Adaptive hypoxic tolerance in the subterranean mole rat *Spalax ehrenbergi*: the role of vascular endothelial growth factor

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Abstract ***Spalax ehrenbergi*** **has evolved adaptations that allow it to survive and carry out normal activities in a highly hypoxic environment. A key component of this adaptation is a higher capillary density in some** ***Spalax*** **tissues resulting in a shorter diffusion distance for oxygen. Vascular endothelial growth factor (VEGF) is an angiogenic factor that is critical for angiogenesis during development and in response to tissue ischemia. We demonstrate here that VEGF expression is markedly increased in those** ***Spalax*** **tissues with a higher capillary density relative to the normal laboratory rat** ***Rattus norvegicus*****. Upregulation of VEGF thus appears to be an additional mechanism by which** ***Spalax*** **has adapted to its hypoxic environment.**

Key words: Capillary density; Hypoxia; Vascular endothelial growth factor; *Spalax ehrenbergi*

1. Introduction

Subterranean mammals have evolved a unique set of structural and functional modifications of their cardiovascular and respiratory systems that insure a successful adaptation to this extreme environment for prolonged periods of time. Nevo and colleagues over the past 30 years have used as a model for understanding these adaptations one such subterranean mammal, the mole rat *Spalax ehrenbergi* [1]. The critical P_{O_2} (the lowest oxygen pressure supporting life) is lower for *Spalax* than for any other rodent studied to date. This capacity to withstand hypoxic conditions even exceeds that manifested by many high altitude and diving mammals [2–11].

The superspecies *Spalax ehrenbergi*, belonging to the family Spalacidae, includes in Israel four biological species that differ not only in their chromosomal number (2n = 52, 54, 58 and 60) but also their habitat [1]. *Spalax* lives predominately in underground tunnels in which the oxygen tension is often severely reduced. A number of strategies that are used by *Spalax* to tolerate this environment have been elucidated. First, *Spalax* can achieve a higher myocardial maximal oxygen consumption under hypoxic conditions [12,13]. Second, *Spalax* demonstrates structural adaptations in tissues such as skeletal muscle that result in a reduction in the diffusion distance of oxygen to the mitochondria and permit efficient oxygen delivery even at low capillary P_{O_2} [12,14]. These adaptations consist of a significant increase in both the mitochondrial density and the capillary density. Third, there is a marked increase in *Spalax* lung diffusion capacity which permits the continued oxygenation of blood to occur in *Spalax* lung even in the presence of a decreased inspired P_{O_2} [3,14–17]. Fourth, specific differences in the abundance of molecules such as myoglobin which augment oxygen delivery particularly at low oxygen tensions have been demonstrated [3,18]. Fifth, the blood properties of *Spalax* have been reported to be adapted to facilitate O_2 transport in burrowing atmospheres via increases in the erythrocyte count [8], reduction in the mean corpuscular volume [14], and changes in the 2,3-diphosphoglycerate:hemoglobin ratio [2]. Finally, the primary structures of *Spalax* hemoglobin [19,20], haptoglobin [21] and myoglobin [22] display many amino acid substitutions as compared with other rodents, albeit the effect of these substitutions on the function of these molecules and their contribution to fitness are unknown. Significantly for the study of these adaptations, Nevo et al. [23] have shown in differential survivorship studies that the four chromosomal species of *Spalax* do not undergo acclimatization upon transfer from their natural habitat to a standardized laboratory environment but rather behave differentially according to their ecogeographic origins.

Recently, Widmer et al. [13] have incorporated these data into a hypothesis that the capacity of *Spalax* to do aerobic work under low O_2 pressures is due in large part to adaptations in the structural design of the cardiorespiratory system. A key component of these adaptations as mentioned above is a higher capillary density which results in a shorter diffusion distance for oxygen to the mitochondria. The molecular basis for this increased blood vessel density, specifically in *Spalax* skeletal muscle, is not understood.

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that is critical for angiogenesis during development [24] and in response to tissue hypoxia [25]. We and others have previously shown that the regulation of VEGF by hypoxia occurs at the level of its steady-state mRNA [25–30]. Therefore, in order to understand the molecular basis for the increased capillary density of *Spalax* we studied the regulation of VEGF mRNA in three species (2n = 52, 58 and 60) of the subterranean superspecies *Spalax ehrenbergi* as compared to the normal laboratory white rat *Rattus norvegicus*. We have cloned and characterized *Spalax* VEGF, measured blood vessel density in *Spalax* and *Rattus* tissues, and have quantitated VEGF mRNA in *Spalax* and *Rattus* tissues under a variety of conditions of graded hypoxia. Taken together our findings suggest an additional molecular mechanism for the hypoxic tolerance of *Spalax* through an increase in the angiogenic factor VEGF.

