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Original article:

The Role of Anti- and Pro-apoptotic Cofactors in Hypoxia/Reoxygenation-Dependent Regulation of MAPKs in the Brain of an Anoxia-Tolerant Model

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Abstract

The cellular and molecular regulation of MAPKs and apoptosis was investigated in a model of hypoxia-tolerance. Survival of neurons in *Chrysemys picta bellii*, an anoxia-tolerant turtle, involves a reduction in energy metabolism. The biochemical/physiological mechanisms of anoxia tolerance have been examined at the level of ion transport and ATP turnover. However, changes in the phosphorylation state of key enzymes and kinases, mainly, MAPKs, may occur during anoxia, thereby reversible protein phosphorylation could be a critical factor and major mechanism of metabolic reorganization for enduring anaerobiosis. If a turtle were to undergo hypoxia akin to that experienced in its native habitat, it was placed in a glass aquarium filled with water to within a half inch of the top. After the turtle was anesthetized, through extended hypoxia or anesthesia, the animal was sacrificed by decapitation. The brain was then excised and placed in anoxic artificial cerebrospinal fluid. Total protein extraction was performed by homogenizing brain in a buffer, followed by threonine and tyrosine phosphorylation determination of MAPKs, and caspase activity. $MAPK^{p38}$ was decreased after reoxygenation following 1 day and 1 week hypoxia. The effect of hypoxia on the phosphorylation of $MAPK^{ERK}$ was biphasic: Enhancement at 5h and inhibition at 6 weeks. Pro-caspases 8/9 were unchanged by hypoxia until increasing at 6 weeks. Both pro-caspases were upregulated by reoxygenation at 1 day or 6 weeks hypoxia. Neither hypoxia nor reoxygenation induced the cleavage of pro-caspases 8/9 into p20 and p10, respectively. Furthermore, hypoxia induced Bax at 3 days and 1 week, and reoxygenation increased Bax ≈ 4-fold at 1 day. Although the expression of Bcl-2 was slightly increased by hypoxia, [Bcl-2] was 3-4-fold smaller in comparison with Bax. These results indicate that hypoxia up-regulates $MAPK^{ERK}$ but not $MAPK^{p38}$; hypoxia/reperfusion increases the expression of caspases and pro-apoptotic cofactors. The patterns of MAPK regulation suggest the significance of these kinases in cellular adaptation to oxygen deprivation with biomedical correlations, and thereby identify novel natural responsive signaling cofactors in *Chrysemys picta bellii* with potential pharmacologic and clinical applications.

Keywords: Anoxia; Apoptosis; Brain; Caspase; *Chrysemys picta*; Hypoxia; MAPK; Neuroprotection; Turtle.

1. Introduction

The Western painted turtles *Chrysemys picta bellii* are unusually tolerant of anoxia in that they survive 24-48h of anoxia at 25^{o}C and 4-6 months at 2-3^{o}C during winter dormancy (Baker et al., 2003; Reese et al., 2002; Ambrosino and Nebreda, 2001; Barone et al., 2001; López-Barneo et al., 2001; Banasiak et al., 2000; Bickler, 1998; Cano and Mahadevan, 1995). Integrative and sustained adaptations during submergence anoxia underlie the animal's capacity to tolerate these conditions for long periods of time (Bobb and Jackson, 2005; Baker et al., 2003; Shin and Buck, 2003; Shin et al., 2003; Barone et al., 2001; Nilson, 2001; Banasiak et al., 2000; Frische et al., 2000; Jackson, 2000a; Satoh et al., 2000; Shi and Jackson, 1997; Shi et al., 1997).

Survival of neurons in these remarkable turtles involves a profound reduction in energy metabolism to approximately 10-20% of the normoxic rate at the same temperature (Baker et al., 2003; Reese et al., 2002; Jackson, 2000a; Ko et al., 2000), suggesting a coordinated reduction of ATP-generating mechanisms and ATP-consuming pathways (Haddad, 2004a; Haddad et al., 2003; Reese et al., 2002; Haddad, 2002; Costanzo et al., 2001; Haddad, 2001; Greenway and Storey, 2000; Greenway and Storey, 1999; Bickler, 1998; Buck et al., 1998).

This metabolic 'arrest' has been shown to lead to suppression of ion channels, thereby allowing decreased excitability, reduced ion translocation and preservation of [ATP] during the energetic stress imposed by anaerobic conditions (Farahani and Haddad, 2003; Lutz et al., 2003; Reese et al., 2002; Costanzo et al., 2001a; Costanzo et al., 2001b; Drew et al., 2001; Enslen and Davis, 2001; López-Barneo et al., 2001; Nilsson, 2001; Costanzo et al., 2000; Frische et al., 2000; Jackson, 2000a; Jackson, 2000b; Jackson et al., 2000; Ko et al., 2000; Lee et al., 2000; Bickler and Buck, 1998; Buck et al., 1998). Suppressed targets include numerous enzymes and molecules that regulate protein synthesis (Haddad, 2004a; Haddad, 2004b; Reese et al., 2002; Bickler et al., 2000; Buck, 2000; Chang et al., 2000; Jackson, 2000a; Garrington and Johnson, 1999; Cano and Mahadevan, 1995).

Another feature that characterizes survival is the ability to buffer an acid-base equilibrium in response to lactate accumulation due to anaerobic glycolysis (Warren et al., 2006; Baker et al., 2003; Costanzo et al., 2001a; López-Barneo et al., 2001; Bickler and Buck, 1998; Cano and Mahadevan, 1995; Han et al., 1994). This mechanism is centered on the release of carbonates from bone and shell to enhance body fluid buffering of lactic acid (Barone et al., 2001; Bickler and Buck, 1998). Therefore, the combination of slow metabolic activity and responsive mineral reserves are crucial to the survival of those animals under extreme conditions (Jackson, 2000a; Jackson, 2000b; Jackson et al., 2000; Ko et al., 2000; Lee et al., 2000).

The biochemical and physiological mechanisms of anoxia tolerance in turtles have been previously examined at the level of ion transport and ATP turnover to better understand the effect of oxygen deprivation (Nilsson and Lutz, 2004; Overgaard and Gesser, 2004; Reese et al., 2003; Reese et al., 2002; López-Barneo et al., 2001; Nicole et al., 2001; Bickler and Buck, 1998; Cano and Mahadevan, 1995; Marshall, 1994; Brooks and Storey, 1991). However, changes in the phosphorylation state of key enzymes and kinases may occur during anoxia, thereby reversible protein phosphorylation could be a critical factor and major mechanism of metabolic reorganization for enduring anaerobiosis (Prentice et al., 2003; Reese et al., 2002; Reese et al., 2001; Barone et al., 2001; Nilsson, 2001; Robinson and Cobb, 1997).

For instance, it has been shown that anoxia mediated changes in the activities of protein kinase A (PKA), PKC and protein phosphatase 1 (Overgaard and Gesser, 2004; Haddad et al., 2003; Scott et al., 2003; Schwartz and Baron, 1999; Singer et al., 1999). Furthermore, anoxia was shown to alter protein synthesis, mRNA accumulation and gene transcription in turtle organs, suggesting that the up-regulation of selective genes is crucial for surviving anoxia (Haddad, 2006; Milton et al., 2006; Haddad, 2004b; Haddad, 2004c; Baker et al., 2003; Reese et al., 2002; Barone et al., 2001; Greenway and Storey, 1999; Su and Karin, 1996; Cano and Mahadevan, 1995).

Members of the mitogen-activated protein kinase (MAPK) superfamily comprise signaling cascades that respond preferentially to certain stresses (Haddad, 2004c; Haddad, 2004d; Scott et al., 2003; Drew et al., 2001; Jackson, 2000a; Jackson 2000b; Satoh et al.,

2000; Schwartz and Baron, 1999; Singer et al., 1999). Many stimuli, including anoxia, oxygenation and hypoxia/re-oxygenation, for example, elicit specific cellular responses through the activation of MAPK signaling pathways (Haddad, 2004b; Haddad, 2004c; Ultsch and Jackson, 1982).

MAPKs are proline-targeted serine-threonine kinases that transduce environmental stimuli to the nucleus and are activated by upstream MAPK kinases (MAPKKs) on both threonine and tyrosine residues within an 'activation loop' (Haddad, 2004b; Widmann et al., 1999; Wasser et al., 1997). MAPKs can phosphorylate and activate other kinases or nuclear proteins, including potential transcription factors (Hetman et al., 1999; Schwartz and Baron, 1999). The regulation of c-Jun NH_2-terminal kinases (JNKs) activation and extracellular signal-regulated protein kinase (ERK) suppression were reported in the organs of hatchling red-eared turtles, *Trachemys scripta elegans* in response to anoxia (Greenway and Johnson, 1999; Greenway and Storey, 1999).

To date, however, anoxia-mediated regulation of signaling pathways mediating MAPK signaling responses is not well characterized in *Chrysemys picta* and other species. These observations prompted me to investigate the regulation of MAPK signaling in response to submergence anoxia *in vivo*. Since $MAPK^{ERK}$ and $MAPK^{p38}$ kinases are involved in adaptive responses involving neuroprotection and injury during hypoxia, I have explored hypoxia-mediated regulation of these kinases in the brain of *Chrysemys picta*.

This work further identified downstream pathways mediating the regulation of anoxia-induced apoptosis, including the expression of pro- and anti-apoptosis cofactors and upstream caspases. Because turtles survive prolonged anoxia without neuronal injury, the hypothesis that $MAPK^{p38}$/$MAPK^{ERK}$ pathways and Bax/Bcl-2-related mechanisms are involved in neuroprotection was subsequently investigated.

2. Materials and Methods

2.1 Chemicals and reagents

Unless indicated otherwise, chemicals of the highest analytical grade were purchased from Sigma-Aldrich (CA, USA). Housing and handling of animals was performed with strict adherence to ethical guidelines set forth in the Animals Legislation Act (USA).

2.2. Animal handling and experimentation

Chrysemys picta bellii were obtained from Lembagen, Oshkosh, WI, and housed according to UCSF Animal Care Facility regulations. Turtles were kept in plexiglass aquaria in several inches of water and were fed frog chow. If a turtle were to undergo hypoxia akin to that experienced in its native habitat, it was placed in a glass aquarium filled with water to within a half inch of the top. The tank was placed in the cold room, which was kept between 3.8 and 4^{o}C. A plexiglass lid was placed on top of the tank and sealed with grease around its edges. The water was constantly bubbled with anoxic gas (95% N_2/5% CO_2) delivered via a tube connected to an airstone at the bottom of the tank.

At the end of the experimental period, the turtle was placed in a canister attached by a hose to an anesthesia vaporizer set to 2%. After the turtle was anesthetized, through extended hypoxia or anesthesia, the animal was sacrificed by decapitation. The brain was then excised and placed in ice-cold anoxic turtle artificial cerebrospinal fluid (aCSF containing (in mM): 86.5 NaCl, 2.6 KCl, 2.5 $CaCl_2$, 0.2 $MgCl_2$, 2.0 NaH_2PO_4, 26.5 $NaHCO_3$, 20 glucose and 10 HEPES), snap frozen and stored at –70^{o}C until extraction.

2.3. Preparation of subcellular extracts for western analysis

Total protein extraction was performed by homogenizing turtle brain tissue in a suitable volume of a buffer (1:40; w/v) containing 20 mM HEPES (pH7.5), 1.5 mM $MgCl_2$, 0.2 mM EDTA and 0.1 M NaCl. Before extraction 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1.2 mM sodium orthovanadate (Na_3VO_4) were added to the buffer. The cellular debris was pelleted by centrifugation at 10,000*g* for 30 min at 4^{o}C, and the collected supernatant was mixed with an equal volume of the same extracting buffer, but containing in addition 40 % (v/v) glycerol.

