Biomarker protein expression in primary cultures of salmon (*Salmo salar* L.) hepatocytes exposed to environmental pollutants

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Primary cultures of salmon (*Salmo salar* L.) hepatocytes were analysed using ^{35}S-methionine/cysteine incorporation and SDS–PAGE gel electrophoresis (1 and 2-D) and Western blotting after treatment with representative environmental pollutants (benzo(*a*)pyrene (BaP), 2,3,3′,4,4′-pentachlorobiphenyl (PCB-105), arsenite (AsO_2^-) and cadmium (Cd)). The results demonstrated striking similarities in changes in protein expression after treatment with the different pollutants. Hsp70 (Hsp72/73) proteins were induced after treatment with all the compounds as shown by ^{35}S-methionine/cysteine labelling. However, high background levels of these proteins were shown with Western blotting and an anti-Hsp70 antibody, indicating a slow turnover of these proteins. The Hsp70s in salmon hepatocytes were extremely susceptible to degradation in urea used in 2-D electrophoresis, resulting in peptide fragments of 45–46 kDa. In addition to these Hsp70 fragments, arsenite induced several proteins of 42, 38, and in the 30–32 kDa range. CYP1A (58 kDa) and an unidentified protein of 16 kDa were furthermore induced after treatment with the organic xenobiotics (BaP, PCB and the model compound β-naphthoflavone, BNF). CYP1A was expressed in a dose-dependent manner, and was resolved into several protein spots in 2-D Western blotting. Elevated levels of metallothionein and haem oxygenase (HO) were indicated in Western blots after treatment with cadmium or arsenite (only HO). The hepatocytes showed cytoplasmic protrusions after treatment with 35 μM arsenite and 100 μM Cd, indicative of cells entering apoptosis.

Keywords: *2-dimensional gel electrophoresis, stress proteins, CYP1A, haem oxygenase, fish cells.*

Abbreviations: *BNF, β-naphthoflavone; DMSO, dimethyl sulphoxide; HO, haem oxygenase; Hsp, Heat shock proteins; MT, metallothionein; PCB, polychlorinated biphenyl; TEM, transmission electron microscopy.*

Introduction

A number of biomarker proteins are induced by specific types of pollutants or act as a general stress indicators in different organisms. Among the first, the cytochrome P4501A (CYP1A) subfamily is induced by polycyclic aromatic hydrocarbons (PAHs), planar polychlorinated biphenyls (PCBs) and chlorinated dioxins in fish and other vertebrates (Stegeman and Hahn 1994, Goksøyr 1995). Metallothionein (MT) binds metals and is similarly induced by heavy metals such as Cu, Zn, Cd and Hg (Hogstrand and Haux 1991). Haem oxygenase (HO) is generally induced by heavy metals and arsenite in, for example rats (Maines and Kappas 1976, Sardana *et al.* 1981). As a general stress indicator, stress proteins or heat shock proteins (Hsps) are involved in protecting eukaryote and prokaryote organisms from damage caused by exposure to different types of environmental stress including elevated temperatures, ultraviolet light, heavy metals and xenobiotics (Sanders 1993, Welch 1993). In mixed exposure situations, interactions between different biomarkers may occur. An interesting example is the induction of HO activity which is accompanied by a decrease in total CYP levels in rat and fish (Maines 1988, Ariyoshi *et al.* 1990).

One strategy of biomarker development involves *in vitro* short term tests and measurements of toxic and genotoxic effects of pollutants, e.g. in cell cultures. Several studies have been performed with primary cultures of isolated fish hepatocytes (reviewed by Baksi and Frazier 1990). Rainbow trout (*Oncorhynchus mykiss*) hepatocytes have, for example, been used in testing effects of dioxin exposure (Pesonen *et al.* 1992), paper mill effluents (Pesonen and Andersson 1992) and oestrogenic potency of chemicals (Pelissero *et al.* 1993).

Hepatocytes are involved in xenobiotic metabolism, and primary cultures from rainbow trout hepatocytes showed retention of cytochrome P450 activity at least 8 days after plating, although it decreased during the first 2 days of culture and then stabilized at approx. 40% (Klaunig *et al.* 1985). A temperature effect was also observed with the highest activity found at 15°C, and the lowest at 25°C (Klaunig *et al.* 1985). In the first 5 days after plating, the ultrastructure of the cells is still intact (Braunbeck and Storch 1992).

The objective of this study was to use primary cultures from Atlantic salmon (*Salmo salar*) hepatocytes to study changes in protein expression after treatment with different environmental pollutants, using radioactive amino acid labelling and 2-D gel electrophoresis. Furthermore, we wanted to investigate whether the observed changes were due to already known biomarkers using available antibodies, or could be candidates for new biomarker proteins.

MATERIALS AND METHODS

Acrylamide and bisacrylamide were purchased from Bio-Rad, Hercules, CA, USA. Ampholytes for isoelectric focusing (Ampholines) and urea were purchased from Pharmacia, Stockholm, Sweden. Collagenase and iodoacetamide were from Sigma, St Louis, MO, USA. Fetal bovine serum, Medium 199 and antibiotica/antimycotica were obtained from Gibco BRL, Life Technologies, Paisley, UK. ^{35}S-methionine/cysteine (PRO-MIX), specific activity 1407 Ci mmol^{-1}, was purchased from Amersham, UK. For autoradiography, Kodak X-Omat AR film (Eastman-Kodak, Rochester, NY, USA) was used. All other chemicals were commercially available analytical grade products.

Fish

Atlantic salmon (*Salmar salar*) were obtained from Sævareid Fiskeanlegg Inc., Hordaland, Norway. The fish were acclimatised in the Industrial Laboratory at the

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Bergen High-technology Centre for more than 1 month before the experiment. The fish were kept at 9°C and were fed commercial dry pellets (FK Ultra, 1.5% per body weight per day) from Felleskjøpet, Stavanger, Norway. Salmon of 250–330 g were used.

