The Journal of Experimental Biology 209, 1765-1776
Published by The Company of Biologists 2006
doi:10.1242/jeb.02152

Regulation of the mitogen-activated protein kinase p44 ERK activity during anoxia/recovery in rainbow trout hypodermal fibroblasts

Carlo G. Ossum*, Tune Wulff and Else K. Hoffmann

Institute of Molecular Biology and Physiology, Department of Biochemistry, The August Krogh Building, University of Copenhagen, Universitetsparken 13, DK-2100 Copenhagen Ø, Denmark

*Author for correspondence (e-mail: cgossum@aki.ku.dk)

Accepted 7 February 2006

Summary

It is well known from various mammalian cells that anoxia has a major impact on the mitogen-activated protein kinase ERK, but a possible similar effect in fish cells has not been investigated. Here we characterise a p44ERK-like protein in the rainbow trout cell line RTHDF and study the effect of (i) serum stimulation, (ii) sodium azide (chemical anoxia) and removal of azide (recovery) and (iii) anoxia (P_{O_2}<0.1%) and recovery. During both chemical and true anoxia p44ERK was inhibited and recovery resulted in robust reactivation of p44ERK activity, far above the initial level. The inhibition was secondary to activation of p38^{MAPK} and the increase was MEK dependent, as SB203580 inhibited the dephosphorylation during anoxia and the presence of PD98059 inhibited phosphorylation of p44ERK during recovery. In addition, we demonstrated that the reactivation of p44ERK during recovery also was dependent on reactive oxygen species and a PP1/PP2A-like phosphatase.

Key words: p38, MAPK, teleost, ROS, phosphatase, PP1, PP2A, calyculin A, hypoxia, recovery, SB203580.

Introduction

Anoxia is a profound cellular stress factor with wide physiological implications. Pathophysiologically, anoxia is the consequence of ischemic disorders, such as stroke and ischemic cardiovascular diseases. With respect to fish, the effects of anoxia are of great importance to their survival and behaviour because of naturally occurring daily and seasonal fluctuations in oxygen availability (Nikinmaa, 2002), as well as during oxygen deprivation of waters (Wu, 2002). As ischemia and consequently anoxia is a major stress factor during harvesting and production of sea food the biochemical events elicited by anoxia might have impact on meat quality. Therefore, the study of anoxia in piscine cell culture is of interest for understanding the basic cellular mechanisms during anoxia and to provide a tool for food technologists in order to improve the quality of fish meat as a human food source.

It is well known from mammalian cells that anoxia has a major impact on the mitogen-activated protein kinases, MAPKs. The MAPK family includes the subfamilies ERK, p38^{MAPK} and c-Jun N-terminal kinase (JNK). The ERKs (Cowan and Storey, 2003; Schaeffer and Weber, 1999) respond to mitogens and survival signals and stimulate cell proliferation, growth and survival. JNK (Barr and Bogoyevitch, 2001) and p38^{MAPK} (New and Han, 1998) are mainly activated by cellular stress. The interplay between the mitogenic and the stress-activated MAPK pathways are considered critical for cell fate, as sustained p38^{MAPK} and JNK activity and suppression of ERK activity result in apoptosis (Xia et al., 1995), whereas ERK1/2 can suppress apoptosis by phosphorylation of Bad (Jin et al., 2002) and procaspase 9 (Allan et al., 2003).

MAPK pathways are generally thought of as three-kinase modules, consisting of a MAP kinase kinase kinase (MAP3K) upstream of a MAPK kinase kinase (MAP2K), which in turn activates the MAP kinase (Kyriakis and Avruch, 2001). The MAP3Ks are often found downstream of small GTPases, such as Ras. This opens up the possibility for a wide range of regulatory inputs, both at the MAP3K level and at the level of MAP2K (Widmann et al., 1999). Upstream of the mammalian MAPKs, ERK1 and ERK2, is Raf (MAP3K) and MAPK/ERK kinase (MEK)1/2 (MAP2K). *In vivo*, ERK is typically activated following activation of receptor protein tyrosine kinases, such as the platelet-derived growth factor receptor and epidermal growth factor receptor that in turn activate the small G protein Ras (Widmann et al., 1999). Regulation of the stress-responsive MAP-kinases p38^{MAPK} and JNK are far more complex (Kyriakis and Avruch, 2001) owing to the presence of multiple MAP3Ks and two different MAP2Ks regulating the kinases, MKK3/6 and MKK4/7 for p38^{MAPK} and JNK, respectively (Harper and LoGrasso, 2001; Kyriakis and Avruch, 2001).

How lack of oxygen, i.e. anoxia and ischemia affect ERK

signalling has been the subject for a number of studies. However, most studies have focussed on specialised mammalian tissues, such as cardiovascular cell types, specialised epithelial cells and neurons. In the whole rat heart, ERK2 is inactivated in response to ischemia, followed by translocation of the inactive kinase to the nuclear compartment (Mizukami and Yoshida, 1997). Interestingly, ERK2 was activated in response to reperfusion, suggesting an interface capable of ERK activation in the nuclear membrane. Using the rat myocyte cell line H9c2, which has been shown to display ERK activities similar to those seen in whole organs during oxidative and metabolic stress (Abas et al., 2000), it was demonstrated that the ischemic nuclear translocation of ERK2 was dependent on both phosphatidyl inositol 3-kinase (PI 3-kinase) and the atypical protein kinase C (PKC) isoform PKCζ (Mizukami et al., 1997; Mizukami et al., 2000). Hypoxia also stimulates ERK1/2 phosphorylation in vascular smooth muscle cells (Blaschke et al., 2002). To our knowledge, there are no studies on the effect of anoxia on ERK signalling in fish cells.

Previously, we demonstrated a rapid, but transient activation of $p38^{MAPK}$ during chemical anoxia in rainbow trout hypodermal fibroblasts (RTHDF) (Ossum et al., 2004). Such an activation of $p38^{MAPK}$ by anoxic insults have previously been reported in perfused hearts (Bogoyevitch et al., 1996; Nakano et al., 2000), ventricular myocytes (Saurin et al., 2000), H9c2 cells (Jung et al., 2004) and in neuronal tissues (Conrad et al., 1999; Harper and LoGrasso, 2001; Zhu et al., 2002). These studies demonstrate multiple functions of $p38^{MAPK}$. In PC12 cells, activation of $p38^{MAPK}$ inhibits expression of cyclin D1 (Conrad et al., 1999) and in primary neurons, $p38^{MAPK}$ stabilises p53 and inhibits transformed mouse 3T3 cell double minute 2 (Mdm2) (Zhu et al., 2002). Studies of vascular tissue demonstrate activation of mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP K-2) downstream of $p38^{MAPK}$ (Bogoyevitch et al., 1996; Nakano et al., 2000). In primary rat astrocytes, $p38^{MAPK}$ signalling has been implicated in induction of Hsp70 (Uehara et al., 1999). Together, these studies demonstrate roles for $p38^{MAPK}$ in both cell death and protection. There are several observations showing cross-talk between $p38^{MAPK}$ and ERK (e.g. Singh et al., 1999). Thus it is probable that the observed activation of $p38^{MAPK}$ could be followed by an inhibition of ERK.

The present study was initiated to determine the effects of chemical anoxia, as well as nitrogen-induced anoxia and recovery on p44ERK activity in RTHDF (Ossum et al., 2004), originating from the rainbow trout *Oncorhynchus mykiss* L. Here, we report that both chemical anoxia induced by sodium azide, as well as true anoxia (P_{O_2}<0.1%) result in inhibition of p44ERK during azide treatment and that p44ERK is dramatically activated in response to recovery. In the chemical anoxia model we find that inhibition of p44ERK activity during anoxia is dependent on $p38^{MAPK}$ activity, whereas its activation during recovery is positively regulated by reactive oxygen species and a calyculin A-sensitive serine/threonine phosphatase. A proposed model describing ERK regulation during anoxia and recovery in RTHDF cells is presented.

Materials and methods

Reagents

All general chemicals were purchased from J. T. Baker (Denventer, Holland) and Sigma-Aldrich Inc. (St Louis, MO, USA). Diphenyleneiodonium chloride (DPI), trypsin, Ponseau S and protease inhibitor cocktail were also obtained from Sigma. Calyculin A, PD98059 and SB203580 and Raf1 kinase inhibitor I (RKI) were purchased from Calbiochem (La Jolla, CA, USA). Stock solutions of 70 μmol l^{-1} calyculin A, 18 mmol l^{-1} PD98059, 10 mmol l^{-1} SB203580, 100 mmol l^{-1} DPI and 10 mmol l^{-1} RKI were prepared, using dimethylsulfoxide (DMSO) as solvent. Pre-cast NuPAGE 10% SDS-polyacrylamide Bis-Tris gels, nitrocellulose membrane filter paper sandwiches and NuPAGE electrophoresis reagents were purchased from Invitrogen (Carlsbad, CA, USA). The Bio-Rad DC Protein Assay Kit was from BioRad Laboratories Inc. (Hercules, CA, USA). BSA solution was obtained from Pierce (Rockford, IL, USA) and solid BSA was from Sigma. All primary antibodies for western blotting were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The secondary antibody, goat anti-rabbit IgG was obtained from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA). BCIP/NBT Membrane Phosphatase Substrate was purchased from KLP (Gaithersburg, MD, USA). All cell culture reagents were from Life Technologies (Naperville, IL, USA).

Cell culture

Rainbow trout (*Oncorhynchus mykiss* L.) hypodermal fibroblasts (RTHDF) were cultured in Leibovitz' L-15, supplemented with 15% (v/v) foetal bovine serum (FBS), penicillin (100 i.u. ml^{-1}) and streptomycin (100 μg ml^{-1}) at 21°C and atmospheric air, as described previously (Ossum et al., 2004). Trypsin solution for cell detachment was made by dissolving 0.1% (w/v) trypsin and 1 mmol l^{-1} disodium EDTA in phosphate-buffered saline (PBS: 137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 8.1 mmol l^{-1} Na_2HPO_4, 1.5 mmol l^{-1} KH_2PO_4) (Ma and Collodi, 1999). All cell culture reagents were used cold from the refrigerator.

All cell culture plastic ware was from TPR (Trasadingen, Switzerland).

Experimental conditions

Experiments were performed with cells grown to approximately 80% confluency in six well plates throughout. Cells were plated 1–2 days prior to experiments.

