The effects of coxsackievirus B3 infection on CYP1A1 and CYP2B gene expression after exposure to brominated flame retardants

Magnus Lundgren
Abstract

The brominated flame retardant, polybrominated diphenyl ethers (PBDE), used in e.g. electronic device, textiles and polymer matrices, are a persistent organic polluters that have been found in human tissue. CYP1A1 and CYP2B are detoxifying enzymes for PBDEs. In this study the effects of a myocardic coxsackievirus B3 (CVB3) infection on gene expression of CYP1A1 and CYP2B in the liver, pancreas and the lungs were investigated. Female balb C mice were CVB3 infected at day 0, orally exposed to 2,2′,4,4′,5-penta-BDE (BDE-99) or the commensally used mixture Bromkal at day 1 and sacrificed at day 3. CYP1A1 and CYP2B gene expression were measured using RT-PCR techniques and EROD and PROD activities were determined in the liver. The CYP2B gene expression in the liver of infected mice was higher after BDE99 treatment compared to the other infected groups. The fact that induction of gene expression did not occur after Bromkal treatment could be due to dioxin contamination of the Bromkal. The CYP2B expression was generally lower in the liver for infected mice than non-infected, although not significantly. None of the mice expressed CYP2B in the pancreas. CYP1A1 was almost exclusively expressed in the infected and PBDE treated mince, indicating an induction of CYP1A1 expression in the pancreas due to PBDE exposure. Infection induces CYP2B gene expression in the lungs. Moreover, the PBDE exposure seemed to be of negligible importance for gene expression. Infection reduced both the EROD and PROD activity in the liver. The enzyme activity corresponded well with CYP2B gene expression but not with the CYP1A1 expression where no difference between the infected and non-infected mice could be observed. This could be due to the inhibitory effect of the immune response on protein synthesis or a result of decreased enzyme activity due to infection.
This degree project is a continuation of a study that started with a degree project by Delila Gasi. The PBDE treatment and virus inoculation of mice has been performed by Gasi as the CYP2B gene expression and PROD activity measurements in the liver. Data from these measurements have been used in this paper. However, procession of these data and discussion of the same have been done by the author. All other results and measurements have been done by the author alone.
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Introduction
Nomenclature, properties and distribution
Brominated flame retardants (BFRs) are produced in large volumes and several of them have been found both in wildlife and humans (Darnerud 2003). The total demand for BFRs is estimated to 150 000 tonnes/year (OECD 1994) of which one third is polybrominated diphenyl ethers (PBDEs). PBDEs are persistent organic pollutants that can remain several years in nature without degradation and have been used for fire-protecting purposes since the 1960s. They are used in plastics, furniture padding, textiles, electronics, building materials and automobiles. For commercial purposes PBDEs are often used as technical mixtures, a combination of congeners of higher and lower bromination levels. However, there is a risk of contamination during the manufacturing process and other substances might also be present (Siddiqi et al. 2003, Darnerud 2003). Commercially used mixtures contain tetra-, penta-, hepta-, octa-, and deca-congeners (Siddiqi et al. 2003). In theory there are 209 congeners of PBDE divided into 10 congener groups (mono- to deca-BDEs). They have a high level of lipophilicity. The log $P_{ow}$ value is in the range of 4.3 to 9.9 (WHO 1994). PBDEs are structurally similar to polychlorinated biphenyls (PCBs) and PBDE congeners follow the nomenclature originally invented for PCB congeners where name is depended on the level of halogens (Darnerud et al. 2001). Försök hitta orginalkällan Deca-BDE is the most commonly used PBDE preparation. It makes up over 80% of the total production of PBDEs. However, the most frequently found congeners in human tissues are 2,2',4,4'-tetra-BDE (BDE-47), 2,2',4,4',5-penta-BDE (BDE-99) and 2,2',4,4'5,5'-hexa-BDE (BDE-153) (figure 1).