Preparation and culture of salmon hepatocytes

Hepatocytes were isolated by a two-step perfusion method described by Berry and Friend (1969) and modified for fish by Andersson *et al.* (1983). The viability of the freshly isolated hepatocytes was always over 90%, determined by trypan blue exclusion. The isolated hepatocytes were seeded on 35 mm Primaria plates (Falcon, Becton Dickinson Labware, Oxnard, CA, USA) (5×10^6 cells per plate) and incubated at 12°C under air in 100% humidity without additional O_2/CO_2 gassing for up to 4 days. The medium used was Medium M199 supplemented with 0.9 g l^{-1} HEPES, 0.35 g l^{-1} $NaHCO_3$, 1.6 g l^{-1} $Na_2HPO_4.12H_2O$, 10 mM NaCl, 0.5% fetal bovine serum, 2 units ml^{-1} penicillin, 2 μg ml^{-1} streptomycin and 1.25 μg ml^{-1} amphotericin.

Treatment of hepatocytes

The organic compounds (benzo(a)pyrene (BaP), 2,3,3',4,4'-pentachlorobiphenyl (PCB-105, Cambridge Isotope Laboratories, UK) and β-naphthoflavone (BNF)) were dissolved in dimethyl sulphoxide (DMSO) and added to the culture after 48 h of preculture. The final concentration of DMSO in the medium never exceeded 0.1%. The concentration ranges of BaP and PCB-105 were 0.5–50 μM, and of BNF 0.05–5 μM. Sodium arsenite and $CdCl_2$ were dissolved in 380 mOsm NaCl. The concentration range of arsenite was 0.07–35 μM and of cadmium 1–100 μM. Control cells received 0.1% DMSO or saline, respectively. After 24 h of exposure to different doses of these compounds, 40 μCi ^{35}S-methionine/cysteine were added per 5×10^6 cells and the labelling continued for 24 h. The cells were then harvested and lysed in a buffer containing 9M urea, 2% Triton X-100, 0.7% mercaptoethanol and 1.4 mg ml^{-1} phenyl methylsulphonyl fluoride or sonicated for 10 s in 0.1 M Na-phosphate, 0.15 M KCl, 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol. Before application on gel, the lysate was centrifuged for 30 min at 13 000 *g* to sediment insoluble material.

Transmission electron microscopic (TEM) studies

The cells were fixed with four volumes of ice-cold glutaraldehyde (2.5%; v/v) in phosphate-buffered saline (PBS) and 0.2 M cacodylate buffer (pH 7.4). Fixed cells were washed in 0.1 M cacodylate buffer (pH 7.4) and post-fixed in 2% OsO_4 (containing 0.1 M cacodylate buffer). Pellets were washed, dehydrated in graded alcohol solutions and embedded in Agar 100 resin. Semi-thin sections were stained in Toluidine Blue. Ultra-thin sections were stained in uranyl acetate and lead citrate, and viewed in a Jeol S100 electron microscope.

Protein determination

Protein concentrations in cell homogenates were determined according to Bradford (1976).

Gel electrophoresis

SDS–PAGE was performed using a discontinous buffer system with 7.5% acrylamide stacking gel and varying concentrations of acrylamide in the resolving gel (Laemmli 1970). Two-dimensional electrophoresis was performed using immobilized pH gradient (IPG) gels with linear gradient pH 4 to 7 and SDS–PAGE 8–18% from Pharmacia LKB; Uppsala, Sweden. The 2-D separation was performed with the Pharmacia Multiphor electrophoresis system (Görg *et al.* 1988). The gels were either first visualized with silver staining, then treated with DMSO/PPO for enhanced fluorography (Bonner and Laskey 1974), dried and visualized on autoradiography films, or transferred to nitrocellulose for Western blotting (see below). The isoelectric point (p*I*) of proteins was determined using the carbamylate calibration kit purchased from Pharmacia LKB.

Western blots

Western blotting was performed using SDS–PAGE and electrophoretic transfer onto nitrocellulose (0.45 mm, BioRad) (Towbin *et al.* 1979). Probing was performed with polyclonal anti-cod CYP1A IgG (Pab-Dy) (Husøy *et al.* 1986), monoclonal anti-human Hsp70 IgG (SPA-820, StressGen Biotechn., Victoria, BC, Canada), referred to as N27 and cross-reacting to both the constitutive and inducible form of Hsp70 (Minota *et al.* 1988), polyclonal anti-rat oxygenase HO-1 IgG (OSA-100, StressGen Biotechn.), or polyclonal anti-perch metallothionein IgG (kindly provided by Dr Christer Hogstrand, Univ. of Kentucky, USA). For chemiluminescence detection of Western blots we used the ECL kit from Amersham.

The results presented reflect experiments that have been repeated three or four times. Western blots were scanned and band areas analysed by Scan Analysis ver. 2.21 (Biosoft, Cambridge, UK).

Results

Morphological observations

Light and electron microscopic observations of the hepatocytes showed that they tend to aggregate with time. One day after plating they had formed band structures which developed to islets of cells after 2–3 days (Figure 1(A and B)). We observed clear morphological changes of the cells after treatment with high doses of arsenite (35 μM) and Cd (100 μM). After 24 h with these treatments, the cells showed blebs similar to cytoplasmic protrusions, which may indicate apoptosis (Kerr *et al.* 1972, Wyllie *et al.* 1980), as shown in figure 1(C and D).

Protein pattern after ^{35}S-labelling

One-dimensional SDS–PAGE of radioactively labelled proteins demonstrated some of the major cellular responses to pollutant exposure in salmon hepatocytes lysed in 9 M urea. It appears that 7 μM arsenite and 100 μM Cd both induce 30–32 and 45–46 kDa proteins and repress the synthesis of other proteins (Figure 2, lanes 11 and 14). The 45–46 kDa protein band also appeared elevated after treatment with BaP (lanes 3–5), PCB (lanes 6–8) and BNF (lanes 16–18).

Separating the proteins by 2-D electrophoresis better resolved the patterns as shown in Figures 3 and 4. Treatment of the hepatocytes with 0.5 μM BaP, 0.5 μM PCB-105 and 5 μM BNF gave induction of proteins of 45–46 kDa and 16 kDa (Figure 3). Five 45–46 kDa protein spots (labelled A–E) were detected, where A has a p*I* of 6.4 and E a p*I* of 6.6. The 16 kDa protein (F) has a p*I* of 6.4.