During experiments, cells were incubated in L15ex, consisting of 140 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 2 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} $MgSO_4$, 1 mmol l^{-1} Na_2HPO_4, 0.4 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} galactose, 10 mmol l^{-1} Hepes and 5 mmol l^{-1} sodium pyruvate (Ossum et al., 2004; Schirmer et al., 1997). Anoxia was induced using L-15ex in which 10 mmol l^{-1} NaCl was replaced with 10 mmol l^{-1} sodium azide (NaN_3). Osmolality was measured and found to 304 and 305 mOsm kg^{-1} for L-15ex and L-15ex with sodium azide, respectively. Recovery (reoxygenation) occurred by

washing the cells twice in PBS, and then incubating in L-15ex without NaN_3.

Nitrogen-induced anoxia was obtained by keeping the Leibowitz' L15 medium with supplements in an airproof chamber under a constant flow of N_2 until anoxia was obtained as registered by an oxygen-sensitive probe. The cells were then exposed to this now anoxic medium while keeping a constant flow of N_2. Recovery (reoxygenation) occurred by removing the cells from the anoxic chamber, then washing the cells twice in PBS and incubating in Leibovitz' L-15ex. Under anoxic conditions, oxygen concentration never exceeded 0.1%.

Cells were serum starved by overnight incubation in Leibovitz' L-15 basal medium, supplemented with antibiotics and 0.1% FBS, where indicated. Serum stimulation was performed by exposing cells to 20% FBS.

When indicated, cells were pre-incubated for 1 h in the presence of 10 μmol l^{-1} SB203580, 10 or 30 μmol l^{-1} PD98059 and 100 nmol l^{-1} DPI. PD98059 was added to the recovery medium at 10 μmol l^{-1} final concentration. Calyculin A was applied for 2 min at 100 nmol l^{-1} final concentration.

SDS-PAGE and western blot analysis

Cells were rinsed once in ice cold PBS and harvested by scraping in 100 μl boiling SDS lysis buffer [1% (v/v) SDS, 1 mmol l^{-1} Na_3VO_4 (from a 200 mmol l^{-1} stock solution), 10 mmol l^{-1} Tris, pH 7.4]. Extracts were homogenised by sonication, followed by removal of cell debris by centrifugation at 16 000 ***g*** for 5 min. Protein concentration was determined spectrophotometrically using the detergent-compatible protein assay Bio-Rad DC Protein Assay Kit and BSA as a standard. Samples for electrophoresis were prepared by adding NuPAGE LDS sample buffer from a 4× stock solution (cat. no. NP0007) supplemented with 10% (v/v) 0.5 M dithiothreitol (DTT), then boiling for 5 min. Proteins were separated by SDS-PAGE, using precast NuPAGE 10% Bis-Tris gels (cat. no. NP0302BOX) and electrotransferred to nitrocellulose membranes of 0.22 μm pore size (cat. no. LC2000), using NuPAGE Mops SDS running buffer (cat. no. NP0001) and NuPAGE transfer buffer (cat. no. NP0006-1), respectively. The gels were loaded with 15–20 μg protein per lane. Equal gel loading and successful transfer of proteins were checked by staining membranes with Ponseau S [0.1% Ponseau S (w/v) 5% acetic acid (v/v)]. Electrophoresis and transfer were performed using the XCell SureLock and the XCell II Blot module (Invitrogen, cat. no. E10002), respectively. After transfer, the nitrocellulose membrane was incubated in blocking buffer [5% non-fatty dry milk in TBS-T (10 mmol l^{-1} Tris-HCl, pH 7.5, 120 mmol l^{-1} NaCl and 0.1% Tween20)] for 1 h at room temperature (RT) or overnight (ON) at 4°C. Primary antibodies were applied for 1 h at room RT or ON at 4°C. The membrane was then washed three times in TBS-T for 5–15 min. The secondary, alkaline phosphatase conjugated antibody was applied for 1 h at RT, followed by washing as described above. Immunoreactive bands were detected using BCIP/NBT. The bands were quantified using a HP Scanjet 4600 (Hewlett Packard Palo Alto, CA, USA) and the software UN-SCAN-IT gel version 5.1 for Windows (Silk Scientific Corp. UT, USA). UN-SCAN-IT gel version 5.1 was also used for estimation of molecular mass.

The primary antibodies against p44ERK, p44/42 MAP kinase antibody (cat. no. 9102) and phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (cat. no. 9101) were diluted 1:1000 in blocking buffer. The phospho-MEK1/2 (Ser217/221) antibody (cat. no. 9121) was diluted 1:500. The secondary antibody was diluted 1:500.

Statistical analyses

Western blot quantification data were statistically analyzed by two-tailed, unpaired Student's *t*-tests. $P<0.05$ was regarded as significant. Quantified results are presented as relative numbers ± s.d. Estimated molecular masses are given as mean ± s.d. *N* indicates the number of independent experiments.

Results

Two serum-responsive ERK isoforms are expressed in RTHDF cells

The presence of ERK proteins in RTHDF cells were analysed by western blotting, using a pan reactive p44/p42 MAPK antibody. Three ERK-like proteins were observed with apparent molecular masses of 38, 42 and 44 kDa (Fig. 1A, lower panel). In this paper, the three ERK proteins are referred to as p44ERK, p42ERK and p38ERK, respectively.

The effect of FBS on phosphorylation of ERK proteins was determined by stimulating serum-starved RTHD fibroblasts with 20% FBS for the indicated periods of time (Fig. 1A, upper panel). Dual phosphorylation of ERK Thr202/Tyr204 indicates its activation (Cano and Mahadevan, 1995; Widmann et al., 1999). The level of dual phosphorylation was measured by western blotting. Two FBS-responsive ERK isoforms with apparent molecular masses of 44 and 38 kDa were detected.

Interestingly, these two isoforms displayed different phosphorylation profiles in response to serum stimulation. Both p44ERK and p38ERK were rapidly activated by serum stimulation of serum starved RTHDF cells. Maximal phosphorylation was observed after 5 min. For p44ERK, there was no detectable difference in the phosphorylation levels between 5 and 10 min of FBS stimulation. After the peak in activity, the phosphorylation level gradually declined. However, p44ERK was still significantly activated after 30 min. The p38ERK isoform demonstrated a biphasic activity. The level of phosphorylation peaked at 5 min, followed by a decrease until 15 min. After 20 min in the presence of FBS, a new peak in p38ERK phosphorylation was observed. The total p44ERK protein did not decay under any of the conditions (Fig. 1A, lower panel).

Dose-dependent inhibition of p44 ERK activity by sodium azide

Sodium azide (NaN_3) treatment is an established model for induction of chemical anoxia (Jørgensen et al., 1999; Ossum

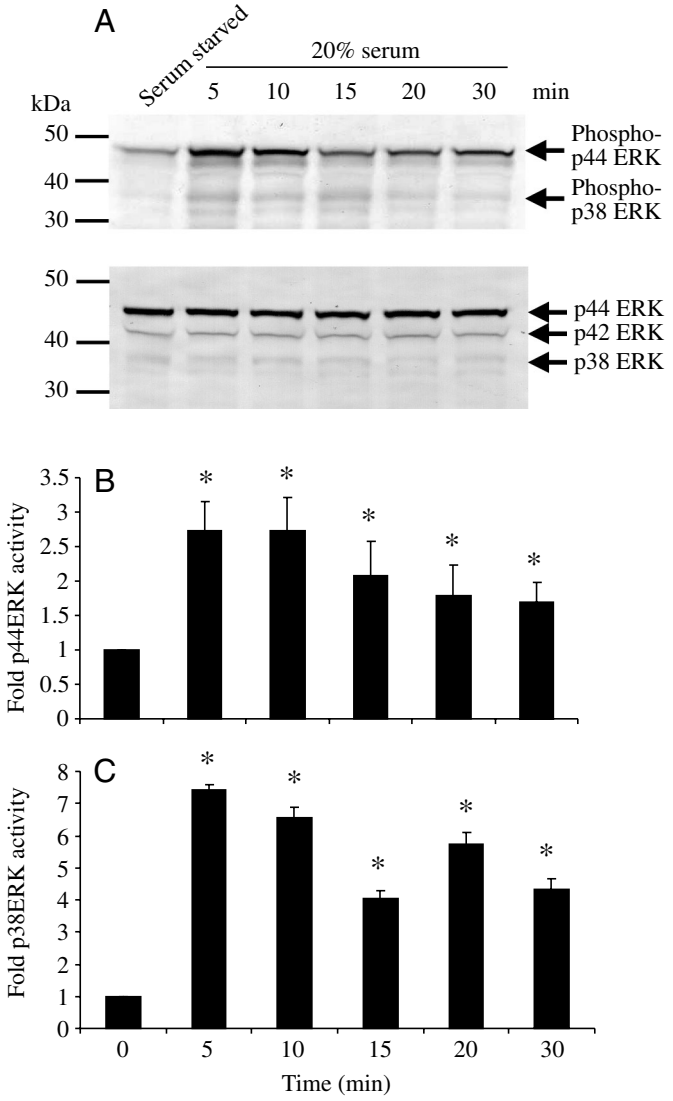


Fig. 1. Effect of serum stimulation on ERK proteins in serum-starved RTHDF cells. (A) Top: western blot analysis of phosphorylation of p44ERK and p38ERK during serum stimulation of serum starved cells. The antibody recognises amino acids Thr202 and Tyr204. Bottom: western blot analysis of total ERK protein. (B) Quantification of the phospho-specific immunoreactions representing active p44ERK in RTHDF cells. (C) Quantification of the phospho-specific immunoreactions representing active p38ERK in RTHDF cells (N=4, *P<0.05).

et al., 2004; Varming et al., 1996). To test the effect of chemical anoxia on p44ERK signalling, RTHD fibroblasts were treated with increasing concentrations of NaN_3 for 30 min and the phosphorylation status of p44ERK was assayed by western blotting (Fig. 2). The ERK activity (phosphorylation) was inhibited in a dose-dependent manner.

