Differential Role for ERK2 in Anoxia-induced Activation of Transcription and Translation of Hsp70 in NIH 3T3 Cells

Carlo G. Ossum, Anders N. Lauritsen, Dorina Gabriela Karottki and Else K. Hoffmann

Department of Biology, University of Copenhagen, Copenhagen

Key Words

ERK • MAPK • Signal transduction • Hsp70 • Anoxia • Gene expression • MEK-independent

Abstract

Hsp70 has the ability to enhance the recovery of stressed cells by its ability to catalyze the reassembly of damaged proteins. Such a chaperoning function is essential for the Hsp70-mediated protection against anoxic stress that causes protein denaturation. We have studied induction of both transcription and translation of Hsp70 during recovery from chemical anoxia and the role of the extracellular signal regulated kinase ERK2 in this induction of Hsp70. 10 mM azide for 30 minutes (chemical anoxia) significantly inhibited the activity of ERK2 (measured as phospho-ERK) but the ERK-2 activity is rapidly increased in a MEK-independent manner, when azide is washed out of the cells. Chemical anoxia and overnight recovery induced Hsp70 expression (analyzed by Western blotting) and this was inhibited by actinomycin D as well as by cycloheximide showing that induction of both translation and transcription was involved. Inhibition of the MAP kinase p38, which was transiently activated during

chemical anoxia, had no effect on the increase in Hsp70 expression whereas an inhibitor of reactive oxygen species and inhibition of the phosphatase PP1 and PP2a inhibited the increase in Hsp70 expression. Inhibition of ERK2 by the MEK inhibitor PD98059 resulted in strong inhibition of Hsp70 protein expression and simultaneous stimulation of *hsp70* transcription.

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Introduction

The heat shock response and the expression and function of heat shock proteins (Hsps) and the 72 kDa Hsp70 in particular, have been extensively studied since the discovery of the heat shock response in the fruit fly *Drosophila bushii* in 1962 [1, 2].

The Hsp70 molecular chaperone system is conserved from bacterial to mammalian cells [3-5]. In eukaryotic cells multiple Hsp70 proteins and co-chaperones have been identified and play multiple roles in the cell [5, 6].

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Carlo Ossum
Department of Biology, The August Krogh Building
Universitetsparken 13, DK-2100 Copenhagen Ø (Denmark)
Tel. +45 35 32 16 95; Fax +45 35 32 15 67
E-Mail cgossum@gmail.com

Seven *hsp70* genes have been identified in the mouse genome, where the inducible *hsp70.1* and *hsp70.3* are nearly identical, only differing in the 3' UTR [6, 7].

Under normal physiological conditions, Hsp70 proteins of the heat shock cognate-group have important housekeeping roles in the folding of non-native proteins by quality control and co- and post translational folding of newly synthesized proteins [3, 5, 6]. In response to cellular stress, the inducible Hsp70 protein genes are rapidly activated by the transactivating transcription factor Heat shock factor (HSF)-1 binding to the heat shock element, HSE [8, 9].

The synthesis of Hsp70 in response to stressful conditions is an important factor for stimulation of cell survival. A main role of Hsp70 is the prevention of apoptosis [10]. Interestingly, Hsp70 inhibits the apoptotic pathway at multiple levels that are both dependent and independent of its chaperone-activity [10-13].

Another group of proteins that are important in the decision of cell fate is the mitogen-activated protein (MAP) kinase super-family [14], which are divided into three main families [15, 16]; the ERKs which are generally growth promoting [16, 17], and JNK [18] and the p38^{MAPK} proteins [19] which are generally stress induced. Interestingly, we and others have demonstrated regulatory crosstalk between MAP kinase pathways [20-23].

How ERK responds to hypoxic and anoxic conditions seems to be highly tissue-dependent. In the cerebral cortex of piglets, hypoxia resulted in a NO-mediated activation of ERK [24] and in the vasculature, ERK plays an important role in vascular remodeling during hypoxia [25, 26]. Thus ERK seems to protect specialized cells, such as neurons and the vascular endothelium during hypoxic stress. However, in less specialized cells, such as fibroblasts from the rainbow trout [21] and NIH 3T3 cells from the mouse (this study), anoxia is an inhibitor of ERK signalling.

