

HIF-1 α cytoplasmic accumulation is associated with cell death in old rat cerebral cortex exposed to intermittent hypoxia

Cinzia Rapino,¹ Giuseppina Bianchi,¹ Camillo Di Giulio,¹ Lucia Centurione,² Marisa Cacchio,¹ Adriano Antonucci² and Amelia Cataldi^{2#}

¹Dipartimento di Scienze Biomediche, and ²Dipartimento di Biomorfologia, [#]Cattedra di Anatomia Umana, Facoltà di Farmacia, Università G. d'Annunzio, Chieti-Pescara, Italy

Summary

Intermittent hypoxia, followed by reoxygenation, determines the production of reactive oxygen species (ROS), which may lead to accelerated aging and to the appearance of age-related diseases. The rise in ROS levels might constitute a stress-stimulus activating specific redox-sensitive signalling pathways, so inducing either damaging or protective functions. Here, we report that in old rat cerebral cortex exposed to hypoxia, the accumulation in the cytoplasm of hypoxic inducible factor 1 α (HIF-1 α) – the master regulator of oxygen homeostasis – concomitant with p66^{Shc} activation and reduced I κ B α phosphorylation is associated with tissue apoptosis or necrosis. In young cerebral cortex, we hypothesize that the hypoxic damage may be reversible, based on our demonstration of elevated HIF-1 α levels, combined with a low level of I κ B α phosphorylation, a decrease in IAP-1 and a lack of major change in Bcl2 family proteins. These observations are associated with a low level of cell death induced by hypoxia, suggesting that HIF-1 α activation in cortical neurons may produce rescue proteins in response to intermittent hypoxia. Key words: aging; brain; oxidative stress; signal transduction.

Introduction

Aging is associated with significant enhancement of cell death both under physiological conditions and after exposure to different stressing challenges that accompany several pathological states (Liu *et al.*, 1998; Nitahara *et al.*, 1998; Otterbein *et al.*, 1998; Razzagne *et al.*, 1999; Sastry & Rao, 2000). In addition, stable and certain continuously dividing cell populations can eliminate dysfunctional elements that show alterations in

homeostasis due to oxidative stress, glycation, DNA damage by apoptosis (Higami & Shimokawa, 2000). Free radicals oxidize many cell proteins, resulting, for example, in reduced catalytic activity of enzymes (Stadtman, 1995). With age, oxygen radicals also cause DNA strand breakage, often resulting in harmful or lethal events (Wickens, 2001).

During development of the nervous system, neuron loss is considered an adaptive mechanism (Oppenheim, 1991). However, during aging neuron cell loss may be evolutionarily non-adaptive, involving alterations in cell homeostasis and in the ability to adapt to environmental alterations (Masoro, 1995). But accurate estimates of relatively small neuronal losses, for example up to 20%, which is typical of the effects of age on selected populations of neurons (Masliah *et al.*, 1993), are notoriously difficult to make, as are losses of synapses and dendritic pruning in selected areas (Anderton, 2002). In fact, although there is a general consensus that neurons in certain brain regions are lost with age, including those in the hippocampus, cerebral cortex and amygdala, other groups of neurons show shrinkage without cell loss. Still other brain regions seem to be protected against shrinkage and neuron loss (Chiu *et al.*, 1984). In addition to the effects of age, neuronal apoptosis can be induced by a variety of stressors, including hypoxia (10–12% O₂) (Bunn & Poyton, 1996; Finkel & Holbrook, 2000; Sharp *et al.*, 2001). The latter involves a reduction of the physiological level of oxygen tension (21% O₂) and gives rise to multiple cellular and systemic responses. These responses include angiogenesis, erythropoiesis and glycolysis (Guillemin & Krasnow, 1997), which occur in acute and chronic vascular and pulmonary diseases and cancer (Wenger, 2002; Carmeliet, 2003). Hypoxia induces different responses at tissue and cellular level depending on its intensity and duration, on cell type and on the oxygen rate tolerance. In addition, intermittent hypoxia (e.g. 12% O₂ for 12 h followed by reoxygenation for 12 h) is a more potent inducer of cell death than continuous hypoxia, probably as a result of a substantial increase in reactive oxygen species (ROS) generation (Cataldi *et al.*, 2004). Increased ROS levels may lead to cell death, accelerated aging and the appearance of age-related diseases (Finkel & Holbrook, 2000; Wickens, 2001). Elevated ROS levels might constitute a stress stimulus activating specific redox-sensitive signalling pathways, with either damaging or protective outcomes. Among the signalling elements activated, attention has been recently focused on HIF-1 (hypoxic inducible factor) (Semenza, 2000; Sharp *et al.*, 2001). HIF-1 is a dimeric transcription factor expressed in many cell types in response to O₂ deprivation (Semenza, 2001, 2004). It is composed of an α subunit, unstable under normoxic conditions, and a common

Correspondence

Amelia Cataldi, Dipartimento di Biomorfologia, Università G. d'Annunzio, Via dei Vestini, 6, 66100 Chieti, Italy. Tel.: +39 0871 3555299; fax: +39 0871 574361; e-mail: cataldi@unich.it

Accepted for publication 29 April 2005

β subunit, also known as aryl hydrocarbon nuclear receptor translocator (ARNT), that heterodimerizes into the nucleus (Wang & Semenza, 1995). In response to hypoxia, HIF-1 α regulation involves transcriptional, post-transcriptional and post-translational changes (Carmeliet *et al.*, 1998; Semenza, 2004). HIF-1 α is a key cellular modulator, selectively stabilized and activated by many different pathways during aging and hypoxia (Abe & Berk, 2002; Michiels *et al.*, 2002).

Because of its role as a regulator of cellular lifespan and of stress-induced apoptotic response in mammals (Migliaccio *et al.*, 1999; Purdom & Chen, 2003), we became interested in the relationship of p66^{Shc} to HIF-1 α . We present data showing the induction of cell death by hypoxia in the aged rat cerebral cortex and associated changes in the expression of HIF-1 α , p66^{Shc} and some of the associated downstream elements involved in responses to age and hypoxic stress.

Results

During aging and after hypoxia exposure, rat cerebral cortex shows changes in vascularization and neuron morphology, along with changes in the number of apoptotic cells. Aging and hypoxia are accompanied by decreasing brain volume and cortical weight, as well as in loss of cortical neurons (Masliah *et al.*, 1993; Zhang *et al.*, 1995; Brody, 1997; Pakkenberg & Gundersen, 1997; Jernigan *et al.*, 2001; White & Barone, 2001; Anderton, 2002).