Time-dependent changes in p44ERK phosphorylation during chemical anoxia and recovery

The time-course of p44ERK activity in RTHDF cells during chemical anoxia and recovery (A/R) was examined. Chemical

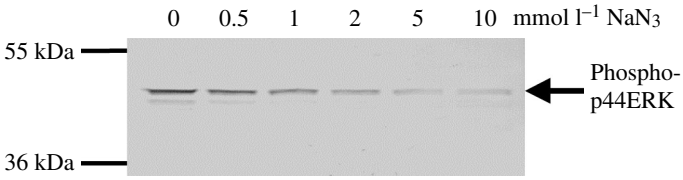


Fig. 2. Dose–response of p44ERK activity to sodium azide in RTHD cells. Western blot analysis of phosphorylated p44ERK after 30 min incubation in L-15ex with increasing concentration of NaN_3 (N=1).

anoxia was induced by 10 mmol l^{-1} NaN_3. We observed a gradual decrease in p44ERK phosphorylation (Fig. 3A), statistically significant after 30 min incubation with sodium azide. Upon recovery, p44ERK was rapidly phosphorylated and the level of phosphorylation continued to increase during 1 h recovery (Fig. 3B). Significant activation of p44ERK was observed after 10 min recovery. The effect of recovery was strongly inhibited when 10 μmol l^{-1} final concentration of the MEK1/2 inhibitor PD98059 was added to the medium during recovery (Fig. 4).

Time-dependent changes in p44ERK phosphorylation during nitrogen-induced anoxia and recovery

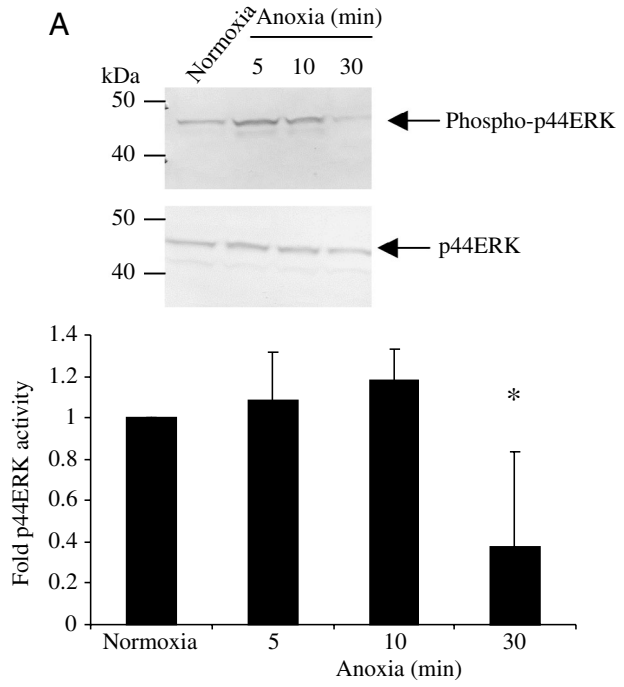
The anoxia experiment was repeated using nitrogen to displace oxygen (Fig. 5). The level of p44ERK phosphorylation slowly decreased, resulting in significant inhibition after 3 h, comparable to the significant decrease after 30 min chemical anoxia. During recovery, phosphorylation of p44ERK occurred faster than in azide-treated cells, with significant and robust activation after 5 min. After 30 min, the level of ERK phosphorylation decreased and reached a plateau that was stable throughout the experiment.

Activation of $p38^{MAPK}$ is required for inhibition of p44ERK during anoxia

In a previous study, we demonstrated that the stress-activated MAPK family member p38 is rapidly activated by anoxia in RTHDF cells (Ossum et al., 2004) but the biochemical role of this activity was not clear. To investigate potential cross-talk between the $p38^{MAPK}$ and the p44ERK pathways, RTHDF cells were incubated with 10 μmol l^{-1} SB203580, a p38α/β-specific inhibitor (English and Cobb, 2002). Inhibition of $p38^{MAPK}$ prevented inhibition of p44ERK during a 30 min anoxic insult (Fig. 6).

Activation of p44ERK during recovery is dependent on active protein phosphatases PP1/PP2A

Calyculin A is a specific inhibitor of the serine/threonine (S/T) phosphatases protein phosphatase-1 (PP1) and protein phosphatase-2A (PP2A) (Sheppeck, II et al., 1997). Under normoxic conditions no significant effect of calyculin A was observed (Fig. 7A). Western blotting demonstrated that pre-treatment of RTHDF cells with 100 nmol l^{-1} calyculin A for 2 min, inhibited recovery-induced activity p44ERK (Fig. 7B).



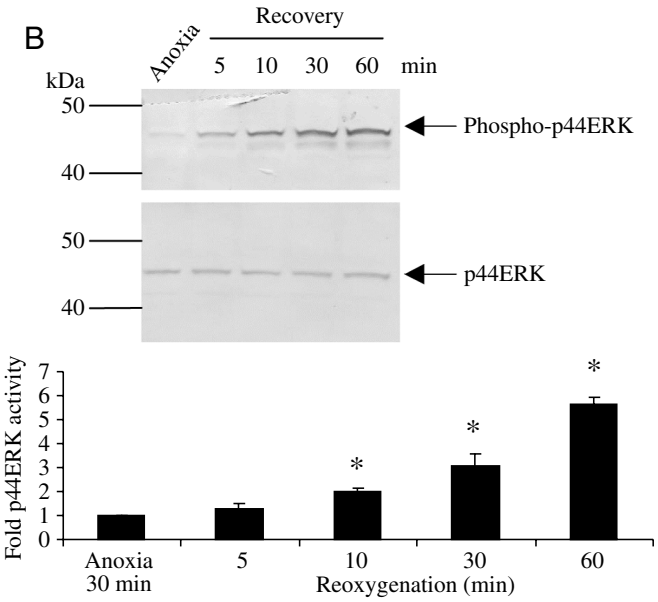


Fig. 3. Time-dependent inhibition of p44ERK activity during chemical anoxia and activation during reoxygenation in RTHD fibroblasts. RTHDF cells were challenged with chemical anoxia and recovery for the indicated periods of time. Chemical anoxia was induced by incubation in L-15ex, containing 10 mmol l^{-1} sodium azide for 30 min. Recovery was achieved by incubating the cells in azide-free L-15ex. (A) Top: western blot analysis of phospho-p44ERK during anoxia (N=4) and total p44ERK (N=1). Bottom: quantification of band intensity showed significant inhibition of p44ERK activity after 30 min, relative to the normoxic control. (B) Top: western blot analysis of phospho-p44ERK during recovery (N=3) and total p44ERK (N=1). Bottom: quantification of band intensity showed that recovery resulted in significant activation of p44ERK after 10 min, relative to anoxic cells. *P<0.005.

Raf-1 is dispensable for stimulation of p44ERK during recovery from chemical anoxia

The S/T kinase Raf-1 is the archetypical MAP3K in the ERK cascade (Kyriakis and Avruch, 2001; Widmann et al., 1999), and is known to be derepressed by dephosphorylation (Ory et al., 2003). To clarify the involvement of Raf-1 in recovery-mediated activation of p44ERK, RTHDF cells were either serum starved and stimulated with FBS or subjected to anoxia/recovery in the absence or presence of 10 μmol l^{-1} Raf1 kinase inhibitor I (RKI). Inhibition of Raf-1 strongly inhibited serum-stimulated phosphorylation of p44ERK in starved cells (Fig. 8A). However, RKI did not affect phosphorylation of p44ERK during recovery from chemical anoxia (Fig. 8B).

Activation of p44ERK by recovery depends on production of reactive oxygen species

A/R are known to result in production of reactive oxygen species (ROS) (Dröge, 2002; Goyal et al., 2004) and it is well known that ROS, such as H_2O_2 are able to activate p44ERK (Cerioni et al., 2003; Dröge, 2002; Guyton et al., 1996; Schmitz et al., 2002). To test for a possible involvement of ROS in A/R, RTHD fibroblasts were pre-incubated with

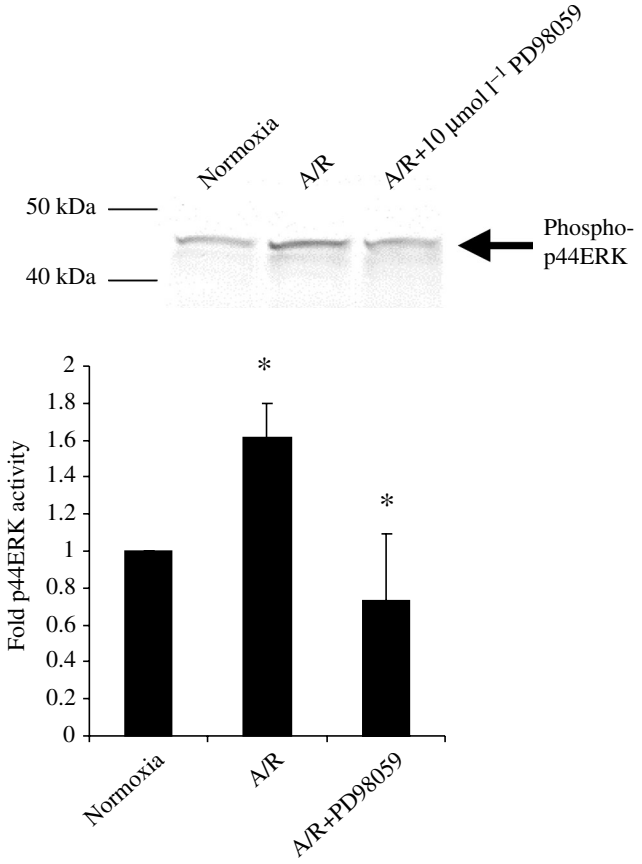


Fig. 4. Activation of p44ERK during recovery is MEK dependent. RTHDF cells were challenged with chemical anoxia (A) and recovery (R) in the absence and presence of 10 μmol l^{-1} of the MEK1/2 inhibitor PD98059 in the recovery medium. A representative western blot and quantification of band intensity is shown, demonstrating the inhibitory effect of 10 μmol l^{-1} PD98059 (N=3, *P<0.05).