Treatment of the cells with 50 μM BaP or 50 μM PCB-105 demonstrated stronger induction of the 45–46 kDa proteins (Figure 4), with somewhat stronger responses to BaP than to PCB-105. The 7 μM dose of arsenite repressed the general protein synthesis, while several proteins were induced (Figure 4(D)). The sizes of the induced proteins were 30–32 (several spots), 38 (H), 42 (G), and 45–46 (A–E) kDa. The proteins of 45–46 kDa appeared to be of the same identity as the proteins induced after treatment with the organic pollutants, although the response was stronger after arsenite exposure.

Immunoblotting

In order to study whether the proteins induced after ^{35}S-labelling are known biomarker proteins or of unknown

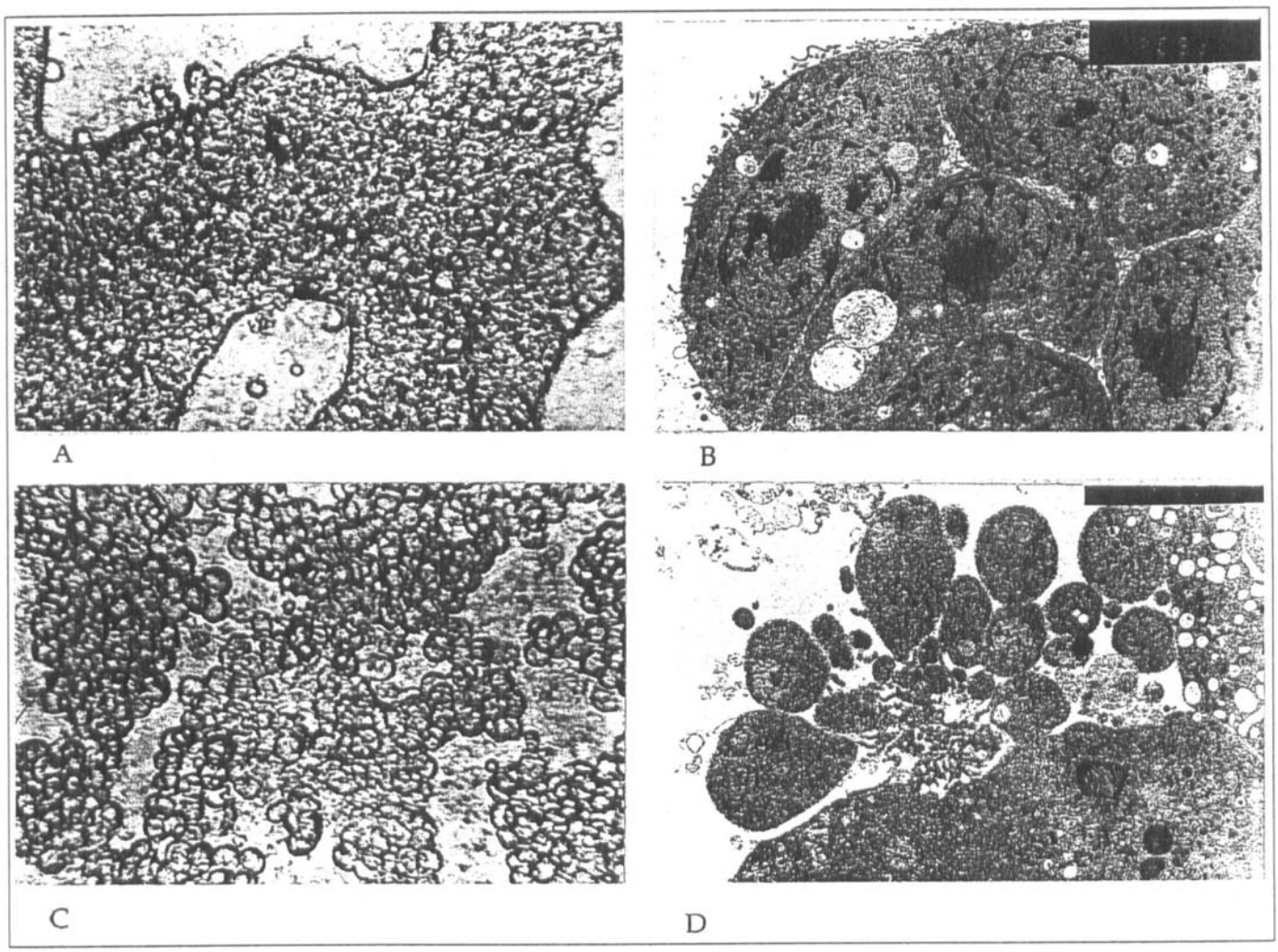

Figure 1. Light and transmission electron microscopic (TEM) observations of the hepatocytes 4 days after plating. Control cells showing aggregate formation, (A) light microscopy, 320×, (B) TEM, 3300×. Cells treated with 35 μM arsenite for 48 h showing cytoplasmic protrusions, (C) light microscopy, 320×, (D) TEM, 6600×.