![Fig. 1 The structural formula of 2,2',4,4',5-penta-BDE (BDE-99)](image)

Exposure
The medium brominated congeners are bioaccumulative and can be found in human adipose tissue, in fish and other animals (Siddiqi et al. 2003). The highest concentrations of same PBDEs in wildlife are found in the aquatic environment. High levels of PBDEs have been found in fatty fishes and water living mammals, especially near point sources of pollution (Darnerud 2003). PBDEs are often used in polymer matrices (e.g. radios, televisions, switches etc.). Since they are not covalently bound to the polymer matrix there is a risk of airborne PBDE release due to diffusion of the polymer. Some building materials may cause spread of PBDE containing fragments at outdoor conditions, for example polyurethane foam. Airborne PBDEs with a high degree of bromination are usually found closer to the source of pollution, whereas less brominated PBDEs can be transported quite a distance (Siddiqi et al. 2003). Although the mechanisms behind the diffusion are not fully known, it is believed that incineration may be an important source of PBDE release in to the environment (Darnerud et al. 2001). Försök hitta orginalkällan, men det står så i artikeln. When combusted there is also a risk of transformation of PBDEs into other more toxic compounds such as dioxins and dibenzo furans (Darnerud 2003). Human exposure routes are mainly through occupational exposure and food intake. In Scandinavian countries an important exposure route for PBDE exposure occurs via fatty fishes (Darnerud 2003). Occupational exposure
can occur in electronic warehouses and computer related working places or via direct work with PBDEs (Siddiqi et al. 2003). However, detectable levels of PBDEs have also been observed in people without any occupational exposure. (Darnerud 2003) PBDEs have been found in human blood, serum, adipose tissue, brain and breast milk. (Siddiqi et al. 2003) The fairly high amounts of PBDEs in breast milk might be an important route of exposure and possible source of toxicity. This may have severe consequences since exposure occurs during a critical phase of neonatal development when the brain is developing and the central nervous system (CNS) is extra vulnerable (Eriksson et al. 2001).

**Distribution**

After oral administration (e.g. PBDEs in food) PBDEs are absorbed from the gastrointestinal tract and are distributed to various target organs of toxicity (Darnerud & Risberg 2006). The route of administration does not seem to affect the distribution. Mice exposed to BDE-47, via intratracheal, oral, intravenous, intraperitoneal or dermal administration, showed a similar tissue distribution regardless of administration route (Staskal et al. 2004). Due to the high octanol/water partition coefficients the distribution of PBDEs is governed by lipophilicity and accumulates in lipid rich tissues. Adipose tissue, skin and liver have the highest concentrations of PBDE but the substance are also found in muscles, lung, kidney, blood and brain (Staskal et al. 2004). However, infection causes changes in PBDE distribution. Studies preformed by Darnerud et al. (2005) showed that mice infected with the human coxsackievirus B3 (CVB3) have higher concentrations of BDE-99 in the liver compared to the non-infected mice and lower concentrations in the lungs and pancreas.

**Toxicity**

Penta-BDEs have low acute toxicity, the oral lethal dose (LD) is 0.5-5g/kg body wt (WHO 1994, Darnerud et al. 2001). However, penta-BDEs can cause toxic effects at lower doses than higher brominated PBDEs. BDE-99 has been reported to cause toxic effects at very low doses compared to other PBDE congeners (Siddiqi et al. 2003, Darnerud 2003). The central nervous system, liver, thyroid gland and reproductive organs have been observed as targets of toxicity (Eriksson et al. 2001, Hallgren & Darnerud 2002, Kuriyama et al. 2005).

**Neurotoxicity**

During the formation of the central nervous system (CNS) a critical phase called the brain growth spurt (BGS) takes place. During BGS the brain undergoes a rapid change, many new motor and sensory functions are acquired and several developmental changes take place, e.g. establishment of neural connections. This crucial period takes place from the third trimester of pregnancy to the age of two years in humans. In rodents the BGS is a neonatal process spanning the first 3-4 weeks of life to the age of two years (Davison & Dobbing 1968). Exposure to neurotoxic agents during this period can induce CNS-effects. Studies performed by Eriksson et al. (2001) show that neonatal exposure of BDE-99 to adult mice caused irreversible changes in spontaneous behaviour and habituation as well as adverse effects on learning ability and memory functions. A similar pattern of neurotoxicity has also been observed for BDE-47. The behavioural aberrations are similar to those of PCBs and it has therefore been speculated that these two groups of chemical can cause interactive effects (Eriksson et al. 2001, Kuriyama et al. 2005).