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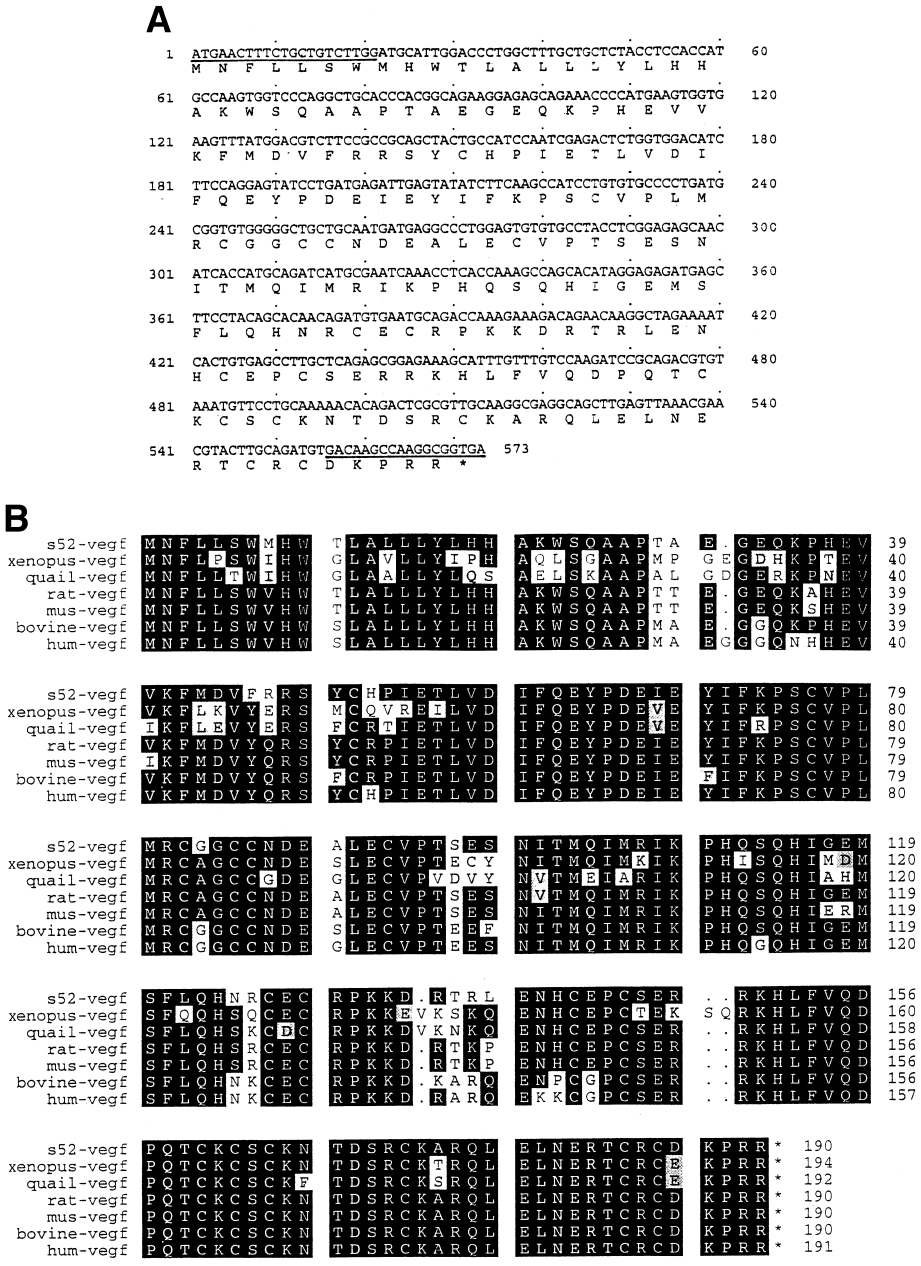

Fig. 1. A: Nucleotide and amino acid sequence of *Spalax ehrenbergi* (2n = 52) for the VEGF 165 amino acid isoform. Primers used for RT-PCR are underlined. B: Comparison of the amino acid sequence of *Spalax ehrenbergi* (2n = 52) VEGF 165 amino acid isoform with that from other species. The PRETTYBOX program from GCG software was used to determine the proper alignment. Black background indicates consensus sequence. White background marks non-conservative amino acid shift. Gray-stripped background marks conservative changes.

2. Materials and methods

2.1. Animals

The study was carried out on white rats (*Rattus norvegicus*) and subterranean mole rats (*Spalax ehrenbergi*). The chromosomal species characteristics of the different species of *Spalax ehrenbergi* have been previously described and include 2n = 52, 2n = 58 and 2n = 60 [1]. The 2n = 52 were from the Kerem Ben Zimra population, the 2n = 58 were from the Mount Carmel Mukhraka population and the 2n = 60 were from the Anza Samaria population. A total of six *Rattus* and six *Spalax* from each of the three chromosomal species were used in these studies. Animals were housed in individual cages, kept under controlled conditions of 22–24°C with seasonal light/dark hours. The *Spalax* used in these experiments were hunted in the field and kept in the animal house for 3–6 months. All animals used in this study were adults and of similar weight (100–150 g). Hypoxia was achieved by incubating the animals in a sealed $70 \times 70 \times 50$ cm chamber divided into separate cells into which defined O_2 or O_2/CO_2 mixtures were delivered at a flow rate of 3.5 l/min. The gas mixturours.