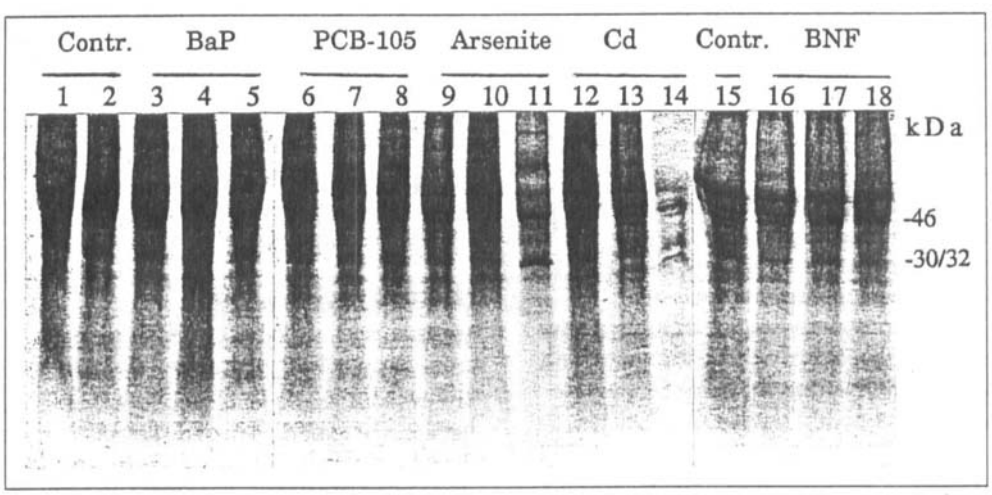

Figure 2. Autoradiography of ^{35}S-methionine/cysteine labelled proteins in salmon hepatocytes lysed in 9 M urea buffer, separated on a 15% SDS–PAGE gel (15 μg protein per lane). Cells were treated with xenobiotic for 48 h and radioactively labelled the last 24 h. Treatment: (1) and (2) control (0.1% DMSO), (3) 0.5 μM BaP, (4) 5 μM BaP, (5) 50 μM BaP, (6) 0.5 μM PCB-105, (7) 5 μM PCB-105, (8) 50 μM PCB-105, (9) 0.7 μM arsenite, (10) 0.07 μM arsenite, (11) 7 μM arsenite, (12) 1 μM Cd, (13) 10 μM Cd, (14) 100 μM Cd, (15) control (0.1% DMSO), (16) 0.05 μM BNF, (17) 0.5 μM BNF, (18) 5 μM BNF.

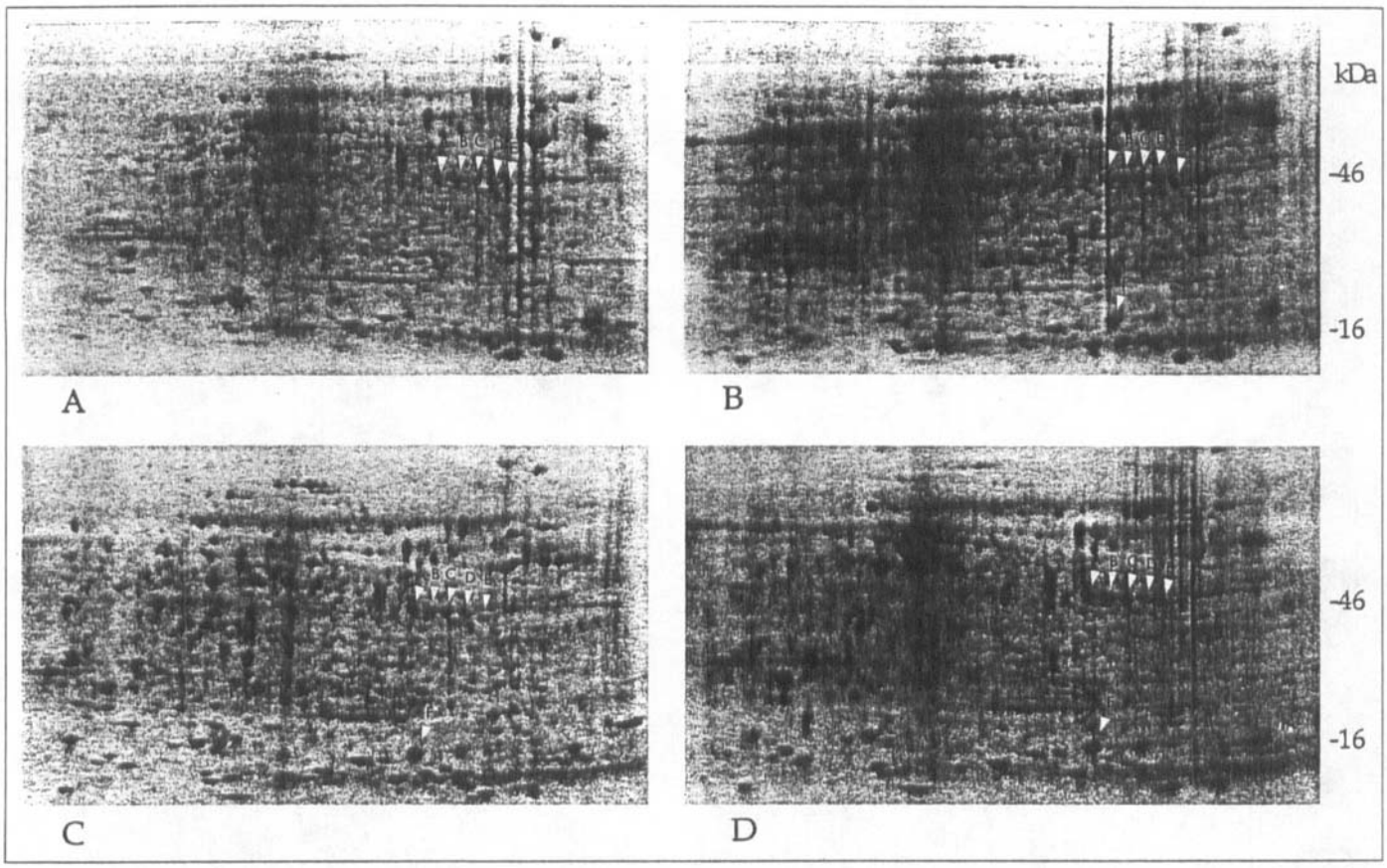

Figure 3. Autoradiography of ^{35}S-methionine/cysteine labelled proteins in salmon hepatocytes separated by 2-D gel electrophoresis (acidic end to the left). Hepatocytes were treated for 48 h with (A) 0.1% DMSO (control), (B) 0.5 μM BaP, (C) 0.5 μM PCB-105, (D) 5 μM BNF. Prominent protein spots induced by the different treatments are indicated with white arrows.

identity, we performed immunoblotting using available antibodies to several biomarker proteins.

CYP1A

Immunoblotting of the samples after 1-D SDS–PAGE with anti-cod CYP1A IgG showed induction of a protein band of 58 kDa after exposure to the organic pollutants BaP, PCB-105 and BNF (Figure 5). The protein was of the expected CYP1A size in Atlantic salmon (Goksøyr and Larsen 1991). The CYP1A antibodies gave almost no cross-reactivity in control hepatocytes, while dose-dependent induction was observed after exposure to the organic pollutants, with the strongest response (5–7-fold induction) occurring after BNF treatment (Figure 5). Immunoblotting of the samples after 2-D gel electrophoresis showed again that the 58 kDa protein was induced but that it was resolved into several spots of p*I* 6.1–6.3 (see Figure 6). These proteins were not prominent after ^{35}S-labelling. The 16 kDa protein observed after treatment with the organic pollutants and ^{35}S-labelling did not cross-react to anti-cod CYP1A.

