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MOLECULAR AND CELLULAR MARKERS OF POLLUTANT EXPOSURE AND LIVER DAMAGE IN FISH

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ABSTRACT

The identification of early onset changes, which may ultimately lead to pathological conditions induced by the injurious effects of chemical contaminants, is a key requirement in assessment of the effects of environmental pollution. It is only by the mechanistic linking of changes in molecular and subcellular processes with the later pathological alterations that it will be possible to establish causal relationships. One approach to this problem is the use of the tools provided by molecular cell biology. This involves the use of molecular probes in live cells isolated from potential target organs, such as liver, coupled with techniques for recognition of specific macromolecules in cells or tissue sections. The use of these methods facilitates the identification of early stages of cell injury, as well as alterations which are restricted to small numbers of cells such as occur in the early stages of neoplastic disease.

The cell surface membrane is often the initial interface with the environment and it is here that changes at the molecular level can be readily identified, such as increases or decreases in membrane-bound receptors. On moving into the cell, the internal membranous systems such as the endocytic-lysosomal apparatus and the endoplasmic reticulum appear to play major roles in processes leading to cell injury and at this level it is possible to relate such changes to the histopathology. Examples of linked alterations from the molecular to the tissue level are described for the livers of flatfish (dab) following experimental exposure to contaminated sediment and compared with data for exposure in the field. In conclusion, the use of molecular and cellular changes as biomarkers of both contaminant exposure and cell damage in impact assessment may have considerable potential, particularly where the mechanistic basis of the alterations is well characterized.

INTRODUCTION

Flatfish are widely used as indicators for the detection of environmental damage by toxic chemical pollutants. One approach is to identify adverse molecular and cellular reactions (biomarkers) to pollutant-induced liver cell injury in fish.[1-7] Such an approach is described in this paper and is further focussed on the the investigation of the linking causal mechanisms involved in molecular damage, cell injury and liver pathology. The aim of this research is to derive a linked sequence of "diagnostic tools", which can be used as "early warning signals" of both exposure to and environmental damage by toxic chemicals, in order to predict potential pathological consequences.

Fish are an important part of the human food chain and both juvenile and adult forms are frequently found in estuaries and coastal zones. However, it is these very areas which are most likely to attract human activity in the form of urbanization and industrialization resulting in the contamination of sediments in estuaries and coastal zones. It is conceivable that this in turn will result in the loss of the living resource through chronic toxicity and impairment of reproductive capability or early deaths of both larval and adult fish. Human health may also be at risk through consumption of contaminated fish products. It is important, therefore, to be able to clearly identify whether fish populations have been affected by toxic chemicals in terms of both environmental resource and human health.

The liver was chosen as it is an integrator of many functions including detoxication/activation of toxic chemicals, digestion and storage, excretion and synthesis of the egg yolk protein vitellogenin. There is also a considerable body of literature on pollutant chemical impact on the cellular pathology of fish liver.[7]

The approach was based on the detection of molecular and cellular changes resulting from chemical contamination. The identification of early onset changes, which may ultimately lead to full-blown disease, is a key requirement in assessment of the effects of environmental pollution. Overtly diseased fish are likely to be rapidly eliminated from the population, hence assessment based on such fish may prove difficult and even if samples are available higher level complications resulting from the primary lesion will be a confounding factor. In fact, it is only through the mechanistic linking of changes in molecular and cellular processes with the later pathological endpoints that it will be possible to establish causal relationships.

Cells are the functional building blocks of life. As such, they consist of an intricate network of molecular machinery which must function with a high degree of co-ordination to maintain the processes of life. Disturbance to part or parts of this molecular machinery will result in functional failure within parts of the cell which may then lead to a cascade of effects culminating in pathological change, such as cell death or tumour formation.

Cell biology provides many tools with which to test for disturbances in cells and the processes leading to liver disease. The approach adopted here uses fluorescent molecular probes that can readily be inserted into live cells.[8] These probes are used to identify and study specific structural components such as receptors or organelles ,as well as to follow dynamic structural and enzymic processes.[3,5] This approach coupled with antibody recognition of specific cellular molecules (immunocytochemistry) has been applied to laboratory investigation of the effects of chemically contaminated sediments on the livers of flatfish (dab, *Limanda limanda*).