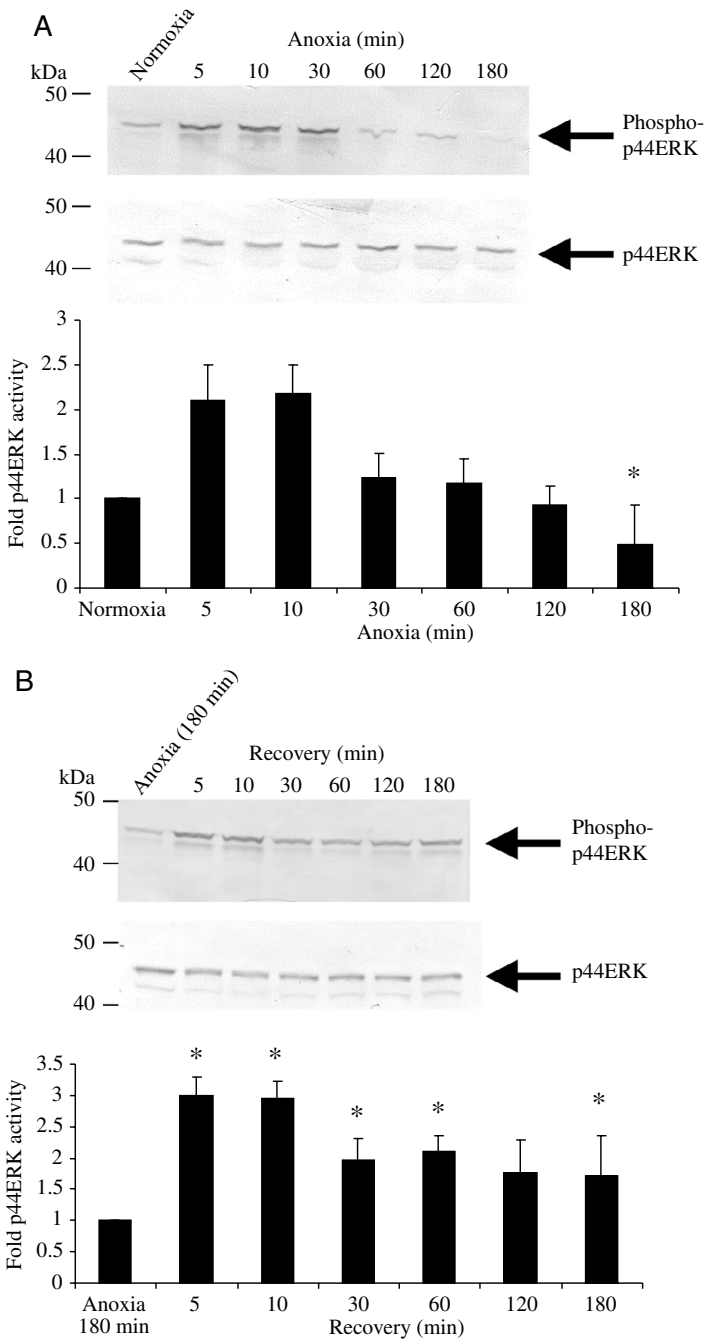


Fig. 5. Time-dependent inhibition of p44ERK activity during nitrogen-mediated anoxia and activation during recovery in RTHD fibroblasts. RTHD fibroblasts were exposed to nitrogen-induced anoxia and recovery for the indicated periods of time. Anoxia was obtained by flushing supplemented Leibovitz' L-15 with nitrogen until the oxygen was removed. Recovery occurred by incubating the cells in atmospheric air. (A) Top: western blot analysis of phospho-p44ERK during anoxia (N=3) and total p44ERK (N=1). Bottom: quantification of band intensity showed significant inhibition of p44ERK activity after 3 h, relative to the normoxic control (*P<0.05). (B) Top: western blot analysis of phospho-p44ERK during recovery (N=3) and total p44ERK (N=1). Bottom: quantification of band intensity showed recovery resulted in significant activation of p44ERK after 5 min, relative to anoxic cells (*P<0.05). The value at 120 min recovery was not significant (P=0.059) because of one very low experimental value.

100 nmol l^{-1} DPI. DPI, which is a specific inhibitor of NAD(P)H oxidase-like enzymes (Matsui et al., 2000; O'Donnell et al., 1993), strongly inhibited recovery-mediated p44ERK phosphorylation (Fig. 9). As a control, RTHDF cells were stimulated with 5 mmol l^{-1} H_2O_2 for 15 min, which resulted in robust activation of p44ERK (data not shown).

Changes in p44ERK phosphorylation during anoxia/recovery are dependent on MEK

The effect of A/R on MEK activity in RTHD fibroblasts was analysed by western blotting and detection of phosphorylated MEK1/2. Paralleling p44ERK, MEK phosphorylation was inhibited by anoxia and stimulated above normoxic levels by recovery (Fig. 10A). When RTHDF cells were incubated with the $p38^{MAPK}\alpha/\beta$-specific inhibitor SB203580 (English and Cobb, 2002) for 1 h prior to the anoxic insult, inhibition of MEK by chemical anoxia was prevented (Fig. 10B). Similarly, incubation with 100 nmol l^{-1} calyculin A for 2 min prior to A/R prevented reactivation of MEK during recovery (Fig. 10C).

Discussion

Expression of three ERK proteins with apparent molecular masses of approximately 38, 42 and 44 kDa was observed in rainbow trout hypodermal fibroblasts (RTHDF) when western blots were probed with an antibody against mammalian ERK1/2. Accordingly, they are referred to as p44ERK, p42ERK and p38ERK. The three ERK-like proteins are likely to be the rainbow trout homologues of mammalian ERK isoforms.

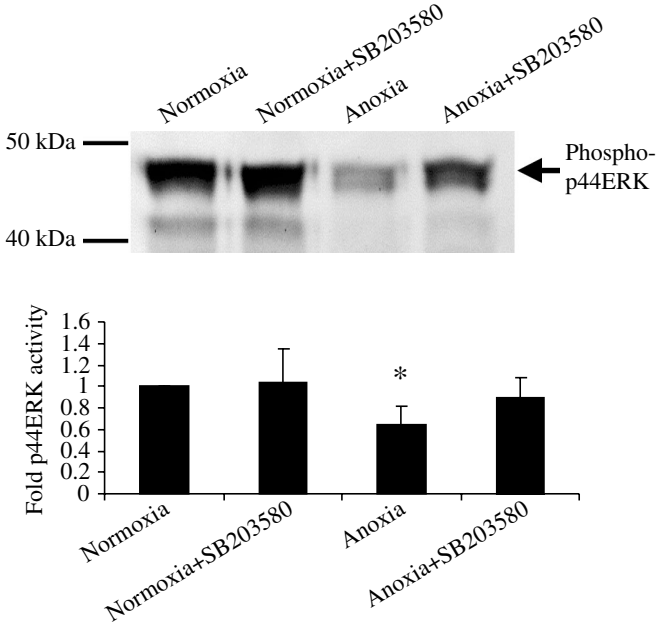


Fig. 6. $p38^{MAPK}$ mediates inhibition of p44ERK during chemical anoxia in RTHDF cells. Prior to 30 min chemical anoxia, RTHDF cells were pre-treated for 1 h with 10 μmol l^{-1} SB203580, inhibiting the MAPK p38α/β. Cell extracts were analysed by western blotting (top) and the immunoreactive bands were quantified (bottom) (N=3, *P<0.05).

The p44- and p38ERK proteins responded to serum stimulation following overnight serum starvation. Interestingly, they differed in their activation profile within the 30 min time frame used in this study. p44ERK was activated approximately threefold after 5–10 min in the presence of FBS followed by a decreasing level of phosphorylation. By contrast, p38ERK was regulated in a biphasic manner in response to FBS. After 5 min, p38ERK was phosphorylated sevenfold, with respect to serum starved cells. This was followed by a decreasing level of phosphorylation, reaching a minimum after 15 min before peaking again at 20 min.

Our observations are consistent with a study of peripheral blood lymphocytes from red drum, *Sciaenops ocellatus* L. and channel cat fish, *Ictalurus punctatus* R., demonstrating the presence of PMA-sensitive ERK proteins of 43 and 46 kDa (MacDougal et al., 1999). Furthermore, p38ERK is probably

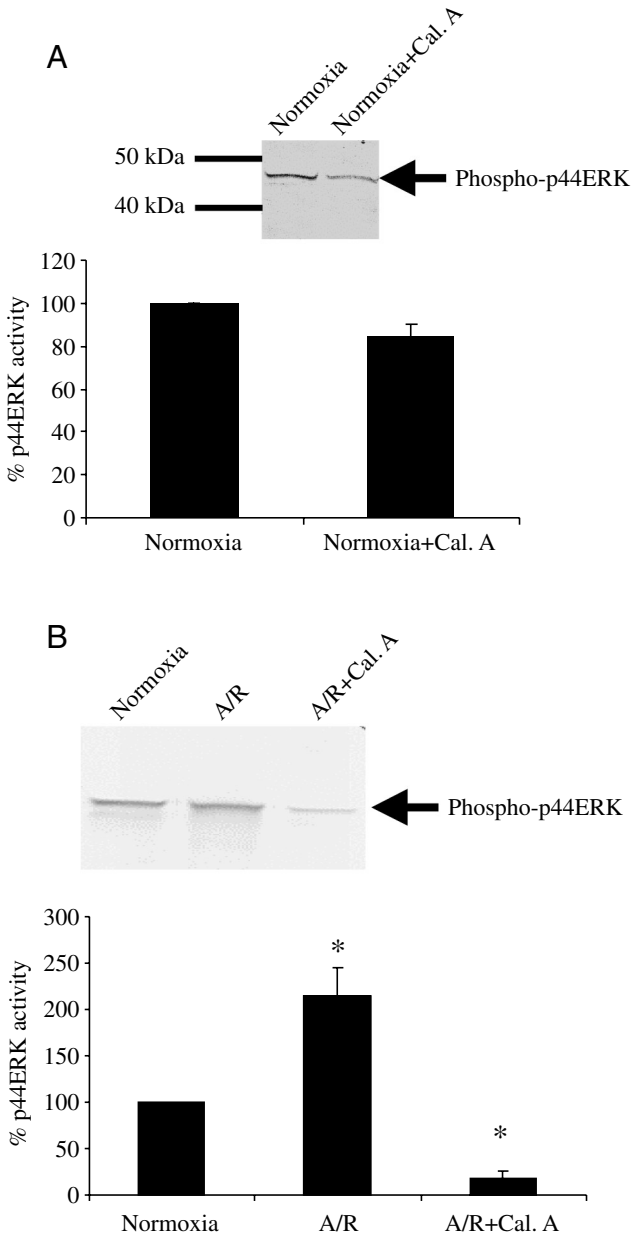


Fig. 7. Reactivation of p44ERK under recovery requires PP1/PP2A activity. RTHDF cells were treated with 100 nmol l^{-1} calyculin A (Cal. A; an inhibitor of PP1/2) for 2 min prior to treatment. Cell extracts were analysed by western blotting (top) and the immunoreactive bands were quantified (bottom). (A) Effect of calyculin A on normoxic p44ERK activity. (B) Effect of calyculin A on p44ERK phosphorylation after chemical anoxia/recovery (A/R) (N=3, *P<0.05).