Threonine and tyrosine phosphorylation of $MAPK^{p38}$ was analyzed according to instructions given in commercially available kits (New England Biolabs, Inc., USA). The kit employs specific anti-phospho-p38 MAPK antibodies against Thr180/Tyr182 sites

that do not cross-react with phosphorylated threonine/tyrosine of extracellular signal-regulated kinase (ERK) 1/2 or c-Jun-NH_2-terminal kinase (JNK). $MAPK^{p38}$ is a member of the MAPK family of kinases that is potently and preferentially activated by various stimuli (Ultsch and Jackson, 1982).

Analysis of Thr^{180}/Tyr^{182} phosphorylation of $MAPK^{p38}$ was performed as follows: Extracted proteins (20-25 μg) were resolved over sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10 %) gels at RT, blotted onto nitrocellulose membrane, and non-specific binding sites were subsequently blocked. The membrane was probed with specific antibody to phosphorylated threonine and tyrosine of $MAPK^{p38}$ for primary detection. Anti-rabbit Ig-biotinylated antibody (Amersham Life Science) was employed for secondary detection followed by the addition of streptavidin-HRP conjugate and visualized on film by chemiluminescence. $MAPK^{p38}$ detection using a specific antibody, which recognizes the non-phosphorylated form, was considered as an internal reference for semi-quantitative loading in parallel lanes for each variable.

In separate experiments, $MAPK^{ERK}$ ($MAPK^{p44/p42}$) westerns were performed using a specific polyclonal antibody that recognizes the phosphorylated and non-phosphorylated forms of ERK (New England Biolabs, Inc., USA). The phospho-antibody specific for $MAPK^{ERK}$ does not cross-react with either $MAPK^{p38}$ or $MAPK^{JNK}$, as supplied by the manufacturer.

Western blots were scanned by NIH MagiScanII and subsequently quantitated by UN-Scan-IT automated digitizing system (Version 5.1; 32-bit), and the ratio of the density of the band to that of the non-phosphorylated form was performed.

2.4. Analysis of pro-caspase expression and cleavage during hypoxia

Brain samples were assayed for the expression of pro-caspase 8 (sc-7890) and pro-caspase 9 (sc-7885) and their cleavage by western immunoblotting analysis, employing specific antibodies (Santa Cruz Biotechnology, CA, USA). Caspase 8 p20 (H-134) is a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 217-350 mapping within the caspase 8 p20 subunit of human origin. H-134 reacts with the active p20 subunit and precursor of caspase 8 (Mch5/MACHa1/FLICE).

Caspase 9 p10 (H-83) is also a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 315-397 mapping within the carboxy terminus of caspase 9 of human origin. H-83 reacts with the active p10 subunit and precursor of caspase 9 (ICE-LAP6). Samples (20-25 μg) were resolved and detected over 10% SDS-PAGE gels and analyzed as described above.

2.5. Analysis of apoptosis cofactor expression during hypoxia

The family of B-cell leukaemia/lymphoma-2 (Bcl-2) related proteins constitutes a class of apoptosis-regulatory gene products that act at the effector stage of cell death. Two functional classes of Bcl-2-related proteins have been identified that share highly conserved homology domains: (i) anti-apoptotic, or antagonistic, members, including Bcl-2, which confer negative control in the pathways of cellular suicide machinery, and (ii) pro-apoptotic, or agonistic, members, including Bcl-associated x protein (Bax), which promote cell death by competing with Bcl-2. While Bax/Bax homodimers induce apoptosis, Bcl-2/Bax heterodimer formation evokes a survival signal.

In order to investigate the role of these signaling cofactors in anoxia-tolerant turtles, the expression of Bcl-2 and Bax on the imposition of hypoxia and the ratio of Bcl-2/Bax as an index of apoptosis regulation under these conditions were determined. Western analysis was performed as above using specific antibodies; Bax (B-9; Santa Cruz Biotechnology, CA, USA) is a mouse monoclonal IgG_{2b} antibody mapping at amino acids 1-171 representing all but the carboxy terminal 21 amino acids of Bax of mouse origin. Bcl-2 (C-2) is a mouse monoclonal IgG_1 antibody raised against a recombinant protein corresponding to amino acids 1-205 of Bcl-2 of human origin. Both antibodies were used to detect specific bands with hypoxia and hypoxia/reoxygenation.

2.6. Statistical analysis and data presentation

Data are the means and the error bars are the SEM. Statistical evaluation of the difference in mean separation was performed by one-way analysis of variance (ANOVA), followed by *post hoc* Tukey's

test, and the *a priori* level of significance at 95% confidence level was considered at $P \leq 0.05$.

3. Results

All turtles thus far experimented upon survived a period of up to 6 weeks of submergence hypoxia, and hypoxia/reoxygenation had no effect on the survival rate of turtles exposed for up to 6h reoxygenation.

3.1. Hypoxia-mediated regulation of MAPK^{p38} and its phosphorylation

The effect of hypoxia submergence and hypoxia-reoxygenation on MAPK^{p38} signaling pathway in the brain of *Chrysemys* is shown in Fig. 1. Exposing turtles to hypoxia for up to 6 weeks had no apparent effect on the phosphorylation/activation of MAPK^{p38} (MAPK$^{phospho\text{-}p38}$), as compared with normoxic turtles (Fig. 1A).

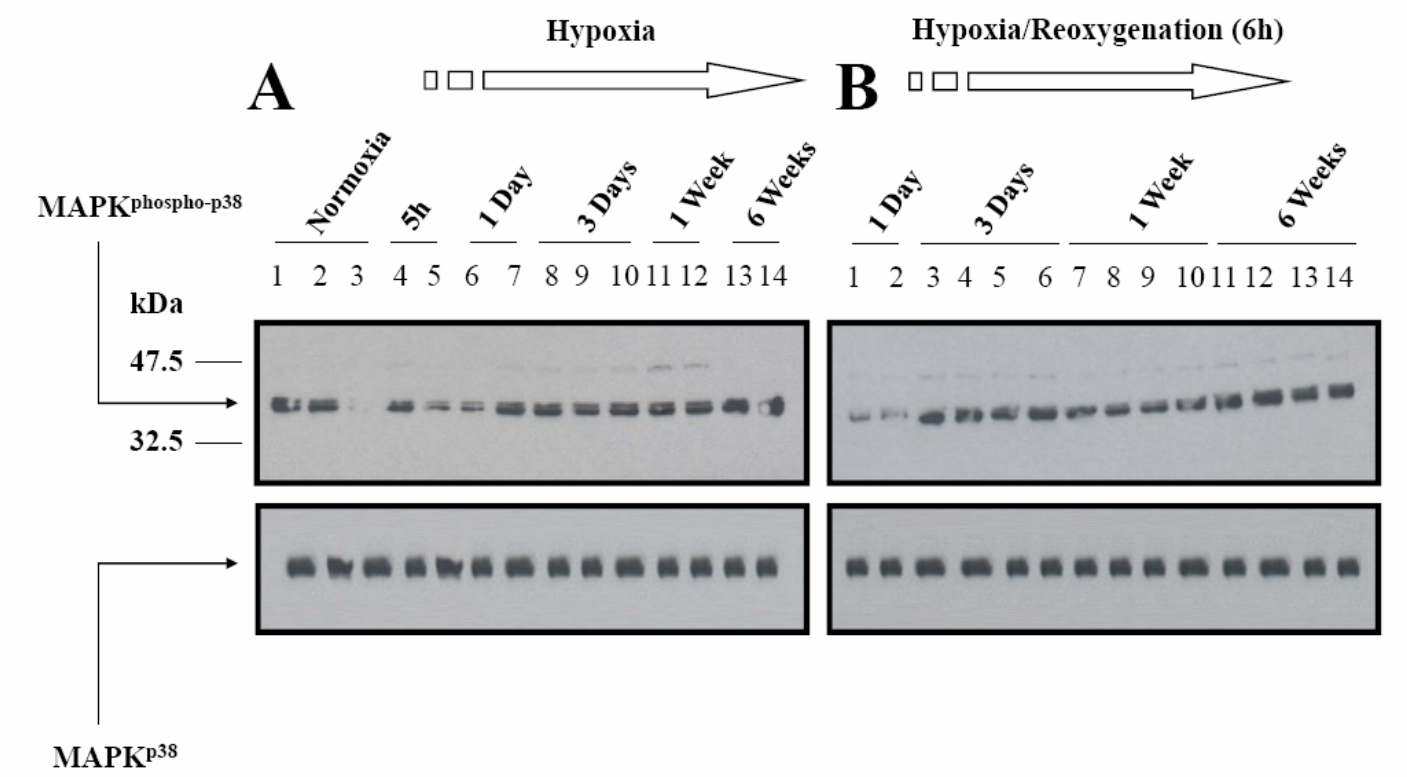

Fig. 1 Hypoxia-mediated regulation of MAPK^{p38} signaling pathway. **(A)** Exposure to hypoxia for a period of time ranging from 5h to 6 weeks did not significantly affect the phosphorylation/activation of MAPK^{p38}, as compared with normoxia control. **(B)** Hypoxia/reoxygenation for 6h variably affected the phosphorylation/activation of MAPK^{p38}, with suppression at 1 day and 1 week of hypoxia followed by oxygenation. This MAPK^{p38} suppression was not observed at either 3 days or 6 weeks of hypoxia. The lower panel shows the expression of the non-phosphorylated form of MAPK^{p38}, as verification for semi-quantitative loading in parallel lanes. The numbers 1-14 allocated to the top of the gel indicates the number of each corresponding lane. *n* = 2-5, which designates the number of turtles and independent experiments performed for each variable.

Exposure to hypoxia for specified periods of time followed by reoxygenation for 6h reduced the phosphorylation/activation of MAPK^{p38} at day 1 ($^{++}$ $P < 0.01$) and 1 week ($^{+}$ $P < 0.01$) of hypoxia, but not at either 3 days or 6 weeks of hypoxia (Fig. 1B). The constitutive, non-phosphorylated level expression of MAPK^{p38} is shown in Figs. 1A and 1B in the lower panel for hypoxia and hypoxia/reoxygenation, respectively, in order to validate semi-quantitative loading in parallel lanes. To further confirm semi-quantitative loading in parallel lanes, the protein abundance of the constitutive expression of β-actin

was performed (Data not shown). The number of experiments undertaken was repeated with various immunoblots taken from 2 – 3 different turtles. Therefore, any variations in band intensity at any given treatment reflect variations in responses of various turtles within same species (endogenous biologic effect). The average of expression is calibrated with histogram analysis (see below).

Quantitative histogram analysis of the effect of hypoxia and hypoxia/reoxygenation on $MAPK^{p\text{-}p38}$ regulation is shown in Fig. 2. For clarity, the degree of $MAPK^{p38}$ phosphorylation was set as 1.00 unit values for the normoxic brains, in both hypoxia and hypoxia-reoxygenation (Fig. 2). The number of turtles in this and any subsequent experiment ranged from between 2 – 3 different turtles. Histogram analysis corresponds with the intensity of protein bands cumulatively taken from each representative gel. Using densitometry analysis, the degree of phosphorylation was determined against this unit value at normoxia. Furthermore, the times (5h – 6 weeks) indicate hypoxia exposure, which was followed with 6h reoxygenation, except that for 5h hypoxia (ND).

