of oxygen and nutrients fails to meet demand and represents hypoxic preconditioning state that could protect metabolically vulnerable neurons in the brain of stroke patients.

An early study by Buffenstein and Woodley[15] revealed that physiological adaptations act as the molecular oxygen sensor that can be directly linked to gene regulation during adaptation to tissue hypoxia by the naked mole-rat. Understanding intracellular pathways that transmit hypoxic stimulus to gene activation could provide the clue on how hypoxia-tolerant neurons sense changes in oxygen dynamics and create signals that have immediate and long-term effects on neuronal survival in stroke patients.

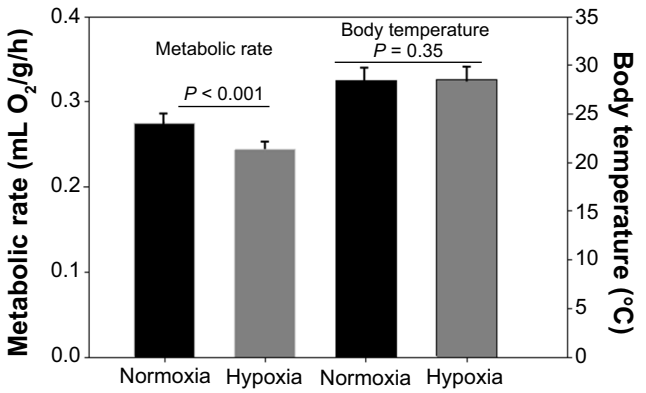

Figure 1. Metabolic rates and Tb of naked mole-rats during normoxia and hypoxia. Naked mole-rats were exposed to 60 minutes of normoxia (21% O_2), followed by 60 minutes of hypoxia (3% O_2). Metabolic rates were not altered during normoxia (ANOVA (F[5,95] = 1.09; P = 0.38)). Core Tbs were not significantly different (ANOVA (F[5,95] = 0.80; P = 0.55)). One-way ANOVA revealed a significant effect of hypoxia (F[5,35] = 3.48; $P < 0.001$) in suppressing metabolic rate during hypoxia.

The Relationship Between Molecular Factors and Hypoxia Tolerance in the Naked Mole-rat

Cumulative evidence indicates that establishing the protective phenotype in response to the deleterious effect of hypoxia depends on a coordinated response at the physiological, molecular, cellular, and tissue levels. It has been shown that the same genes that are upregulated during hypoxia preconditioning are also upregulated during tissue repair,[16] suggesting that focusing on the gene targets that detect tissue hypoxia will be a novel approach to counteract tissue hypoxia in stroke patients. The demonstration that there are specific molecular changes during chronic hypoxia in the naked mole-rats provides insights into the molecular mechanisms that are associated with the resistance to tissue hypoxia. This is possible because telomere shortening as well as the free radical theories that provide classical explanations for the biology of resistance to tissue hypoxia and longevity[17] are both connected to oxygen availability. Interestingly, the longevity ability in the naked mole-rat simulates phenotypically starvation conditions, including hypoxia.

Several molecules associated with hypoxia tolerance in the naked mole-rats have been documented. For instance, real-time polymerase chain reaction (PCR) comparative analysis of gene expression of erythropoietin (Epo), a key regulator of circulating erythrocytes and hypoxia-inducible factors (HIF-1α) in the mole-rat, revealed a significantly elevated, quicker, and longer response to different oxygen levels in the mole-rat when compared with white rats.[18] After a four-hour exposure to hypoxia at 6% oxygen, Epo level in mole-rats was three-fold elevated than in white rats, and after 24 hours of 10% oxygen, Epo level in mole-rats was remarkably six-fold maximally expressed than in white rats. The HIF-1α level during normoxia was two-fold elevated in mole-rats than in white rats. HIF-1α expression in mole-rats was found to achieve maximal expression after four-hour hypoxia at 3% oxygen. There was a two-fold increase compared with normoxia, whereas no significant change was detected in HIF-1α level in white rats at any of the conditions studied. These findings suggest that Epo level is a major factor that facilitates the hypoxia tolerance ability of the naked mole-rat. Although there is no direct connection between HIF-1a or Epo and aging, the influence of the HIF or Epo system on hypoxia tolerance in the longest-living rodent suggests the potential in developing these molecules into pharmacological agents for the clinical management of tissue hypoxia during stroke.