Hsp70

Western blotting of 1-D gels with anti-Hsp70 (Hsp72/73) IgG showed a strong cross-reacting band of 72 kDa, in addition to staining of 52, 42 and 40 kDa bands when lysed in the non-urea buffer (Figure 7, lanes 1–5). Lysis in the 9 M urea buffer (as used in 2-D electrophoresis) resulted in strong staining at 45–46 kDa (Figure 7, lanes 6–9), demonstrating that these polypeptides are in fact degraded Hsp70 proteins. Accordingly, Western blotting of the 2-D gels with Hsp70 antibodies showed that the proteins of 45–46 kDa induced after organic and heavy metal exposure (A–E), cross-reacted strongly with anti-human Hsp70 (Figure 6). However, the differences in the levels of Hsp70 cross-reacting proteins of 45–46 kDa were low between control and treated cells owing to high background levels observed in the untreated samples, although some of the more basic spots (D and E) may appear to be induced in the exposed cells. Densitometric analysis of 1-D blots showed the strongest induction (1.8-fold) to occur in 7 μM arsenite-treated cells.

Haem oxygenase (HO)

Western blots of the heavy metal-treated samples gave a major cross-reacting band of approx. 30 kDa to anti-rat HO-1, although this antibody gave a high background staining. The level of this protein band appeared elevated (20–40% by densitometric analysis) after arsenite treatment and treatment with 10 μM Cd (Figure 8, lanes 2–4 and 6). Cadmium (100 μM) apparently gave repression of protein synthesis as shown in Figure 2, lane 14. Two-dimensional blotting with this antibody demonstrated staining of the 30 kDa protein spot I (p*I* = 6.4) in Figure 3(D) after 7 μM arsenite treatment (not shown).

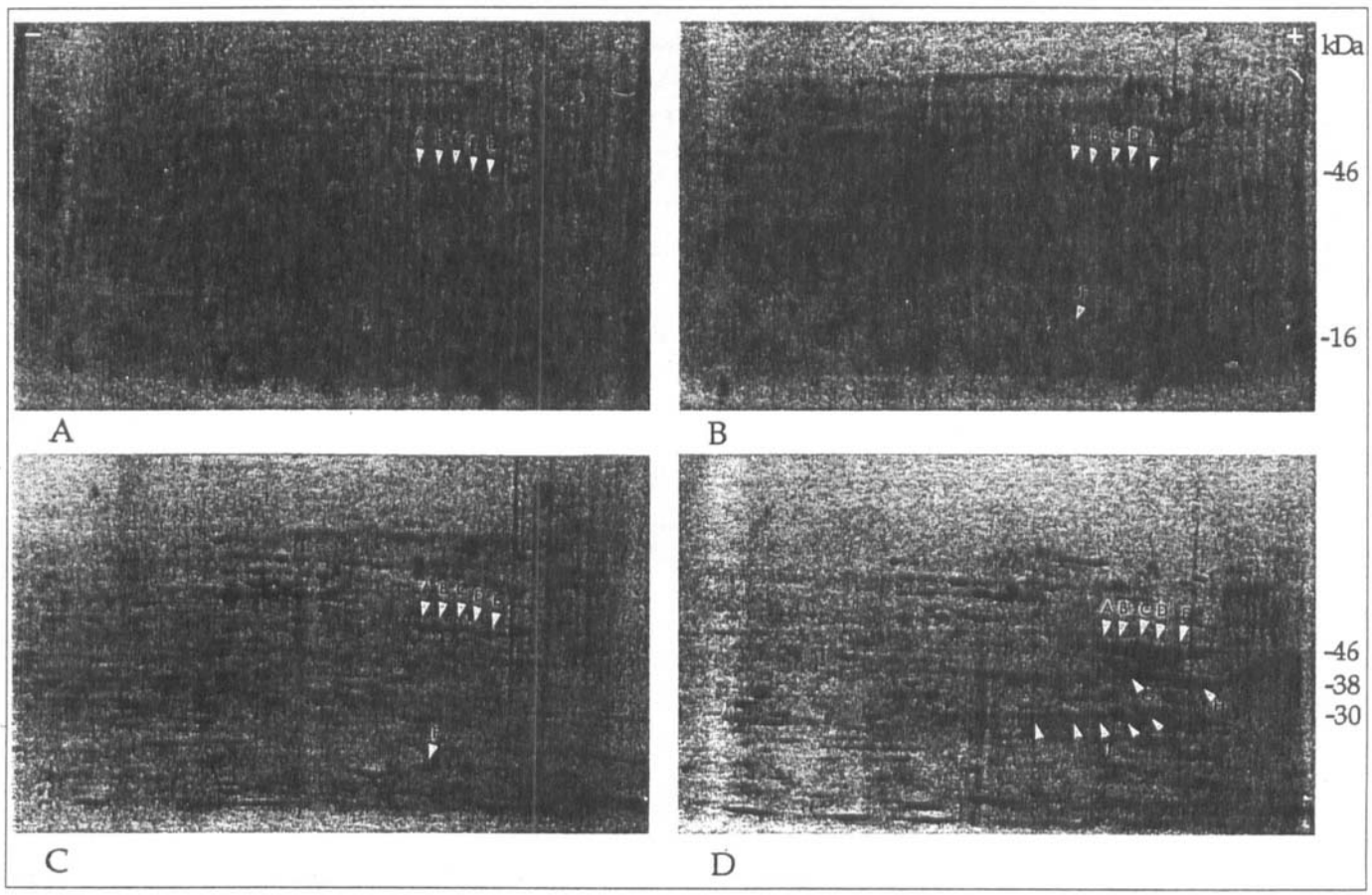

Figure 4. Autoradiography of ^{35}S-methionine/cysteine labelled proteins in salmon hepatocytes separated by 2-D gel electrophoresis (acidic end to the left). Hepatocytes were treated for 48 h with (A) 0.1% DMSO (control), (B) 50 µM BaP, (C) 50 µM PCB-105 (D) 7 µM arsenite. Prominent protein spots induced by the different treatments are indicated with white arrows.