In this study, we have investigated the effect of chemical anoxia on the activity and regulation of ERK2 and expression of Hsp70 on both the mRNA and protein level. Interestingly, we demonstrate that chemical anoxia inhibits ERK2 signalling and that recovery from chemical anoxia results in increased ERK2-phosphorylation in a MEK-independent manner, induction of *hsp70* mRNA and expression of Hsp70 protein. We observed a dual role for ERK2, in that ERK2 stimulates the synthesis of Hsp70 protein, but inhibits the induction of *hsp70* mRNA.

Inhibitor	Target	Stock solution	Final concentration	Incubation time
Actinomycin D	Transcription	1 µg/µl	0.5 µg/ml	1 hour
Cycloheximide	Translation	10 µg/µl	5 µg/ml	1 hour
SB203580	p38 ^{MAPK}	10 mM	10 µM	1 hour
PD98059	MEK1/2	10 mM	10 µM	1 hour
Diphenyleneiodonium, (DPI)	NADP(H) oxidase-like proteins	1 mM	1 µM	1 hour
Calyculin A	PP1/PP2A	70 µM	100 nM	2 min

Table 1. Inhibitors used in this study. Overview of the inhibitors used and their target proteins, stock solutions, final concentrations and incubation times.

Materials and Methods

Chemicals and reagents

Unless otherwise stated, reagents were analytical grade and purchased from J.T. Baker (Denver, Holland) or Sigma-Aldrich Inc. (St. Louis, MO, USA). Diphenyleneiodonium chloride (DPI), Actinomycin D and cycloheximide were obtained from Sigma. Calyculin A, PD98059 and SB203580 and were purchased from Calbiochem (La Jolla, CA, USA). The 10 mg/µl cycloheximide and 1 mg/µl actinomycin D were purchased as ready-made solutions (DMSO) and stock solutions of 70 µM Calyculin A, 10 mM PD98059, 10 mM SB203580, 1 mM DPI, were prepared using DMSO as solvent. Pre-casted 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). The anti-phospho-p42/p44 antibody (ERK1/2)/(pThr202 and pTyr204) was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA) and the anti-Hsp70 antibody was from Sigma. The secondary antibodies, goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Sigma and Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA), respectively. BCIP/NBT Membrane Phosphatase Substrate was purchased from (KLP, Gaithersburg, MD, USA). All cell culture reagents were from Life Technologies (Naperville, IL, USA). SuperScript II reverse transcriptase, oligo(dT)₁₂₋₁₈, random primers deoxynucleotide kit were purchased from Invitrogen and molecular biology grade water were from Eppendorf. Deoxynucleotides were prepared as a stock solution containing 10 mM of each nucleotide in molecular biology grade water. Brilliant QPCR master mix was purchased from Stratagene (Cedar Creek, TX, USA) and primers and Taqman probes were from MWG (Ebersberg, Germany). All cell culture reagents were purchased from Life Technologies (Naperville, IL, USA) and the cell culture plastic wear were from TPR (Trasadingen, Switzerland).

Primer	Sequence (5'→3')	Florescent labeling (5'/3')
hsp70 FW	AGATAAAGGAGATCGCTGAGGC	none
hsp70 Rev	TGATGATCCGCAGCACGTTC	none
Hsp70 probe	TCACGGTGCCTGCCTACTTCAACGACTCTCA	FAM/TAMRA
β-actin FW	AGAGCTATGAGCTGCCTGAC	none
β -actin Rev	GGATGCCACAGGATTCCATAC	none
β -actin probe	CCCTGAGGCTCTTTTCCAGCCTTCCTTCTT	FAM/TAMRA

Table 2. Primers and probes used in quantitative real-time PCR. Sequences are given in 5' to 3' direction. Taqman probes were labeled with fluorescein (FAM) in the 5' end and the quencher tetramethylrhodamine (TAMRA) in the 3' end.