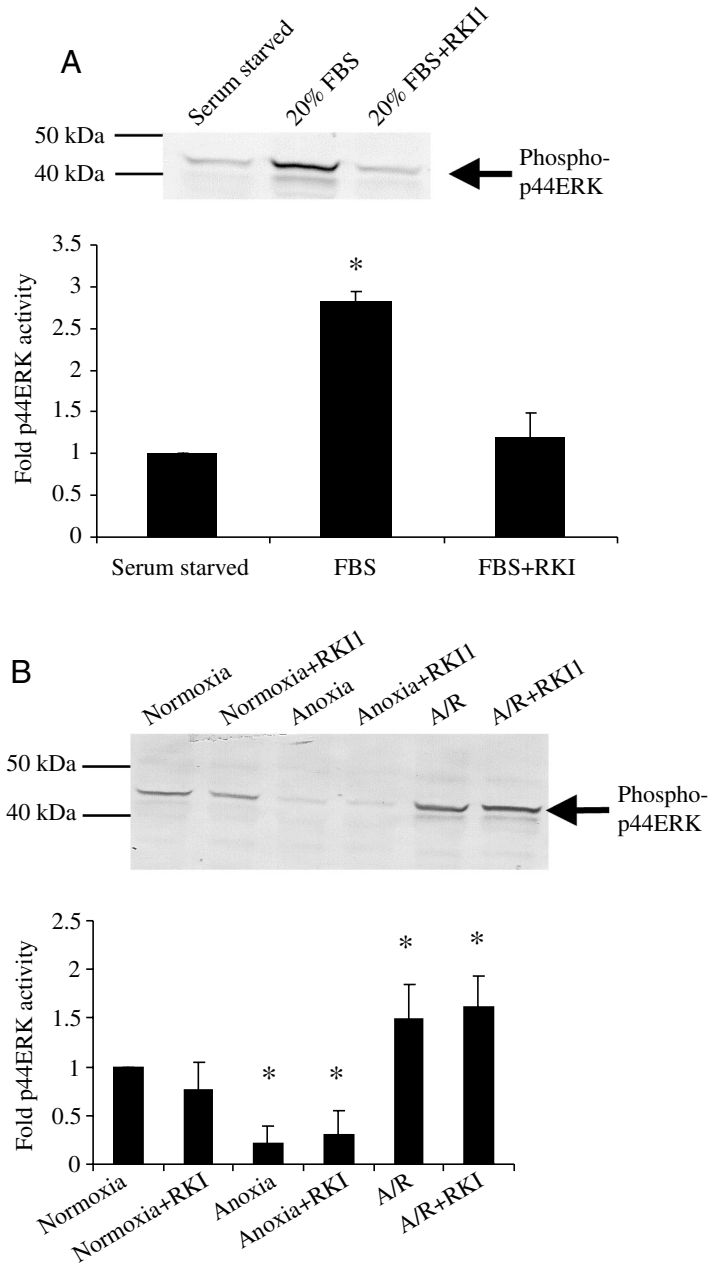


Fig. 8. Activation of p44ERK by serum (FBS) is dependent on Raf-1, but activation of p44ERK during recovery is independent of Raf-1. (A) Western blot analysis (top) and quantification (bottom) of phospho-p44ERK after serum stimulation of serum-starved cells, in the absence and presence of 10 μmol l^{-1} Raf-1 inhibitor, RKI. (B) Western blot analysis (top) and quantification (bottom) of the effect of 10 μmol l^{-1} Raf-1 inhibitor on p44ERK phosphorylation during chemical anoxia and recovery (A/R) (N=3, *P<0.05).

the same molecule as the H_2O_2-responsive, 38 kDa ERK-like protein observed in the rainbow trout hepatoma cell line RTH 149 (Burlando et al., 2003). Together with these studies, our data points to the existence of divergent ERK-like proteins among fishes.

We and others have previously used 10 mmol l^{-1} sodium azide as an inducer of chemical anoxia (Jørgensen et al., 1999; Ossum et al., 2004; Varming et al., 1996). In our previous study of chemical anoxia in RTHD fibroblasts (Ossum et al., 2004) a regime of 30 min of anoxia was established. Using this time period, a dose-dependent inhibition of basal ERK activity by sodium azide was found.

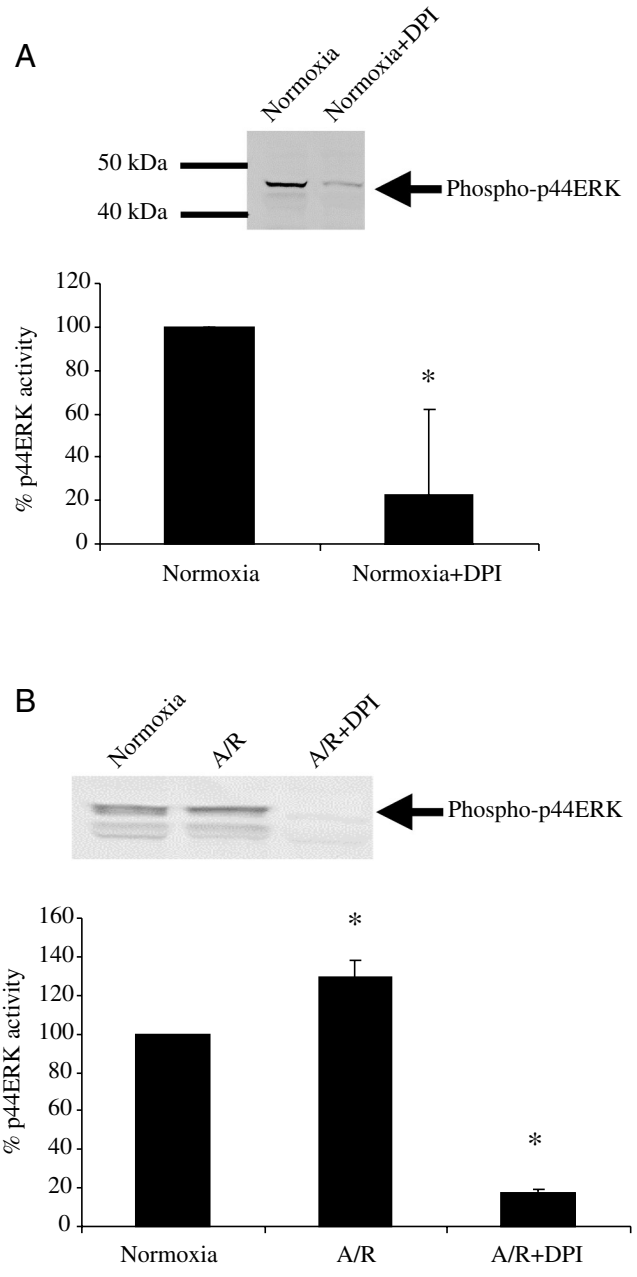


Fig. 9. Reactive oxygen species are required for reactivation of p44ERK activity during recovery. (A) Western blot analysis (top) and quantification (bottom) of basal p44ERK activity after 1 h treatment of RTHDF cells with 100 nmol l^{-1} DPI (N=3, *P<0.05). (B) Western blot analysis (top) and quantification (bottom) of phospho-ERK1-specific bands after 1 h treatment of RTHDF cells with 100 nmol l^{-1} DPI prior to chemical anoxia/recovery (A/R) (N=3, *P<0.05).

In the present study, it was demonstrated that chemical anoxia results in a time-dependent dephosphorylation of p44ERK in RTHDF cells, statistically significant after 30 min. Upon recovery, p44ERK was rapidly phosphorylated. Significant phosphorylation, with respect to anoxic cells, was observed after 10 min recovery. After 60 min recovery, the phosphorylation of p44ERK is six times higher than in anoxic cells. Activation of p44ERK is MEK dependent, as demonstrated using PD98059, which is a pharmacological inhibitor of MEK1/2 (English and Cobb, 2002). A similar decrease in ERK activity during cyanide-induced anoxia was observed, using the heart-derived, myogenic rat cell line H9c2 (Jung et al., 2004).

To validate chemical anoxia as a substitute for real oxygen deprivation, the anoxia/recovery experiment was repeated using nitrogen to displace oxygen. The same gradual inhibition of p44ERK during anoxia and phosphorylation during recovery were observed. However, the inhibition of p44ERK in response to nitrogen-induced anoxia was slower, resulting in significant inhibition only after 3 h. During recovery, phosphorylation of p44ERK occurred faster than in azide-treated cells, with significant and robust activation after 5 min. After 30 min, the level of ERK phosphorylation decreased and reached a plateau that was stable throughout the experiment. A similar, stable plateau level was observed in a single experiment with chemical anoxia (data not shown). We conclude that sodium azide can substitute for real anoxia in this experimental setting and that azide-induced anoxia has a stronger and more lasting effect compared to the removal of O_2 using nitrogen.

During anoxia, induced by both azide and nitrogen there was a trend towards an initial stimulation of p44ERK at 5 and 10 min, however, not statistically significant in either situation.

In a previous study we demonstrated a rapid, but transient activation of the stress-responsive $p38^{MAPK}$ in RTHDF cells (Ossum et al., 2004). The physiological significance of this observation, however, was not clear. In the present study, we obtained data indicating that activation of $p38^{MAPK}$ is necessary for attenuation of p44ERK signalling and involves the dephosphorylation of MEK.