MATERIALS AND METHODS

The experimental investigation involved the exposure of flatfish (body length 14-20.5 cm, males and females) to either a contaminated sediment (210 ppm total hydrocarbons) or a relatively clean reference sediment (75 ppm total hydrocarbons) for a period of 144 days at field ambient temperatures. The sediment depth was approximately 15 cm and the particle size characteristics of both sediments were very similar. At the end of this period the fish were killed and their livers removed. Part of the liver was used to prepare tissue sections for cellular pathology and antibody-recognition tests for

clathrin (a protein involved in the process of receptor-mediated endocytosis, which imports specific extracellular substances into the cell), low density lipoprotein (LDL, a cholesterol rich lipid-protein complex used by the cell for synthesis of new membranes), cell surface receptor for epidermal growth factor (EGF, a chemical signal that triggers cell growth and division), and *ras*-oncoprotein (the product of a "cancer gene").[4,9,10] The remainder of the liver was used to prepare isolated hepatocytes (the main type of liver cell) using a mechanical and enzymic process of disaggregation. These individual living cells were then subjected to analysis using fluorescent molecular probes for a range of structural and functional features. These included intracellular organelles, such as endoplasmic reticulum (ER), Golgi apparatus and lysosomes, cytochrome P-450 associated enzymic reactions involved in detoxication of contaminant chemicals (7-ethoxyresorufin-o-deethylase EROD and 3-cyano,7-ethoxycoumarin-o-deethylase CNECOD), production of chemically reactive forms of oxygen (oxyradicals), glutathione (GSH), which protects against reactive xenobiotic derivatives and radicals, and, finally, processes of bulk molecular uptake involving invagination of the cell surface membrane (endocytosis).[5,9-16]

Livers were excised and the hepatocytes were isolated by mechanical dissaggregation in a mixture of collagenase and lipase as described by Lowe *et al.*(1992). Isolated hepatocytes (20μl of cell suspension) were allowed to attach to cleaned glass slides for 15 min at 15°C prior to exposure to the probe solutions. All cells used in this study had a viability of >98% as tested using eosin Y exclusion. Fluorescent molecular probes used in this study included 3, 3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) and rhodamine B hexyl ester (R6) for ER, dihydrorhodamine 123 (DiHR123) for superoxide radicals, 7-ethoxyresorufin for EROD, monochlorobimane for glutathione (GSH),

NBD-colcemid for microtubules, acridine orange for lysosomal integrity, Texas Red conjugated albumin for endocytosis, DiI-low density lipoprotein (LDL-DiI) and BODIPY-concanavalin A (con A-BODIPY) for cell surface receptor binding. With the exception of the albumin, LDL-DiI and con A-BODIPY all probes were prepared as stock solutions in DMSO and used at a dilution of 10^{-4} in culture medium (amended Hanks balanced salt solution). [5] The final concentrations and incubation times were as follows :-

$DiOC_6(3)$, 10 min, 250ng.ml^{-1}; R6, 10 min, 250ng.ml^{-1}; 7-ethoxyresorufin, 10 min, 2.4μg.ml^{-1}; dihydrorhodamine 123, 15 min, 43μM; monochlorobimane, 10 min, 2300 ng.ml^{-1}; NBD-colcemid, 10 min, 270ng.ml^{-1}; acridine orange, 10 min, 1000 ng.ml^{-1} [3-8]. The reaction for oxyradicals was stopped after 10 min by the addition of N-t-butyl-α-phenylnitrone (PBN) to give a final concentration of 100mM. [5] Texas Red-albumin was dissolved directly in culture medium to give a final concentration of 40μg.ml^{-1} and cells were incubated in this medium for 60 min at 20°C. LDL-DiI and Con A-BODIPY were added directly to the culture medium and incubated for 10 min at final concentrations of 25μg.ml^{-1} and 10μg.ml^{-1} respectively.