2.2. Tissue sampling

Animals were killed by intramuscular injection of Imalgene 100 (100 mg/ml ketamine, 5 mg/kg body weight injection, Rhone-Merieux, France). Tissue was immediately extracted and frozen in liquid nitrogen (for RNA analysis) or placed in 10% buffered formalin for morphometric analysis. For molecular analysis skeletal muscle (rhomboideus capitis) or whole brain was used. For morphometric analysis skeletal muscle (rhomboideus capitis) or specific brain sections from cerebral cortex, white matter, hippocampus and cerebellar cortex were studied.

2.3. Microvessel quantitation

Skeletal muscle and brain microvascularity were determined by

quantifying the density of capillary structures [13]. Capillary structures were identified immunohistochemically by staining with factor VIII-related antigen. Evaluation of vascularity was performed in microscopic fields where the myofibrils were sectioned transversely. Representative sections of cerebral cortex (temporal lobe), white matter (temporal lobe), hippocampus and cerebellar cortex were evaluated. A single microvessel was defined as any immunostained endothelial cell that was separated from adjacent microvessels, other cell types or connective tissue elements. The number of vessel profiles was counted at 400× magnification. The area of field of vision at this magnification is 0.55 mm. Six fields were counted for each tissue sample and the average number of counts was recorded as vessels per high power field.

2.4. Immunohistochemical methods

Sections from formalin-fixed paraffin-embedded tissue blocks were cut at 5 μm, deparaffinized and dehydrated with xylene and graded alcohol. Microwave/pressure cooker pretreatment (two cycles of 10 min each) was performed in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide at room temperature for 10 min. Sections were blocked with 10% goat serum for 30 min and then incubated with polyclonal rabbit anti-factor VIII-related antigen (EURODPC, Gwynedd, UK) for 1 h at room temperature. The labeled streptavidin-biotin-peroxidase method using the Histostain-Plus kit and AEC substrate from Zymed Laboratories (South San Francisco, CA, USA) was employed. Light counterstaining was performed with hematoxylin.

2.5. Isolation and sequencing of Spalax VEGF cDNA

Total RNA was prepared from *Spalax* 2n = 52 brain using RNA-clean system (Angewandte Gentechnologie, Heidelberg, Germany). First strand cDNA was prepared with oligo dT and reverse transcriptase (BRL, USA) and subsequently used in a PCR reaction (95°C, 1 min denaturation; 52°C, 50 s annealing; 72°C, 1 min elongation for 30 cycles) using oligonucleotides from the amino- and carboxy-termini of *Rattus* VEGF (sense 5′-atgaactttctgct(cg)tcttg-3′ and antisense 5′-tcaccgccttggcttgtc-3′). The PCR product was subcloned into pGEM-T easy vector (Promega, Madison, WI, USA) and *Spalax* VEGF was identified by colony hybridization using a partial *Rattus* VEGF cDNA as probe. Two independent clones were sequenced in both orientations (Weizmann Institute of Science, Rehovot, Israel). Analysis and characterization of the sequence was carried out using GCG software.

2.6. Analysis of VEGF mRNA in tissue

mRNA was prepared from tissue using RNA-Stat 60 (Tel-Test B, Inc., USA). Quantitation of VEGF mRNA was performed by RNase protection assay [28]. We have previously described the use of this riboprobe for the detection of *Rattus* VEGF mRNA [28]. Based on our nucleotide sequence of *Spalax* VEGF we were able to determine the size of the protected fragment in the RNase protection assay using the identical riboprobe. For *Rattus* VEGF mRNA a riboprobe containing exons 5, 7 and 8 of *Rattus* VEGF will protect a 239 bp fragment for VEGF isoform 165. Based on the sequence we have determined for *Spalax* VEGF this same riboprobe will protect a 145 bp fragment for *Spalax* VEGF isoform 165. Normalization was performed using 18S rRNA. Quantitation was performed using a Molecular Dynamics Phosphorimager.

2.7. Data analysis

Mean ± S.E.M. values are depicted unless indicated otherwise [31]. Student's *t*-test was used for comparisons between data sets and $P < 0.05$ was considered significant.

3. Results

3.1. Isolation of Spalax VEGF cDNA

A full length *Spalax* cDNA for VEGF of the 165 amino acid splice variant was obtained by RT-PCR of brain tissue from the 2n = 52 species using oligonucleotides from the translational initiation and termination sites of the previously published *Rattus* VEGF. The nucleotide sequence and the predicted amino acid sequence are presented in Fig. 1A. Fig. 1B depicts the amino acid sequences of VEGF of several vertebrates from different classes. Analysis of the amino acid sequence reveals that the percentage identity with the *Spalax* VEGF is 75.8% for *Xenopus*, 75% for the quail, 94.7% for the *Rattus*, 95.2% for the mouse, 91% for the bovine, and 91.5% for the human. There are three amino acid substitutions unique to *Spalax* and another four residues are unique to *Spalax* in Rodentia.