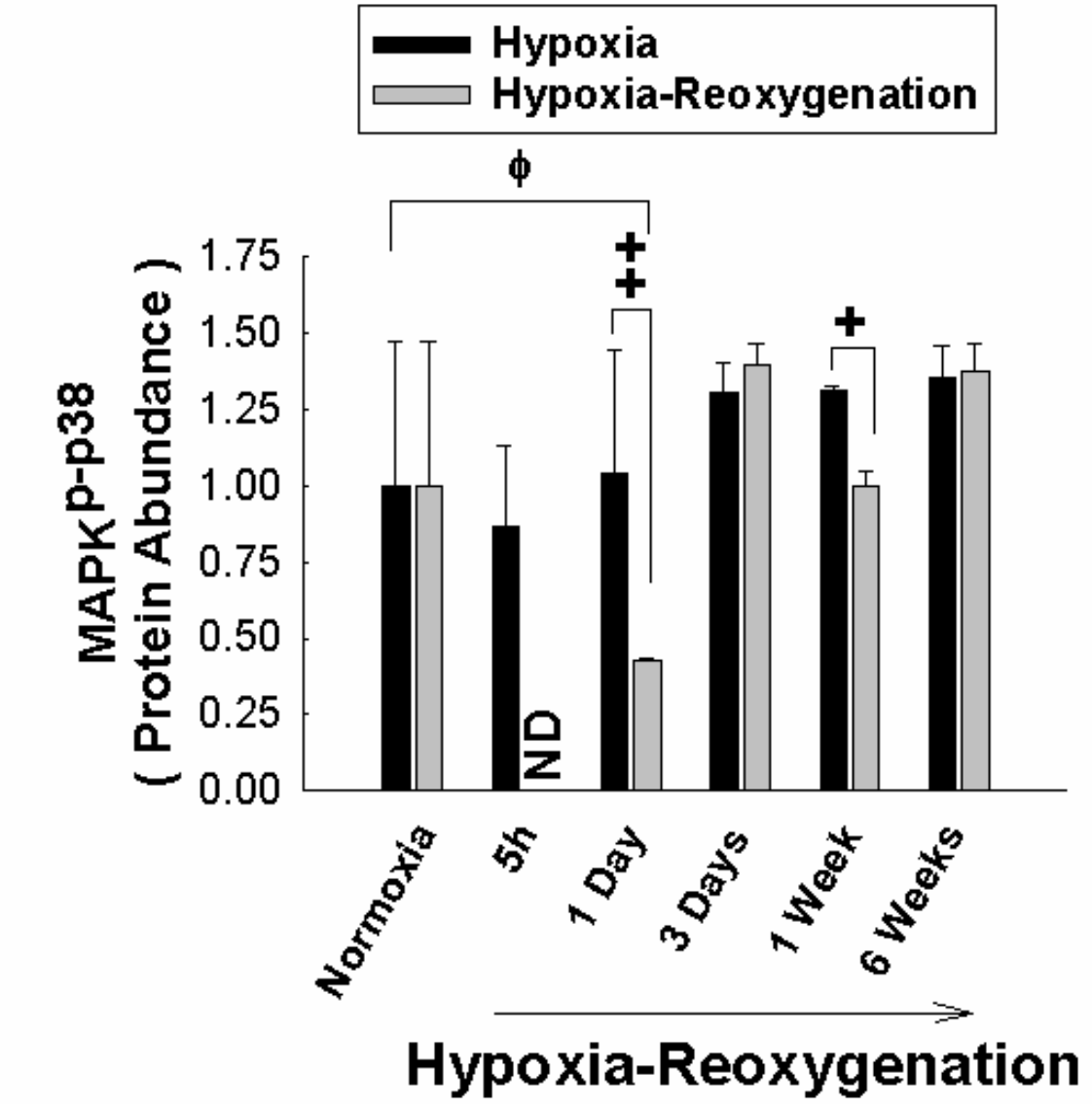

Fig. 2 Histogram analysis of the relative abundance of the phosphorylated form of $MAPK^{p38}$ under hypoxia/reoxygenation (6h) (The level of expression at normoxia was adjusted to 1 unit and all other values were expressed relative to this unit). *ND*, not determined.

The level of phosphorylation of $MAPK^{p38}$ with reoxygenation at 1 day of hypoxia was significantly reduced (^{Ø} $P < 0.05$), as compared with normoxic brains (Fig. 2). The level of phosphorylation of $MAPK^{p38}$ with reoxygenation at 1 day and 1 week of hypoxia was significantly reduced ($^{+}$ $P < 0.05$; $^{++}$ $P < 0.01$), as compared with hypoxic brains during the same periods (Fig. 2).

3.2. Hypoxia-mediated regulation of $MAPK^{ERK}$ and its phosphorylation

The effect of hypoxia submergence and hypoxia/reoxygenation on $MAPK^{ERK}$ ($MAPK^{p44/p42}$) signaling pathway in the brain of *Chrysemys* is shown in Fig. 3. Exposing turtles to hypoxia for up to 6 weeks variably and in a biphasic manner allowed the phosphorylation/activation of $MAPK^{ERK}$ ($MAPK^{phospho\text{-}ERK}$), as compared with normoxic turtles (Fig. 3A). Exposure to hypoxia for 5h induced the phosphorylation/activation of $MAPK^{ERK}$ (** $P < 0.01$); this activation returned to baseline levels between 1 day and 1 week of hypoxia, and was suppressed at 6 weeks (** $P < 0.01$) (Fig. 3A). Interestingly, the observation that after 6 weeks of hypoxia $MAPK^{phospho\text{-}ERK}$ was no longer detectable, ostensibly due to the fact that contiguous, rather than intermittent, exposure to hypoxia may act as a factor in downregulating the expression of $MAPK^{ERK}$.

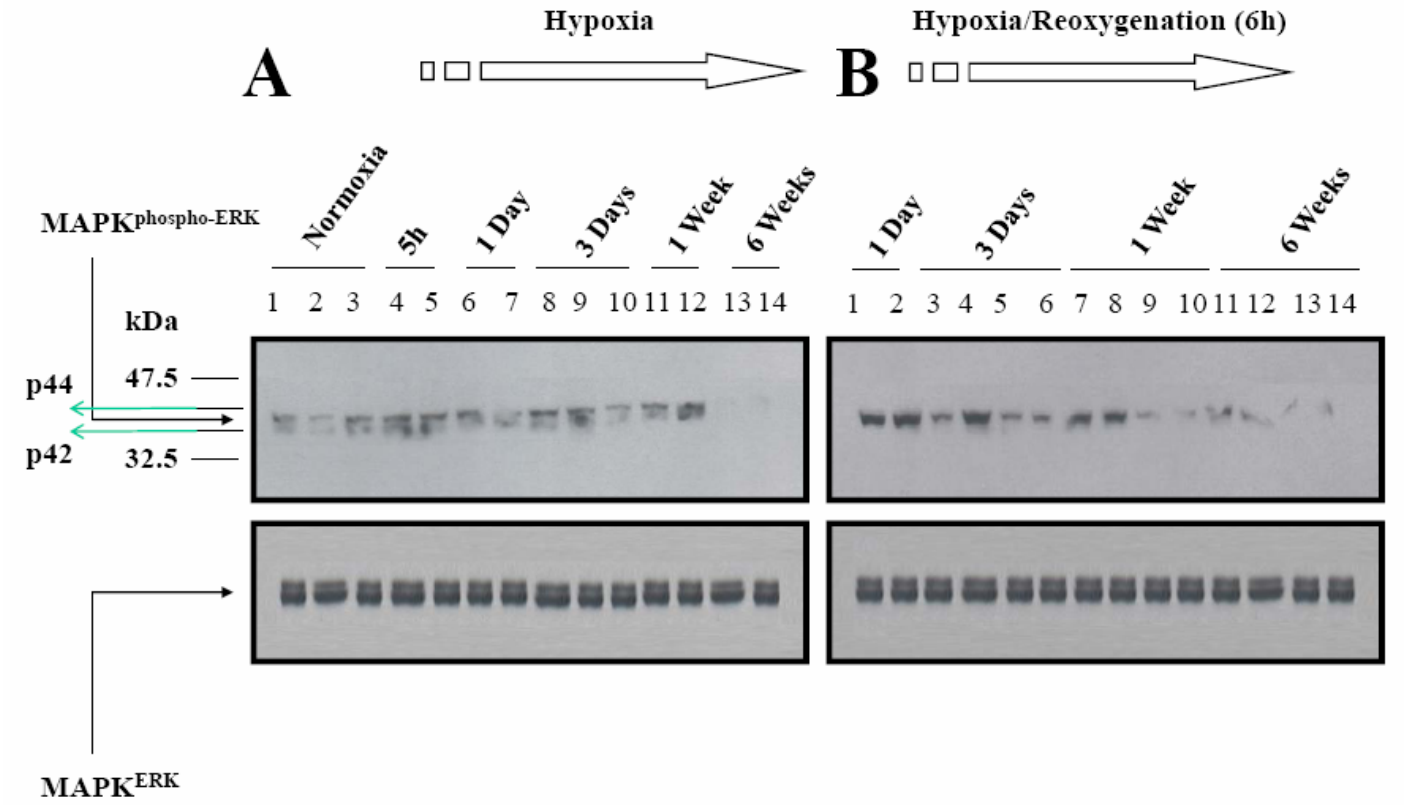

Fig. 3 Hypoxia-mediated regulation of $MAPK^{p44/p42}$ ($MAPK^{ERK1/2}$) signaling pathway. **(A)** Exposure to hypoxia for a period of time ranging from 5h to 6 weeks variably and in a biphasic manner affected the phosphorylation/activation of $MAPK^{p44/p42}$, as compared with normoxia control. Whereas 5h of hypoxia significantly increased the phosphorylation/activation of $MAPK^{p44/p42}$, 1 day $\rightarrow$ 1 week was not significantly different from normoxia, and at 6 weeks of hypoxia the phosphorylation/activation of $MAPK^{p44/p42}$ was suppressed, as compared with normoxia and 5h. **(B)** Hypoxia/reoxygenation for 6h mildly affected the phosphorylation/activation of $MAPK^{p44/p42}$, with suppression at 6 weeks of hypoxia followed by oxygenation. This $MAPK^{p44/p42}$ suppression was not observed at 1 day $\rightarrow$ 1 week of hypoxia. The lower panel shows the expression of the non-phosphorylated form of $MAPK^{p44/p42}$, as verification for semi-quantitative loading in parallel lanes. The numbers 1-14 allocated to the top of the gel indicates the number of each corresponding lane. *n* = 2-5, which designates the number of turtles and independent experiments performed for each variable.

Exposure to hypoxia for specified periods of time followed by reoxygenation for 6h did not affect the phosphorylation/activation of $MAPK^{ERK}$, except at 6 weeks of hypoxia where a partial, but significant ($^{+}$ $P < 0.05$), restoration was observed (Fig. 3B). The constitutive, non-phosphorylated level expression of $MAPK^{ERK}$ is shown in Figs. 3A and 3B in the lower panel for hypoxia and hypoxia/reoxygenation, respectively, in order to validate semi-quantitative loading in parallel lanes. Data were reproduced in at least 2 – 3 different turtles.

Histogram analysis of the effect of hypoxia and hypoxia/reoxygenation on $MAPK^{ERK}$ ($MAPK^{p44/p42}$) regulation is shown in Fig. 4. The level of phosphorylation of $MAPK^{ERK}$ with reoxygenation at 6 weeks of hypoxia was found significantly reduced (^{Ø} $P < 0.05$), as compared with normoxic brains (Fig. 4). Exposure to hypoxia (5h) induced the phosphorylation of $MAPK^{ERK}$ (** $P < 0.01$). The level of phosphorylation of $MAPK^{ERK}$ with reoxygenation at 6 weeks of hypoxia was significantly increased ($^{+}$ $P < 0.05$), as compared with hypoxic brains during the same period (Fig. 4). Data were reproduced in at least 2 – 3 different turtles.