**Liver toxicity**

The liver is the first organ that encounters nutrients, vitamins and minerals, but also other substances such as various xenobiotics. The liver is an important organ in maintaining metabolic
homeostasis and venous blood from the gastrointestinal tract passes through the liver before entering the systemic circulation. Toxic agents can adversely affect the enzymatic reactions in the liver such as biotransformation of xenobiotics, metabolic processes, protein synthesis and formation and secretion of bile. Loss or decreased efficiency of these functions due to acute or chronic exposure to toxicants could severely affect the health status of an individual. Lipophilic compounds such as PBDEs can diffuse over epithelial membranes into hepatocytic cells where it could cause cell death, formation of scar tissue and in worst-case fatal acute hepatotoxicity (Klaassen et al. 2001 b). The liver is an important organ concerning metabolism and detoxification of xenobiotics. The hepatocytes are highly active in expressing phase I and II enzymes. Cytochromes of all subtypes are expressed in the hepatocytes and the highest concentration of most cytochrome enzymes in the human body are found in the liver (Klaassen et al. 2001 a, Klaassen et al. 2001 b).

**Thyrotoxicity**
Thyroid hormones (THs) play an important role in the development of many organs, e.g. the brain. A theory has been forwarded that alterations in the CNS in some cases could be the result of exposure to thyroid hormone disrupting chemicals (Hallgren & Darnerud 2002). Thyroxine (T₄) is an, among vertebrates, iodinated amino acid produced in the thyroid gland. In general T₄ is an endocrine hormone that mainly stimulates the general metabolism. It is a non-polar molecule bound to carrier proteins e.g. the plasma TH-transporter transthyretin (TTR) that is transported via the blood circulation. There is a state of equilibrium between the T₄ bound to carrier proteins and the free T₄ (FT₄), where FT₄ is the biologically active form (Voet & Voet 2004). It has been confirmed that penta-BDE reduce T₄ serum levels in mice and rats (Fowles et al. 1994, Darnerud et al. 2005, Stoker et al. 2004, Zhou et al. 2001, Zhou et al. 2002, Hallgren & Darnerud 2002) and that PBDE metabolites compete with FT₄ for TTR binding sites in vitro (Meerts et al. 2000). PBDEs have also been shown to have negative synergistic effects on FT₄ levels. Hallgren and Darnerud (2002) have shown that BDE-47 also has synergistic effects with chlorobiphenyls and chlorinated paraffin. T₄ can be converted into triiodothyronine (T₃), and T₃ is responsible for most of the receptor mediated hormonal effects. Production of thyroid stimulating hormone (TSH) in the pituitary gland affects the levels of T₃ and T₄, thereby influencing the thyroid induced effects. T₄ is a feedback regulator of TSH production and is excreted via the bile through glucuronidation. However, PBDE does not seem to affect the TSH plasma level (Hallgren & Darnerud 2002, Fowles et al. 1994, Zhou et al. 2001).

**The cytochrome P450-system**
The human body is constantly exposed to xenobiotics, but normally they do not accumulate because the body has an ability to eliminate these potentially toxic substances by biotransformation. During biotransformation a lipophilic xenobiotic is gradually converted via enzymatic reactions into a hydrophilic compound that can be excreted via the urine or faeces. This process is schematically divided into two phases, phase I and phase II. The phase I reactions are mainly hydrolysis, reduction or oxidation of a xenobiotic, whereas the phase II reactions are often conjugation reactions e.g. conjugation with glutathione or amino acids. Cytochrome P450 (P450) family of enzymes catalyses many phase I reactions within a broad range of xenobiotics and plays a key role in the metabolism of drugs. The P450 enzymes are divided into several subgroups with various substrate specificity. These enzymes are present in all tissues in all mammals but the highest concentrations are found in the liver (Klaassen et al. 2001 a). They are mainly located in the endoplasmic reticulum that is connected to the cell nucleus membrane. When a cell is
homogenised the endoplasmic reticulum forms small vesicles called microsomes. Purified microsomes are often used when studying biotransformation in vitro (Birgersson et al. 1995).