Metallothionein

Using polyclonal anti-perch MT IgG an increased level of a cross-reacting protein band with apparent M_r of approx. 15 kDa after cadmium treatment (Figure 9, lanes 7–9) was visually observed, but no change appeared after treatment with arsenite (lanes 2–4).

Discussion

In this study we have used primary cultures of salmon hepatocytes to test short term sublethal effects of environmental pollutants. The endpoints have been changes in protein expression measured by autoradiography of 1-D or 2-D gels after ^{35}S-labelling and by Western blotting of such gels using antibodies towards known biomarker proteins. Our objectives have been to try to identify changes in protein expression that could be diagnostic of the different exposures, for later evaluations in *in vivo* situations.

After 24-h exposure to high doses of arsenite and cadmium, the hepatocytes showed cytoplasmic protrusions which is a characteristic morphological feature of cells entering apoptosis (Kerr *et al.* 1972, Wyllie *et al.* 1980). Cadmium-induced apoptosis has been reported previously in rat (Tanimoto *et al.* 1993), dog (Hamada *et al.* 1994) and carp (*Cyprinus carpio*) (Iger *et al.* 1994). Cadmium at low and high concentrations killed a human T-cell line by apoptosis and necrosis, respectively (El Azzouzi *et al.* 1994). Apoptosis induced by arsenite treatment alone is, as far as we know, a novel observation, although Buchman *et al.* (1993) reported that porcine endothelial cells sequentially exposed to bacterial endotoxin and arsenite died by apoptosis, not necrosis.

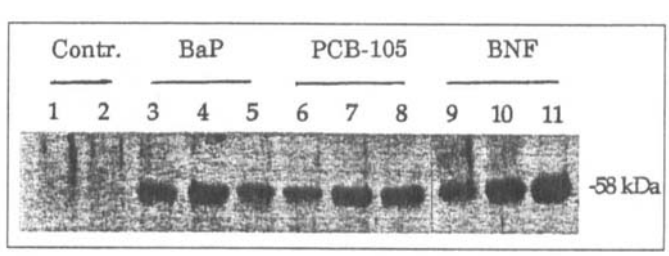

Figure 5. Western blot of salmon hepatocytes exposed to different organic xenobiotics and probed with polyclonal anti-cod CYP1A IgG. 9% SDS–PAGE with 15 µg protein added per lane. Hepatocytes were treated for 48 h with: (1) and (2) 0.1% DMSO (control), (3) 0.5 µM BaP, (4) 5 µM BaP, (5) 50 µM BaP, (6) 0.5 µM PCB-105, (7) 5 µM PCB-105, (8) 50 µM PCB-105, (9) 0.05 µM BNF, (10) 0.5 µM BNF, (11) 5 µM BNF.