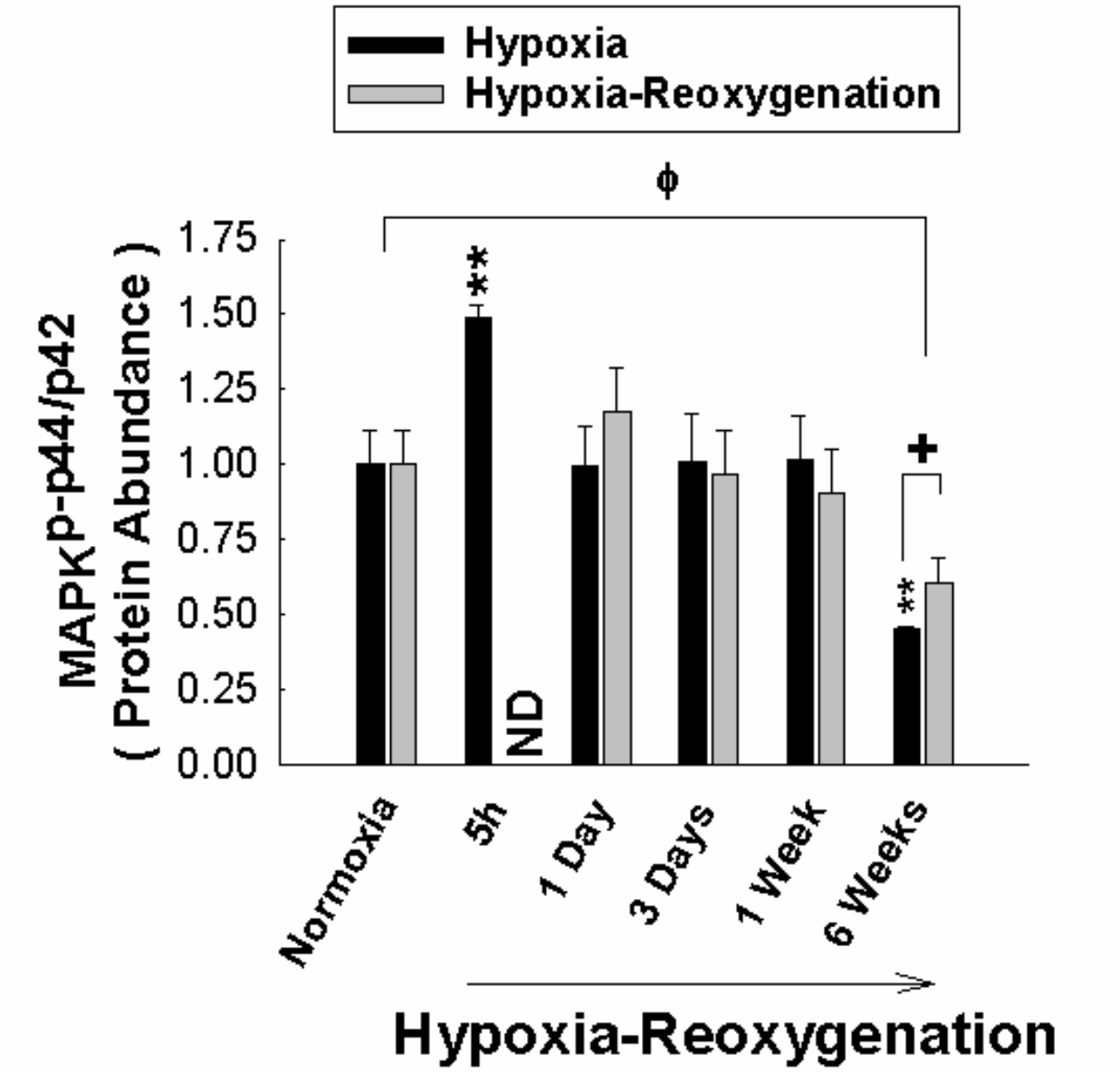

Fig. 4 Histogram analysis of the relative abundance of the phosphorylated form of $MAPK^{p44/p42}$ under hypoxia/reoxygenation (6h) (The level of expression at normoxia was adjusted to 1 unit and all other values were expressed relative to this unit). *ND*, not determined.

3.3. Hypoxia-mediated regulation of caspase-8 activation

The effect of hypoxia and hypoxia-reoxygenation on caspase 8 (H-134) cleavage and activation is shown in Fig. 5. Hypoxia exposure has no effect on the protein expression of pro-caspase 8 (≈ 55 kDa), as compared with normoxia, except at 6 weeks of hypoxia, where a sharp decline was observed (*** $P < 0.001$) (Fig. 5A). As a valid control for pro-caspase-8 expression (equal gel loading), the protein levels of β-actin were not significantly different among treatments (data not shown).

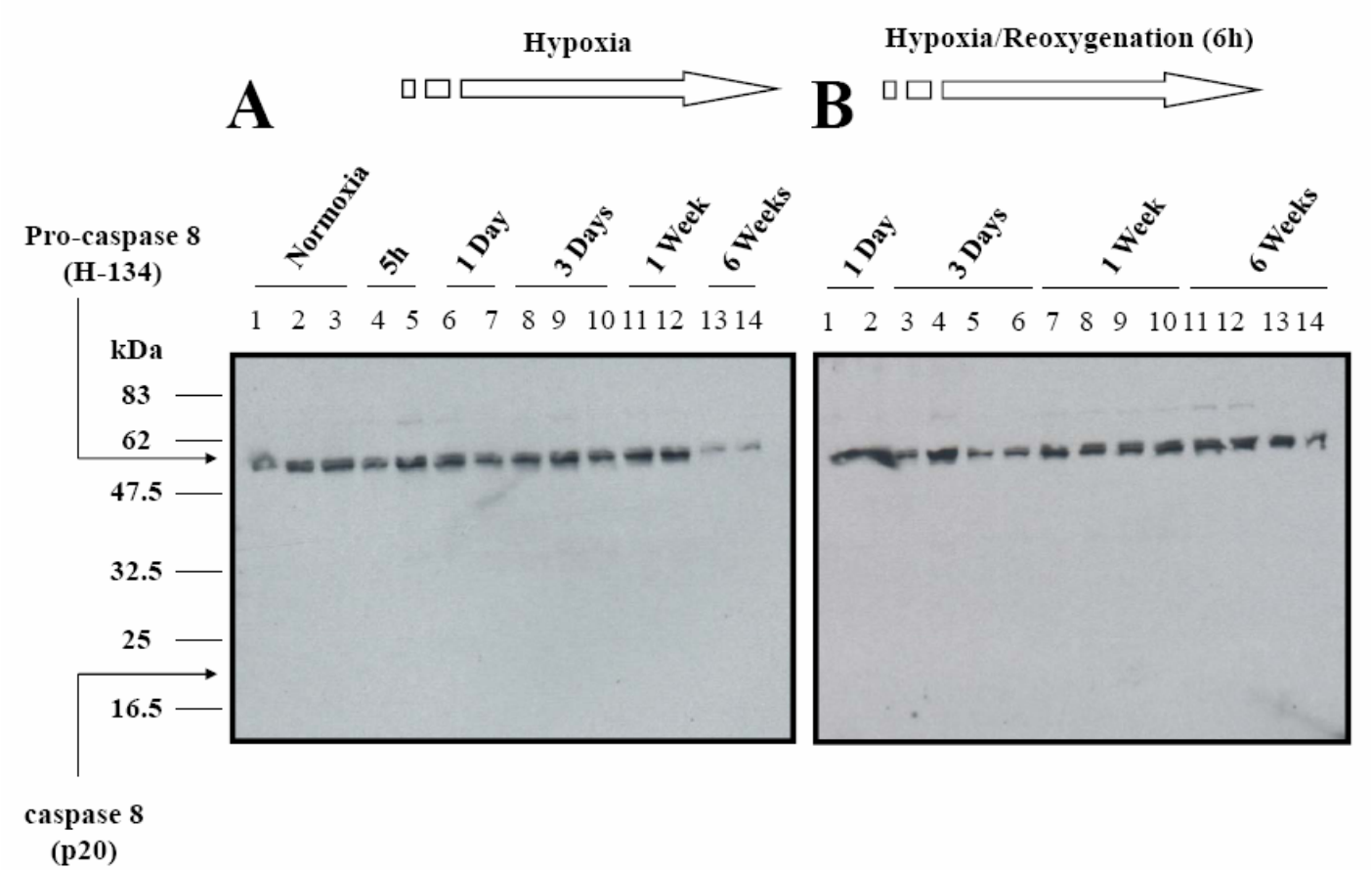

Fig. 5 Hypoxia-mediated regulation of caspase 8 expression and activation (cleavage). **(A)** Exposure to hypoxia for a period of time ranging from 5h to 6 weeks did not affect the expression of pro-caspase 8 (H-134), as compared with normoxia control, except at 6 weeks of hypoxia where the relative abundance of H-134 was lower. However, across the range of hypoxia spanned there was no conversion of pro-caspase 8 into the active form (p20). **(B)** Hypoxia/reoxygenation for 6h mildly affected the relative abundance of pro-caspase 8, with elevation at 1 day and 6 weeks of hypoxia followed by oxygenation. This increase was not observed at 3 days → 1 week of hypoxia. Neither did reoxygenation allow activation of pro-caspase 8 and appearance of p20 active subunit across the hypoxia-reoxygenation spanned curve. The numbers 1-14 allocated to the top of the gel indicates the number of each corresponding lane. *n* = 2-5, which designates the number of turtles and independent experiments performed for each variable.

Exposure to hypoxia for specified periods of time followed by reoxygenation for 6h has no apparent effect on the level of pro-caspase 8, except at 1 day ($^{+}$ $P < 0.05$) and 6 weeks ($^{++}$ $P < 0.01$) where the constitutive level of pro-caspase 8 partially increased (Fig. 5B). Neither hypoxia nor hypoxia/reoxygenation induced the cleavage of pro-caspase 8 into the active form, as there was no detectable band for the active form of caspase 8 (p20 subunit) (Figs. 5A and 5B).

Histogram analysis of the effect of hypoxia and hypoxia/reoxygenation on caspase-8 (H-134) cleavage is shown in Fig. 6. The level of caspase-8 (H-134) at 1 week and 6 weeks of hypoxia-reoxygenation was significantly increased ($^{+}$ $P < 0.05$; $^{++}$ $P < 0.01$), as compared with hypoxic brains during the same periods (Fig. 6). Data were reproduced in at least 2 – 3 different turtles.