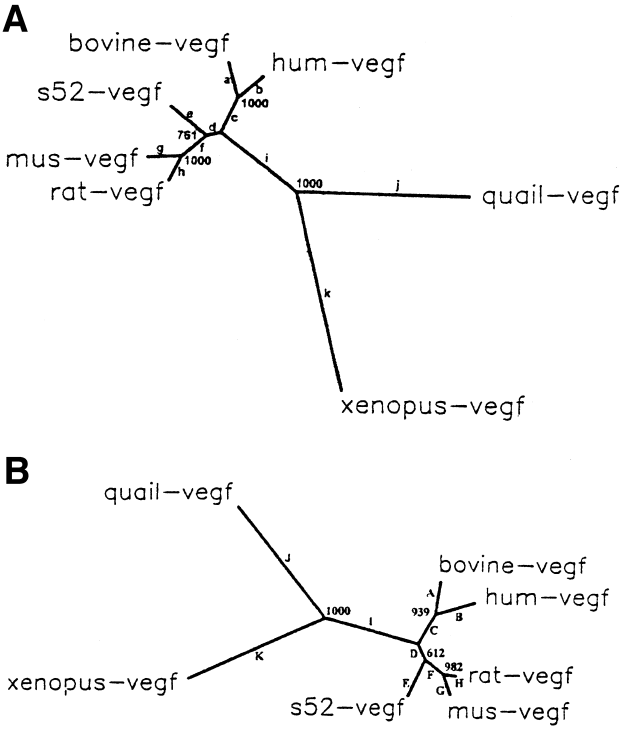

Fig. 2. Phylogenetic trees. The unrooted trees describe the evolutionary relationship among the VEGF 165 isoform of the specified species (S52 is *Spalax ehrenbergi*, 2n = 52). A: Nucleic acid tree based on Kimura [32] two parameter distance. B: Amino acid tree based on Kimura [32] protein distance. Both trees are derived from the GCG software. The numbers in the junctions are bootstrapping based on 1000 replications. The lengths of the branches are in A: a = 23; b = 20.6; c = 24.3; d = 9; e = 28.3; f = 19.7; g = 21.1; h = 17.4; i = 60.1; j = 108.5; k = 128.1 and in B: A = 25.7; B = 31.9; C = 27.2; D = 13.4; E = 31.4; F = 18.3; G = 16.5; H = 9.6; I = 75.4; J = 109.3; K = 113.5.

We have used the Kimura distance [32] to generate two phylogenetic trees (Fig. 2A,B). Fig. 2A is based on the distances between nucleic acids and Fig. 2B is based on distances between amino acids. The method examines each pair of aligned sequences item by item and counts the number of exact matches, partial matches and gap symbols. If the sequences are nucleic acids, transitions and transversions are also tallied. Both trees cluster mammals together separated from amphibians (*Xenopus*) and birds (quail). Within mammals rodents are separated from bovine and humans and within rodents *Spalax* diverged earlier than the *Mus* from *Rattus* with significantly high bootstrap values. The phylogenetic pattern found here is in accordance with those obtained from hemoglobin [19,20] and ribonuclease A [33].