Cell counting, performed in haematoxylin–eosin-stained sections on 77 000- μm^2 fields, shows a reduced density of neurons in hypoxic and normoxic old cerebral cortex, as compared with young in the same experimental conditions (Fig. 1, Table 1).

In normoxic young cerebral cortices, the diameter of capillary vessels ranges between 3.26 and 10.38 μm and between 4.09

Table 1 Cell number/field determined on haematoxylin–eosin-stained sections of rat cerebral cortex in different experimental conditions. Data are the means \pm SD of five independent counts made using light microscopy (magnification $\times 40$)

Group	Cell number
Normoxic young	201.4 \pm 21.08
Hypoxic young	252.2 \pm 19.08
Normoxic old	188.6 \pm 15.82
Hypoxic old	141.2 \pm 17.74

and 27.80 μm after exposure to hypoxia. The total number of capillaries also increases after exposure to hypoxia (15 vs. 32). The normoxic old cerebral cortex discloses vessel diameter ranges between 4.43 and 29.03 μm and between 4.38 and 34.31 μm after hypoxia. No substantial modification has been shown in the number of blood vessels between hypoxic young and old (32 vs. 38). Lastly, the percentage area occupied by capillary vessels increases in both hypoxic samples with respect to normoxic ones (Fig. 2).

The incidence of cell death was examined by counting TUNEL-positive neurons. Figure 3 shows that the number of TUNEL-positive cells increases in hypoxic compared with normoxic young as well as old cortex.

Because of its role in responses to cellular stress, the expression of HIF-1 α was evaluated by immunohistochemistry. Figure 4 shows increased expression of HIF-1 α following hypoxia in both young and old cortex (8–10-fold; Fig. 4E). In addition, HIF-1 α is translocated to the nucleus, presumably indicating activation of the protein, in hypoxic young cortex. However, this response is not detectable in aged cortex where HIF-1 α accumulates in the cytoplasm after hypoxia.

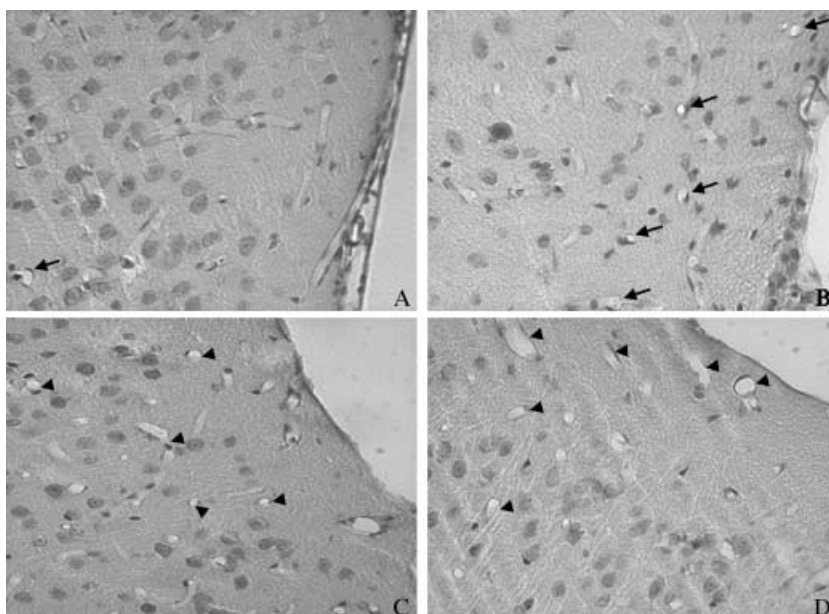


Fig. 1 Haematoxylin–eosin counterstained rat cerebral cortex in different experimental conditions. Micrographs represent one of five slides observed per sample. Magnification $\times 40$. (A) Normoxic young, (B) hypoxic young, (C) normoxic old, (D) hypoxic old. Arrows indicate blood vessels. Arrowheads indicate the different capillary vessels diameter disclosed in the two experimental conditions.