Cell culture

NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium, DMEM, supplemented with 10% (v/v) foetal bovine serum, 100 units penicillin/ml and 100 µg streptomycin/ml. The cells were incubated in a 5% CO₂ atmosphere at 37°C and 95% humidity. Passaging was performed every 4-5 days and the cells maintained at approximately 80% confluency.

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 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 1 mM MgSO₄, 1 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 1 mM CaCl₂, 5 mM galactose, 10 mM HEPES and 5 mM sodium pyruvate [27, 28]. Chemical anoxia was induced using 10 mM NaN₃ in L-15ex, where 10 mM NaCl was replaced with 10 mM NaN₃. 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 incubated in L-15ex without NaN₃.

In the inhibitor experiments, cells were pre-treated as described in Table 1.

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 mM Na₃VO₄ (from a 200 mM stock solution), 10mM Tris, pH 7.4) and immediately boiled for 5 min. Extracts were homogenised by sonication, followed by removal of cell debris by centrifugation at 16,000×g for 5 minutes. 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 supplemented with 10% (v/v) 0.5 M dithiothreitol (DTT), then boiled for 5 minutes. Proteins were separated by SDS-PAGE, using precast NuPAGE 10% Bis-Tris gels and electrotransferred to nitrocellulose membranes of 0.22 µm pore size, using NuPAGE MOPS SDS Running buffer and NuPAGE Transfer buffer, 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 Ponceau S [0.1% Ponceau S (w/v) 5% acetic acid (v/v)]. Electrophoresis and transfer were performed using the XCell SureLock and the XCell II Blot module (Invitrogen), respectively. After transfer, the nitrocellulose membrane was incubated in blocking buffer [5% non-fatty dry milk in TBS-T (10 mM Tris-Cl, pH 7.5, 120 mM NaCl and 0.1% polyoxyethylenesorbitan monolaurate, "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 3 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 6.1 for Windows (Silk Scientific Corp. UT, USA).

The primary antibody against phospho-p44/42 MAP kinase (Thr202/Tyr204) polyclonal antibody was diluted 1:1000 and the anti-Hsp70 monoclonal antibody was diluted 1:5000 in blocking buffer. The secondary antibody goat anti-rabbit IgG were diluted 1:500 and goat anti-mouse IgG was diluted 1:3000.

Both careful determinations of protein concentration and staining of the nitrocellulose membranes with Ponceau S post protein transfer were performed to ensure loading of equal amounts of protein per lane per gel.

cDNA synthesis and quantitative real-time PCR

After experiments cells were rinsed once in cold PBS and total RNA harvested using Mammalian total RNA purification kit from Sigma according to the manufacturer's instructions. cDNA was synthesized using SuperScript II reverse transcriptase, oligo(dT)₁₂₋₁₈ and random primers according to the instructions from Invitrogen.

The quantitative real-time PCR (qPCR) were performed using Brilliant QPCR master mix from Stratagene and the PCR-reaction carried out at 55°C using the MX4000 machine from Stratagene. Primers and Taqman probes (Tab. 2) targeting mouse *hsp70* and β-actin mRNAs were purchased from MWG. The probes were labeled with the reporter fluorescein (FAM)

Fig. 1. Sodium azide inhibits ERK2 in a dose-dependent manner. Western blot analysis of ERK2-phosphorylation in NIH 3T3 cells incubated with increasing concentrations of NaN_3 . NIH3T3 cells were grown to 80% confluency (the same cell confluency was ensured throughout the study) and incubated with the indicated concentrations of NaN_3 in L15-ex for 30 min. Phosphorylated ERK2 is indicated by p ERK2 in the figure and in the following figures.

in the 5' end and the quencher tetramethylrhodamine (TAMRA) in the 3' end. β -actin was used as reference gene. Primer and probe concentrations were optimized (data not shown). Final probe and primer concentrations in the experiments were 600 nM og 125nM, respectively. As internal reference dye 30 nM 6-Carboxyl-X-Rhodamine (ROX) were used according to the Brilliant QPCR master mix manual.

The obtained data were analyzed by relative quantification according to the method of Pfaffl [29] and presented as relative numbers with respect to the untreated control.