3.2. Qualitative measurement of blood vessel density in Spalax and Rattus muscle and brain

We directly compared the relative microvessel density in

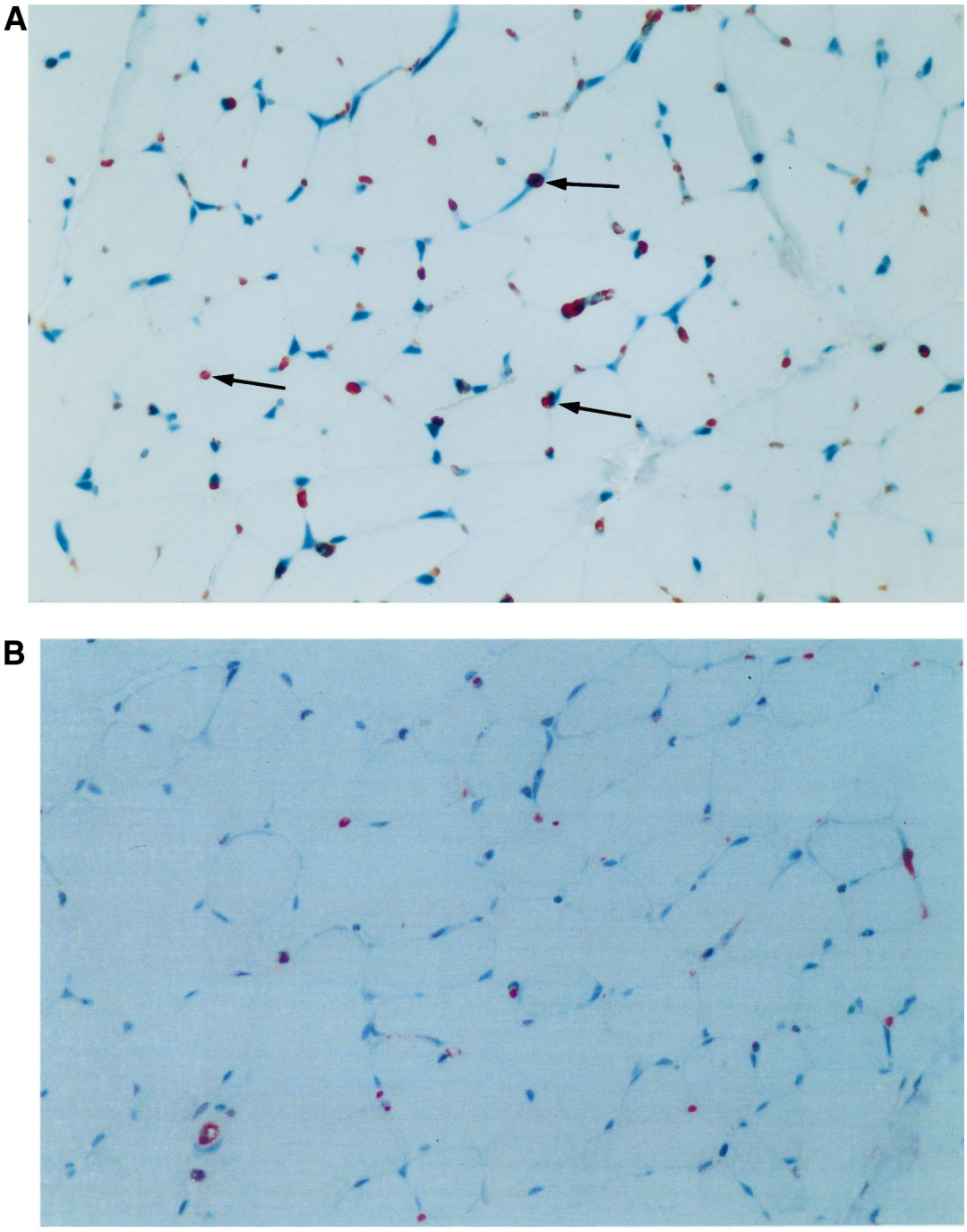

Fig. 3. Factor VIII-related antigen immunohistochemically stained skeletal muscle and cerebral cortical sections (400×). Vascular endothelial cells are stained orange red. A representative example of a stained endothelial cell is denoted with an → in panel A. An approximately two-fold increase in endothelial cell staining is seen in *Spalax* skeletal muscle (A) as compared to *Rattus* skeletal muscle (B) (157 ± 11.7 versus 73 ± 12.8 microvessels per high power field ($P < 0.05$)). No significant difference is seen in endothelial cell staining in *Spalax* cerebral cortex (C) as compared to *Rattus* cerebral cortex (D) (57 ± 4.0 versus 62 ± 4.8 microvessels per high power field (*P* N.S.)).

Spalax skeletal muscle and brain tissue using factor VIII-related Ag immunostaining. *Spalax* has previously been reported to demonstrate a substantially increased vascular density in skeletal muscle compared to *Rattus* [22]. Using a different, well described method to quantitate vascular density [34], we find a similar marked increase (two-fold) in vessel density in the *Spalax* compared to the *Rattus* muscle. Using the same procedure comparable brain sections from the *Spalax* and *Rattus* failed to reveal any significant difference in vascular density. The microvessel density in the *Spalax* brain was significantly lower than in the *Spalax* muscle. Photomicrographs of representative fields from *Spalax* and *Rattus* muscle and brain are shown in Fig. 3A–D.

3.3. *Measurement of VEGF mRNA in Spalax and Rattus under ambient conditions*

Spalax and *Rattus* VEGF mRNA isoforms 165 were quantitated by RNase protection assay (Fig. 4) [29]. We measured