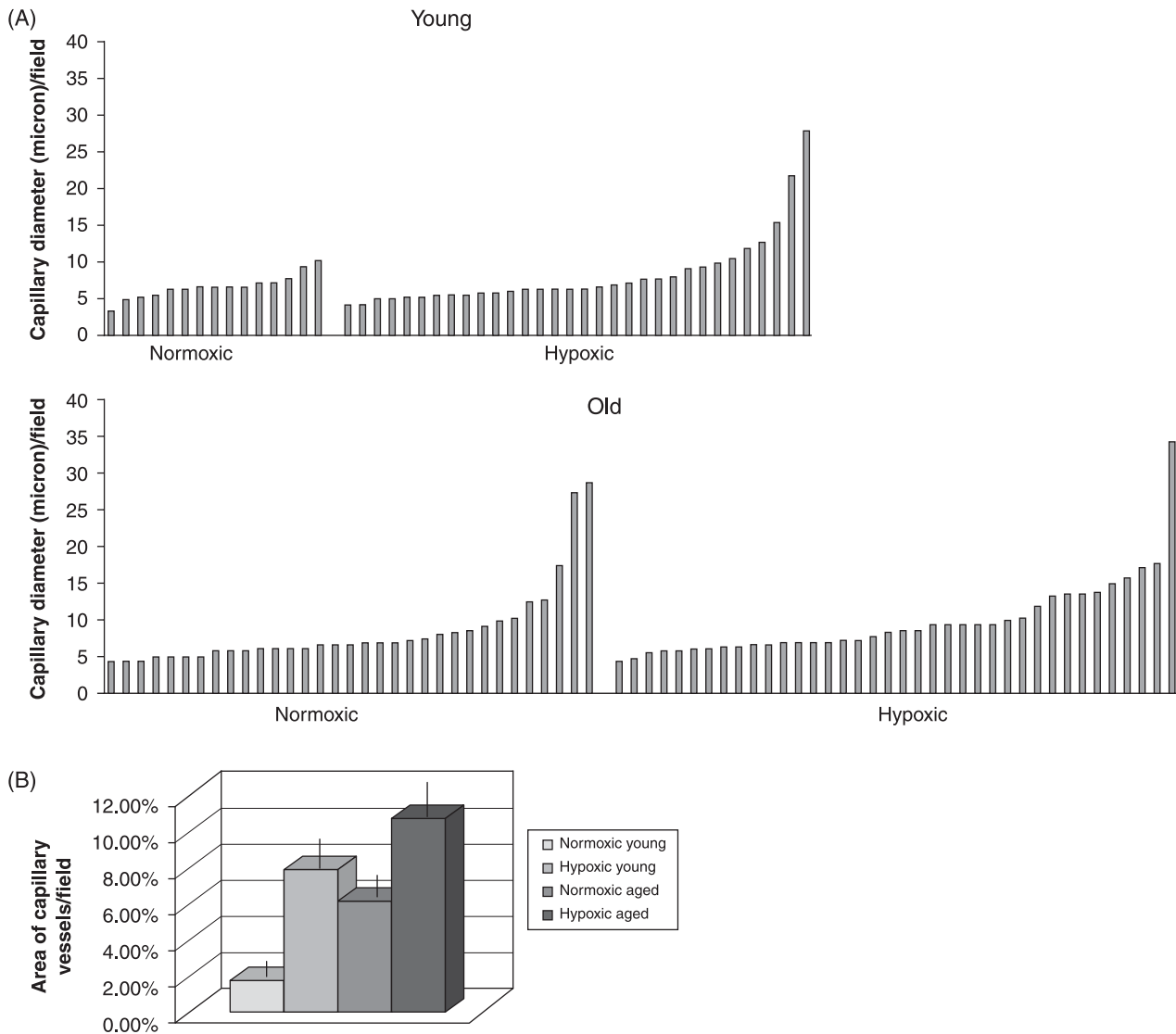


Fig. 2 (A) Frequency histograms of capillary vessel diameter acquired at 20 \times magnification using the MetaMorph Software System. (B) Mean percentage (\pm SD) of the area occupied by capillary vessels determined on five slides per sample measured by the MetaMorph Software System.

Because p66^{Shc} plays a role in stabilizing HIF-1 α during hypoxia (Jung *et al.*, 2002), p66^{Shc} expression was assessed, and its activation analysed using antibodies detecting proteins phosphorylated on tyrosine residues, as previously reported (Ugi *et al.*, 2002). Figure 5 demonstrates higher expression of p66^{Shc} in both young and old cortices after hypoxia, while tyrosine phosphorylation is significantly increased in old cortex following hypoxia.

Figure 6 depicts a substantial I κ B α phosphorylation in normoxic and hypoxic old cortices as compared with the young cortices, while the hypoxic young cortex shows reduced I κ B α phosphorylation. In hypoxic old cortices this result is paralleled by a decline in Bcl-2 expression, without Bax and IAP-1 modifications. Moreover, a slight increase in Bax level, no modification of Bcl-2 expression and a decrease in IAP-1 are seen in hypoxic young cortices (Fig. 7).

Discussion

The reduced density of cortical neurons that we have shown may reflect a loss of neurons involving both large neurons and a mixture of small cells and glia, which are difficult to distinguish from each other. Such loss may be due to either apoptosis, which is known to occur during aging (Higami & Shimokawa, 2000), or necrosis, which results from damage and causes inflammation (Kerr *et al.*, 1972).

Endogenous oxidative stress results in DNA damage that leads to changes in gene expression and contributes to aging processes. Exogenous oxidative stress, including that induced by intermittent hypoxia, results in modifications of some signal transduction pathways, leading to apoptotic or necrotic responses and damage to cells and tissues. In young rat cerebral cortex we report an increase in cell death following hypoxic exposure, which resembles a physiological oxygen sensing function

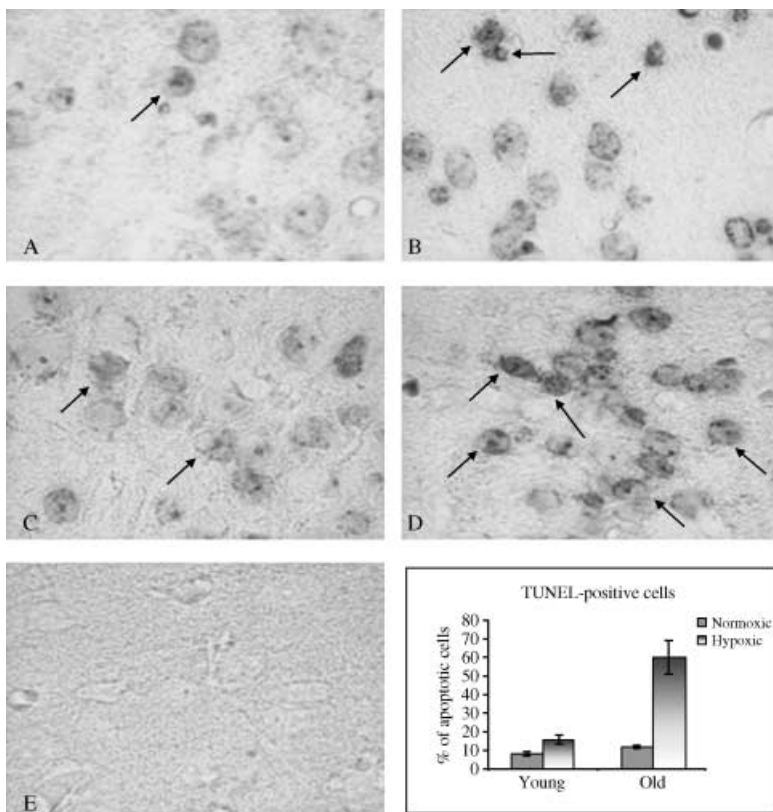


Fig. 3 TUNEL-positive nuclei in rat cerebral cortex in different experimental conditions. (A) Normoxic young, (B) hypoxic young, (C) normoxic old, (D) hypoxic old, (E) negative control. Arrows indicate positive nuclei. (F) Histogram showing per cent TUNEL-positive cells. The presence of DNA fragmentation was quantified by counting labelled nuclei in the light microscope at $\times 40$ magnification. TUNEL-positive nuclei were expressed as percentage of total. Values are means \pm SD; $n = 3$ for all groups.