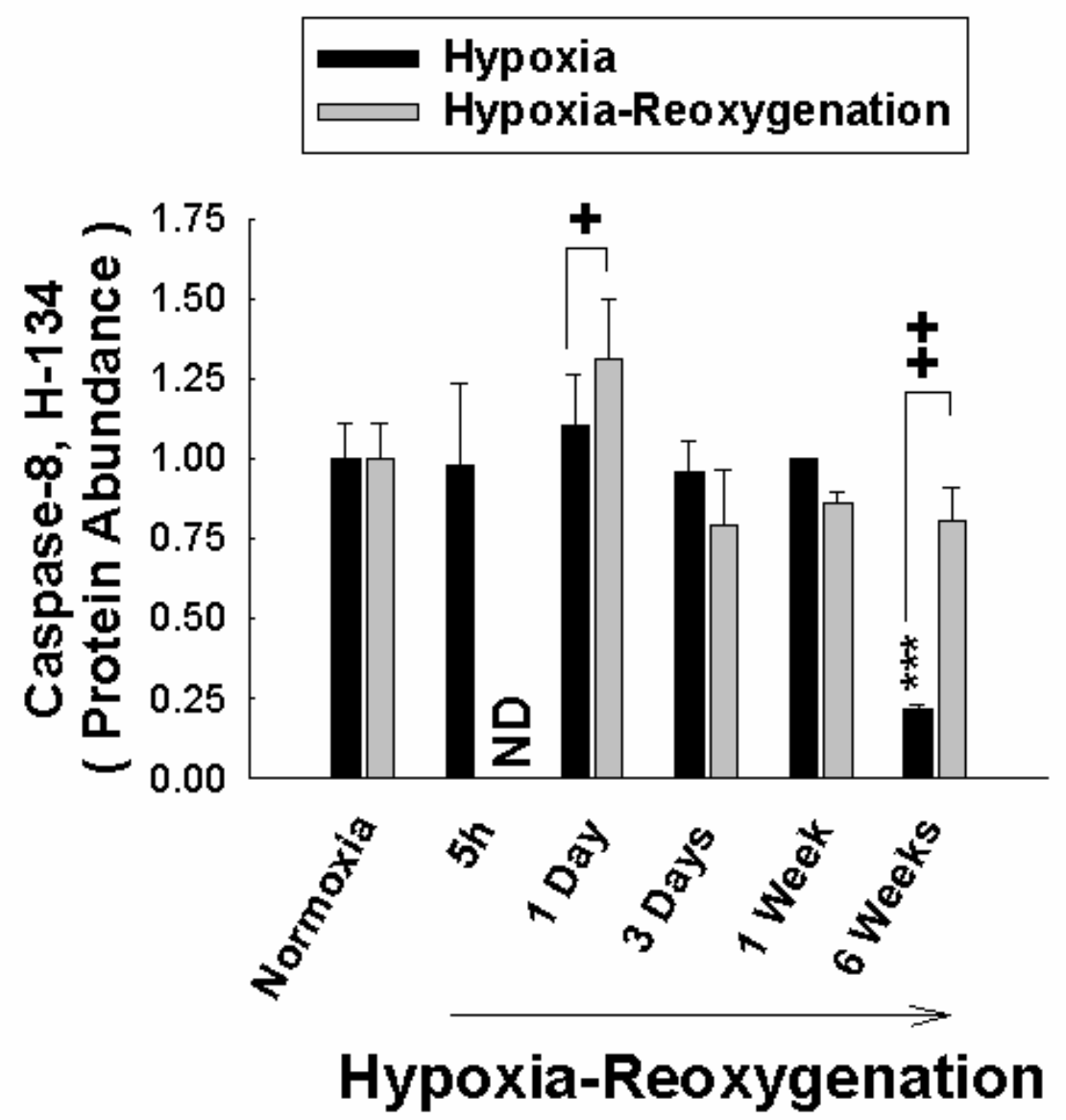

Fig. 6 Histogram analysis of the relative abundance of pro-caspase-8 (H-134) under hypoxia/reoxygenation (6h) (The level of expression at normoxia was adjusted to 1 unit and all other values were expressed relative to this unit). *ND*, not determined.

3.4. Hypoxia-mediated regulation of caspase-9 activation

Hypoxia exposure has no effect on the protein expression of pro-caspase 9 (≈ 50 kDa), as compared with normoxia, except at 6 weeks of hypoxia, where a sharp decline was observed (*** $P < 0.001$) (Fig. 7A). Exposure to hypoxia for specified periods of time followed by reoxygenation for 6h partially increased the constitutive level of pro-caspase 9, specifically at 3 days (^{Ø} $P < 0.05$), 1 week (^{Ø} $P < 0.05$) and 6 weeks (^{Ø} $P < 0.05$) of hypoxia (Fig. 7B). Neither hypoxia nor hypoxia-reoxygenation induced the cleavage of pro-caspase 9 into the active form, as there was no detectable band for the active form of caspase 9 (p10 subunit) (Figs. 7A and 7B). As a valid control for pro-caspase-9 expression (equal gel loading), the protein

levels of β-actin were not significantly different among treatments (data not shown).

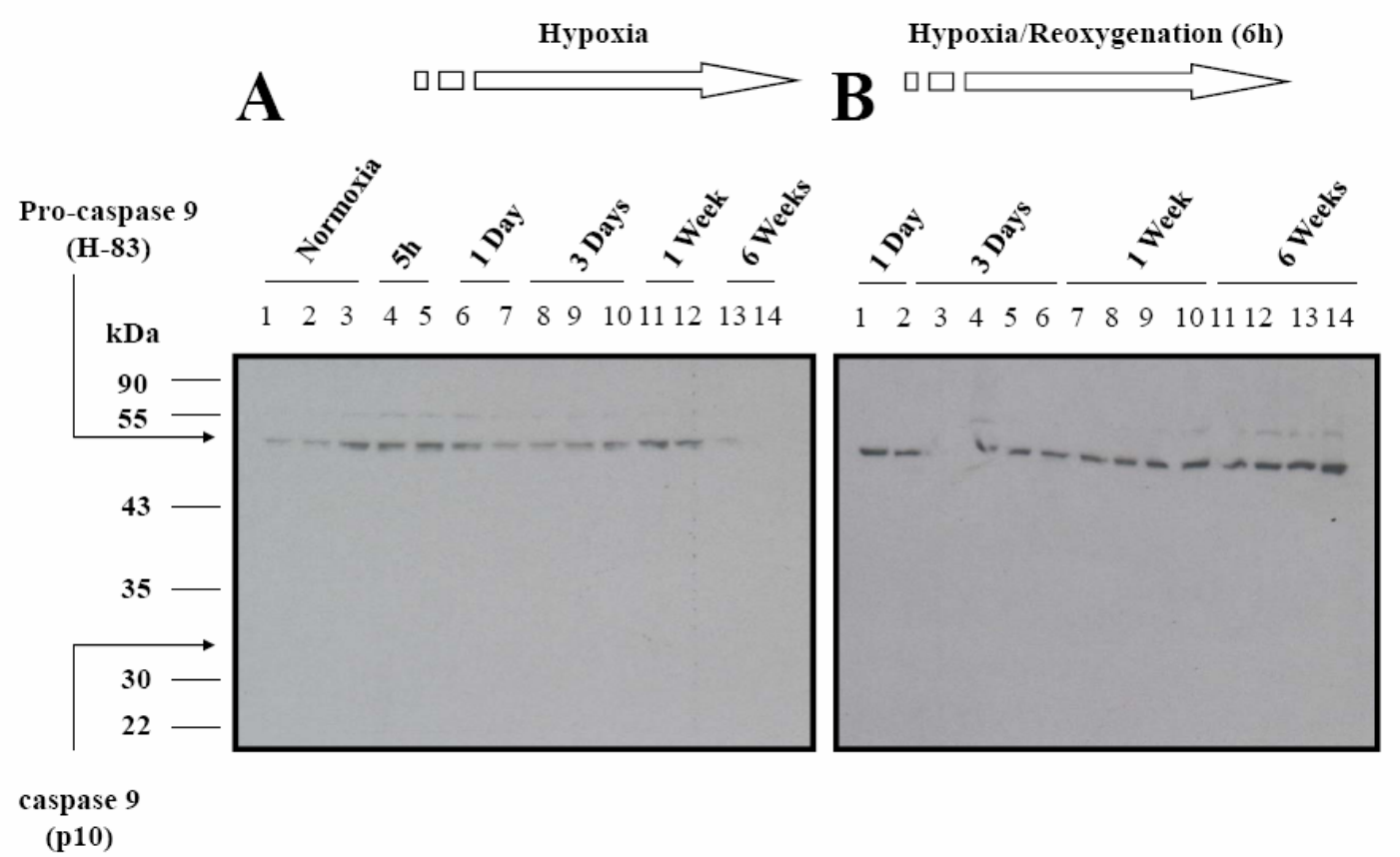

Fig. 7 Hypoxia-mediated regulation of caspase 9 expression and activation (cleavage). **(A)** Exposure to hypoxia for a period of time ranging from 5h to 6 weeks did not affect the expression of pro-caspase 9 (H-83), as compared with normoxia control, except at 6 weeks of hypoxia where the relative abundance of H-83 was lower. However, across the range of hypoxia spanned there was no conversion of pro-caspase 9 into the active form (p10). **(B)** Hypoxia/reoxygenation for 6h mildly affected the relative abundance of pro-caspase 9, with elevation at 6 weeks of hypoxia followed by oxygenation. This increase was not observed at 1 day $\rightarrow$ 1 week of hypoxia. Neither did reoxygenation allow activation of pro-caspase 9 and appearance of p10 active subunit across the hypoxia-reoxygenation spanned curve. The numbers 1-14 allocated to the top of the gel indicates the number of each corresponding lane. *n* = 2-5, which designates the number of turtles and independent experiments performed for each variable.

Histogram analysis of the effect of hypoxia and hypoxia/reoxygenation on caspase-9 (H-83) cleavage is shown in Fig. 8. The level of caspase-9 (H-83) at 6 weeks of hypoxia-reoxygenation was significantly increased ($^{+++}$ $P < 0.001$), as compared with hypoxic brains during the same period (Fig. 8). Data were reproduced in at least 2 – 3 different turtles.

Hypoxia-mediated regulation of apoptosis signaling cofactors, Bcl-2 and Bax

The effect of hypoxia and hypoxia/reoxygenation on protein expression of the anti-apoptotic cofactor Bcl-2 is shown in Fig. 9. Bcl-2 expression was minor in response to hypoxia, although the abundance of Bcl-2 reached statistical significance between 1 day and 1 week of hypoxia (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), in comparison with normoxic turtles (Fig. 9A). Hypoxia/reoxygenation decreased the mild expression of Bcl-2 observed with hypoxia (^{Ø} $P < 0.05$) (Fig. 9B). As a valid control for Bcl-2 expression (equal gel loading), the protein levels of β-actin were not

significantly different among treatments (data not shown).