All incubations were performed in the dark in a humidity chamber at 15°C. Cell preparations were coverslipped, sealed with a liquid paraffin-lanolin mixture and epifluorescence images captured on high-resolution video tape for subsequent analysis of fluorescence intensity.[3] An FITC filter-block was used for visualising ER, oxyradical generation, lysosomes receptor-ligand binding and microtubules; a blue-violet filter block was used for CNECOD and GSH; a rhodamine block was used for EROD; and a Texas Red block was used for endocytosis.[3]

A laserscan confocal microscope (SARASTRO / Molecular Dynamics) with Silicon Graphics imaging capabilities was used in the detailed study of the localization of the fluorescent bioprobes. An additional probe was used to test for ER; this was rhodamine B hexylester (R6) (250ng.ml^{-1}, 10 min), which requires a rhodamine block for visualization (Teresaki, M., unpublished data).[11]

Fluorescent emission was measured against sets of graded images for the appropriate probe, which were prepared using image analysis (Silicon Graphics). Twenty cells (diameter 12 ± 1μm) were measured per liver sample and the Mann-Whitney U-test was used for statistical analysis of the data. In the studies on endocytosis and presence of *ras*-oncoprotein cells, were scored as being positive or negative. Data is presented as % of positive livers and the Fisher exact probability test used in statistical analysis.

Immunocytochemical detection of clathrin, LDL and receptors for EGF was performed on water-soluble methacrylate sections using polyclonal antibodies with appropriate controls (Lowe, unpublished data). The *ras*-oncoprotein was tested for using frozen sections fixed in methanol for 10 min using a polyclonal antibody for N-*ras*-oncopeptide.[4] All three methods employed the fluorescent second antibody technique.[4]

RESULTS

The results showed that the two probes used to detect endoplasmic reticulum in the hepatocytes both had the same type of distribution in a tubular and lamellar network as demonstrated using confocal imaging. This finding supports the premise that the probes are localising in the ER and not in other

intracellular structures. Fluoresence produced by EROD activity and superoxide generation showed some apparent association with the ER but the distribution was often diffuse. Monochlorobimane reacts specifically with glutathione (GSH) and, here, the fluorescence showed diffuse localization in the cytoplasm.[11] Fluorescence for NBD-colcemid binding to microtubules tended to be distributed throughout the cytoplasm. Acridine orange fluoresces orange-red in lysosomes due to proton trapping and subsequent polyanionic binding. At low concentrations of trapping and binding the fluorescence is green. Texas Red-albumin was localized in vesicles believed to be endosomes and possibly also some lysosomes.

The results of the experimental investigation showed evidence of major molecular disturbances within the liver cells of fish exposed to contaminated sediment (Figs. 1-3). These included a proliferation of endoplasmic reticulum membrane, an increase in the activity of the associated detoxication enzymes (EROD and CNECOD), a decrease in intracellular glutathione, atrophy of the Golgi system which is central to the intracellular vesicular transport of proteins, as well as loss of integrity of the lysosomal system (important in the processing of macromolecules ingested by endocytosis and in the recycling of cellular constituents). Additional changes included a marked reduction in non-specific endocytosis and an increase in clathrin and cell surface receptors for binding epidermal growth factor (EGF) and LDL (Fig. 3). The amount of endocytosed LDL was also greatly increased in the liver cells.

A positive reaction for *ras*-oncoprotein was detected in small groups of liver cells in all livers from both reference and exposed fish. The intensity of the fluorescence was stronger in the exposed fish.

DISCUSSION

The results show that relatively subtle changes within cells can be identified and these are helping in the construction of a mechanistic picture of the pathological processes induced by chemical contaminants such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs).[3-6]

Molecular probes inserted into live isolated liver cells showed biochemical alterations as well as changes in some of the membranous compartments within the liver cells. These included an increase in the amount of endoplasmic reticulum (ER) in liver cells of contaminant exposed fish. The ER is responsible for manufacture of proteins and detoxication of xenobiotics (ie organic chemical contaminants). Activity of two ER associated detoxication enzymes, CNECOD and EROD, were significantly elevated in live cells from the contaminant exposed fish. Elevated activity of this enzyme is widely accepted as a likely biomarker of exposure to toxic xenobiotics.[17] A consequence of xenobiotic exposure is the oxidative stress imposed on animals as a result of one-electron metabolism giving rise to oxyradicals.[13] Also, many organic xenobiotics can be metabolised to redox-cycling intermediates which proliferate the production of oxyradicals.[18] Oxyradicals are also believed to be a major factor in attacking intracellular components which results in cell injury.[18,19] However, it is not possible on the basis of the data presented here to directly link the increase in oxyradical production with observed cellular injury, although it is possible that reported lysosomal damage in these fish, described below, may have been caused by such a mechanism.