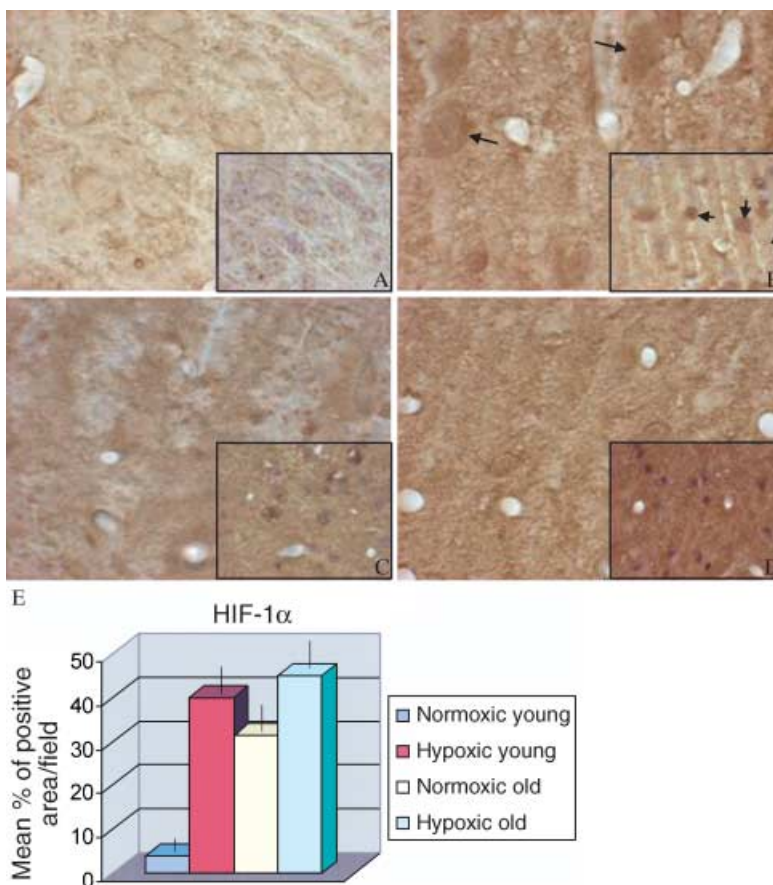


Fig. 4 Immunohistochemical analysis of HIF-1 α expression in paraffin-embedded sections of rat cerebral cortex in different experimental conditions. Arrows indicate HIF-1 α nuclear translocation in hypoxic young sample. Magnification $\times 100$. (A) Normoxic young, (B) hypoxic young, (C) normoxic old, (D) hypoxic old. Insets indicate haematoxylin-counterstained nuclei. (E) Densitometric analysis of HIF-1 α -positive area/field ($77\,000\ \mu\text{m}^2$) expressed as percentage \pm SD measured by the MetaMorph Software System (hypoxic young vs. normoxic young $P < 0.05$).

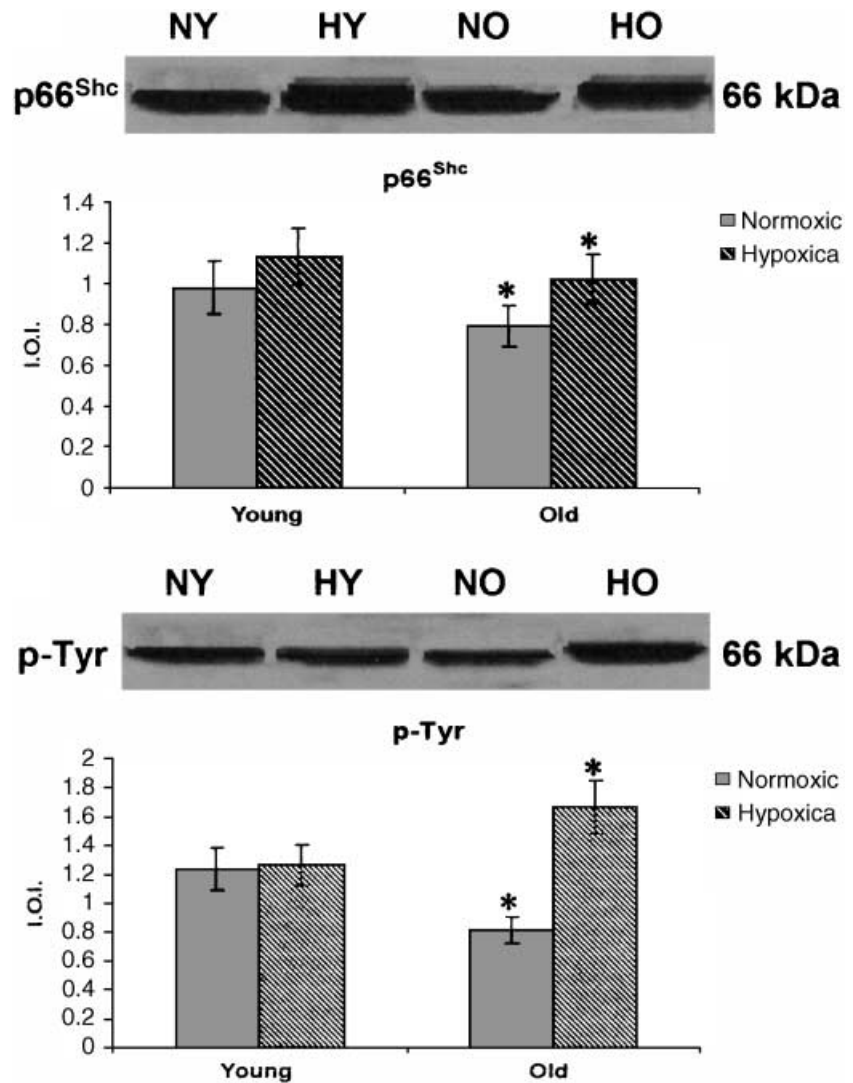


Fig. 5 Western blotting analysis and densitometric evaluation of p66^{Shc} expression and activation in rat cerebral cortex under different experimental conditions. Membranes were exposed to mouse p66^{Shc} monoclonal antibody, stripped and reprobed with rabbit p-Tyr polyclonal antibody, which recognizes tyrosine phosphorylated proteins. Samples (20 μ g) have been normalized by incubation in the presence of mouse β -actin monoclonal antibody. Results shown are representative of three separate experiments and are expressed as mean \pm SD, $n = 3$ at each experimental point. Normoxic old p66^{Shc} Tyr phosphorylation vs. hypoxic old p66^{Shc} Tyr phosphorylation: * $P < 0.05$.

that is enhanced approximately six-fold during aging (Prabhakar & Kumar, 2004). Cell death was assessed by the TUNEL technique, which labels necrosis and reversible DNA damage as well as apoptosis (Portera-Caillan *et al.*, 1994). We found that exposure to intermittent hypoxia (12 h of hypoxia followed by 12 h of reoxygenation) seems to be a more potent inducer of cell death than continuous hypoxia (Yuan *et al.*, 2004). This may be because reoxygenation triggers ROS production in the attempt to restore normal O₂ levels (Kang *et al.*, 2000).

