The function of hypoxia-inducible factor 1 (HIF-1) is impaired in senescent mice

Galit Frenkel-Denkberg[a], David Gershon[a,*], Andrew P. Levy[b]

[a] *Department of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel*
[b] *Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel*

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Abstract Senescent organisms respond poorly to hypoxic stress. The transcription factor hypoxia-inducible factor 1 (HIF-1) plays a critical role in the coordinated genetic program that is induced in all tissues to adapt to hypoxic stress by binding to a specific DNA hypoxia-responsive recognition element (HRE). This study was designed to address whether aging is associated with an alteration in HIF-1 production and function. Young and old mice were exposed to hypoxia for various lengths of time. We found a severe impairment in the capacity of the old animals to form a HIF-1-HRE complex. This attenuation in the capacity to form HIF-1-HRE complexes in senescent tissues may explain the decreased ability of such tissues to respond to hypoxic stress.

Key words: Aging; Stress; Hypoxia; Angiogenesis; Collateral circulation

1. Introduction

One of the major characteristics of aging is the decline in the capacity to respond to stress [1]. Hypoxia is a typical stress to which senescent organisms respond poorly. One example of this phenomenon in man is manifested as a decreased ability to tolerate and adapt to ischemic vascular disease in the elderly [2]. Recently, it has been demonstrated that this impaired ability to adapt to ischemia in the elderly is in part due to a reduction in the amount of angiogenesis responsible for collateral blood vessel development [3]. This age-dependent impairment of angiogenesis was shown to be mediated by a decline in the production of vascular endothelial growth factor (VEGF) [3]. The molecular alterations responsible for this decline have not been fully elucidated.

Hypoxia-inducible factor 1 (HIF-1) [4] is a transcription factor responsible for a coordinated genetic program [5] mediating such diverse but related functions as increased respiratory rate, increased erythropoiesis, increased glycolysis and increased angiogenesis all in response to organismal hypoxic stress. These physiological responses to hypoxia are mediated by specific gene products whose transcription rates are increased by HIF-1, notably the genes for tyrosine hydroxylase [6], erythropoietin (Epo) [7], glycolytic enzymes [8] and VEGF [9], respectively. Accordingly, in order to understand the molecular basis for the age-dependent impairment in responding to hypoxic stress, the present study was designed to address whether aging is associated with an alteration in HIF-1 production and function.

2. Materials and methods

2.1. Animals

Young and old C57 Bl/6J female mice used in these studies were 2–3 months and 24 months of age, respectively. The animal experimentation protocols followed approved institutional guidelines.

2.2. Hypoxia

Hypoxia was achieved by subjecting animals to a mixture of 93% N_2 and 7% O_2 for periods of 30 and 60 min in impermeable plastic bags.

2.3. Preparation of nuclear extracts

Following exposure to hypoxia, the animals were killed by cervical dislocation. Brains, lungs, kidneys and livers were frozen in liquid nitrogen and kept at −80°C until further use. Frozen tissues were homogenized in three volumes of TKM buffer (5 mM Tris-HCl, pH 7.5, 2.5 mM KCl, 5.0 mM $MgCl_2$, 0.5 mM DTT, 0.4 mM PMSF, 2 μg/μl aprotinin, 1 μg/ml sodium vanadate and 2 μg/ml leupeptin) containing 0.25 M sucrose in a Potter-Elvenheim homogenizer [10]. The homogenate was filtered through gauze and the filtrate brought up to 1.62 M sucrose and 0.5% NP-40. The preparation was then centrifuged at 60 000 × *g* for 60 min and the pellet suspended in buffer D (10 mM Tris-HCl, pH 7.9, 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 0.4 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml sodium vanadate and 2 μg/ml leupeptin). Nuclear proteins were extracted according to Parker and Topol [11] with minor modifications. The suspension of nuclei in buffer D was brought up to 0.4 M $(NH_4)_2SO_4$ and incubated at 4°C for 30 min. Subsequently, this preparation was centrifuged in a Ti-50 rotor at 130 000 × *g* for 120 min. The supernatant proteins were precipitated by the addition of 0.33 g/ml $(NH_4)_2SO_4$ and incubated at 4°C for 30 min followed by centrifugation at 27 000 × *g* for 30 min.