The observation that $p38^{MAPK}$ activity is sufficient to attenuate ERK signalling is consistent with recent studies (Ding and Adrian, 2001; Jung et al., 2004; Lee et al., 2002; Li et al., 2003; Liu and Hofmann, 2004; Singh et al., 1999; Westermarck et al., 2001). In the very first report observing $p38^{MAPK}$-to-ERK cross-talk, it was shown that inhibition of p38α/β by SB203585 alone is sufficient to induce expression of low density lipoprotein receptor via activation of the ERK1/2 pathway in HepG2 cells (Singh et al., 1999). Further studies demonstrated that exposure of human skin fibroblasts to arsenite or adenoviral transduction of a constitutive active allele of MKK3b blocked ERK signalling (Li et al., 2003;

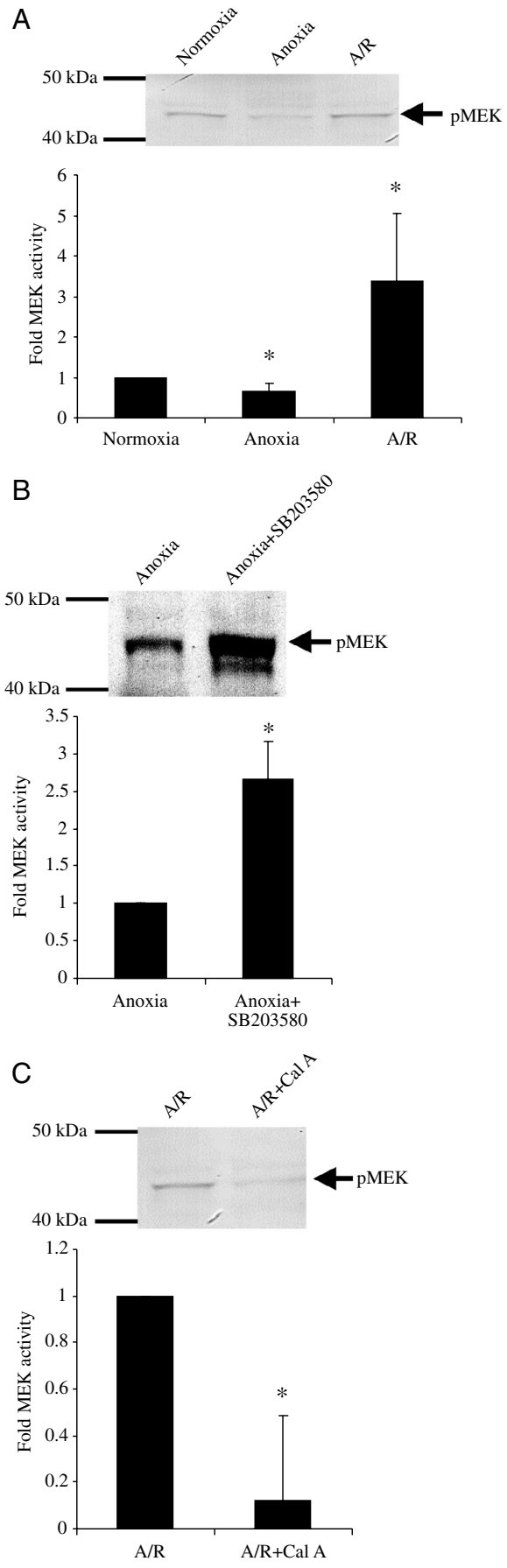


Fig. 10. p44ERK is regulated at the level of MEK during chemical anoxia/recovery (A/R). Phosphorylation of MEK1 during A/R was analysed by western blotting (top) and phospho-specific bands were quantified (bottom). (A) Anoxia inhibits MEK in RTHD fibroblasts (N=4, *P<0.05). (B) Pre-treatment with 10 μmol l^{-1} SB203580 for 1 h prevented inhibition of MEK during anoxia (N=3, *P<0.05). (C) Pre-treatment with 100 nmol l^{-1} calyculin A (Cal. A) for 2 min blocked reactivation of MEK during recovery (N=4, *P<0.05).

Westermarck et al., 2001). Using H9c2 cells, Jung et al. (Jung et al., 2004) observed activation of JNK and $p38^{MAPK}$ with the concurrent inhibition of ERK, using cyanide as a metabolic inhibitor. Also, a similar $p38^{MAPK}$-dependent mechanism has been described for induction of cell cycle arrest in myoblasts committed to differentiation (Lee et al., 2002). Lee et al. demonstrated that both the p38α/β-inhibitor, SB203580, and a dominant negative mutant of MKK6 inhibit differentiation and result in activation of the Raf/MEK/ERK pathway and subsequent proliferation. Finally, $p38^{MAPK}$-dependent signalling was discovered to inhibit stimulation of ERK1/2 in rat ventricular myocytes during H_2O_2-induced apoptosis (Liu and Hofmann, 2004). These studies, together with the present data, might suggest that $p38^{MAPK}$ is involved in regulatory mechanisms converging at ERK, which are common to environmental and physiological stimuli. This is shown schematically in Fig. 11, middle panel.

Interestingly, Westermarck et al. (Westermarck et al., 2001) and Liu and Hofmann (Liu and Hofmann, 2004) both presented evidence indicating the serine/threonine protein phosphatase 1 (PP1) and/or PP2A as the downstream effector of $p38^{MAPK}$. Using calyculin A, which is a highly specific inhibitor of PP1 and PP2A at nanomolar concentrations (Honkanen and Golden, 2002), we observed that a calyculin A-sensitive activity was required for stimulation of p44ERK during recovery of anoxic cells. However, calyculin A did not prevent dephosphorylation of p44ERK in the presence of sodium azide.

There is clear evidence in the literature that the calyculin A-sensitive phosphatases PP1 and/or PP2A can affect the MAPK pathway *via* different mechanisms specified by the cellular context (Janssens and Goris, 2001; Millward et al., 1999; Silverstein et al., 2002). Accumulating evidence strongly indicate a role for PP1/PP2A in inhibition of ERK signalling (Alessi et al., 1995; Goméz and Cohen, 1991; Liu and Hofmann, 2004; Westermarck et al., 2001). However, to our knowledge, only a few prior studies demonstrate a role for PP2A in stimulation of the mammalian ERK1/2 pathway. PP1/PP2A was shown to be a positive regulator of the kinase Raf-1 *in vivo* (Abraham et al., 2000; Mitsuhashi et al., 2003). PP2A seems to stimulate Raf/MEK/ERK signalling by dephosphorylating binding sites for 14-3-3 proteins, which are involved in suppression of Raf-1 and the scaffold protein kinase suppressor of Ras (KSR) (Ory et al., 2003). The present inhibitor studies, however, demonstrated that Raf-1 is dispensable for phosphorylation of p44ERK during recovery of RTHDF cells. By contrast, inhibition of Raf-1 prevented

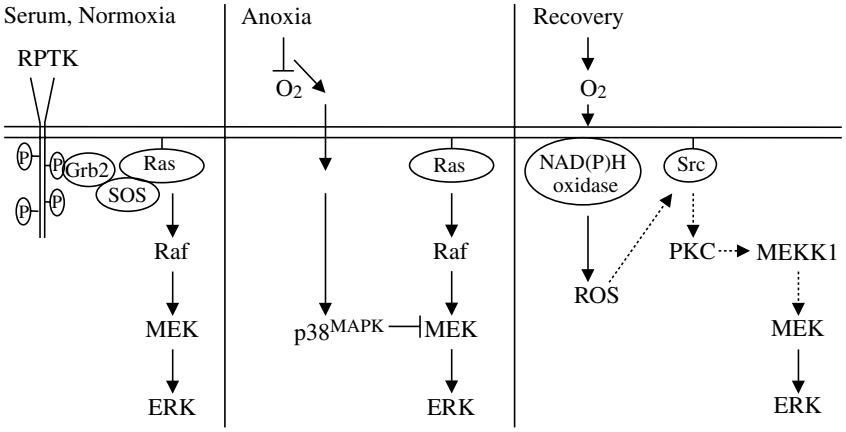


Fig. 11. Working model for the regulation of p44ERK activity in RTHD fibroblasts during chemical anoxia/recovery. Our data suggest that $p38^{MAPK}$ inhibits ERK activity at the level of MEK during chemical anoxia. During recovery, we demonstrated the requirement for ROS, produced *via* a NAD(P)H oxidase-like activity for reactivation of ERK in a Raf-independent manner. We speculate that ROS results in activation of Src, which in turn activates PKC upstream of MEKK1.

FBS-induced p44ERK phosphorylation in serum-starved RTHD fibroblasts. We, therefore, suggest that a calyculin-sensitive S/T phosphatase activity is necessary for activation of p44ERK during recovery from chemical anoxia. However, the identity of the presumptive phosphatase and its pathway are currently unknown and requires further studies.

Our data indicate that p44ERK phosphorylation during recovery is critically dependent on ROS-mediated signalling. Activation of p44ERK following removal of sodium azide was blocked by pre-treatment with DPI. This result strongly suggests that a NAD(P)H oxidase-like enzyme is involved in intracellular ROS production. Owing to their small size, diffusion properties and controllable production, reactive oxygen derivatives are ideally suited for a role as second messengers (Hancock et al., 2001). An appealing notion is that, in response to extreme environmental changes, production of ROS could represent a mechanism for rapid and robust ERK signalling. When relieved from potentially lethal anoxic stress conditions, NAD(P)H oxidase-mediated ERK activation could prevent cell death by stimulating survival pathways and antagonising apoptotic signalling. Indeed, low activity NAD(P)H oxidase homologues, e.g. NOX enzymes, which act as signalling molecules but not for host defence, are shown to be present in a variety of non-phagocytic cells (Babior, 1999; Lambeth, 2004). Mitogenic signalling has been shown to be mediated by ROS in Ras-transformed NIH 3T3 cells (Irani et al., 1997) and evidence suggests that non-phagocytic cells have the ability to regulate ROS production according to changes in P_{O_2} (Jones et al., 2000). Furthermore, H_2O_2 seems to regulate a genetic programme involving a large number of genes associated with signal transduction and cell cycle progression (Arnold et al., 2001). The latter observation is in agreement with the findings that generation of superoxide is involved in activation of nuclear factor kappa B (NF-κB) by platelet-derived growth factor (PDGF) (Marumo et al., 1997; Sun and Oberley, 1996) and AP-1 (Sun and Oberley, 1996). Our observations, taken together with the above studies and the fact that H_2O_2 is an established ERK activator in various mammalian systems, support a role for NAD(P)H oxidase as a mediator of rapid responses to changes in oxygen availability.