The naked mole-rat is an extreme wild species that is constantly exposed to severe levels of hypoxia. It differs dramatically from high-altitude mammals that are often limited in their vertical rise and hypoxic exposure.[18] The mole-rats are also different from aquatic anoxic-tolerant species that face anoxia for extended periods of time. This difference is because the naked mole-rats live all its life mostly underground in sealed burrows[19] and can tolerate hypoxia of about 3% oxygen.[20] Permanent activity under persistent hypoxia requires unique molecular mechanisms to regulate metabolic activities within the brain tissue. Existing lines of evidence indicate that erythrocyte supply is critical for the hypoxia tolerance ability of the naked mole-rats and that Epo expression is adapted to oxygen fluctuations in the brain of the naked mole-rats.

To thwart the possible deleterious effects of hypoxia in the brain, the mole-rat can initiate Epo synthesis at the mRNA level regulated by transcription and posttranscriptional mechanisms as the immediate molecular response. This response is orchestrated by HIF-1, which is the mammalian chief facilitator of oxygen homeostasis.[21] During Epo synthesis, the HIF-1α subunit responds to hypoxia, and its activation is directly connected with oxygen-dependent accumulation of HIF-1α protein.[21] The synthesis of Epo mRNA during the first 24 hours of hypoxia in the naked mole-rat is adequate to induce a suitable rate of erythropoiesis for the maintenance of a stable state under longer hypoxic conditions. Epo is known to have nonerythropoietic functions in the naked mole-rat because it protects metabolically vulnerable neurons in the brain from ischemic or hypoxia damage.[22] Taken together, Epo is generally known to play a vital role in the modulation of the ventilatory acclimatization of mole-rats to hypoxic exposure.[23] Thus, understanding the triad mechanisms that control its synthesis in mole-rats can provide potential clues for a pharmacological target in the clinical management stroke.

c-fos is a known powerful metabolic indicator, as it triggers the upregulation of downstream genes, such as tyrosine hydroxylase gene, during hypoxia.[24] The activation of c-fos is important for the survival of neurons, because it indicates the selective susceptibility of a specific brain region when exposed to tissue hypoxia.[25] In a series of experiments, we qualitatively compare c-fos levels in hippocampus (CA1) following exposure to hypoxia and normoxia conditions (Fig. 2) for five days. These experiments were approved by the ETSU committee for animal care research.

Our results revealed that the c-fos-positive cells increased in naked mole-rats that were repeatedly exposed first to normoxia followed by 3% O_2 for 60 minutes. The significant effect of chronic hypoxia in activating c-fos when compared with mole-rats that were exposed to normoxia indicates the functional significance of c-fos during extreme hypoxia conditions. Changes in c-fos expression are directly linked with hypoxia, probably because these changes may provide adaptive responses to hypoxia. The increase in c-fos expression in naked mole-rats during hypoxia indicates that the brain of the naked mole-rat is responsive to hypoxia at the level of gene expression involving immediate early gene (IEG) products, such as c-fos. It is possible that c-fos may represent an active response mechanism that is upregulated rapidly in response to hypoxia at the transcription level. This may contribute to the first line of defense against tissue hypoxia. For this reason, organized modulation of c-fos expression might be significant for initiating phenotypic protective changes in the central nervous system of a stroke patient.