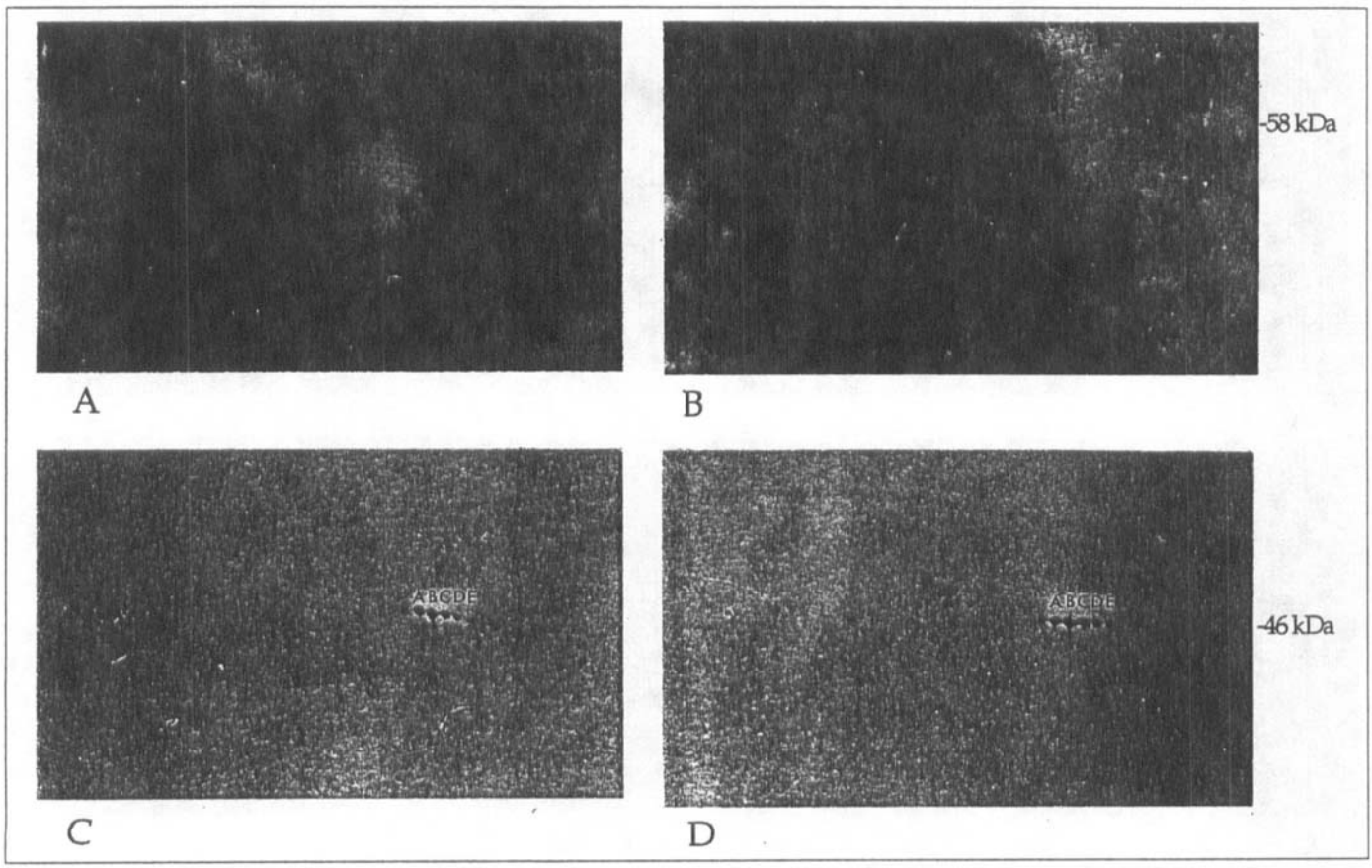

Figure 6. Western blots of 2-D gels of salmon hepatocytes. (A) control (0.1% DMSO) and (B) cells treated with 5 μM BaP, probed with polyclonal anti-cod CYP1A IgG. (C) control (saline) and (D) cells treated with 0.7 μM arsenite, probed with anti-human Hsp70. Spots with the same migration pattern as in autoradiography are identified with A–E as in Figures 3 and 4.

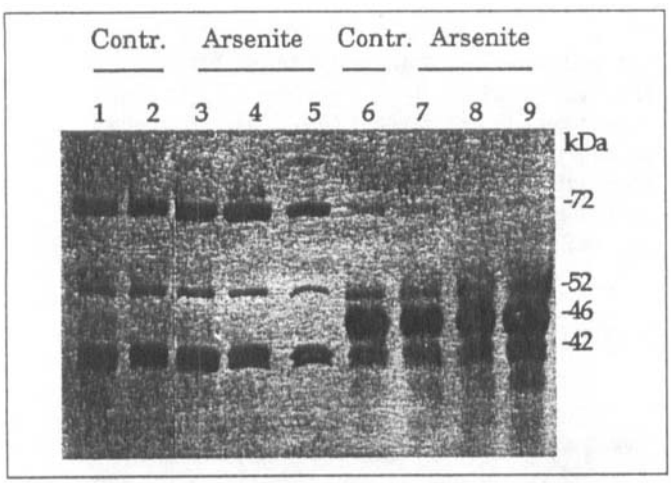

Figure 7. Susceptibility of Hsp70 in salmon hepatocytes to breakdown in lysis buffer. The Western blot was probed with monoclonal anti-human Hsp70 IgG. SDS–PAGE (15%) with 15 μg protein added per lane. Hepatocytes were treated for 48 h with (1) and (2) control, (3) 0.7 μM arsenite, (4) 7 μM arsenite, (5) 35 μM arsenite, (6) control, (7) 0.07 μM arsenite, (8) 0.7 μM arsenite, (9) 7 μM arsenite. Samples in lanes 1–5 were sonicated for 10 s in 0.1 M Na-phosphate, 0.15 M KCl, 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol, while samples in lanes 6–9 were lysed in 9M urea, 2% Triton X-100, 0.7% mercaptoethanol and 1.4 mg ml^{-1} phenyl methylsulphonyl fluoride.

The major and surprising common feature of the different treatments observed by ^{35}S-labelling and 2-D electrophoresis, was induction of several proteins of 45–46 kDa. This was observed both after treatment with the organic pollutants and with the metals. Arsenite was the strongest inducer of these proteins and BaP a stronger inducer than PCB-105. Western blotting with anti-Hsp70 gave strong cross-reactivity with the 45–46 kDa proteins. In order to test whether these proteins were degradation products of Hsp70, we performed 1-D SDS–PAGE and Western blotting of hepatocytes sonicated in

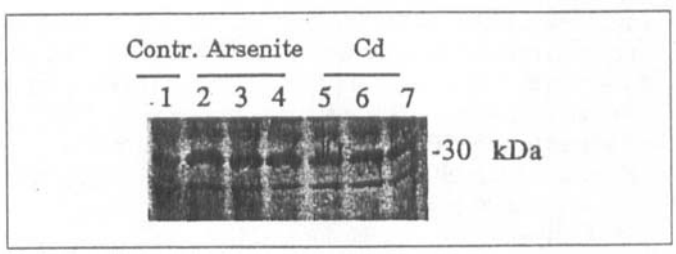

Figure 8. Western blot of salmon hepatocytes probed with polyclonal anti-rat haem oxygenase-1 IgG SDS–PAGE (15%) with 15 μg protein added per lane. Hepatocytes were treated for 48 h with: (1) control, (2) 0.07 μM arsenite, (3) 0.7 μM arsenite, (4) 7 μM arsenite, (5) 1 μM Cd, (6) 10 μM Cd, (7) 100 μM Cd.

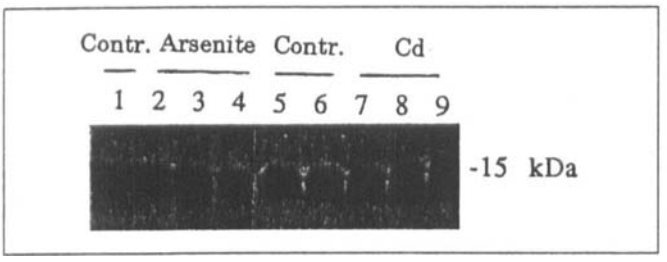

Figure 9. Western blot of salmon hepatocytes probed with polyclonal anti-perch metallothionein IgG. SDS–PAGE (18%) with 15 µg protein added per lane. Hepatocytes were treated for 48 h with: (1) control, (2) 0.7 µM arsenite, (3) 7 µM arsenite, (4) 35 µM arsenite, (5 and 6) control, (7) 1 µM Cd, (8) 10 µM Cd, (9) 100 µMCd.

urea-free buffer or lysed in 9 M urea buffer as used in 2-D electrophoresis. The results show that the 72 kDa band from urea-free conditions was degraded almost totally into a 45–46 kDa band when lysed in urea buffer (Figure 7). In 2-D electrophoresis, we obtained the same pattern regardless of lysis buffer, because the isoelectric focusing requires high urea concentrations. Hsp70 is reported to be highly unstable and capable of self degradation also in studies of other species like the fruit fly *Drosophila* and carp *Cyprinus carpio* (Mitchell *et al.* 1985, Ku and Chen 1991). One of the main degradation products observed was a 43 kDa peptide. These authors showed that Hsp70 can be degraded under gel running after boiling in SDS. The amino acid sequence of the 70-kDa Hsp from *Drosophila* is published (Ingolia *et al.* 1980), and it contains in the carboxyl end the appropriate arrangements of serine, cysteine, histidine and aspartic acid residues to become a serine type protease if it were folded properly (Mitchell *et al.* 1985). Many polypeptide chains may have the potential for refolding during or after denaturation, to yield a product with protease activity (Mitchell *et al.* 1985). This may explain the observed breakdown of Hsp70 from salmon hepatocytes.