Statistical analyses

All quantification data were statistically analyzed by two-tailed, unpaired Student's *t*-test. $P < 0.05$ were regarded significant and shown with an asterisk in the bar diagrams. Quantified results are presented as relative numbers $\pm$ S.D. "n" indicates the number of independent experiments.

Results

Sodium azide inhibits ERK2, recovery stimulates it

The effect of 10 mM sodium azide, NaN_3 as inducer of chemical anoxia is a well established method for simulation of anoxia of cells in culture [21, 28, 30, 31]. In these studies 10 mM NaN_3 was applied to induce anoxia in different cell types. To verify the usability of this model system for studies of ERK2 in the NIH 3T3 cell line, cells were incubated with increasing concentrations of sodium azide for 30 min and the activity of ERK2 was detected by Western blotting, using a phosphospecific antibody (Fig. 1). A dose-dependent inhibition of ERK2 was observed during the anoxic insult and the phosphorylation was strongly inhibited when incubated with 5 and 10 mM NaN_3 . Thus 10 mM sodium azide was used to induce chemical anoxia throughout this study.

NIH 3T3 cells were challenged with chemical anoxia for 30 min followed by recovery for 5 and 10 min. The ERK2-phosphorylation was analyzed by Western blotting and the band intensities quantified (Fig. 2). Chemical

Fig. 2. Sodium azide inhibits ERK2 reversibly. Western blot analysis of ERK2-phosphorylation in NIH 3T3 cells after chemical anoxia and recovery. Upon recovery significant phosphorylation of ERK2. The anoxic control was harvested immediately after the anoxic insult. Recovery was achieved by washing the cells twice in sodium azide followed by incubation in azide-free L15ex for the indicated periods of time. The non-treated control was incubated in azide-free L15ex throughout the experiment. The anoxic control was quantified relative to the non-treated control and the recovered samples were quantified relative to the anoxic sample. (n=3)

anoxia significantly inhibited the activity of ERK2, measured by its dephosphorylation. Recovery induced a rapid and robust stimulation of ERK2 and the kinase was significantly activated already after 5 min after washing out the NaN_3 .

Transient activation of $p38^{\text{MAPK}}$ during chemical anoxia

NIH 3T3 cells were incubated with NaN_3 and harvested at 5, 10 and 20 min (Fig. 3). As expected, a very low background activity was observed. However,

Fig. 3. p38^{MAPK} is transiently activated during chemical anoxia. NIH 3T3 cells were incubated with 10 mM NaN₃ in L-15ex and harvested at the indicated time points. The phosphorylation status of p38^{MAPK} was analysed by Western blotting. Significant p38^{MAPK} was observed after 5 min, with a peak at 10 min. After 20 min, the level of p38^{MAPK} was declining. Phosphorylated p38^{MAPK} is indicated by pp38^{MAPK} in the figure. (n=3)

upon exposure to chemical anoxia, p38^{MAPK} was significantly activated after 5 and 10 min. At 20 min chemical anoxia the p38^{MAPK}-activity was declining, as measured by the level of protein phosphorylation.

Regulation of ERK2 during recovery

To gain insight into the regulation of ERK2 during the recovery phase, we tested three different inhibitors shown to inhibit ERK-activation during recovery after chemical anoxia in rainbow trout cells [21]. We tested the MEK1/2-inhibitor PD98059 [32], diphenyleneiodonium chloride (DPI), an inhibitor of NADP(H)-oxidase-like enzymes [33], and the protein phosphatases PP1/PP2a inhibitor calyculin A [33]. Concentrations and incubation times are shown in Tab.1. To our surprise, none of the inhibitors affected phosphorylation, meaning activation of ERK2 (Fig 4).