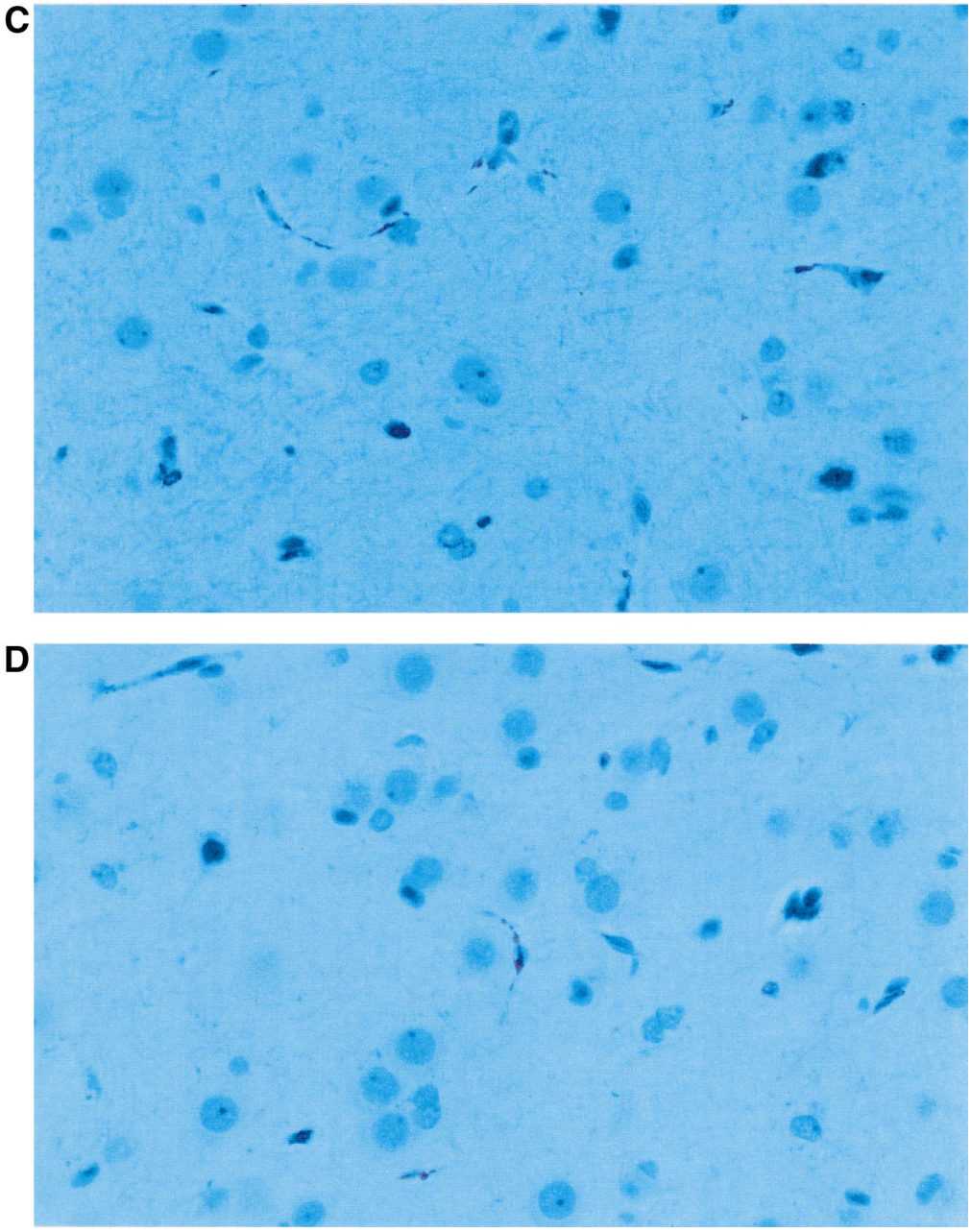

Fig. 3 (*continued*).

VEGF mRNA levels in muscle, brain, lung and heart tissue under normoxic conditions (21% O_2). A plot of VEGF mRNA versus capillary density for brain, heart, lung and muscle resulted in a linear regression with correlation coefficient of 0.95 (Fig. 5).

3.4. *Determination of the induction of VEGF mRNA in Spalax and Rattus under conditions of hypoxia*

We exposed *Spalax* or *Rattus* to conditions of controlled O_2 for periods of 2.5–11 h. *Rattus* were near death by 4 h of 6% O_2 or 2.5 h of 3% O_2. *Spalax* on the other hand demonstrated no deleterious effects under either 6% or 3% O_2 for up to 11 h. We found that VEGF mRNA was significantly increased by 2.2 ± 0.3 ($n = 4$, $P < 0.05$) and 1.6 ± 0.1 ($n = 4$, $P < 0.05$) times in *Rattus* muscle and brain respectively. For these studies we pooled all three chromosomal species of *Spalax* because no significant differences were found between the different *Spalax* species studied with respect to VEGF mRNA induction by hypoxia. We observed a 2.3 ± 0.2 ($n = 12$, $P < 0.05$) fold increase in *Spalax* brain and a small but significant decrease in VEGF mRNA in *Spalax* muscle with hypoxia of 0.7 ± 0.1 ($n = 12$, $P < 0.05$). No significant differences were observed between animals exposed to 3% or 6% O_2 nor were any significant differences observed in the presence of hypercapnia (6% CO_2) in addition to the hypoxia.

4. Discussion

Ecological mammalian niches which involve frequent diving [35], living at a high altitude [36] or living in an underground

habitat [1] all expose the animal to an environment low in oxygen. Diving mammals are exposed to hypoxia for brief periods followed by a return to a normoxic environment. High altitude mammals often are limited in their vertical ascent and hypoxic exposure more by the availability of adequate food supplies and often migrate from high altitudes to lower altitudes in response to this need. *Spalax*, on the other hand, is unique among most subterranean animals in that it spends much of its life underground in sealed burrows under differential levels of hypoxia. The only time that it may leave its underground territory is after weaning when the newborns are chased out of the mother's territory. Moreover, at least two of the species of *Spalax* studied here, the 2n = 52 and the 2n = 58, inhabit heavy soil with up to 800 mm of rain which dramatically limits soil ventilation and gas permeability [1].