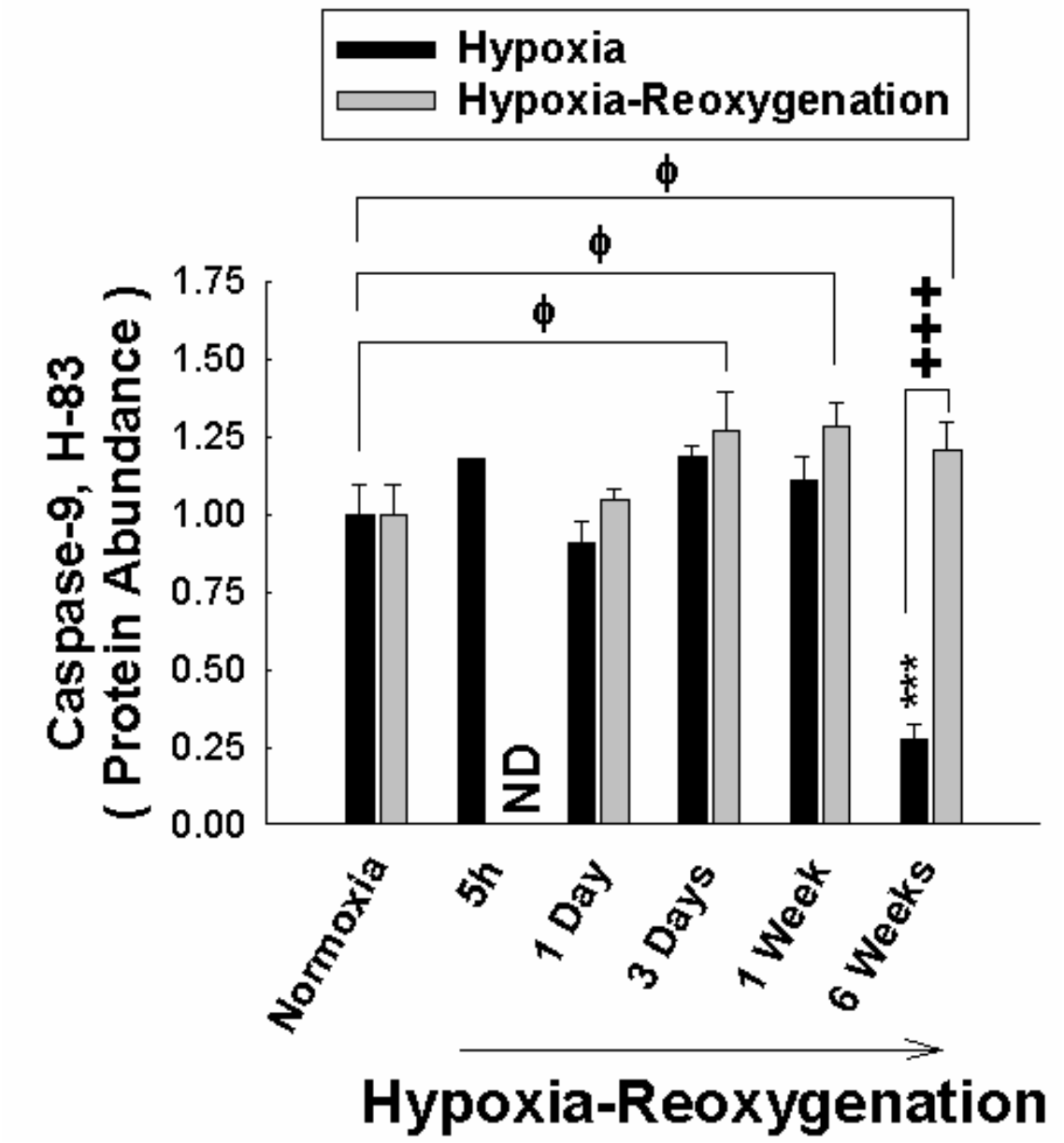

Fig. 8 Histogram analysis of the relative abundance of pro-caspase-9 (H-83) under hypoxia/reoxygenation (6h) (The level of expression at normoxia was adjusted to 1 unit and all other values were expressed relative to this unit). *ND*, not determined.

Histogram analysis of the effect of hypoxia and hypoxia/reoxygenation on Bcl-2 is shown in Fig. 10. The level of Bcl-2 was mildly increased at 1 day (* $P < 0.05$), 3 days (** $P < 0.05$) and 1 week (* $P< 0.05$) of hypoxia, as compared with normoxia. Hypoxia/reoxygenation decreased the mild expression of Bcl-2 observed with hypoxia at 1 day, 3 days, 1 week and 6 weeks (^{Ø} $P < 0.05$). This effect is significantly lowered as compared with normoxia at 1 day ($^{++}$ $P < 0.01$), 3 days ($^{+++}$ $P < 0.001$), 1 week ($^{++}$ $P < 0.01$) and 6 weeks of hypoxia ($^{+}$ $P < 0.05$) (Fig. 10). Data were reproduced in at least 2 – 3 different turtles.

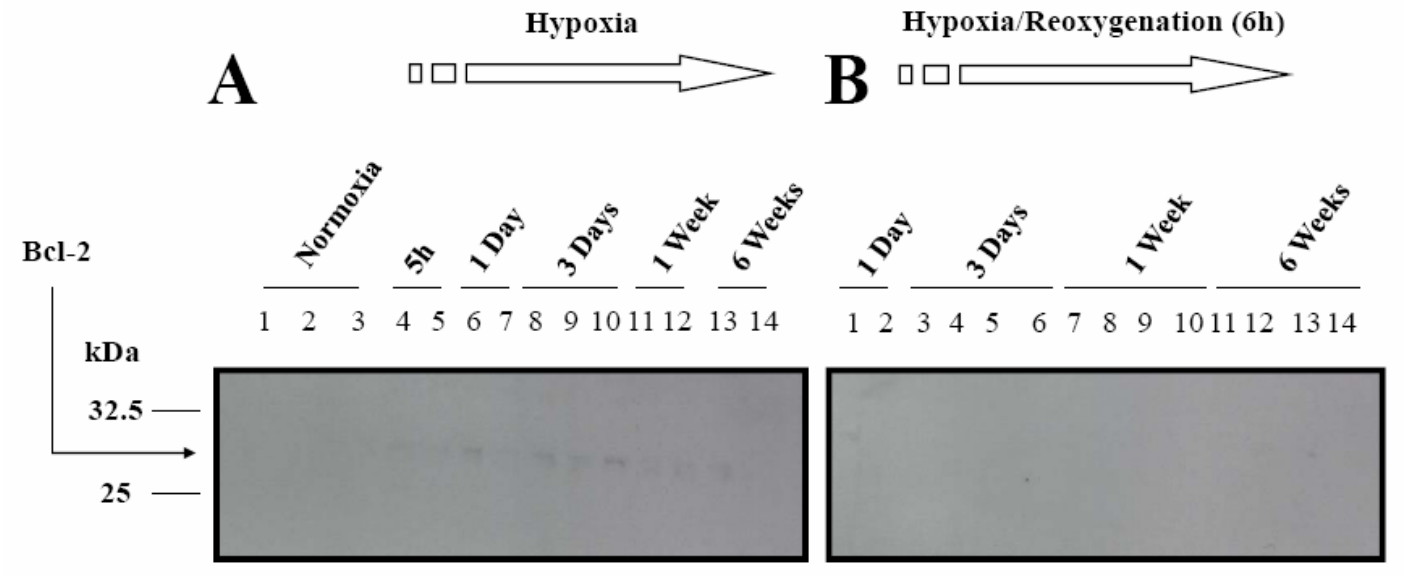

Fig. 9 Hypoxia-mediated regulation of anti-apoptotic cofactor, Bcl-2. **(A)** Exposure to hypoxia for a period of time ranging from 5h to 6 weeks mildly up-regulated the protein expression of Bcl-2 at 1 day - 1 week of hypoxia, as compared with normoxia control. **(B)** Hypoxia/reoxygenation for 6h has no effect on Bcl-2 expression. The numbers 1-14 allocated to the top of the gel indicates the number of each corresponding lane. *n* = 2-5, which designates the number of turtles and independent experiments performed for each variable.

The abundance of Bax increased at least linearly with the imposition of hypoxia, to attain statistical significance at 3 days (** $P < 0.01$) and 1 week (*** $P < 0.001$) of hypoxia, as compared with normoxic turtles (Fig. 11A). Hypoxia/reoxygenation up-regulated the expression of Bax at 1 day ($^{Ø} P < 0.05$), 3 days ($^{Ø} P < 0.05$) and 1 week ($^{Ø} P < 0.05$) of hypoxia, as compared with normoxic controls (Fig. 11B). As a valid control for Bax expression (equal gel loading), the protein levels of β-actin were not significantly different among treatments (data not shown).

Histogram analysis of the effect of hypoxia and hypoxia/reoxygenation on Bax is shown in Fig. 12. Hypoxia/reoxygenation significantly increased the expression of Bax at 1 day $^{Ø} P < 0.05$), 3 days ($^{Ø} P < 0.05$) and 1 week ($^{Ø} P < 0.05$). This effect is significantly increased as compared with hypoxia at 1 day ($^{++} P < 0.01$) (Fig. 12).

The % variations of the ratios of Bcl-2/Bax with hypoxia and hypoxia-reoxygenation are shown in Figs. 13A and 13B, respectively. In normoxia, the constitutive expression of Bcl-2 exceeds that of Bax (58.9/41.0 %). The ratio variations become prominent with hypoxia (Fig. 13A) and hypoxia-reoxygenation (Fig. 13B). For comparison of the protein abundance of both Bcl-2 and Bax, calibration curves were undertaken relative to the constitutive expression of β-actin, solely to indicate semi-quantitative loading in parallel lanes (Data not shown).

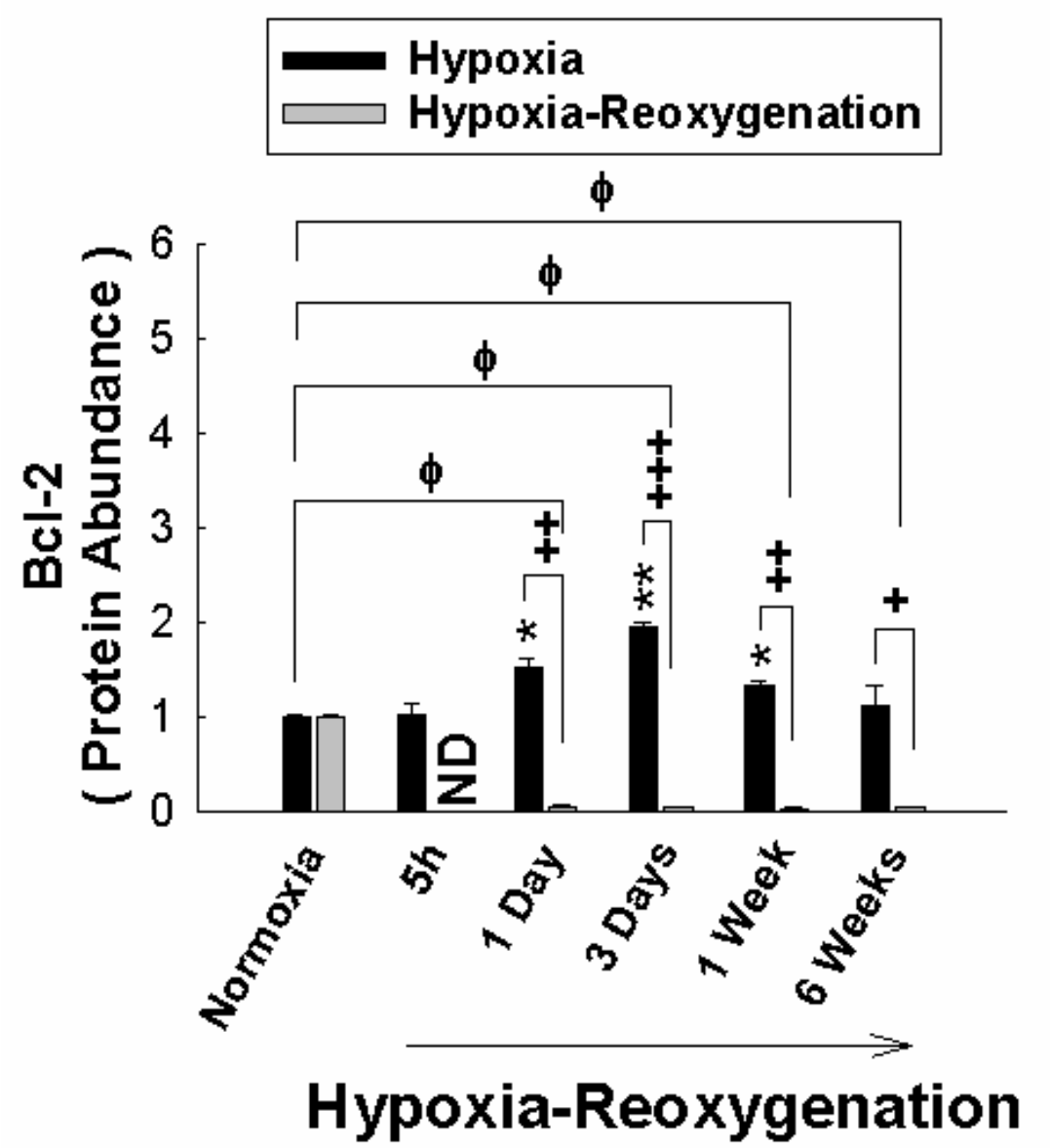

Fig. 10 Histogram analysis of the relative abundance of Bcl-2 under hypoxia/reoxygenation (6h) (The level of expression at normoxia was adjusted to 1 unit and all other values were expressed relative to this unit). *ND*, not determined.