Another membranous compartment, the lysosomes, was also adversely affected.

Lysosomes are responsible for degrading and recycling both endogenous and exogenous macromolecules and are highly sensitive to the toxic effects of xenobiotics and metals.[1-3,5] The functional integrity of this compartment was significantly impaired in the contaminant exposed fish.

The lysosomal compartment is closely linked with the **Golgi apparatus**, which packages newly synthesised proteins and targets them to their correct destination, and the **endocytic system** (responsible for bulk transport into the cell by invagination of the cell surface). The endocytic system transports macromolecules from the cell surface to the lysosome for degradation and involves a complex molecular sorting system for the recycling of cell surface receptors. Non-specific endocytosis of labelled protein was significantly reduced in cells from exposed fish. However, there was a marked increase in the cellular content of a special form of fat (lipid) known as **low-density lipoprotein (LDL)**; this LDL enters the cell by endocytosis following binding to a specific cell surface receptor. The inference is that endocytosis of LDL is enhanced and this in turn contributes to the accumulation of LDL observed in the liver cells of exposed fish. Much of this lipid accumulation was associated with enlarged lysosomes and constitutes a toxic lipidosis (Kohler, pers. comm.). This type of degenerative change appears to be an important step on the path to liver disease.[7]

An increase in cell surface receptors for **epidermal growth factor** (EGF-stimulates cell growth) in exposed fish may be indicative of attempted regeneration of the damaged liver.[7,9] Once again, the EGF-receptor complex, like LDL, enters the cell by receptor-mediated endocytosis.[9] This type of endocytosis requires a specific protein (**clathrin**), which is also elevated in exposed fish.[9]

These findings clearly demonstrate degenerative changes in liver cells from the contaminant exposed fish. Lysosomal damage, Golgi atrophy, accumulation of LDL, lysosomal enlargement, toxic lipidosis and a positive reaction for *ras*-oncoprotein are all **biomarkers of liver degeneration** in the dab used in this study. The increased endoplasmic reticulum, its associated EROD and CNECOD activity together with the depletion of glutathione are considered to be probable **biomarkers of exposure** to contaminant xenobiotics. The presence of *ras*-oncoprotein in the liver cells of reference fish indicates either prior exposure of the animals in the field or, else more likely, a sufficient xenobiotic content in the reference sediment.

The conclusion is that fish from the contaminant exposed group were impacted by organic xenobiotics which induced proliferation of ER and increases in EROD and CNECOD in the liver cells. Furthermore, the cells were injured in a manner which is comparable to xenobiotic and radical attack in mammalian cells and damage to the liver was of such an extent as to impair integrated function. [3] The results obtained from the use of the fluorescent molecular probes described above indicates their potential in identifying molecular and subcellular biomarkers of xenobiotic-induced cell injury in isolated liver cells. The methods used in this study have already been used in the field and the findings are consistent with those of the present study.[3-6] Such techniques could be readily modified for larger scale biomonitoring using a fluorescence microtitre reader. This would permit the rapid screening of large numbers of samples and microscopy would only be required for cell counts, viability tests and to validate the localization of the fluorescence. The immunocytochemical techniques could be readily applied to routine samples for histopathology and would greatly extend the informational content for a

little extra work.