HIF-1 α is considered a master regulator of O₂ variations by governing adaptive patterns of gene expression (Chandel & Schumacker, 2000). Indeed, whereas in normoxic conditions HIF-1 α is continuously expressed and degraded by the ubiquitin-proteasome system (Jewell *et al.*, 2001), under hypoxia, degradation is suppressed, HIF-1 α migrates to the nucleus and rescue proteins are produced by the cell.

Stabilization of HIF-1 α is regulated by a signal transduction pathway involving p66^{Shc} that plays a key role in certain pathophysiological mechanisms. The Shc isoforms represent a family

of stress-regulated proteins acting as sensors for ROS and other genotoxic stresses. In particular, p66^{Shc} is considered to be a longevity gene (Migliaccio *et al.*, 1999; Ventura *et al.*, 2002). Immunohistochemical demonstration of accumulation of HIF-1 α in the neuronal cytoplasm in old rat cerebral cortex, concomitant with p66^{Shc} activation, can lead to inhibition of HIF-1 α transcriptional activity (Abe & Berk, 2002), which may result in production of proteins counteracting the effects of hypoxia (Chavez La Manna, 2003). In particular, p66^{Shc} is an immediate substrate of tyrosine kinases playing an important role in linking activated tyrosine kinase receptors to downstream hypoxic and other signalling pathways, including recruitment of the RAS extracellular signal regulated kinase (ERK) cascade or Raf-1 kinase (Lowenstein *et al.*, 1992; Abe & Berk, 2002; Jung *et al.*, 2002). In hypoxic young cortex, we have demonstrated increased expression and HIF-1 α translocation to the nucleus, generally considered as evidence of protein activation, along with no modification of Bcl-2 level, a slight increase in Bax and decrease in IAP-1 levels, all of which might contribute to the observed small number of

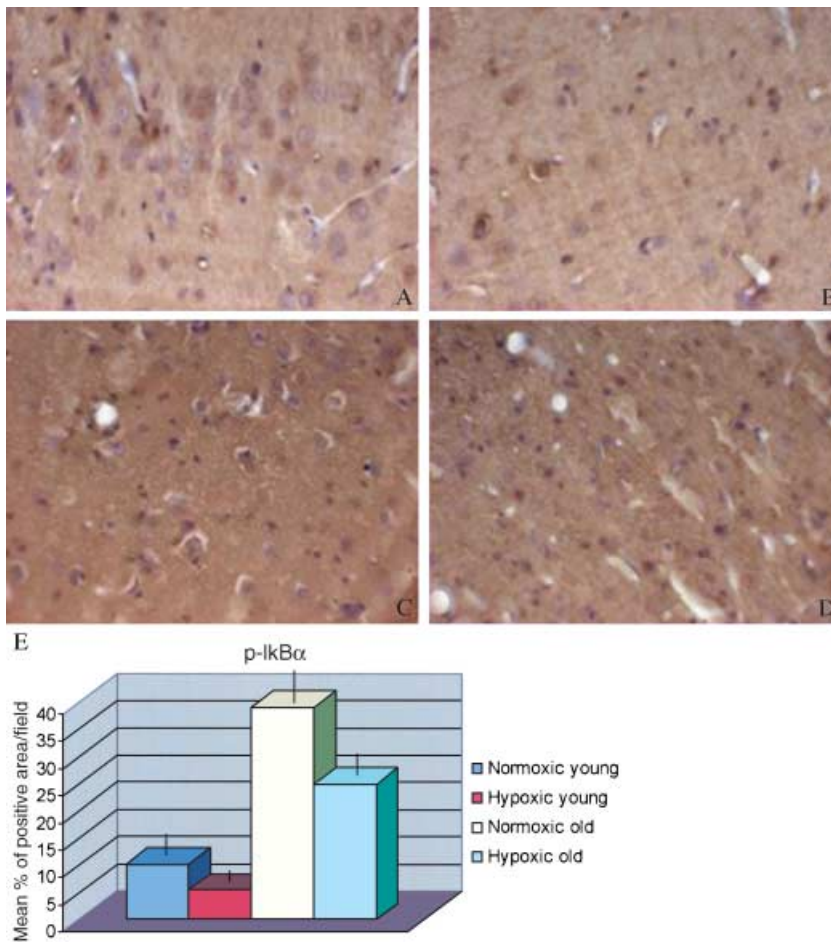


Fig. 6 Immunohistochemical analysis of p-IkB α in paraffin embedded sections of rat cerebral cortex in different experimental conditions; magnification $\times 40$. (A) Normoxic young, (B) hypoxic young, (C) normoxic old, (D) hypoxic old. (E) Densitometric analysis of p-IkB α -positive area expressed as percentage area \pm SD measured by MetaMorph Software System (hypoxic young vs. normoxic young $P < 0.05$).

apoptotic events (Yang & Korsmeyer, 1996). IKB- α expression is thought to act as an important signal in neurodegenerative diseases (Leroualch & Behl, 1998), leading to neuron and glial cell apoptosis through hydrogen peroxide formation (Vollgraf *et al.*, 1999). IKB- α phosphorylation and the lack of modification in IAP-1 levels that we have shown in the old cortex exposed to hypoxia may therefore be considered as an attempt by the aged cerebral cortex to counteract oxidative damage by eliminating dead cells (Korhonen *et al.*, 1997). By contrast, in the young cortex the hypoxic damage could be reversible, achieved by the balance we show between the low level of IKB- α phosphorylation, the decrease in IAP-1 and HIF-1 activation, which appear to represent a concerted attempt to counteract hypoxia.

Experimental procedures

Animals

Two groups, each composed of ten male Sprague–Dawley rats, 3 and 24 months old, weighing approximately 250 and 400 g, respectively, were used according to the UK (Scientific Procedures) Act, 1986 and associated guidelines. Only animals free of acute or chronic illness were employed. Five animals from each group

were kept at 21% oxygen (normoxia), while five young and five old rats were exposed to intermittent hypoxic challenge (12 h at 10% oxygen followed by 12 h at 21% oxygen) for 8 days in a large plexiglass chamber (80 \times 40 \times 65 cm). Oxygen was monitored by an oxymeter (Beckman Coulter, Fullerton, CA, USA) and the air in the chamber was circulated with a pump. CO₂ was removed from the chamber air with baralyme and was continuously monitored with a capnograph (Beckman Coulter, Fullerton, CA, USA). Boric acid was given to the litter to minimize emission of urinary ammonia. The ambient temperature was maintained at 25 $^{\circ}$ C. Rats were anaesthetized with pentobarbital sodium salt (Nembutal, Sigma Aldrich, St. Louis, MO, USA; 40 mg kg⁻¹) and the cerebral cortex was excised from each rat.