We then as a control experiment tested the same panel of inhibitors for their ability to inhibit ERK2-activation by stimulating serum-starved cells with 20% (v/v) FBS. We found that the MEK1/2-inhibitor PD98059 and calyculin A strongly inhibited the serum response (Fig. 5). This confirm the surprising result that none of these inhibitors inhibits ERK2 activation during the recovery phase

Chemical anoxia followed by recovery induces Hsp70 protein expression

The effect of chemical anoxia on Hsp70 protein expression was tested by incubation of NIH 3T3 cells in increasing concentrations of NaN₃ in L15ex for 30 min followed by recovery overnight in fresh culture medium. Expression of Hsp70 protein during the recovery period was analyzed by Western blotting (Fig. 6). All concentrations of sodium azide tested resulted in significant expression of Hsp70 protein and the level of protein expression was dose-dependent.

Fig. 4. Activation of ERK2 during the recovery phase is independent of MEK1/2. *Panel A:* NIH 3T3 cells were pre-treated incubated with PD98059, DPI and calyculin A. None of the inhibitors affected the phosphorylation of ERK2 during recovery. *Panel B:* Total ERK2 protein. *Panel C:* Quantification and statistical treatment of Western blot data presented in panel A. Level of ERK-phosphorylation under anoxia was undetectable in this series of experiments, and consequently not quantified. (n=3)

Effect of inhibitors on Hsp70 protein expression

To elucidate if Hsp70 protein expression is regulated at the transcriptional or translational level, or both, we treated NIH 3T3 cells with the transcriptional inhibitor actinomycin D and cycloheximide, an inhibitor of translation. Also, we wanted to investigate the involvement of MAP kinase signalling pathways in activation of Hsp70 protein expression by pre-incubation with a selection of chemical inhibitors.

We tested if actinomycin D and cycloheximide could induce Hsp70 by them self, as inhibition of key cellular functions as transcription and translation possibly could activate the heat shock response. However, 1 hour treatment of NIH 3T3 cells 0.5 µg/ml actinomycin D or 1 µg/ml cycloheximide in azide free L-15ex followed by overnight recovery did not result in any expression of Hsp70 (Fig. 7 and Tab. 1). Cells were also pre-incubated with our panel of inhibitors (Fig. 7 and Tab. 1) and subjected to 30 min chemical anoxia followed by overnight recovery. Expression of Hsp70 protein was determined by Western blot analysis. As shown if Fig. 7, actinomycin D and cycloheximide both inhibited Hsp70 expression. 10 mM PD98059, 1 µM diphenylene iodonium and 100 nM calyculin A had all a strong and significant inhibitory effect on the sodium azide-mediated Hsp70 protein expression. Pre-incubation with 10 µM SP203580, an inhibitor of p38^{MAPK} [32], however, did not affect the heat shock response after chemical anoxia.

Analysis of hsp70 mRNA level

In the mouse genome there are two nearly identical *hsp70* genes, *hsp70.1* and *hsp70.3* which differ only in their 3' UTR [7]. We chose to analyze the effect of chemical anoxia on the total *hsp70* mRNA using quantitative real-time PCR. Taqman probes were used and our primer/probe design did not discriminate between *hsp70.1* and *hsp70.3* (see Tab. 2). Cells were exposed to chemical anoxia and harvested after 10 and 30 min or exposed to chemical anoxia for 30 min followed by recovery for up to 120 min (Fig. 8). No induction of *hsp70* mRNA was observed during the periods of anoxia, but after 10 min recovery, *hsp70* mRNA expression had reached a significant level which was sustained during the time-course of the experiment.

Further, it was tested if actinomycin D indeed inhibited transcription of *hsp70* and if the observed effect of the MEK inhibitor PD98059 could be caused by inhibition of the transcription *hsp70* mRNA or rather the translation rate of Hsp70 protein. NIH 3T3 cells were

Fig. 5. Effect of pharmacological inhibitors on serum-induced ERK2 phosphorylation in serum starved cells. NIH 3T3 cells were grown to 80% confluency and serum starved for 24 hrs. *Panel A:* After starvation the cells were pre-incubated with PD98059, DPI and calyculin A according to Tab. 1. After inhibitor treatment the cells were stimulated with 20% FBS for 5 min and phosphor-ERK2 was analysed by Western blotting. PD98059 and calyculin A strongly inhibited phosphorylation of ERK2 in response to serum. DPI, however, did not affect the serum-induced ERK2-activation. *Panel B:* Total ERK2 protein. (n=1)