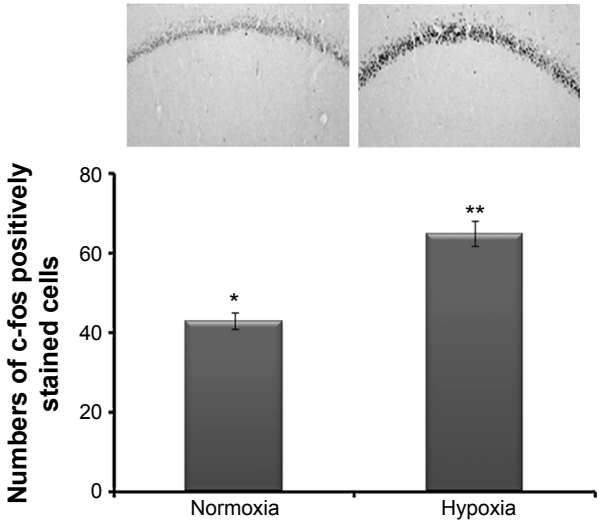

Figure 2. Immunoreactivity and quantification of c-fos-positive cells during normoxia or hypoxia condition. c-fos immunohistochemistry analysis was performed on the hippocampus (CA1) of normoxia and hypoxia naked mole-rats ($n = 12$) for five days. Top figure represents immunoreactivity in CA1, while bottom figure indicates quantification of c-fos-positive cells. Scale bar = 100 μm. Quantification of c-fos-positive cells was done in the CA1 of normoxia naked mole-rats (left panel) and hypoxia-tolerant naked mole-rats (right panel). Numbers of c-fos-positive cells in hypoxia-tolerant naked mole-rats were higher (**$P < 0.005$) when compared with mole-rats kept in normoxia (*$P < 0.005$).

Vascular endothelial growth factor (VEGF) is also a potential target of neuroprotection in hypoxia-tolerant mole-rats.[26] A study by McCloskey et al[27] measured the VEGF levels of mole-rats exposed to 3% oxygen to determine anti-tumor mechanism in them. They observed a significant increase in VEGF in mole-rats after hypoxic stress. The results suggest that the inherent ability of the naked mole-rat to resist tissue hypoxia prevented cellular upregulation of VEGF and programmed cell death under hypoxic stress. Indeed, the decline of VEGF expression during hypoxia correlates with an aged-dependent impairment of angiogenesis.

There are important conceptual issues about VEGF and other molecules in non-hypoxia-tolerant models that make them important factors to consider in the clinical management of tissue hypoxia during stroke. For instance, the VEGF is an angiogenic protein with therapeutic potential in hypoxia-brain injury disorders, including stroke. VEGF exerts its effects predominantly via receptor tyrosine kinases VEGFR-1(flt-1), VEGFR-2(flk-1/KDR), and neuropilins-1 and -2. Endogenous neuronal VEGF regulation in the hypoxic brain plays a neuroprotective role in the pathophysiologic processes that follow stroke, while exogenous VEGF, directly administered or overexpressed by gene delivery, into the brains of rodents reduces ischemic brain infarct and decreases hypoxic/ischemic neuronal cell death.

VEGF also has direct effects on neuronal cell types. In explant cultures of dorsal root ganglia, VEGF stimulates axonal outgrowth and promotes the survival of neurons and satellite cells.[28] VEGF also has neurotrophic effects on cultured neurons of the CNS. In mesencephalic explants of rats, VEGF promotes the growth and survival of dopaminergic neurons and astrocytes.[29] The increase in the numbers or length of neurites in cultured neurons or cortical explants caused by VEGF involves signaling through VEGFR-2/flk-1, MAP kinase, and phosphatidylinositol 3′-kinase/Akt pathway. VEGF abrogates cell death of hippocampus neurons subjected to serum withdrawal or hypoxia in vitro by activating VEGFR-2, PI3K/Akt, and the transcription factor NF. VEGF also protects cultured cerebral cortical neurons from hypoxia by decreasing activation of caspase-3. Whereas a great deal is known of the cytoplasmic signaling pathway activated by VEGF, much less is known of the mechanisms through which VEGF communicates with the cell nucleus. An area of potential interest is to identify the pathways through which cytoplasmic signaling downstream of VEGFR-2/Flk-1 transmits signals into the nuclei of neurons and glial cells.

Conclusion

Naked mole-rats, with their several strange thermoregulatory and behavioral attributes, provide an intriguing model for studies of mammalian metabolic regulation as a novel approach in the treatment of hypoxia-induced brain injury. The mechanisms used could represent a strategy that can be used to develop pharmacological agents to tip the balance away from cell death toward cell survival during stroke. Brain metabolic regulation in an ischemic stroke patient would induce a state of neuroprotection by mitigating energy-consuming processes. This will help brain cells deal with the sudden disruption of oxygen delivery. The induction of metabolic suppression may as well provide an additional time period of protection until specialized treatment can reopen occluded blood vessels and allow extraction of oxygen in the brain of an ischemic stroke patient.

Author Contributions

Conceived and designed the experiments: TIN, AIN. Analyzed the data: JOS, AA. Wrote the first draft of the manuscript: TIN. Jointly developed the structure and arguments for the paper: TIN, JOS, AA, AIN. Made critical revisions and approved final version: TIN, JOS, AA, AIN. All authors reviewed and approved of the final manuscript.

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