Light microscopy and immunohistochemistry

Formaldehyde-fixed cerebral cortices were embedded in paraffin and left frontal sections stained with haematoxylin–eosin (Cataldi *et al.*, 2004). Neuronal cell density was determined by direct visual counting of ten fields for each of five slides per sample at 40 \times magnification. To detect p-IkB α and HIF-1 α proteins, sections were blocked in PBS containing 5% normal goat serum, 0.1% bovine serum albumin and 0.1% Tween-20. They

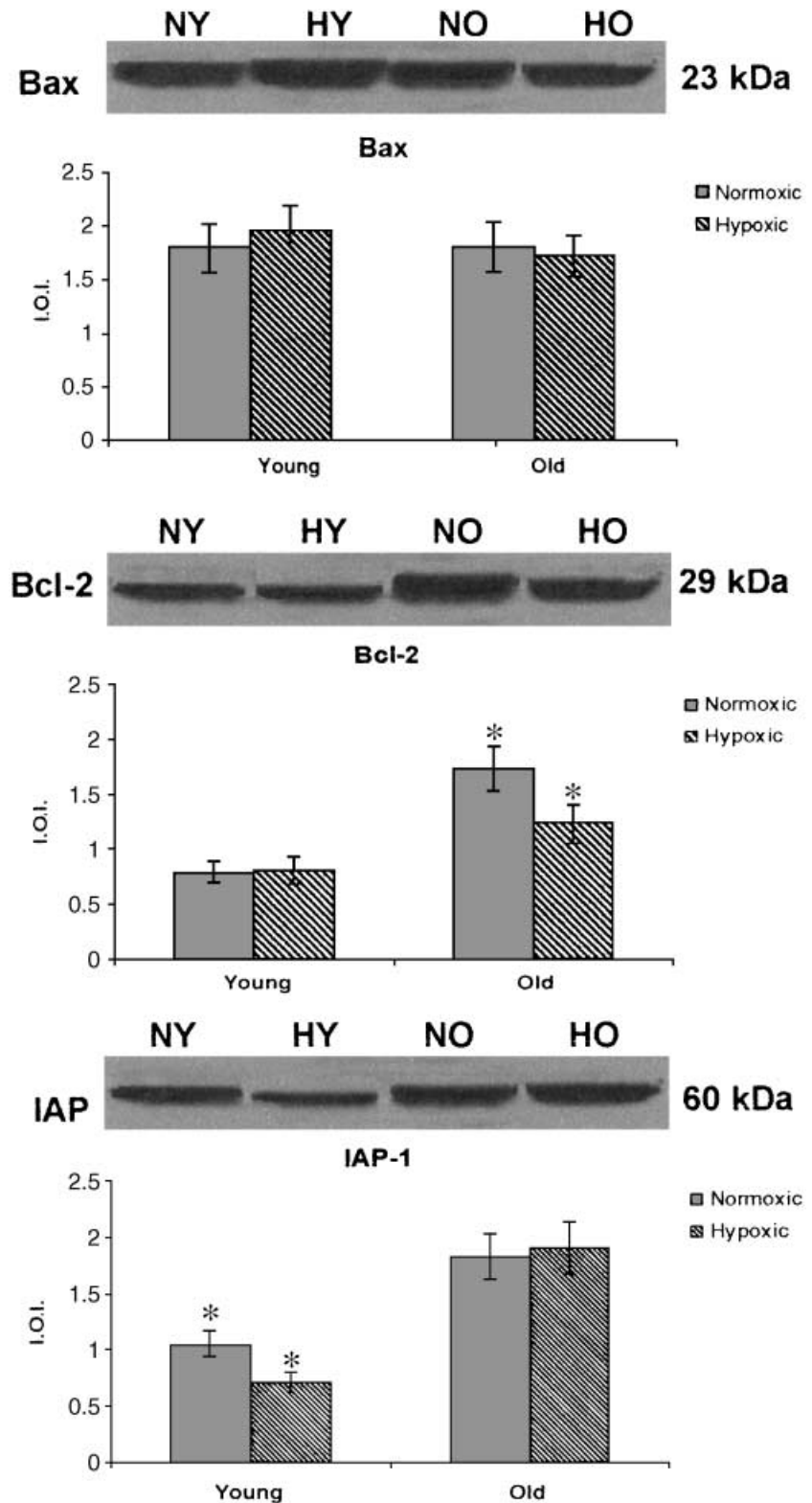


Fig. 7 Western blotting analysis and densitometric evaluation of Bax, Bcl-2 and IAP-1 expression in rat cerebral cortex in different experimental conditions. Samples (20 μ g) were normalized by incubation in the presence of mouse β -actin monoclonal antibody. Results shown are representative of three separate experiments ($n = 3$) and are expressed as mean integrated optical intensity (I.O.I.) \pm $\Sigma \Delta$ (normoxic old Bcl-2 vs. hypoxic old Bcl-2: * $P < 0.05$; hypoxic young IAP-1 vs. normoxic young IAP-1: * $P < 0.05$).

were then incubated for 1 h in the presence of mouse HIF-1 α or p-IkB α monoclonal antibodies (Santa Cruz Biotechnology, CA, USA) and then 1 h in the presence of biotinylated secondary antibodies followed by streptavidin-peroxidase developed using

diaminobenzidine chromogen (DAB) (Biomedica Corp., CA, USA) and nuclei were counterstained with haematoxylin. Negative controls, performed by omitting the primary antibody (data not shown), disclosed no positive reaction.

Computerized morphometry and image analysis

MetaMorph Software System (Universal Imaging Corp., Molecular Device Corp., PA, USA; Crisel Instruments, Rome, Italy) was used to acquire digital images and to measure capillary vessel area and diameters on haematoxylin–eosin-stained sections and to evaluate HIF-1 α and p-IkB α expression on immunolabelled sections. Morphometric computerized analysis of area and capillary vessel diameters was performed after calibrating the program for the objective magnification used (20 \times).