**CYP1A1**

All mammalian species have CYP1A enzymes (CYP1A1 and CYP1A2). In mice and humans CYP1A1 catalyse the O-dealkylation of 7-ethoxyresorufin and CYP1A2 catalyse the O-dealkylation of 7-methoxyresorufin. CYP1A1 also catalyses hydroxylation and epoxidation of polycyclic aromatic hydrocarbons. There are species differences in tissue expression of CYP1A1, i.e., normally, it is expressed at low levels in the human liver but constitutively expressed in the liver of rhesus monkeys and guinea pigs (Klaassen et al. 2001 a).

**CYP2B**

Although CYP2B enzymes are present in low concentrations in humans they are important in the biotransformation of many xenobiotics. (Meerts et al. 2000) The CYP2B and CYP2B1/2 are the murine enzymes corresponding to the human CYP2B6 (Klaassen et al. 2001 a, NCBI 2005). In vitro studies have shown CYP2B to be an important subgroup in metabolising PBDE. Some CYP2B metabolites of PBDE are able to compete with thyroxine for binding to the plasma transporter protein transthyrin.

Both CYP1A1 and CYP2B are inducible enzymes. Exposure to xenobiotics stimulates gene expression and protein synthesis of CYP1A1 and CYP2B. The expression and synthesis of these enzymes are not only dependent on specific differences and environmental factors (e.g. xenobiotics) but also on individual genetic differences (Klaassen et al. 2001 a). Induction of P450s and higher EROD activity has been confirmed after oral exposure of penta-BDE in rodents (Fowles et al. 1994, Zhou et al. 2001).

**Infection**

The immune response can be divided in two types of responses, the innate response and the adaptive response. The innate immunity (also knows as natural or native immunity) is a physical and biochemical defence system. It is always present and is rapidly responding to microbial infections. The innate immune response has no memory function and the response is the same every time of infection, regardless if it is the first encounter with the antigen or not. Innate immunity involves the complement system, phagocytic cells (neutrophils and macrophages), natural killer cells (NK-cells), cytokines and physical barriers such as epithelia and antimicrobial substances. (Abbas & Lichtman 2003) Macrophages play an important roll in the defence system against viruses and bacteria due to their ability of presenting antigens for T-helper cells. They are activated by the Th1 cytokines IFNγ and TNF. (Ilbäck 2005) NK-cells have the ability to kill virus-infected cells and tumour cells. Cytokines are proteins produced by activated immune cells that regulate and coordinate activities of the innate response. The innate system provides an effective and quick response but many microorganisms can resist the innate immune defence.

Although the adaptive immunity (specific immunity) is a somewhat slower response it is more specific. It is triggered by the innate immune response and has the capacity to distinguish between antigens, even closely related molecules and microorganisms. The adaptive immune system has a memory function and the response becomes faster and higher in magnitude after each exposure to a specific antigen. There are two types of adaptive responses, the humoral response and the cell-mediated response. The humoral response is antibody mediated. B lymphocytes (B-cells) produce
antibodies that recognise microbial antigens in the blood circulation and lymphatic system and neutralise them. The cell-mediated response is T lymphocyte mediated. T lymphocytes activate macrophages to kill phagocytised microbes and infected cells. (Abbas & Lichtman 2003)