The signalling molecules downstream of the NAD(P)H oxidase-like enzyme and upstream of p44ERK are currently unknown. A working model is presented in Fig. 11, right panel. We speculate that ROS-mediated activation of p44ERK occurs by a pathway including the c-Src family of protein tyrosine kinases and PKC to MEK *via* MEKK1. Supporting this notion is the findings that hypoxia/recovery and oxidative stress, elicited by H_2O_2 activate Src-family kinases upstream of ERK (Aikawa et al., 1997; Seko et al., 1996; Suzaki et al., 2002). PKCβ has been shown to be activated by ROS and to functionally interact with MEKK1 (Datta et al., 2000; Kaneki et al., 1999) and MEKK1 is a known MEK kinase in the ERK pathway (Hagemann and Blank, 2001). As well, PKCα has been shown to trigger Ras and Raf-independent MEK/ERK activation in HepG2 cells (Wen-Sheng, 2005). In addition, H_2O_2 stimulation of COS-7 cells results in tyrosine phosphorylation and activation of the classical PKC isoforms α, βI and γ, the novel PKC isoforms δ and ϵ and the atypical PKC isoform PKCζ (Konishi et al., 1997). Finally, H_2O_2 has been implicated in ligand-independent activation of the platelet-derived growth factor receptor β by a mechanism depending on c-Src and PKCδ (Saito et al., 2002). In the studies of Datta (Datta, 2000) and Kaneki (Kaneki, 1999), MEKK1 stimulated JNK. However, JNK was not activated in our system either by chemical anoxia or recovery (K. Krogh-Jeppesen, C.G.O. and E.K.H., unpublished results). Thus we speculate that MEKK1 may be the MAP3K responsible for the observed phosphorylation of p44ERK during recovery.

In conclusion, we have described a 44 kDa p44ERK protein in RTHDF cells. When cells are challenged with chemical anoxia, phosphorylation of p44ERK was inhibited secondary to activation of $p38^{MAPK}$-dependent mechanism. Recovery resulted in robust upregulation of p44ERK activity, in a PP1/PP2A- and ROS-dependent manner. A similar, but slower inhibition and a similar, but faster recovery of pp44ERK was seen under nitrogen-induced anoxia.

List of abbreviations

A/R	anoxia/recovery
DPI	diphenyleneiodonium chloride

ERK	extracellular signal-regulated kinase
FBS	foetal bovine serum
JNK	c-Jun N-terminal kinase
KSR	kinase suppressor of Ras
MAPK	mitogen-activated protein kinase
MAP2K	mitogen-activated protein kinase kinase
MAP3K	mitogen-activated protein kinase kinase
MEK	MAPK/ERK kinase
NF-κB	nuclear factor kappa B
PDGF	platelet-derived growth factor
PI 3-kinase	phosphatidyl inositol 3-kinase
PKC	protein kinase C
PP1/PP2A	protein phosphatase 1 and 2A
ROS	reactive oxygen species
RTHDF	rainbow trout hypodermal fibroblasts
TBS-T	TRIS-buffered saline with Tween 20

The research was supported by grants from the Danish Network in Aquaculture and Fisheries Research (www.fishnet.dk) financed by the Danish Ministry for Food, Agriculture and Fisheries and the Danish Agricultural and Veterinary Research Council to E.K.H., as well by the NOVO Nordic Foundation. We thank Dr scient. Ian Lambert (University of Copenhagen) for critically reading the manuscript.

References

Abas, L., Bogoyevitch, M. A. and Guppy, M. (2000). Mitochondrial ATP production is necessary for activation of the extracellular-signal-regulated kinases during ischaemia/reperfusion in rat myocyte-derived H9c2 cells. *Biochem. J.* **349**, 119-126.

Abraham, D., Podar, K., Pacher, M., Kubicek, M., Welzel, N., Hemmings, B. A., Dilworth, S. M., Mischak, H., Kolch, W. and Baccarin, M. (2000). Raf-1-associated protein phosphatase 2A as a positive regulator of kinase activation. *J. Biol. Chem.* **275**, 22300-22304.

Aikawa, R., Komuro, I., Yamazaki, T., Zou, Y., Kudoh, S., Tanaka, M., Shiojima, I., Hiroi, Y. and Yazaki, Y. (1997). Oxidative stress activates extracellular signal–regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats. *J. Clin. Invest.* **100**, 1813-1821.

Alessi, D. R., Gomez, N., Moorhead, G., Lewis, T., Keyse, S. and Cohen, P. (1995). Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100 in various cell lines. *Curr. Biol.* **5**, 283-295.

Allan, L. A., Morrice, N., Brady, S., Magee, G., Pathak, S. and Clarke, P. R. (2003). Inhibition of caspase-9 through phosphorylation at Thr 125 by ERK MAPK. *Nat. Cell Biol.* **5**, 647-654.

Arnold, R. S., Shi, J., Murad, E., Whalen, A. M., Sun, C. Q., Polavarapu, R., Parthasarathy, S., Petros, J. A. and Lambeth, J. D. (2001). Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1. *Proc. Natl. Acad. Sci. USA* **98**, 5550-5555.

Babior, B. M. (1999). NADPH oxidase: an update. *Blood* **93**, 1464-1476.

Barr, R. K. and Bogoyevitch, M. A. (2001). The c-Jun N-terminal protein kinase family of mitogen-activated protein kinases. *Int. J. Biochem. Cell Biol.* **33**, 1047-1063.

Blaschke, F., Stawowy, P., Goetze, S., Hintz, O., Gräfe, M., Kintscher, U., Fleck, E. and Graf, K. (2002). Hypoxia activates β_1-integrin via ERK1/2 and p38 MAP kinase in human vascular smooth muscle cells. *Biochem. Biophys. Res. Comm.* **296**, 890-896.

Bogoyevitch, M. A., Gillespie-Brown, J., Ketterman, A. J., Fuller, S. J., Ben-Levy, R., Ashworth, A., Marshall, C. J. and Sugden, P. H. (1996). Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. *Circ. Res.* **79**, 162-173.

Burlando, B., Magnelli, V., Panfoli, I., Berti, E. and Viarengo, A. (2003). Ligand-independent tyrosine kinase signalling in RTH 149 trout hepatoma cells: Comparision among heavy metals and pro-oxidants. *Cell. Physiol. Biochem.* **13**, 147-154.

Cano, E. and Mahadevan, L. C. (1995). Parallel signal processing among mammalian MAPKs. *Trends Biochem. Sci.* **20**, 117-121.

Cerioni, L., Palomba, L. and Cantoni, O. (2003). The Raf/MEK inhibitor PD98059 enhances ERK1/2 phosporylation mediated by peroxynitrite via enforced mitochondrial formation of reactive oxygen species. *FEBS Lett.* **547**, 92-96.

Conrad, P. W., Rust, R. T., Han, J., Millhorn, D. E. and Beitner-Johnson, D. (1999). Selective activation of p38α and p38γ by hypoxia. *J. Biol. Chem.* **274**, 23570-23576.

Cowan, K. J. and Storey, K. B. (2003). Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. *J. Exp. Biol.* **206**, 1107-1115.

Datta, R., Yoshinaga, K., Kaneki, M., Pandey, P. and Kufe, D. (2000). Phorbol ester-induced generation of reactive oxygen species is protein kinase Cβ-dependent and required for SAPK activation. *J. Biol. Chem.* **275**, 41000-41003.

Ding, X.-Z. and Adrian, T. E. (2001). MEK/ERK-mediated proliferation is negatively regulated by p38 MAP kinase in the human pancreatic cancer cell line, PANC-1. *Biochem. Biophys. Res. Comm.* **282**, 447-453.

Dröge, W. (2002). Free radicals in the physiological control of cell function. *Physiol. Rev.* **82**, 47-95.

English, J. M. and Cobb, M. H. (2002). Pharmacological inhibitors of MAPK pathways. *Trends Pharmacol. Sci.* **23**, 40-45.

Goméz, N. and Cohen, P. (1991). Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases. *Nature* **353**, 170-173.

Goyal, P., Weissmann, N., Grimminger, F., Hegel, C., Bader, L., Rose, F., Fink, L., Ghofrani, H. A., Schermuly, R. T., Schmidt, H. H. et al. (2004). Upregulation of NAD(P)H oxidase 1 in hypoxia activates hypoxia inducible factor 1 via increase in reactive oxygen species. *Free Radic. Biol. Med.* **36**, 1279-1288.

Guyton, K. Z., Liu, Y., Gorospe, M., Xu, Q. and Holbrook, N. J. (1996). Activation of mitogen-activated protein kinase by H_2O_2. *J. Biol. Chem.* **271**, 4138-4142.

Hagemann, C. and Blank, J. L. (2001). The ups and downs of MEK kinase interactions. *Cell. Signal.* **13**, 863-875.

Hancock, J. T., Desikan, R. and Neill, S. J. (2001). Role of reactive oxygen species in cell signalling pathways. *Biochem. Soc. Trans.* **29**, 345-350.

Harper, S. J. and LoGrasso, P. (2001). Signalling for survival and death in neurons. The role for stress-activated kinases, JNK and p38. *Cell. Signal* **13**, 299-310.

Honkanen, R. E. and Golden, T. (2002). Regulators of serine/threonine protein phosphatases at the dawn of a clinical era? *Curr. Med. Chem.* **9**, 2055-2075.

Irani, K., Xia, Y., Zweier, Y. L., Sollott, S. J., Der, C. J., Fearon, E. R., Sundaresan, M., Finkel, T. and Goldschmidt-Clermont, P. J. (1997). Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. *Science* **275**, 1649-1652.

Janssens, V. and Goris, J. (2001). Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and singallin. *Biochem. J.* **353**, 417-439.

Jin, K., Mao, X. O., Zhu, Y. and Greenberg, D. A. (2002). MEK and ERK protect hypoxic cortical neurons via phosphorylation of Bad. *J. Neurochem.* **80**, 119-125.

Jones, R. D., Hancock, J. T. and Morice, A. H. (2000). NADPH oxidase: a universal oxygen sensor? *Free Radic. Biol. Med.* **29**, 416-424.

Jørgensen, N. K., Pedersen, S. F., Damgaard, I., Schousboe, A. and Hoffmann, E. K. (1999). Increases in $[Ca^{2+}]_i$ and changes in intracellular pH during chemical anoxia in mouse neocortical Neurons in primary culture. *J. Neurosci. Res.* **56**, 358-370.

Jung, Y.-S., Jung, Y.-S., Kim, M.-Y. and Kim, E. (2004). Identification of caspase-independent PKCε-JNK/p38 MAPK signaling module in response to metabolic inhibition in H9c2 cells. *Jpn. J. Physiol.* **54**, 23-29.