One strategy taken by some species for surviving prolonged periods of hypoxia is a marked downregulation of energy requiring processes – such as myocardial and skeletal muscle contractile activity. This is manifested by a slowing of the heart rate and motor activity and a decrease in the basal metabolic rate of the organism [37–40]. However, maintenance of normal physical activity under conditions of chronic hypoxia requires a strategy to increase oxygen delivery to the tissues, especially those skeletal muscles used for tunneling and burrowing. *Spalax* achieves this essentially by decreasing the diffusion distance for oxygen in the skeletal muscle tissue by increasing the capillary density.

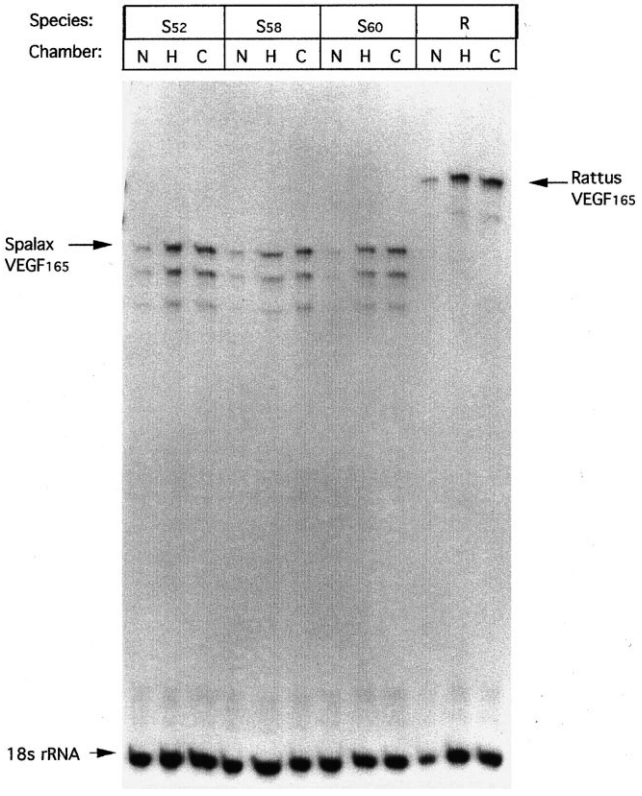

Fig. 4. Representative RNase protection assay (RPA) to quantitate *Spalax ehrenbergi* (three chromosomal species, 2n = 52, 58 and 60) and *Rattus norvegicus* VEGF mRNA. The VEGF antisense riboprobe will protect a 239 bp fragment from *Rattus* VEGF mRNA and a 134 bp fragment from *Spalax* RNA. This RPA analyzes *Spalax* and *Rattus* brain VEGF mRNA from animals incubated in a closed environmental chamber containing 21% O_2 (N), 6% O_2 (H) or 6% O_2/6% CO_2 (C). An antisense riboprobe for 18S rRNA was used for sample normalization.

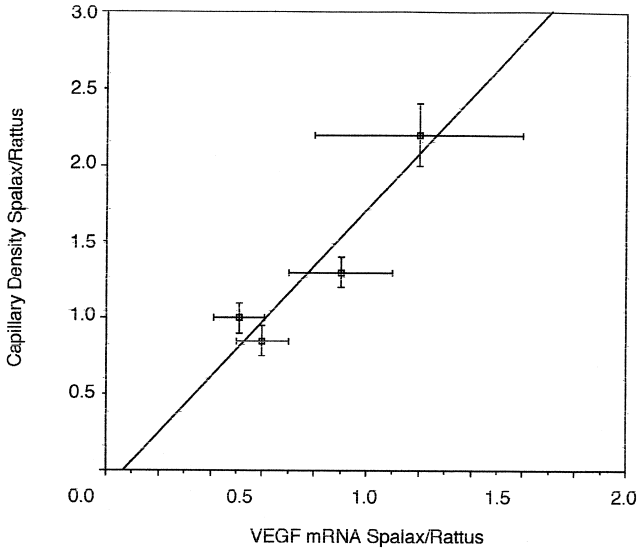

Fig. 5. Correlation between the relative blood vessel density and VEGF mRNA levels in *Spalax* and *Rattus* skeletal muscle, lung, heart and brain determined using Cricket Graph software. The ratio of VEGF mRNA in *Spalax* as compared to *Rattus* in skeletal muscle, lung, heart and brain was 1.2 ± 0.2, 0.9 ± 0.1, 0.6 ± 0.1, and 0.5 ± 0.1 respectively. The ratio of the blood vessel density in *Spalax* as compared to *Rattus* in skeletal muscle, lung, heart and brain was 2.2 ± 0.4, 1.3 ± 0.2, 0.85 ± 0.1 and 1.0 ± 0.1 respectively. Correlation coefficient is 0.95. For determination of VEGF levels $n = 4$ for each of the tissues. Determination of microvessel density in brain and muscle is as described in the text. Values for microvessel density for heart and lung are from Widmer et al. [13].