Image analysis of protein expression was performed by establishing a discrimination threshold for the brown immunolabelling, then measuring the thresholded area for each light microscopical field and expressing it as a per cent of area scanned. Threshold settings were kept constant for the different experimental groups.

MetaMorph data were logged to Microsoft Excel where mean values and standard deviations were calculated and expressed as percentages of the total area.

TUNEL staining

TUNEL (terminal-deoxynucleotidyl-transferase-mediated dUTP nick end-labelling) is widely used for the identification and quantification of dead and apoptotic cells (Portera-Caillan *et al.*, 1994). Paraffin-embedded tissue sections were dewaxed, rehydrated and pre-incubated with proteinase K (20 $\mu\text{g mL}^{-1}$) in 10 mmol L^{-1} Tris/HCl, pH 7.6, after which they were exposed to TUNEL mixture, according to the manufacturer's instructions (Boehringer Mannheim, Germany). After two rinses in PBS, slides were coverslipped using glycerol solution (10 $\mu\text{g mL}^{-1}$) and analysed under an optical microscope (Leica Cambridge Ltd, Cambridge, UK). Five slides from each sample were assessed; neuron counts were made on five fields per slide. Negative controls, created by omitting the incubation with the enzymatic mixture, disclosed no positive reaction (see Fig. 3F).

Western blotting

Cerebral cortices deriving from each of five animals were homogenized in RIPA buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS, 2 $\mu\text{g mL}^{-1}$ leupeptin, 1 $\mu\text{g mL}^{-1}$ aprotinin, 10 mM EDTA, 1 mM vanadate, 57 mM PMSF). Total tissue lysates were subject to SDS PAGE electrophoresis and blotted onto nitrocellulose membranes (Hybond ECL, Amersham International, Bucks., UK) as previously described (Cataldi *et al.*, 2004). Membranes were blocked in 5% non-fat milk in 10 mmol L^{-1} Tris buffer, pH 7.5, containing 100 mmol L^{-1} NaCl and 0.1% Tween-20. They were then probed with mouse monoclonal antibodies against Bcl-2 (28 kDa), Bax (20 kDa), IAP-1 (inhibiting apoptosis protein) (53 kDa) and p66^{Shc} (1 : 100) overnight. A rabbit p-Tyr polyclonal antibody (Santa Cruz Biotechnology, CA, USA) was used to identify tyrosine phosphorylated p66^{Shc}. After the primary antibody, membranes were incubated in the presence of specific mouse or rabbit IgG conjugated to

horseradish peroxidase. Immunoreactive bands were detected using ECL detection (Amersham International).

Image processing and analysis

Densitometric analysis of Western blots was performed using a Sony videocamera connected to a Leica Quantimet 500 plus software (Leica Cambridge Ltd, Cambridge, UK). Integrated optical intensity (IOI) was measured using ISO transmission density Kodak CAT 152-3406 (Eastman Kodak Company, Rochester, USA) as a standard. Results are expressed as mean \pm SD. Statistical analysis was performed using the analysis of variance (ANOVA) considering age and hypoxia as variables. Probability of the null hypothesis of < 0.1% ($P < 0.05$) is considered statistically significant.

References

- Abe J, Berk BC (2002) Hypoxia and HIF-1 α stability: another stress sensing mechanism for Shc. *Circ. Res.* **91**, 4–6.
- Anderton BH (2002) Ageing of the brain. *Mech Ageing Dev.* **123**, 811–817.
- Brody H (1997) Cell counts in the cerebral cortex and brainstem. In *Alzheimer's Disease: Senile Dementia and Related Disorders*, pp. 345–351. Williams Wilkins, Philadelphia.
- Bunn HF, Poyton RO (1996) Oxygen sensing and molecular adaptation to hypoxia. *Physiol. Rev.* **76**, 839–885.
- Carmeliet P (2003) Angiogenesis in health and diseases. *Nat. Med.* **9**, 653–660.
- Carmeliet P, Dor Y, Herbert JM, Fukumura Brusselmanns K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E (1998) Role of HIF-1 α in hypoxia mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* **394**, 485–490.
- Cataldi A, Bianchi G, Rapino C, Sabatini N, Centurione L, Di Giulio C, Bosco D, Antonucci A (2004) Molecular and morphological modifications occurring in rat heart exposed to intermittent hypoxia: role for Protein Kinase C α . *Exp. Gerontol.* **39**, 395–405.
- Chandel NS, Schumacker PT (2000) Cellular oxygen sensing by mitochondria: old questions, new insight. *J. Appl. Physiol.* **88**, 1880–1889.
- Chavez JC, La Manna JC (2003) HIF-1 α accumulation in the rat brain in response to hypoxia and ischemia is attenuated during ageing. In *Oxygen Transport to Tissue* (eds Wilson D, *et al.*), pp. 337–341. New York: Kluwer Academic/Plenum Publishers.
- Chiu HC, Bondareff W, Zarow C, Slager N (1984) Stability of neuronal number in human nucleus basalis in Meynert with age. *Neurobiol. Aging* **5**, 83–88.
- Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239–247.
- Guillemin K, Krasnow MA (1997) The hypoxic response: huffing and HIFing. *Cell* **89**, 9–12.
- Higami Y, Shimokawa I (2000) Apoptosis in the ageing process. *Cell. Tissue Res.* **301**, 125–132.
- Jernigan TL, Archibald SL, Femena-Notestine C, Gaust AC, Stout JC, Bonner J, Hesselink JR (2001) Effects of age on tissues and regions of the cerebrum and cerebellum. *Neurobiol. Ageing* **22**, 581–594.
- Jewell UR, Kvicukova T, Scheid A, Wenger RH, Gassmann M (2001) Induction of HIF-1 α in response to hypoxia is simultaneous. *FASEB J.* **15**, 1312–1314.