Stress proteins are generally induced by physical and chemical stress that creates denaturation or incorrect folding of proteins in a way that activates heat shock factor(s) (HSFs), resulting in expression of the stress genes (Voellmy 1994). HSFs are monomers that oligomerize to trimers upon activation, undergo phosphorylation, are transported to the nucleus, bind to the heat shock elements (HSE) and activate gene transcription (Sorger and Nelson 1989). But stress proteins are also induced in response to exposure to compounds not normally associated with protein denaturation, such as various xenobiotics, therapeutic drugs, prostaglandins, haemin and oestrogens (Hiwasa and Sakiyama 1986, Theodorakis *et al.* 1989, Amici *et al.* 1992, Sanders 1993, Yang *et al.* 1995). Why such compounds should result in induction of the cellular stress response is more difficult to understand, unless the observed response is a secondary effect, linked to other signal transduction pathways, as suggested by Voellmy (1994).

We observed an induction of stress proteins, most notably Hsp70, in response to BaP and PCB exposure, aromatic hydrocarbons that normally act through the Ah-receptor. This stress response could be a secondary response to products of Ah-receptor mediated gene activation. Alternatively, the stress genes induced could contain xenobiotic responsive elements (XREs) in their regulatory region. An argument supporting this hypothesis is that the regulatory region of human Hsp70 contains several elements that may interact with other transcription factors (Wu *et al.* 1987, Williams *et al.* 1989). Also, Villaboa *et al.* (1995) observed that agents capable of increasing cAMP, attenuated Hsp70 induction of cadmium in human myeloid cells. The consensus sequence for XRE is 5′-T-GCGTCG-3′, where the four base sequence 5′-CGTG-3′ is required for binding of the nuclear heterodimer (Whitlock 1993). Very few genomic regulatory Hsp sequences are available, and the consensus XRE sequence is not present in the regulatory sequence of rainbow trout constitutive Hsp70 (Zafarullah *et al.* 1992). Interestingly, however, the regulatory sequence of *Chlamydomonas reinhardtii* Hsp70 (Müller *et al.* 1992), contains two full XREs and three imperfect XREs (5′-GCGTG-3′).

Although induction of Hsp70 was observed after ^{35}S-labelling in our salmon hepatocytes, Western blotting with Hsp70 did not reveal this change in BaP and PCB-treated cells. This was because of high background staining in the control samples in both 1-D and 2-D gels. However, 7 µM arsenite treatment resulted in a two-fold induction of Hsp70 by immunostaining. Similar analysis of fresh samples from livers of Atlantic salmon and flounder, *Platichthys flesus*, also gave high background levels in control fish (not shown). These results indicate that the turnover of Hsp70 in the cells is slow and that the Hsp70 response may not be a useful biomarker in immunodetection methods. However, in 2-D immunoblotting, it was observed that the more basic proteins (D and E) showed stronger responses than the A–C spots. The antibody used recognizes both inducible and constitutive forms of Hsp70 (Minota *et al.* 1988), indicating that the inducible form is represented by the D and E spots. An antibody for the inducible form of Hsp70 (SPA-810, Stressgen) has been tested, but did not cross-react properly in our experiments (not shown). However, both Hsp72 and Hsp73 is shown to be induced after different types of metabolic stress (Beckmann *et al.* 1992), suggesting that both the so-called constitutive and inducible forms may be influenced by different types of stress.

Immunoblotting of the samples with anti-CYP1A demonstrated strong induction after treatment with all the organic compounds compared with the control, confirming the designation of CYP1A as a useful biomarker of these compounds in fish (Goksøyr 1995). Western blotting of the 2-D gel showed resolution of the 58 kDa protein to several spots with p*I* of 6.2–6.4. This is a new observation that could be explained by charge heterogeneities in the CYP1A protein because of *in vivo* post-translational modifications, such as phosphorylation, acetylation, or addition of charged carbohydrate groups (O'Farrell 1975). Mapping changes in protein expression in xenobiotic-treated mice using 2-D gel electrophoresis, Anderson *et al.* (1987) reported failure to detect CYP1A induction, but explained this by postulating that the CYP proteins were too basic to be detected in their

system. We have shown that although the CYP1A protein in salmon is strongly induced by xenobiotic treatment and clearly detectable within the p*I* range of our 2-D gels (4–7), the CYP1A protein constitutes only a minor and undetectable proportion of the cellular proteins stained by ^{35}S-methionine labelling.

Induction of proteins in the molecular range of 30–34 kDa after metal exposure is observed both in mammals (Caltabiano *et al.* 1986) and fish (Kothary and Candido 1981). Less is known about the identity of these proteins. We observed that the 30–32 kDa band after 1-D electrophoresis is resolved to several proteins when run on 2-D gel. One of these (I, Figure 4(D)) cross-reacts with anti-rat HO-1. HO is shown to be induced by heavy metals, UVA radiation and hydrogen peroxide in human fibroblasts (Keyse and Tyrrell 1989). The other proteins induced by arsenite in this range, as well as other unidentified proteins such as the G and H spots at 42 and 38 kDa, respectively (although these cross-react weakly with the Hsp70 antibody), and the F spot induced by organic xenobiotics, all appear to be strongly induced by pollutant exposure. These may very well be just as interesting to employ as biomarkers as the already known responsive proteins. One way to study this, is to isolate, characterize and sequence the proteins in question, and to prepare the appropriate antibodies for use in later evaluation of their potency. Fish-specific antibodies for such proteins would be promising candidates for new biomarkers of pollution exposure and effect in marine organisms.

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