Capillary growth both developmentally [24] and in response to hypoxia [25] has been demonstrated to be critically dependent on the angiogenic factor VEGF. Therefore we decided to focus our studies on the regulation of VEGF in *Rattus* and *Spalax* tissues. To facilitate these studies we first cloned *Spalax* VEGF and found that it was remarkably similar to VEGF from other rodents but with a number of unique amino acid substitutions. Whether these substitutions result in an altered bioactivity for the protein remains to be determined.

We then set out to ask (i) Is there a relationship between the greater capillary density in certain *Spalax* tissues such as skeletal muscle and the amount of VEGF in those tissues? (ii) Is VEGF regulated by hypoxia in some unique way in *Spalax* tissues?

We provide here a plausible molecular explanation for the increased capillary density in *Spalax* skeletal muscle compared to *Rattus* skeletal muscle. Taken together with our measurements of VEGF mRNA in these tissues these studies show a clear and significant correlation ($r = 0.95$) between the relative levels of VEGF mRNA and the increased capillary density in *Spalax* as compared to *Rattus*. It will be interesting and future studies are considered to test if the findings shown here for *Spalax* are present in other subterranean mammals as well as in normoxic rodents of comparable body size and evolutionary distance.

VEGF is regulated by hypoxia at the level of its steady-state mRNA [25–30]. The regulation of VEGF mRNA in hypoxic *Rattus* in vivo was similar to that previously reported [41] with a significant increase noted in all tissues examined. We

also observed a marked increase in VEGF mRNA in brain from hypoxic *Spalax*. Unexpectedly, however ,we found no increase but rather a statistically significant decrease in skeletal muscle VEGF mRNA taken from *Spalax* subjected to hypoxia. (A similar small but not statistically significant decrease with hypoxia was also observed with *Spalax* lung tissue VEGF mRNA levels.) One possible explanation for why *Spalax* muscle does not increase VEGF mRNA with hypoxia is that in the in vivo hypoxia experiments the *Spalax* muscle does not reach a hypoxic threshold for induction of VEGF as opposed to *Rattus* which does reach this threshold. Conceivably, this could be due to any of a number of physiological adaptations utilized by *Spalax* in response to hypoxia as enumerated earlier in Section 1. A second possibility is that the molecular mechanisms controlling VEGF expression in *Spalax* muscle are different from those in *Rattus*. We believe that the first possibility is unlikely because if the in vivo hypoxic stress we have imposed on *Spalax* did not reach a hypoxic threshold in its skeletal muscle then we would expect to find no change in VEGF expression (levels similar to no hypoxic stress). However, we have reported a significant decrease in VEGF expression with hypoxia in *Spalax* muscle suggesting that the tissue is indeed responding, albeit unexpectedly, to this stimulus. It should be noted that the mRNAs for over 95% of all expressed genes are decreased in response to hypoxia in vitro and in vivo [38]. Thus in *Spalax* muscle VEGF is behaving in response to hypoxia similar to most other genes. This would suggest a different regulatory mechanism for VEGF in *Spalax* muscle in response to hypoxia. This hardly seems surprising in light of the fact that *Spalax* muscle clearly has a different mechanism for constitutive VEGF regulation than *Rattus*.

Hypoxia has been shown to induce VEGF mRNA in many cell types via transcriptional activation [26,27,29,42] and mRNA stabilization [25–28,43]. Determining the mechanism for the apparent paradoxical response in *Spalax* is a main focus of ongoing studies. These studies will require the cloning of the *Spalax* homologues of the *cis*-acting sequences (i.e. HIF-1 and HuR binding sites in *Spalax* VEGF mRNA) and the *trans*-acting factors (i.e. HIF-1, HuR) which have been previously shown to mediate the induction of VEGF mRNA by hypoxia in other species and cell types [43]. We have compared the regulation of another HIF-1-regulated gene in both *Spalax* and *Rattus*, erythropoietin, and preliminary studies have failed to detect any differences in its regulation between *Rattus* and *Spalax* under normoxic or hypoxic conditions in vivo which may suggest that changes in the HIF-1 molecule itself do not explain the differences reported in this study. We do not know what advantage downregulation of VEGF mRNA by hypoxia in *Spalax* skeletal muscle might provide. Perhaps this is related to the increased vascular permeability function [44] of VEGF which could be deleterious to a muscle that is used intensively for burrowing in times of acute hypoxia.

Spalax has evolved multiple mechanism that have allowed for its successful survival in a hypoxic environment. One such mechanism exploited by *Spalax* principally in its skeletal muscle is an increase in capillary density. Our studies here with VEGF taken together with earlier results on hemoglobin [19,20], haptoglobin [21] and myoglobin [22], open a window to a more detailed molecular explanation for the physiological adaptation of *Spalax* to hypoxia.

Molecular studies from *Spalax* are currently hampered by the inability to breed *Spalax* in captivity. We hope that the development of stable cell lines from *Spalax* tissues will allow these studies to proceed more rapidly and to be standardized more easily. Ultimately we believe that this information on *Spalax* VEGF will be instrumental in the development of new strategies to regulate capillary density in ischemic syndromes, malignancies and environmental extremes such as outer space.

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