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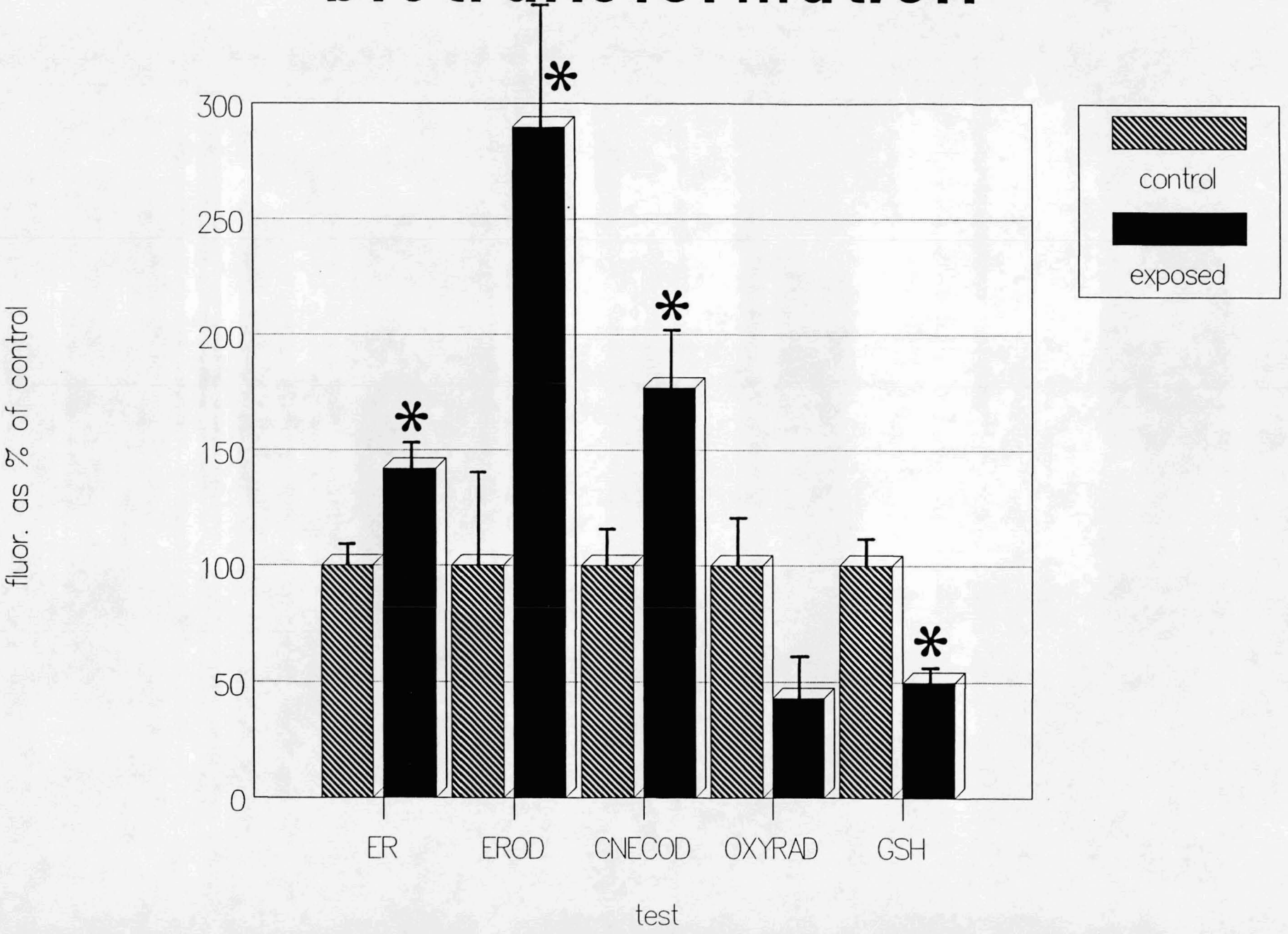
Figure Legends

Fig. 1. Biotransformation of Xenobiotics: Fluorescent molecular probes in liver cells of fish exposed to contaminated sediment (210 ppm total hydrocarbons; exposed) and reference sediment (75 ppm total hydrocarbons; control) for 144 days. Parameters measured include endoplasmic reticulum (ER), the detoxication enzymes EROD and CNECOD, oxyradicals (OXYRAD) and glutathione (GSH). * - indicates statistically significant difference ($P < 0.05$, mean and SEM, n=10) from the control.