2.4. Electromobility shift assay (EMSA)

EMSA was performed according to Semenza and Wang [12]. The mutant and wild-type probe containing a HIF-1 binding site were from the rat VEGF hypoxia-responsive recognition element (HRE) [9]. The binding reaction was performed in a final volume of 20 μl binding buffer (final concentration of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5% glycerol and 1 mM sodium vanadate). The reaction mixture also contained 5 μg of nuclear extract protein and 0.1 μg denatured calf thymus DNA. The reaction mixture was incubated at room temperature for 5 min prior to the addition of 0.2 ng (10^4 cpm) radioactive probe. After an additional 15 min, the mixture was electrophoresed at 180 V on a 4% acrylamide gel in 0.3 × TBE. The gel was dried and the amount of HIF-HRE complex quantitated by phosphorimager analysis.

2.5. Antibodies and Western blot

Monoclonal antibody to HIF-1α was provided by R.H. Wenger. Western blot and ECL (Amersham) were performed according to the manufacturer's protocol.

*Corresponding author. Fax: (972)-4-8221106.
E-mail: dgershon@tx.technion.ac.il

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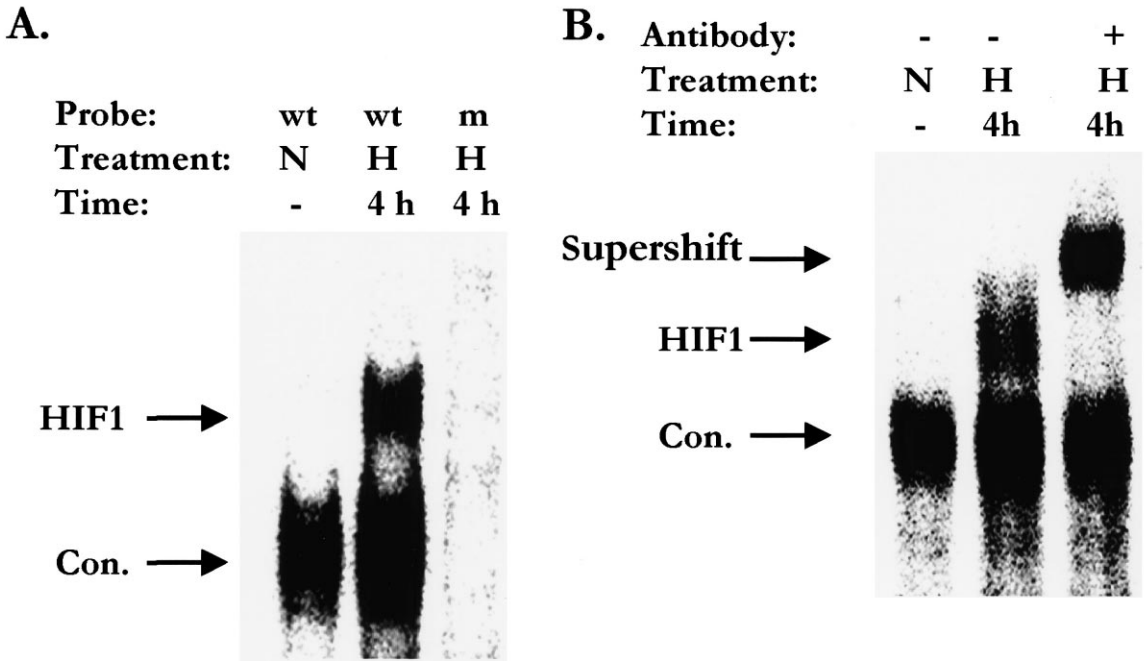

Fig. 1. Establishment of specificity of binding of HIF-1 to the HRE. (A) DNA EMSA performed with hypoxic and normoxic nuclear extracts using the wild-type or mutant HRE (wt, wild-type HRE sequence; m, mutant HRE sequence; N, normoxia; H, hypoxia; Con., constitutive). (B) Supershift of HIF-HRE complex with HIF-1α antibody.

3. Results

3.1. Establishment of specificity of the EMSA system for HIF-1

Using a radioactively labelled 36 bp DNA fragment corresponding to the rat HRE, we were able to demonstrate the existence of a hypoxia-inducible protein complex in nuclear extracts (Fig. 1A), similar to that previously described [9]. Specificity of binding due to HIF-1 was demonstrated using a DNA fragment in which the HIF-1 site was mutated (Fig. 1A). Excess cold competitor HRE eliminated the formation of the complex as previously described [9] (data not shown). Finally, antiserum directed against HIF-1α was able to efficiently supershift the hypoxia-inducible complex (Fig. 1B).