Infection can generally be divided into three phases, the incubation period, the acute phase and the recovery period. It is mainly during the acute phase that the hosts defence mechanisms, the innate and adaptive immune response, is mobilised. During the acute phase of infection the metabolic rate increases with approximately 13% per degree body temperature. This causes an increased need for certain nutrients. Malnutrition has been shown to worsen the symptoms of infectious diseases. Malnutrition among mice during infection with coxsackievirus B results in increased virus persistence and more serious symptoms. The symptoms vanish when the mice are fed a normal diet. The body’s response to the increased energy needs results in changed metabolism of fat, protein and carbohydrates. The protein synthesis is down regulated, including the cytochrome P450 system. This causes a decreased detoxification and excretion of xenobiotics. A decreased biotransformation rate might result in long-term accumulation of xenobiotics in target organs of exposure and infection. The immune system is sensitive for exposure to certain chemicals such as polycyclic aromatic hydrocarbons (PAHs), halogenated aromatic hydrocarbons (e.g. PBDE and TCDD) and non-essential trace elements. These chemicals are found in food and wildlife. (Ilbäck 2005)

Eventually the increased need for energy and lower energy intake due to loss of appetite can lead to degradation of muscle protein, both red and white skeletal muscle and even heart muscle. Hormones that inhibit protein synthesis and stimulate proteolysis in muscles are proinflammatory cytokines (IL-1, IL-6) and TNF. The released amino acids from protein degradation are used for synthesis of cytokines and antibodies or in the gluconeogenesis. The high-energy demands decrease the glucose level in the blood. The production of glucose in the gluconeogenesis compensates the lower glucose level in the blood and is crucial for survival. If the production is disturbed e.g. because of toxic damage to the liver of xenobiotics this might also result in deterioration of host defence reactions that could have fatal consequences to survival. (Ilbäck 2005)

_Coxsackievirus_

Coxsackieviruses belong to the enterovirus genus in the Picornaviridae family and are organised in two serogroups, A and B. They are non-enveloped viruses with a single-stranded RNA genome (Huber & Ramsingh 2004, Whitton 2002, Wessely 2004). When cellular infection takes place the RNA is released and translated into polypeptides using the host cells protein translation system. Coxsackievirus B3 (CVB3) infection can induce CD27/CD70-caused apoptosis (cell death) in pancreas (exocrine cells) and heart tissue by interaction between the CVB3 capsid protein V2 and the proapoptotic protein Siva (Henke et al. 2000).

Coxsackievirus A consists of 23 serotypes and these normally give rise to less severe clinical syndromes. Coxsackievirus B (CVB) consists of 6 serotypes and are associated with several human diseases such as heart diseases, idiopathic chronic pancreatitis and insulin-dependent type I diabetes mellitus (IDDM) (Huber & Ramsingh 2004, Whitton 2002, Trianaffilou et al. 2004). The cause of IDDM is however controversial. A relationship between CVB infection and IDDM is only supported by epidemiological and serological studies and has not been confirmed in murine models where the islets of Langerhans seem to be unaffected after infection with CVB. It has been shown
that CVBs can infect pancreatic cells and induce cytokine production, something that might influence the pathogenesis of IDDM (Trianañflou et al. 2004). CVB3 infection can change tissue distribution of xenobiotics. In a study performed by Darnerud et al. (2005) infection with CVB3 changed the organ distribution of BDE-99.

**Purpose of study**
The aim of this study was to investigate the effects of coxsackievirus B3 infection on gene expression of CYP1A1 and CYP2B in liver, pancreas and lungs of non-exposed and PBDE exposed mice. Moreover, gene expression and related enzymatic activities were studied in the liver samples after PBDE exposure in non-infected and CVB3 infected mice. Investigated PBDEs were 2,2’,4,4’,5-pentabromodiphenyl ether (BDE99) and the commercial PBDE mixture Bromkal 70-5 DE.

**Material and methods**

**Mice**
Balb C mice (females) were purchased from Scanbur B&K, Stockholm, Sweden and were kept at the infection department, Biomedical Centre, Uppsala, Sweden under a 12 hour light/dark cycle. The mice had free access to food (R3, Ewos, Södertälje, Sweden) and tap water *ad libitum*.