Fig. 6. Hsp70 expression is induced by NaN_3 in a dose-dependent manner. Western blot analysis of Hsp70 expression in NIH 3T3 cells after 30 min incubation with increasing concentrations of NaN_3 followed by overnight recovery. All concentrations tested significantly induced Hsp70, in a dose-dependent manner. (n=3)

Fig. 7. Differential effect of pharmacological inhibitors on Hsp70 protein expression after chemical anoxia. Cells were pre-incubated with the various inhibitors according to Tab. 1 prior to NaN_3 -induced anoxia for 30 min. Recovery was achieved by washing the cells twice in sodium azide followed by incubation overnight. The non-treated control cells and the control cells pre-incubated with actinomycin D and cycloheximide were incubated in azide-free L15ex throughout the experiment. Actinomycin D and cycloheximide could possibly induce a stress response per se, thus tested and statistically compared to non-treated cells. The anoxic control was quantified relative to the normoxic control (#, $P < 0.05$) and the recovered samples were quantified relative to the anoxic control. Statistically significant samples are indicated by *. (n=3)

Fig. 8. Quantitative real-time PCR of *hsp70* during chemical anoxia and recovery. Subconfluent NIH3T3 cells were exposed to chemical anoxia and recovery and harvested at different time-points during anoxia and recovery. Total RNA was extracted from the samples and the cellular levels of *hsp70* mRNA was detected by quantitative real-time PCR using TaqMan probes (see table 2). (n=3)

Fig. 9. *hsp70* mRNA transcription is inhibited by actinomycin D and stimulated by PD98059. Subconfluent cells were subjected to chemical anoxia and recovery. When pre-treated for 1 hour with the transcription-inhibitor actinomycin D expression of *hsp70* mRNA was blocked. The MEK1/2-inhibitor PD98059 significantly stimulated the expression of *hsp70* mRNA. (n=3).

pre-treated with actinomycin D and PD98059 and exposed to 30 min chemical anoxia and 1 hour recovery. It was observed that actinomycin D was a strong inhibitor

of *hsp70* mRNA transcription and that inhibition of MEK/ERK signalling resulted in a stimulated transcription of *hsp70* mRNA (Fig. 9).

Discussion

In this study we have investigated transcription and translation of Hsp70 and activity of ERK2 in NIH 3T3 fibroblasts during anoxia and recovery in a chemical anoxia-model, using sodium azide as an inducer.

Observation of ERK activity and Hsp70 expression during chemical anoxia and recovery

First, dose-response experiments were performed, where NIH 3T3 cells were incubated in increasing concentrations of sodium azide for 30 min. It was observed that ERK2-phosphorylation was clearly reduced in the presence of 2 mM azide and a strong inhibition was observed when the cells were treated with 5 and 10 mM NaN₃. Hsp70, on the other hand, was significantly induced at all concentrations tested, with the level of protein expression increasing in a dose-dependent fashion. Thus, we chose 10 mM sodium azide for induction of anoxia in the subsequent experiments, as this concentration resulted in the strongest inhibition of ERK2 and the highest expression of Hsp70 protein.

Inhibition of ERK2-signalling during chemical anoxia and regulation of its phosphorylation during the recovery phase

When the cells were challenged with 10 mM azide for 30 min the activity of ERK2 was significantly inhibited relative to the untreated control. This is similar to our earlier observation in studies of fibroblast cells from rainbow trout [21]. Previous studies on ERK-signalling under anoxic conditions are mostly concerned with specialized tissues, such as neuronal cells [34], cardiomyocytes and vasculature [26, 35] due to their medical importance in relation to ischemic diseases. In contrast to what we find in fibroblast studies of both neuronal and cardiovascular tissues demonstrates that ERK-signalling is stimulated by exposure to hypoxic and anoxic conditions [24, 36]. Further, the ERK-activity seems to play a protective role in these essential cell types by its capacity to inhibit apoptosis by phosphorylation of pro-apoptotic proteins such as Bad [37, 38] and caspase-9 [39]. Thus there seems to be a cell- and tissue-dependency in determining the specific mode of ERK-regulation and function. Therefore, we suggest the existence of a mechanism ensuring that ERK is activated during hypoxia in highly specified cells that are essential to the organism, while inhibition of ERK occurs in dispensable cells with higher proliferative potential, such as fibroblasts. As ischemic and anoxic conditions are