3.2. Hypoxia results in an attenuation of HIF-1 binding activity in extracts from old compared to young animals

EMSA assays were performed with nuclear extracts prepared from the tissues of young and old animals subjected to 30 or 60 min hypoxia. We found a highly reproducible marked age-associated decrease in HIF-HRE complex formation in brain, lung, kidney and liver (Fig. 2A–D).

3.3. The attenuation of HIF-1 binding activity with aging is not due to decreased HIF-1 levels

In order to determine if the decreased amount of HIF-HRE complex formed in aged animals was due to decreased HIF-1 levels, we performed Western blot analysis with an antibody to HIF-1 on the same nuclear extracts used in the EMSA. In the brain, we found, unexpectedly, that the level of HIF antigen was higher in older animals than in younger animals under normoxic conditions (Fig. 3A). Under hypoxic conditions, HIF antigen was increased in the young animals, but remained the same in the old animals. These results support the contention that the failure to increase the HIF-HRE complex in older animals is not due to less HIF protein but rather due to loss of the ability of HIF to bind to the HRE. In the liver, we found no difference between the level of HIF-1 antigen present in young and old animals exposed to hypoxia (Fig. 3B). These results further support the notion that the decrease in HIF-HRE complex seen in old animals is not due to a decrease in HIF protein.

4. Discussion

Aging is associated with a marked impairment in hypoxia-induced angiogenesis and anemia-induced erythropoiesis due to a reduced production of VEGF [3] and Epo [13], respectively. HIF-1 mediates the transcriptional activation of both VEGF and Epo [9]. This study provides for the first time a molecular explanation for the impaired formation of these cytokines by hypoxia. Specifically, we have shown that in aged animals, HIF-1 has a reduced ability to bind to the HRE present in these genes. Such a post-translational loss of function has previously been described with aging for other proteins [14,15]. This was related to an age-associated impairment in protein degradation [16,17] possibly due to an alteration in the functioning of the ubiquitin-proteasome complex [18]. Interestingly, HIF-1α is regulated by hypoxia at the level of its protein stability [5] with the tumor suppressor VHL functioning as the ubiquitin ligase for HIF-1α [19].

Identification of the molecular mechanism responsible for the loss of the ability to adapt to hypoxic stress provides a molecular target for a rational pharmacological strategy to restore the ability to respond to this stress in the elderly and to concomitantly reduce morbidity from many disorders. Indeed, in light of the known increase in susceptibility to oxidative stress in the elderly [1] and the ability of oxidative stress