**Virus**
A myocardidic strain of coxsackievirus B3 was used in the study. The virus was propagated in HeLa cells grown in Eagle minimal essential medium. The medium was supplemented with antibiotics and 5% fetal calf serum. Virus amounts in HeLa cells were determined as plaque-forming units (pfu). The stock solution was determined to $10^7$-$10^8$ pfu/ml and was diluted with phosphate-buffered saline to get $2 \times 10^3$ pfu/0.2 ml. The stock solution was stored at -20°C. Each mouse was inoculated intraperitoneally with 0.2 ml of this solution.

**Chemicals**
Pure (99%) 2,2’,4,4’,5-penta-BDE (BDE-99) was obtained as a gift from Dr. Hellmuth Lilienthal, University of Bochum, Germany. Purity was analysed with HRGC/HRMS by Ökometric, Germany. Bromkal 70-5 DE (Klak Chemische Fabrik, Germany), a technical PBDE mixture containing 37% 2,2’,4,4’-tetra-BDE (BDE-47) and 35% 2,2’,4,4’,5-penta-BDE (BDE-99). Both substances were dissolved in corn oil (20 mg/ml). Controls were administrated corn oil.

**Study design**
After a two-week acclimatisation period the mice were randomly divided into six groups, (n=5). Infected and non-infected mice were untreated (controls) or treated with BDE99 or Bromkal, (table 1). The mice were infected (i.p.) at day 0, orally treated with BDE-99 and Bromkal at day 1 (20 mg/kg bw) and sacrificed at day 3. Gene expression of CYP1A1 and CYP2B was determined in the liver, pancreas and lungs. EROD and PROD activity were measured in the liver.

<table>
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<td><strong>Non-infected mice</strong> (n=15)</td>
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<td>Control n=5</td>
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<tr>
<td>Treated with BDE-99 n=5</td>
</tr>
<tr>
<td>Treated with Bromkal n=5</td>
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* One mouse showed no clinical signs of disease.
**Statistics and calculations**
The non-parametric Mann-Whitney test (MiniTab 12 for Windows) was used to test statistical differences between the groups, i.e. effects of infection and/or treatment with BDE99 or Bromkal. Significance was accepted at p<0.05. In order to compare gene expression levels a model based on the CT-values of the specific gene of interest and the housekeeping gene was used. \( _{C}T \) is the difference between the CT values of the gene of interest and the housekeeping gene. \( \Delta C_{T} \) is \( _{C}T \) minus the mean value of the differences in the control group. The \( 2^{-\Delta C_{T}} \) values were used for comparing the gene expression data between groups.

**Primers and primer design**
The gene sequences of CYP2B and CYP1A1 were obtained using the nucleotide search at the National Centre for Biotechnology Information (NCBI) homepage. The obtained gene sequences (CYP2B: NM_009998, CYP1A1: NM_009992) were compared with the genome of the mouse, *Mus musculus*, using the BLAST function at the same homepage. Primer sequences (forward primer and reverse primer) and probe sequences were obtained using the Primer Express 2.x program, *(table 2)*. The primer sequences were compared with the genome of the mouse, *Mus musculus*, using the BLAST function at the NCBI homepage. Primers were purchased from Thermo Electron GmbH, Germany *(www.thermo.com)*. Probes were purchased from Tib Molbiol GmbH, Germany.

**Table 2.** Primer and probe sequences used for CYP 1A1 and CYP2B.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reverse primer</th>
<th>Forward primer</th>
<th>Probe</th>
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<tr>
<td>CYP1A1</td>
<td>5'- GGTGGCTGTTCCTGTGATTCA -3'</td>
<td>5'- AAAGTGGAGGCGAGCAACATG -3'</td>
<td>5'- TAGCCAGAAAACACAGATC -3'</td>
</tr>
<tr>
<td>CYP2B</td>
<td>5'- GCCCTTCTCAACAGGACAAATT -3'</td>
<td>5'- GCCAATGCTTTCACCAAGACA -3'</td>
<td>5'- TGATCAAAGGTCTGTTGGAAAGCGCAT -3'</td>
</tr>
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**RT-PCR**
Preparation and isolation of total RNA from the liver, pancreas and lungs were made using the Qiagen RNA/DNA Mini kit (Cat. No. 14123, Qiagen) according to manufactures instructions. Synthesis of cDNA was done using the Omniskript Reverse Transcription kit (Cat. No. 205113, Qiagen) according to manufactures instructions. Primer optimisations were performed using heat-label Uracil-DNA-Glycosylase (Cat. No. 1775367, Roche), RNas inhibitor 2500 U (Cat. No. 556881, Cabiochem), LightCycler Capillaries (Cat. No. 1909339, Roche), Real-Time PCR (LightCykler, Roche) and primer concentrations 50nM, 300nM and 900nM, *(table 3)*.