- Jung F, Haendeler J, Hoffmann J, Reissner A, Derubach E, Zeiher AM, Dimmeler S (2002) Hypoxic induction of hypoxia-inducible factor is mediated via the adaptor protein Shc in endothelial cells. *Circ. Res.* **91**, 38–45.
- Kang PM, Haustetter A, Aoki H, Usheva A, Irumo S (2000) Morphological and molecular characterization of adult cardiomyocyte apoptosis during hypoxia and reoxygenation. *Circ. Res.* **87**, 118–125.
- Kerr JFR, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br. J. Cancer* **26**, 239–257.
- Korhonen P, Helenus M, Salmusén A (1997) Age-related changes in the regulation of transcription factors NF- κ B in rat brain. *Neurosci. Lett.* **25**, 61–64.
- Leroualch F, Behl C (1998) Transcription factor NF- κ B: friend or foe of neurons. *Mol. Psychiatry* **3**, 15–20.
- Liu L, Azhar G, Gao W, Zhang X, Wei JY (1998) Bcl-2 and Bax expression in adult rat hearts after coronary occlusion: age-associated differences. *Am. J. Physiol.* **33**, 1232–1242.
- Lowenstein EJ, Daly RJ, Batzer AG, Li W, Margolis B, Lammers R, Ulrich A, Skolnik EY, Bar-Sagi D, Schlessinger J (1992) The SH2 and SH3 domain containing protein GRB2 links receptor tyrosine kinases to Ras signalling. *Cell* **70**, 431–442.
- Masliyah E, Mallory M, Hansen L, De Teresa R, Terry RD (1993) Quantitative synaptic alterations in the human neocortex during normal ageing. *Neurology* **43**, 192–197.
- Masoro EJ (1995) Ageing: current concepts. In *Handbook of Physiology—Ageing Sec.* (ed. Masoro, EJ), pp. 3–21. New York: Oxford University Press.
- Michiels M, Minet E, Mottet D, Raes M (2002) Regulation of gene expression by oxygen: NF- κ B and HIF-1 two extremes. *Free Rad. Biol. Med.* **33**, 1232–1242.
- Migliaccio E, Giorgio M, Mele S, Pellicci G, Reboldi P, Pandolci PP, Lanfranconi L, Pellicci PG (1999) The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature* **402**, 309–313.
- Nitahara JA, Cheng W, Liu Y, Li B, Leri A, Li P, Mogul D, Gambert SR, Kajstura J, Anversa P (1998) Intracellular calcium, DNase activity and myocyte apoptosis in aging Fischer 344 rats. *J. Mol. Cell. Cardiol.* **30**, 519–535.
- Oppenheim RW (1991) Cell death during development of the nervous system. *Annu. Rev. Neurosci.* **14**, 453–501.
- Otterbein LE, Chin BY, Manteil LL, Stansberry L, Horowitz S, Choi AM (1998) Pulmonary apoptosis in aged and oxygen tolerant rats exposed to hyperoxia. *Am. J. Physiol.* **275**, L14–L20.
- Pakkenberg B, Gundersen HJ (1997) Neocortical neuron number in humans: effect of sex and age. *J. Comp. Neurol.* **384**, 312–320.
- Portera-Caillan C, Martin LJ, Hedreen JC, Price DFL, Kiliatsos VE (1994) Cell death following excitotoxic injury in the striatum is ultrastructurally necrotic but also involves DNA laddering. *Soc. Neurosci. Abstract* **20**, 114–122.
- Prabhakar NR, Kumar GK (2004) Oxidative stress in the systemic and cellular responses to intermittent hypoxia. *Biol. Chem.* **385**, 217–221.
- Purdum S, Chen QM (2003) p66 Shc: at the crossroad of oxidative stress and the genetics of aging. *Trends Mol. Med.* **9**, 206–210.
- Razzagne MS, Shimokawa I, Koji T, Higami Y, Taguchi T (1999) Life-long caloric restriction suppresses age-associated Fas expression in the Fischer 344 rat kidney. *Mol. Cell. Biol. Res. Commun.* **1**, 82–85.
- Sastry PS, Rao KS (2000) Apoptosis and the nervous system. *J. Neurochem.* **74**, 1–20.
- Semenza GL (2000) HIF-1: mediator of physiological and pathophysiological response to hypoxia. *J. Appl. Physiol.* **88**, 1474–1480.
- Semenza GL (2001) HIF-1 and mechanisms of hypoxia sensing. *Curr. Opin. Cell. Biol.* **13**, 167–171.
- Semenza GL (2004) Hydroxylation of HIF-1: oxygen sensing at the molecular level. *Physiology* **19**, 176–182.
- Sharp FR, Bergeron M, Bernandin M (2001) Hypoxia inducible factor in the brain. *Adv. Exp. Med.* **502**, 273–291.
- Stadtman ER (1995) The status of oxidatively modified proteins as a marker of ageing. In *Molecular Aspects of Ageing* (eds Esser K, Martin GM), pp. 129–144. New York: Wiley.
- Ugi S, Sharma PM, Ricketts W, Imamura T, Olefsky JM (2002) Phosphatidylinositol 3-kinase is required for insulin-stimulated tyrosine phosphorylation of Shc in 3T3-L1 adipocytes. *J. Biol. Chem.* **277**, 18592–18597.
- Ventura A, Luzi L, Pacini S, Baldari CT, Pellicci PG (2002) The p66^{Shc} longevity gene is silenced through epigenetic modifications of an alternative promoter. *J. Biol. Chem.* **277**, 22370–22376.
- Vollgraf U, Wagner M, Richter-Landsberg C (1999) Activation of AP1 and NF- κ B transcription factors is involved in hydrogen peroxide apoptotic cell death of oligodendrocytes. *J. Neurochem.* **73**, 2501–2509.
- Wang GL, Semenza GL (1995) Purification and characterization of HIF-1. *J. Biol. Chem.* **270**, 1230–1237.
- Wenger RH (2002) Cellular adaptation to hypoxia: O₂ sensing protein hydroxylases, hypoxia inducible transcription factors and O₂ regulated gene expression. *FASEB J.* **16**, 1151–1162.
- White LD, Barone S (2001) Qualitative and quantitative estimates of apoptosis from birth to senescence. *Cell Death Differ.* **8**, 345–356.
- Wickens AP (2001) Ageing and the free radical theory. *Resp. Physiol.* **128**, 379–391.
- Yang E, Korsmeyer SJ (1996) Molecular thanatopsis: a discourse on the Bcl2 family and cell death. *Blood* **88**, 386–401.
- Yuan G, Adhikary G, McCormic AA, Holcroft JJ, Kumar GK, Prabhakar NR (2004) Role of oxidative stress in intermittent hypoxia-induced immediate early gene activation in rat PC12 cells. *J. Physiol.* **557**, 773–783.
- Zhang L, Kokkonen G, Roth GS (1995) Identification of neuronal programmed cell death in situ in the striatum of normal adult rat brain and its relationship to neuronal death during ageing. *Brain Res.* **677**, 177–179.