A hypothetical model for hypoxia-MAPK signaling is depicted in Fig. 14. Although the $MAPK^{JNK}$ pathway has not been discussed in this paper, it was included given preliminary results of the distribution of the phosphorylated form in different organs of the turtle (Haddad et al., 2007, unpublished observations; UCSF). This model was derived in part to depict the significance of hypoxia and hypoxia-reoxygenation in the regulation of cell death or survival in anoxia-tolerant organisms from the perspective of MAPK signaling pathways. Further experimental work, however, is mandated to verify whether the aforementioned pathways are closely linked in modules relating to or are associated with hypoxia-tolerant organisms and models.

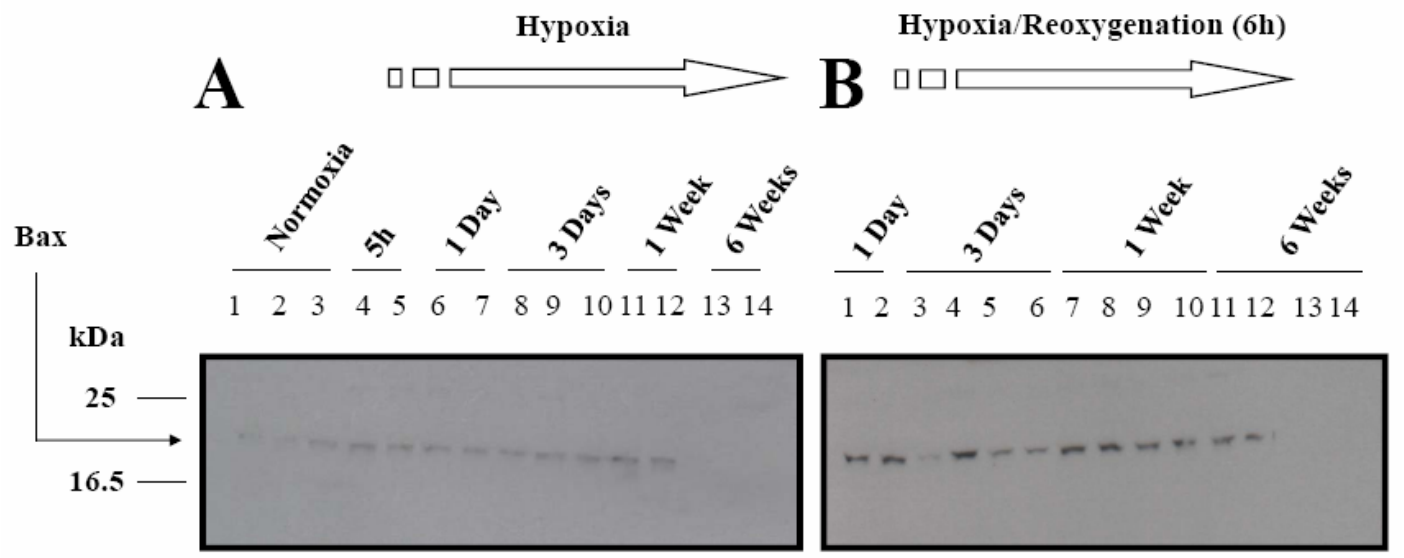

Fig. 11 Hypoxia-mediated regulation of pro-apoptotic cofactor, Bax. **(A)** Exposure to hypoxia for a period of time ranging from 5h to 6 weeks up-regulated the protein expression of Bax at 3 days and 1 week of hypoxia, as compared with normoxia control. **(B)** Hypoxia/reoxygenation for 6h variably affected Bax expression, with up-regulation at 1 day, 3 days and 1 week of hypoxia followed by oxygenation. The numbers 1-14 allocated to the top of the gel indicates the number of each corresponding lane. *n* = 2-5, which designates the number of turtles and independent experiments performed for each variable.

4. Discussion

This study has investigated *in vivo* hypoxia-mediated regulation of MAPK signaling pathways and caspase/apoptosis cofactor expression in anoxia-tolerant turtles (Haddad, 2004c). In summary, these results indicate that the regulation of MAPK signaling pathways in anoxia-tolerant turtles is hypoxia-sensitive and that the regulation of apoptosis-related cofactors during hypoxia is caspase-insensitive and involve, at least in part, a Bax-dependent mechanism. Because turtles survive prolong anoxia without neuron injury, the upregulation of MAPKs and Bax/Bcl-2-related mechanisms may be involved in neuroprotection.

Since *Chrysemys* is amongst the most anoxia-tolerant vertebrate presently known, it therefore offers a unique opportunity to understand mechanisms of anoxia tolerance (Haddad, 2004b; Nilsson et al., 2004; Reese et al., 2002; Hochachka and Lutz, 2001; Banasiak et al., 2000; Bickler et al., 2000; Buck, 2000; Frische et al., 2000; Jackson, 2000a; Jackson, 2000b; Satoh et al., 2000; Singer et al., 1999; Cano and Mahadevan, 1995). However, little is known about the molecular mechanisms and pathways underlying anoxia tolerance in those turtles. Bickler and colleagues (Bickler et al., 2000; Bickler, 1998; Bickler and Buck, 1998), for instance, have reported a role for hypoxia-mediated silencing of NMDA receptors in turtle neurons.

Recently, Greenway and colleagues (Greenway and Storey, 2000; Greenway and Storey, 1999) reported that MAPKs are sensitive to anoxia and freezing cycles in hatchling red-eared sliders, *Trachemys scripta elegans*. In that study, $MAPK^{ERK}$ was not stress-activated except in the brain of frozen turtles. In addition, $MAPK^{JNK}$ was only transiently activated by anoxia, and the expression of c-Fos and c-Myc transcription factors was organ-specific, consistent with other observations (Milton et al., 2006; Jackson et al., 2000). These results indicated that MAPKs are hypoxia-sensitive, suggesting the significance of these kinases in cellular adaptation to oxygen deprivation.

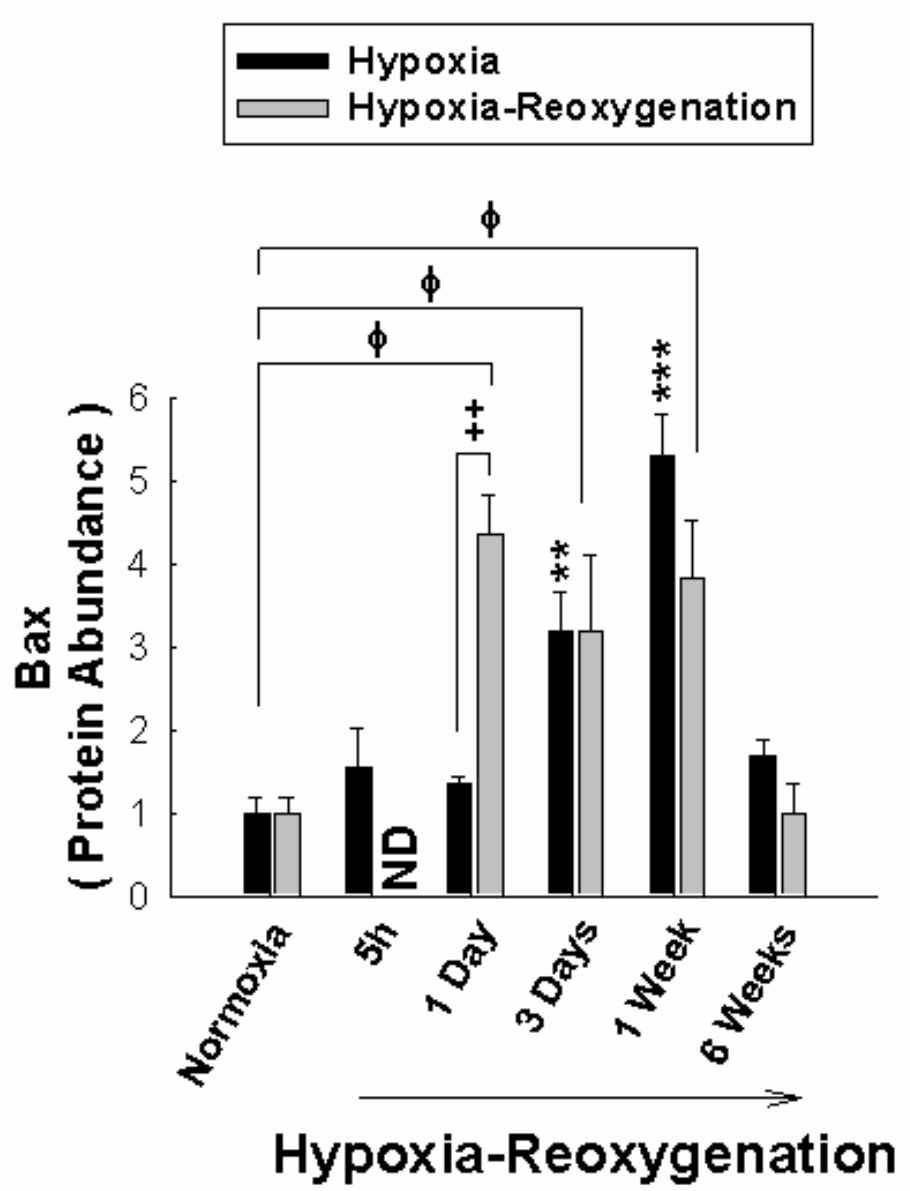

Fig. 12 Histogram analysis of the relative abundance of Bax under hypoxia/reoxygenation (6h) (The level of expression at normoxia was adjusted to 1 unit and all other values were expressed relative to this unit). *ND*, not determined.

Amongst the known signal transduction pathways that control cell fate (apoptosis) are the MAPK cascades (Haddad, 2005; Reese et al., 2001; Robinson and Cobb, 1997). A relationship between the activation of these modules and the regulation of caspases and Bax/Bcl-2 expression is not clear, and the molecular mechanisms involved are not known. For instance, it has been reported that $MAPK^{ERK}$ activation was associated with estrogen-mediated neuroprotection following glutamate toxicity in mammalian cortical neurons (Reese et al., 2003; Costanzo et al., 2001).

Similarly, neuroprotection by brain-derived neurotrophic factor (BDNF) was mediated by $MAPK^{ERK}$ and phosphatidylinositol 3-kinase in cortical neurons (Reese et al., 2003). Furthermore, neuroprotection mediated by glial cell line-derived neurotrophic factor (GDNF) required the involvement of a reduction of NMDA-induced calcium influx in a $MAPK^{ERK}$-dependent mechanism (Scott et al., 2003; Bickler et al., 2000; Singer et al., 1999).