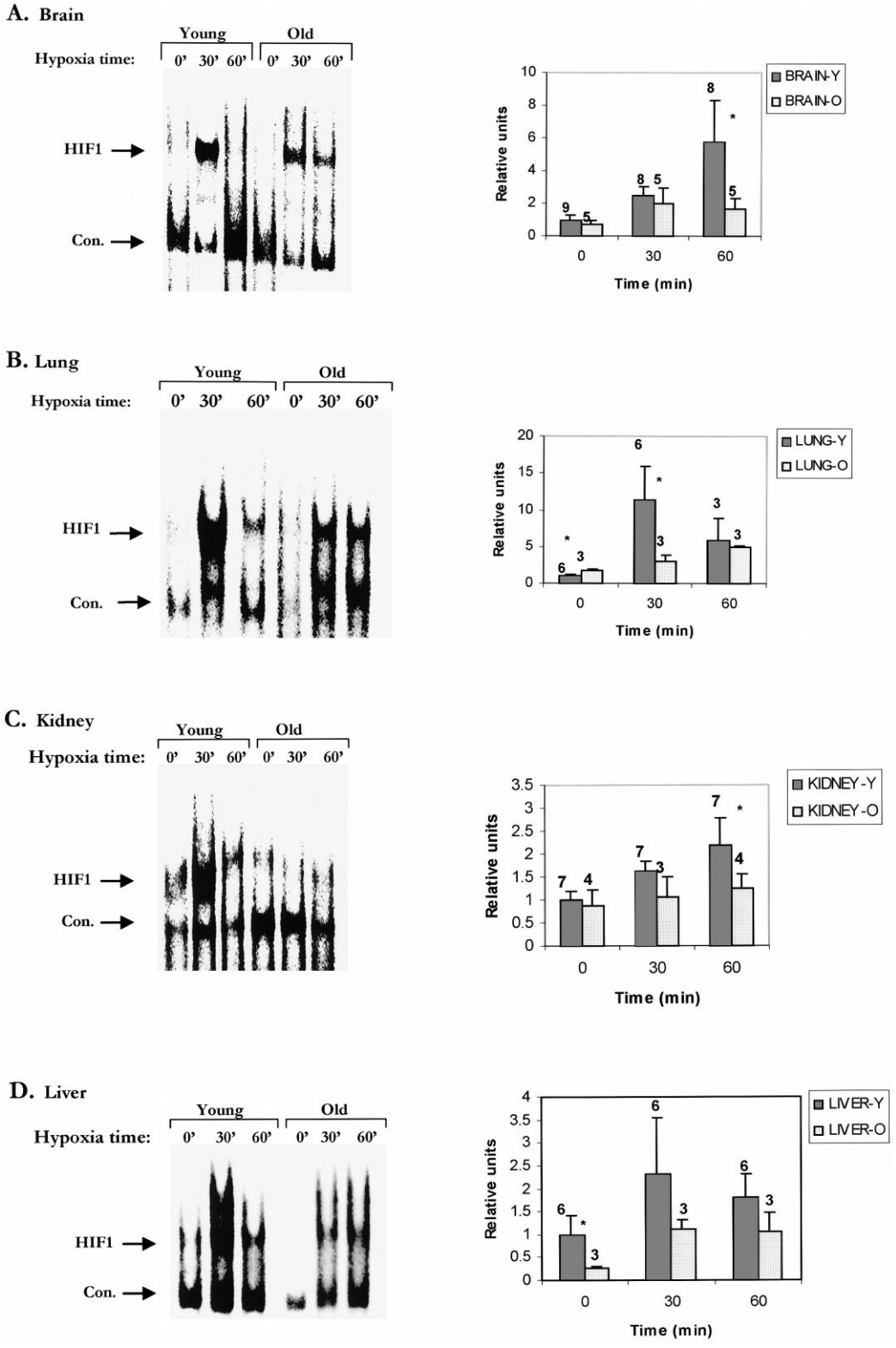

Fig. 2. Quantitation of HRE-HIF complex formation by EMSA in young and old animals exposed to hypoxia. Representative EMSAs are from brain (A), lung (B), kidney (C) and liver (D). The results are quantitated in the histogram (Y, young; O, old). The data for each time point represent the mean ± S.E.M. obtained from at least three animals (the actual number of animals is indicated on the histogram) with the results normalized to the zero time point for young animals. *, Significant difference ($P < 0.05$) between young and old animals at the time point indicated.

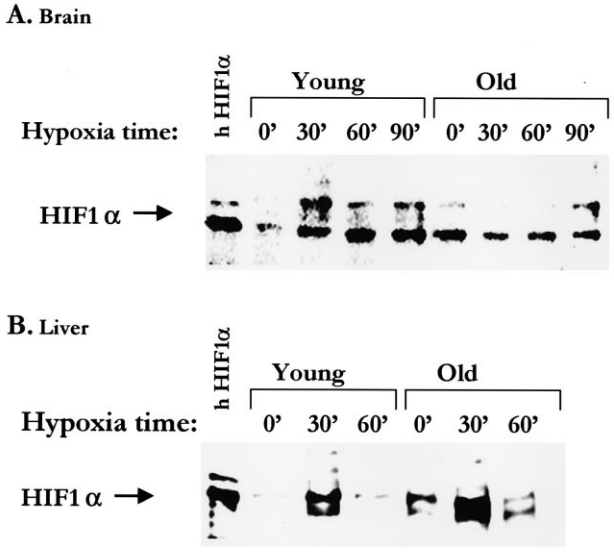

Fig. 3. Western blot of HIF-1α antigen in nuclear extracts taken from (A) brain and (B) liver of young and old animals exposed to hypoxia. Equivalent amounts of nuclear extract were used for all lanes except for the first lane in which recombinant HIF-1α protein was used.

to diminish HIF-1 binding [5], it is tempting to speculate that anti-oxidant therapy might prevent the loss of function of HIF-1 in the elderly.

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