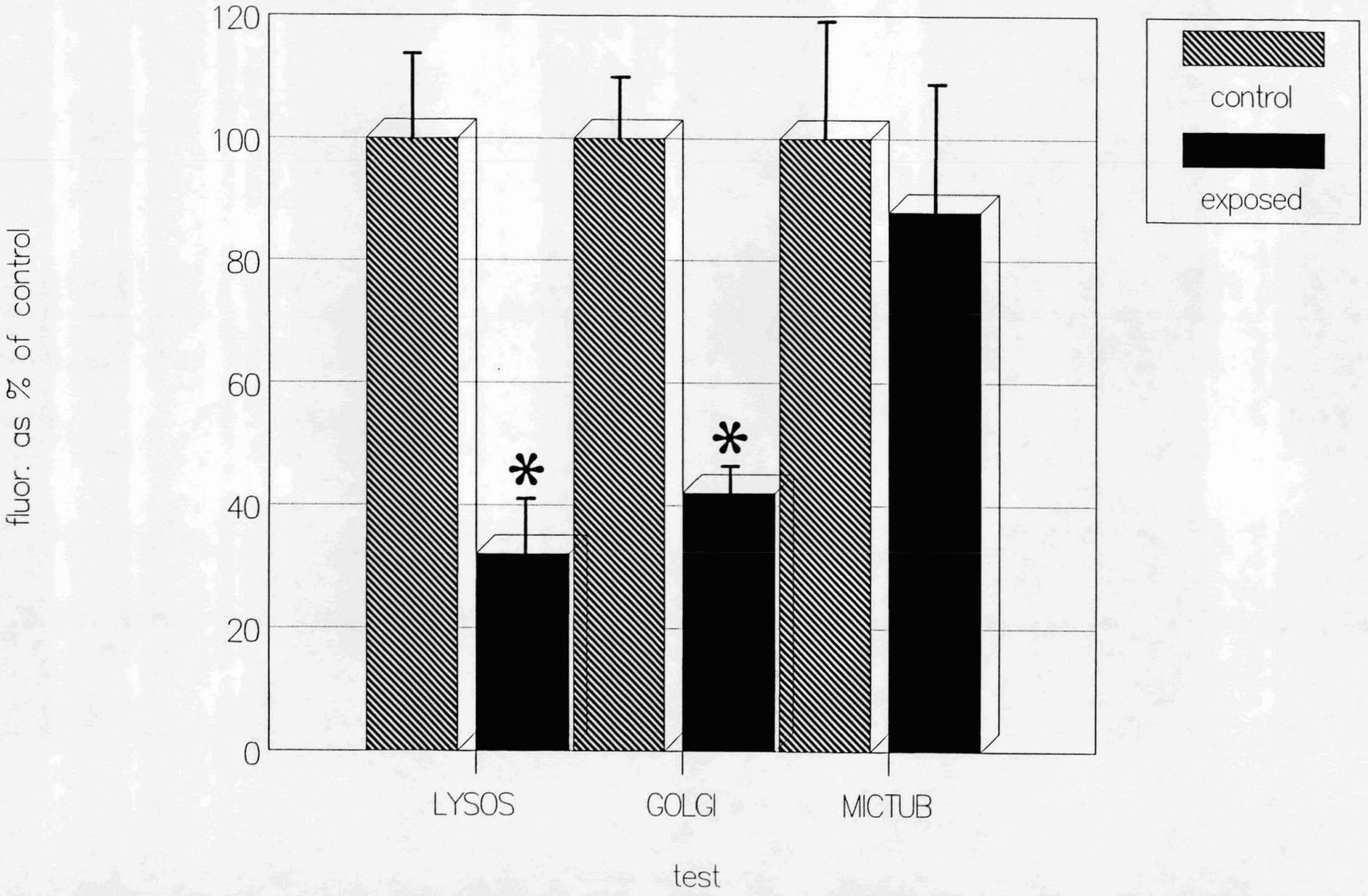
Fig. 2. Organelles: Fluorescent molecular probes in liver cells of fish treated as in Fig. 1. Parameters measured include lysosomal integrity, Golgi membranes and microtubules. * - indicates statistically significant difference ($P < 0.05$, mean and SEM, n=10) from the control.

Fig. 3. Endocytosis: Fluorescent molecular probes in liver cells of fish treated as in Fig. 1. Parameters measured include incidence of liver cell samples showing a positive reaction for non-specific invagination of Texas Red albumin (ENDOCYT), cell surface receptors for low density lipoprotein (LDL REC) and concanavalin A (CON A REC). * - indicates statistically significant difference ($P < 0.002$, Fisher exact probality test, n=10) from the control.

biotransformation



organelles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