Kaneki, M., Kharbanda, S., Pandey, P., Yoshida, K., Takekawa, M., Liou, J.-R., Stone, R. and Kufe, D. (1999). Functional role for protein kinase Cβ as a regulator of stress-activated protein kinase activation and monocytic differentiation of myeloid leukemia cells. *Mol. Cell. Biol.* **19**, 461-470.

Konishi, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikkawa, U. and Nishizuka, Y. (1997). Activation of protein kinase C by tyrosine phosphorylation in response to H_2O_2. *Proc. Natl. Acad. Sci. USA* **94**, 11233-11237.

Kyriakis, J. M. and Avruch, J. (2001). Mammalian mitogen-activated

protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.* **81**, 807-869.

Lambeth, J. D. (2004). NOX enzymes and the biology of reactive oxygen. *Nat. Rev. Immunol.* **4**, 181-189.

Lee, J., Hong, F., Kwon, S., Kim, S. S., Kim, O. D., Kang, S. H., Lee, S. J., Ha, J. and Kim, S. S. (2002). Activation of p38 MAPK induces cell cycle arrest via inhibition of Raf/ERK pathway during muscle differentiation. *Biochem. Biophys. Res. Comm.* **289**, 765-771.

Li, S.-P., Junttila, M. R., Han, J., Kähäri, V.-M. and Westermarck, J. (2003). p38 mitogen-activated protein kinase pathway suppresses cell survival by inducing dephosphorylation of mitogen-activated protein/extracellular signal-regulated kinase kinase 1,2. *Cancer Res.* **63**, 3473-3477.

Liu, Q. and Hofmann, P. A. (2004). Protein phosphatase 2A-mediated cross-talk between p38 MAPK and ERK in apoptosis of cardiac myocytes. *Am. J. Physiol.* **286**, H2204-H2212.

Ma, C. and Collodi, P. (1999). Preparation of primary cells from lamprey. *Methods Cell Sci.* **21**, 39-46.

MacDougal, K. C., Mericko, P. A. and Burnett, K. G. (1999). Antigen receptor-mediated activation of extracellular related kinase (ERK) in B lymphocytes of teleost fishes. *Dev. Comp. Immunol.* **23**, 221-230.

Marumo, T., Schini-Kerth, V. B., Fisslthaler, B. and Busse, R. (1997). Platelet-derived growth factor – stimulated superoxide anion production modulates activation of transcription factor NF-κB and expression of monocyte chemoattractant protein 1 in human aortic smooth muscle cells. *Circulation* **96**, 2361-2367.

Matsui, T., Suzuki, Y., Yamashita, K., Yoshimaru, T., Suzuki-Karasaki, M., Hayakawa, S., Yamaki, M. and Shimizu, K. (2000). Diphenyleneiodonium prevents reactive oxygen species generation, tyrosine phosphorylation, and histamine release in RBL-2H3 mast cells. *Biochem. Biophys. Res. Comm.* **276**, 742-748.

Millward, T. A., Zolnierowicz, S. and Hemmings, B. A. (1999). Regulation of protein kinase cascades by protein phosphatase 2A. *Trends Biochem. Sci.* **24**, 186-191.

Mitsuhashi, S., Shima, H., Tanuma, N., Matsuura, N., Takekawa, M., Urano, T., Kataoka, T., Ubukata, M. and Kikuchi, K. (2003). Usage of tautomycetin, a novel inhibitor of protein phosphatase 1 (PP1), reveals that PP1 is a positive regulator of Raf-1 *in vivo*. *J. Biol. Chem.* **278**, 82-88.

Mizukami, Y. and Yoshida, K. I. (1997). Mitogen-activated protein kinase translocates to the nucleus during ischemia and is activated during reperfusion. *Biochem. J.* **323**, 785-790.

Mizukami, Y., Hirata, T. and Yoshida, K. I. (1997). Nuclear translocation of PKCζ during ischemia and its inhibition by wortmannin, an inhibitor of phosphatidylinositol 3-kinase. *FEBS Lett.* **401**, 247-251.

Mizukami, Y., Kobayashi, S., Überall, F., Hellbert, K., Kobayashi, N. and Yoshida, K. I. (2000). Nuclear mitogen-activated protein kinase activation by protein kinase Cζ during reoxigenation after ischemic hypoxia. *J. Biol. Chem.* **275**, 19921-19927.

Nakano, A., Baines, C. P., Kim, S. O., Pelech, S. L., Downey, J. M., Cohen, M. V. and Critz, S. D. (2000). Ischemic preconditioning activates MAPKAPK2 in the isolated rabbit heart: evidence for involvement of p38 MAPK. *Circ. Res.* **86**, 144-151.

New, L. and Han, J. (1998). The p38 MAP kinase pathway and its biological function. *Trends Cardiovasc. Med.* **8**, 220-229.

Nikinmaa, M. (2002). Oxygen-dependent cellular functions – why fishes and their aquatic environment are a prime choice of study. *Comp. Biochem. Physiol.* **133A**, 1-16.

O'Donnell, B. V., Tew, D. G., Jones, O. T. and England, P. J. (1993). Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. *Biochem. J.* **290**, 41-49.

Ory, S., Zhou, M., Conrads, T. P., Veenstra, T. D. and Morrison, D. K. (2003). Protein phosphatase 2A positively regulates Ras signaling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites. *Curr. Biol.* **13**, 1356-1364.

Ossum, C. G., Hoffmann, E. K., Vijayan, M. M., Holt, S. E. and Bols, N. C. (2004). Characterization of a novel fibroblast-like cell line from rainbow trout and responses to sublethal anoxia. *J. Fish Biol.* **64**, 1103-1116.

Saito, S., Frank, G. D., Mifune, M., Ohba, M., Utsunomiya, H., Motley, E. D., Inagami, T. and Eguchi, S. (2002). Ligand-independent *trans*-activation of the platelet-derived growth factor by reactive oxygen species requires protein kinase C-δ and c-Src. *J. Biol. Chem.* **277**, 44695-44700.

Saurin, A. T., Martin, J. L., Heads, R. J., Foley, C., Mockridge, J. W., Wright, M. J., Wang, Y. and Marber, M. S. (2000). The role of differential activation of p38-mitogen-activated protein kinase in preconditioned ventricular myocytes. *FASEB J.* **14**, 2237-2246.

Schaeffer, H. J. and Weber, M. J. (1999). Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell. Biol.* **19**, 2435-2444.

Schirmer, K., Chan, A. G. J., Greenberg, M. B., Dixon, D. G. and Bols, N. C. (1997). Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. *Toxicol. Vitro* **11**, 107-109.

Schmitz, M. L., Bacher, S. and Dröge, W. (2002). Molecular analysis of mitogen-activated protein kinase signaling pathways induced by reactive oxygen intermediates. *Methods Enzymol.* **352**, 53-61.

Seko, Y., Tobe, K., Takahashi, N., Kaburagi, Y., Kadowaki, T. and Yazaki, Y. (1996). Hypoxia and hypoxia/reoxygenation activate Src family tyrosin kinases and $p21^{ras}$ in cultured rat cardiac myocytes. *Biochem. Biophys. Res. Comm.* **226**, 530-535.

Sheppeck, J. E., II, Gauss, C.-M. and Chamberlin, A. R. (1997). Inhibition of the Ser-Thr phosphatases PP1 and PP2A by naturally occuring toxins. *Bioorg. Med. Chem.* **5**, 1730-1750.

Silverstein, A. M., Barrow, C. A., Davis, A. J. and Mumby, M. C. (2002). Actions of PP2A on the MAP kinase pathway and apoptosis are mediated by distinct regulatory subunits. *Proc. Natl. Acad. Sci. USA* **99**, 4221-4226.

Singh, R. P., Dhawan, P., Golden, C., Kapoor, G. S. and Mehta, K. D. (1999). One-way cross-talk between $p38^{MAPK}$ and $p42/p44^{MAPK}$. *J. Biol. Chem.* **274**, 19593-19600.

Sun, Y. and Oberley, L. W. (1996). Redox regulation of transcription activators. *Free Radic. Biol. Med.* **21**, 335-348.

Suzaki, Y., Yoshizumi, M., Kagami, S., Koyama, A. H., Taketani, Y., Houchi, H., Tsuchiya, K., Takeda, E. and Tamaki, T. (2002). Hydrogen peroxide stimulates c-Src-mediated big mitogen-activated protein kinase 1 (BMK1) and the MEF2C signaling pathway in PC12 cells. *J. Biol. Chem.* **277**, 9614-9621.

Uehara, T., Kaneko, M., Tanaka, S., Okuma, Y. and Nomura, Y. (1999). Possible involvement of p38 MAP kinase in HSP70 expression induced by hypoxia in rat primary astrocytes. *Brain Res.* **823**, 226-230.

Varming, T., Drejer, J., Frandsen, A. and Schousboe, A. (1996). Characterization of a chemical anoxia model in cerebellar granule neurons using sodium azide: protection by nifedipine and MK-801. *J. Neurosci. Res.* **44**, 40-46.

Wen-Sheng, W. (2005). Protein kinase Cα trigger Ras and Raf-independent MEK/ERK activation for TPA-induced growth inhibition of human hepatoma cell HepG2. *Cancer Lett.* doi:10.1016/j.canlet.2005.07.034.

Westermarck, J., Li, S. P., Kallunki, T., Han, J. and Kähäri, V. M. (2001). p38 mitogen-activated protein kinase-dependent activation of protein phosphatase 1 og 2A inhibits MEK1 and MEK2 activity and collagenase 1 (MPP-1) gene expression. *Mol. Cell. Biol.* **21**, 2373-2383.

Widmann, C., Gibson, S., Jarpe, M. B. and Johnson, G. L. (1999). Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human. *Physiol. Rev.* **79**, 143-180.

Wu, R. S. S. (2002). Hypoxia: from molecular responses to ecosystem responses. *Mar. Pollut. Bull.* **45**, 35-45.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. and Greenberg, M. E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270**, 1326-1331.

Zhu, Y., Mao, X. O., Sun, Y., Xia, Z. and Greenberg, D. A. (2002). p38 mitogen-activated protein kinase mediates hypoxic regulation of Mdm2 and p53 in neurons. *J. Biol. Chem.* **277**, 22909-22914.