extremely harmful in that these conditions rapidly lead to energy deprivation, such a mechanism would allow for redistribution of energy, i.e. glucose, to the most essential organs of the animal, namely the brain and the heart. This hypothesis may be further strengthened by the finding that the ERK2-activity is rapidly increased in the fibroblasts when the azide was washed out of the cells and is thereby able to elicit its protective functions as soon as the anoxic condition is over.

We have previously shown that MEK, PP1/PP2a and reactive oxygen species (ROS) are involved in activation of ERK in the rainbow trout cell line RTHDF when recovering from an anoxic insult [21]. Also, the involvement of ROS [21, 40-45], PP1/PP2a in the ERK-pathway is well established in many model systems [21, 46-49]. However, this does not seem to be the case in the mouse NIH 3T3 fibroblasts. Our data strongly indicate that ERK2 is activated independently of MEK, ROS and PP1/PP2a factors in the recovery phase.

As a positive control the inhibitors PD98059, DPI and calyculin A were tested for their ability to inhibit serum-mediated ERK-phosphorylation in serum starved cells. PD98059 and calyculin A strongly inhibited the serum response, while DPI did not. In the chemical anoxia system neither of the drugs was able to inhibit the phosphorylation of ERK during recovery.

A number of previous studies indicate that ERK can be activated independently of MEK. This include PKC-mediated signal transduction [50] and kinases downstream of PI3K/Akt, such as PDZ-binding kinase, PBK [51]. PBK is a recently cloned and characterized 35 kDa Ser/Thr kinase which is expressed in malignant cells [52] and other highly proliferative cell types, but not in almost non-proliferating tissues, such as the brain [53, 54]. However, a Tyr-kinase is still missing in the puzzle, as PBK could be the Thr-kinase phosphorylating ERK2.

Aksamitiene and colleagues [51] demonstrates a crosstalk mechanism between the PI3K/Akt and Ras/ERK pathways and suggest that PBK may be activated downstream of Akt involved in activation ERK.

The stress activated MAPK p38 is transiently activated during chemical anoxia

The stress-activated MAP kinase p38^{MAPK} was transiently activated during chemical anoxia. We did not investigate this observation further, but we speculate that the p38^{MAPK}-activity is necessary for dephosphorylation of ERK during chemical anoxia, as p38^{MAPK}-dependent dephosphorylation of ERK has been shown by us and others [20, 21, 23, 55].

Regulation of Hsp70 expression as a result of chemical anoxia and recovery

It has been shown in Madin-Darby canine kidney cells that non-translated pools of *hsp70* mRNA may exist and that different stresses may utilize either this pool or induce *de novo* transcription of *hsp70* genes [56]. We thus investigated whether the observed increase in Hsp70 after an anoxic insult and overnight recovery resulted from increased *de novo* transcription or increased translation. Inhibition of transcription and translation was achieved by pre-treating the cells with actinomycin D and cycloheximide, respectively. Neither inhibitor induced Hsp70 protein by itself; however, both inhibitors blocked induction of Hsp70 after an anoxic insult and overnight recovery. Thus we can conclude that both *de novo* transcription and translation is necessary for expression of Hsp70 protein in NIH 3T3 fibroblasts during recovery from chemical anoxia.

Sheikh-Hamad and colleagues [56] demonstrate that p38^{MAPK} is necessary for induction of *hsp70* mRNA in response to osmotic stress. When testing the effect of the p38^{MAPK} inhibitor SB203580, no effect was observed in our system. Thus we conclude that the requirement for p38^{MAPK} activity observed in Madin-Darby canine kidney cells are either dependent on the cell type or the type of stress the cells are exposed to. However, we find the concentrations of SB203580 that are used by Shaykh-Hamad and co-workers to be extremely high (50 and 100 μ M), and may have various unspecific effects. We have routinely used a final concentration of 10 μ M in both NIH 3T3 fibroblast and the rainbow trout cell line RTHDF in previously published studies [21, 57]. This concentration was clearly demonstrated to inhibit p38 activity in the NIH3T3 fibroblasts used in the present study [57].