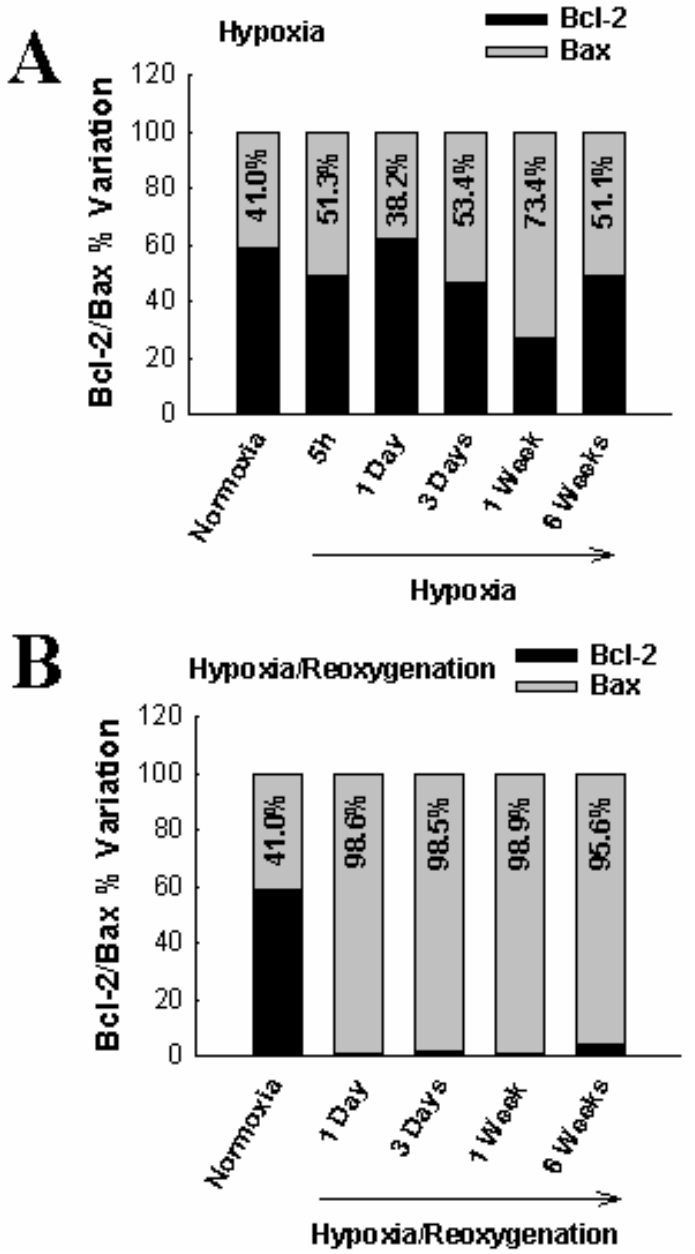

Fig. 13 The ratio (%) of Bcl-2/Bax in hypoxia and hypoxia/reoxygenation. **(A)** Bcl-2/Bax % variation with hypoxia. The number in % designates the variation of Bax relative Bcl-2 at different time point of hypoxia. **(B)** Bcl-2/Bax % variation with hypoxia-reoxygenation. The number in % designates the variation of Bax relative Bcl-2 at different time point of hypoxia/reoxygenation. The numbers 1-14 allocated to the top of the gel indicates the number of each corresponding lane. *n* = 2-5, which designates the number of turtles and independent experiments performed for each variable.

In contrast, Satoh and colleagues (Satoh et al., 2000) recently showed that inhibition of $MAPK^{ERK}$ with U0126 mediated neuroprotection against oxidative stress in neuronal cells. On the other hand, selective inhibition of the $MAPK^{p38}$ pathway reduced brain injury and neurological deficits in cerebral focal ischemia (Nilsson, 2001; Satoh et al., 2000). Moreover, it has been shown that synergistic activation of $MAPK^{p38}$ and caspase-3-like proteases was involved in calyculin A-induced apoptosis in cortical neurons (Shin et al., 2003; Widmann et al., 1999). In addition, selective inhibition of the $MAPK^{p38}$ pathway has been proposed as a therapeutic

strategy for pre-clinical evaluation (Reese et al., 2001; Costanzo et al., 2000; Robinson and Cobb, 1997).

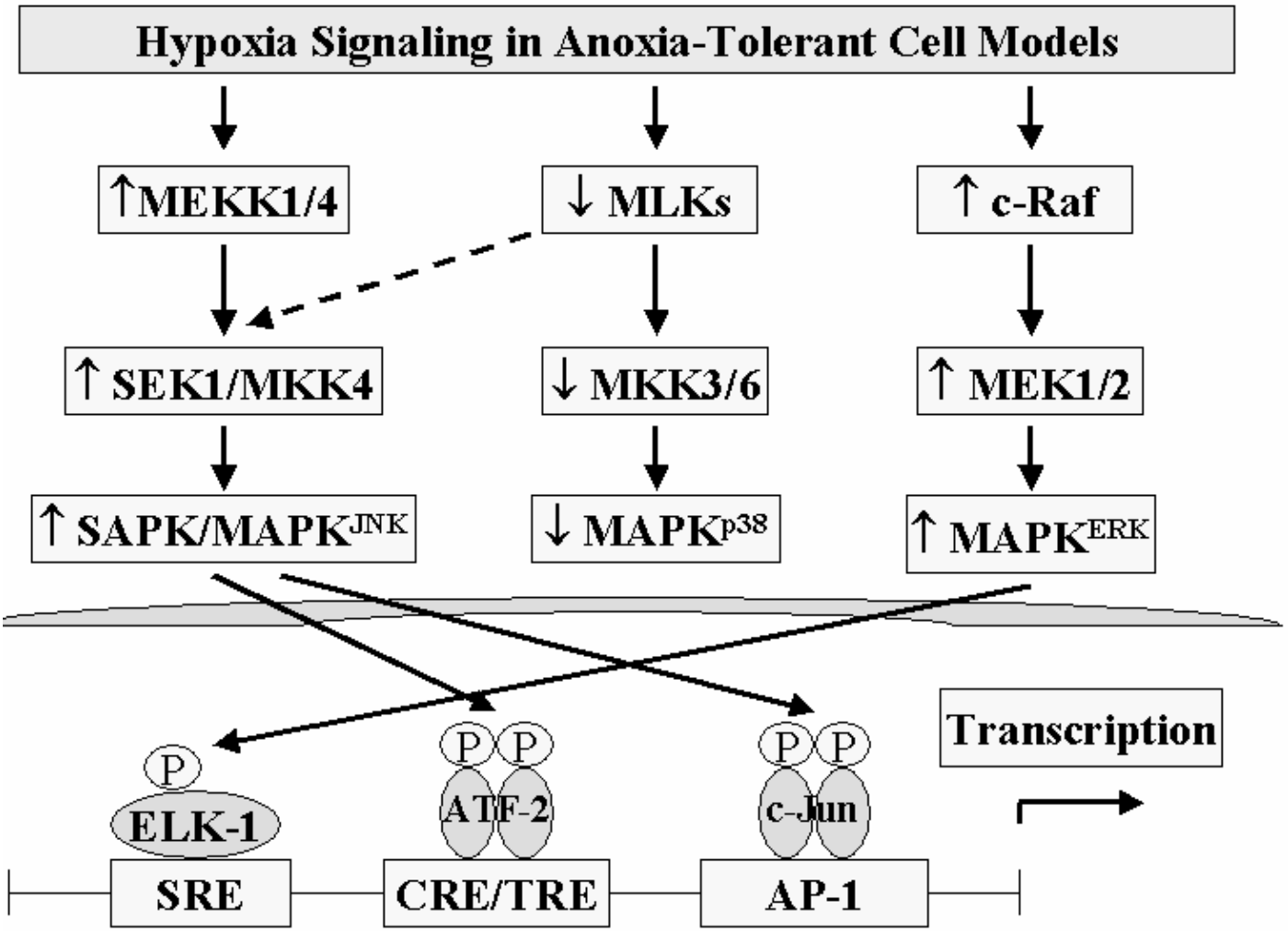

Fig. 14 A hypothetical schema for hypoxia-mediated regulation of MAPK signaling pathways in anoxia-tolerant organisms and models, such as the Western painted turtle, *Chrysemys picta bellii*. This model is based in part on unpublished observations carried out by the author at UCSF, with particular emphasis on the neuroinjurious effects of MAPK^{p38} (MKK) and MAPK^{JNK} (SEK; SAPK), and neuroprotective effects of MAPK^{ERK} and upstream kinases (MEK) associated with this pathway. For further explanation, the reader's attention is focally directed to Haddad, 2004d.

The results therein reported agree with these observations in regard to the differential (biphasic) regulation of MAPK signaling in anoxia-tolerant turtles. For instance, I have observed no changes in MAPK^{p38} phosphorylation with either hypoxia or reoxygenation. Furthermore, hypoxia, but not reoxygenation, up-regulated the phosphorylation of MAPK^{ERK}. Of note, neither hypoxia nor reoxygenation induced the cleavage of pro-caspases 8 and 9; nevertheless, hypoxia increased the expression of Bax and, to a lesser extent, Bcl-2, an effect augmented with reoxygenation for Bax (Haddad, 2004c).

Despite the significance of this response of MAPKs to oxygen deprivation, the molecular basis for this differential regulation remains to be elucidated. Interestingly, this study has revealed not only that MAPK signaling pathways are hypoxia-responsive, particularly MAPK^{ERK}, but also the regulation of apoptosis-related cofactors in anoxia-tolerant brains is

hypoxia-sensitive and involves, at least in part, a Bax-dependent mechanism (Haddad, 2005).

Whether the signaling pathways regulating MAPKs in hypoxia are directly linked to the expression of pro- and anti-apoptotic cofactors cannot be ruled out; however, it is conspicuous that the regulation of MAPK signaling is involved in mediating a neuroinjurious (Haddad, 2005; Prentice et al., 2003) or neuroprotective (Haddad, 2004b; Haddad, 2004c) response following oxygen deprivation. Furthermore, it wasn't clear from this data alone whether the effect on MAPKs could be cold (3-4^{o}C) sensitive or the effect could be synergistic with hypoxia (Baker et al., 2003). However, it was recently reported that cold exposure had no effect on the activity of NMDA receptors in the anoxia-tolerant turtle, *Chrysemys* (Bickler et al., 2000); the effect in the brain was exclusively hypoxia-dependent, thus reinforcing the observation that MAPKs are hypoxia-sensitive or responsive.

Current studies are aimed at identifying the upstream and downstream components of the MAPK signaling machinery *in vitro* and *in vivo* in order to locate potential targets for neuroprotection during hypoxia. The molecular basis for a possible relationship between MAPK signaling and the regulation of cell death in *Chrysemys picta* is, therefore, crucial for identifying cellular modules naturally activated in hypoxia-tolerant vertebrates (Dinkelacker et al., 2005; Packard and Packard, 2005).

In recapitulation, i) hypoxia did not up-regulate the phosphorylation of MAPK^{p38}, but hypoxia/reoxygenation suppressed this pathway during 1 day and 1 week of hypoxia; ii) the effect of hypoxia on the phosphorylation of MAPK^{ERK} was biphasic (early up-regulation and late suppression); iii) neither hypoxia nor hypoxia/reoxygenation induced the cleavage of pro-caspase 8 and 9 into their corresponding active subunits, p20 and p10, respectively (Chou et al., 2000; Chou et al., 1997); iv) hypoxia induced the expression of the pro-apoptotic cofactor, Bax, and hypoxia/reoxygenation increased Bax ≈ 4-fold during early hypoxic submergence; and v) the expression of Bcl-2, an anti-apoptotic cofactor, was minimal in response to hypoxia, the level of which was 3-4-fold smaller in comparison with Bax.

Therefore, hypoxia up-regulated the phosphorylation of MAPK^{ERK} but not MAPK^{p38}; in addition, the regulation of apoptosis-related cofactors is hypoxia-sensitive and requires, at least in part, a Bax-dependent mechanism during hypoxia and hypoxia/reoxygenation. These results indicate a discordant response of MAPK signaling pathways in response to hypoxia and that the expression of apoptosis signaling cofactors is hypoxia-responsive (Haddad, 2005; Haddad, 2004c). The patterns of MAPK regulation suggest the significance of these kinases in cellular adaptation to oxygen deprivation, and thereby identify novel natural responsive signaling cofactors in *Chrysemys picta bellii*.

Acknowledgements

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