Interestingly, inhibition of MEK1/2 and subsequently ERK2 by PD98059 resulted in strong inhibition of Hsp70 protein expression, compared to the anoxic control. This was surprising, as ERK is shown to be involved in negative regulation of HSF-1 by the sequential phosphorylation of HSF-1 by ERK and GSK β [58-61]. Also, it has been demonstrated that exposure of porcine aortic cells to benzo[a]pyren results in inhibition of Hsp70 expression secondary to activation of ERK [62]. Thus we expected to observe an increase in the level of Hsp70 protein. Others, however, have shown a stimulatory function for ERK with respect to Hsp70 expression, thus ERK is necessary for Hsp70 expression in 9L rat brain tumor cells when exposed to low concentration of cadmium [63] and in response to hyperthermia in astrocytes [64].

The possibility exists, that inhibition of ERK may result in a general inhibition of translation resulting in the observed inhibition of Hsp70 protein expression. We did not test this experimentally, but it is an issue for further studies of ERK-signalling in this model system. There is however to our knowledge no evidence in the literature that ERK has a general stimulatory effect on translation thus this possibility is regarded as rather unlikely. However ERK plays a critical role in regulation of mRNA stability of the VEGF transcript [65] and it is possible that it might also affect the mRNA stability of the *hsp70* transcript.

Reactive oxygen species and phosphatases are involved in Hsp70 expression

Our observation that PD98059, DPI and calyculin A inhibit Hsp70 expression points to a role for ERK, ROS and PP1/PP2a in regulation of Hsp70 protein expression. A significant role for ROS in the expression of Hsp70 is further strengthened by the observation that ROS production starts early in the recovery phase after chemical anoxia in NIH 3T3 cells (Ossum C.G., Lauritsen A.N., Hoffmann, E.K. unpublished results). ERK has been shown to play a necessary role in *hsp70* mRNA translation in Pac2 cells from the zebrafish, *Danio rerio*, when subjected to heat stress [66].

Analysis of hsp70 mRNA

At the mRNA level our data are in agreement with studies that demonstrate an inhibitory role for ERK and GSK β [58-61], as we observed a significant increase in *hsp70* mRNA transcription when the NIH 3T3 cells were pre-treated with PD98059.

Concluding remarks and perspectives

The exact molecular mechanism for the phenomena observed is unknown and further studies are in the pipeline to address a number of important question. However, based on the data presented in this paper and the literature cited herein, we suggest the existence of negative regulatory mechanism in NIH 3T3 cells controlling ERK2, possibly mediated by p38^{MAPK} and PP1/PP2a; chemical anoxia inhibits ERK2 and upon removal of sodium azide ERK2 is stimulated possibly by a PBK-dependent phosphorylation. Both the hypothesis that p38^{MAPK}-signalling is necessary for the dephosphorylation of ERK2 during the anoxic phase and the possible role of the PI3K/Akt/PBK pathway in the phosphorylation of ERK2 after reoxygenation needs further investigations

and moreover we will look into the possible involvement of tyrosine kinases, such as the Src-family and the multifunctional focal adhesion kinase, FAK. To undertake such studies in the current cell model and other model systems are very important in order to gain further understanding of the complexity of ERK-signalling in relation to anoxia and recovery.

We also demonstrate that ERK-signalling, production of reactive oxygen species and the action of phosphatases are required for induction of Hsp70 expression after an anoxic insult and overnight recovery in our model system. Additional experiments should be undertaken in order to determine whether ROS and the phosphatases

are acting on the level of transcription or translation. On the mRNA level, inhibition of ERK-signalling stimulates *hsp70* mRNA transcription, probably by suspension of ERK and GSK β -mediated inhibition of HSF-1, but this is yet to be proven.

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