**Table 3.** Concentrations of primers and combinations of these used in primer optimisation for both CYP1A1 and CYP2B primers.

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<tr>
<th>Concentration</th>
<th>Forward</th>
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<td>Forward</td>
<td>Reverse</td>
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<tr>
<td>50nM Reverse</td>
<td>300nM</td>
<td>900nM</td>
</tr>
<tr>
<td>300nM Forward</td>
<td>300nM</td>
<td>300nM</td>
</tr>
</tbody>
</table>

11
Optimisation was performed using a non-specific fluorescent probe, LightCycler FastStart DNA Master SYBR Green 1 kit (Cat. No. 3003230, Roche). The combination of primers with the lowest CT-value was selected for further measurements (CYP1A1: RP 900nM, FP 900nM, probe 225nM, CYP2B: RP 900nM, FP 900nM, probe 225nM). The CT-value is the number of cycles where the fluorescence of the probe is first detectable, e.g., a low CT-value means that the amplification of DNA starts at an early cycle corresponding to a high gene expression and/or successful optimisation. Primer optimisation was controlled by electrophoresis using agarose gel (3%) that confirmed the amplicon length. Both optimisation and amplification were run at 55°C for 40 cycles (optimisation) and 45 cycles (amplifications).

Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene since standard curves are missing for both CYP1A1 and CYP2B. A housekeeping gene is used in order to determine the background/standard gene expression.

PROD measurements
The PROD activities were measured using the method originally described by Pohl and Fouts (1980) and modified by Sinjari et al. (1993). The protein concentrations were determined using the method described by Hartree (1971).

EROD measurements
The EROD activities were measured using the method originally described by Pohl and Fouts (1980) and modified by Sinjari et al. (1993). The protein concentrations were determined using the method described by Hartree (1971).

Results
All infected mice except two showed clinical signs of disease, (e.g., ruffled hair and inactivity), but no mortality had occurred at day 3. These two animals that did not show any symptoms of disease at day three of infection were excluded from the study. In all other infected mice clinical signs of the disease appeared at day 2 and the peak of the infection occurred at day 3.

The liver
Gene expression of CYP1A1 did not show any significant differences between the groups. However, in the non-infected mice the gene expression values in BDE99 exposed animals tended to be higher than the values in Bromkal exposed mice. A lower gene expression was observed after exposure to BDE99 and Bromkal in both infected and non-infected mice compared to untreated infected and untreated non-infected mice, (figure 2). In general the CYP1A1 expression tended to be lower in the infected untreated mice compared to the non-infected untreated mice. A similar difference was observed between infected and non-infected mice treated with BDE99, but not with Bromkal.
The livers from the coxsackievirus B3 (CVB3) infected mice exposed to BDE99 showed a significantly higher (p=0.0200) CYP2B gene expression compared with the livers from the CVB3 infected control group and the CVB3 infected group exposed to Bromkal among (figure 3). No other significant differences were detectable among the groups of infected mice. However, among non-infected mice gene expression of CYP2B in the liver tended to be higher in livers from BDE99 and Bromkal exposed mice.

Pancreas
It was interesting to note that CYP1A1 was only expressed in the pancreas of eight mice, (table 4). Seven of these eight individuals that expressed CYP1A1 were mice infected with CVB3. Three of these six individuals had been treated with BDE99 and three with Bromkal. Although the gene expression of CYP1A1 in the non-infected untreated mouse was clearly present it was very small and the model for compensation of CT-values with the housekeeping gene could not be used. Thus, CVB3 infection seems to induce CYP1A1 in the pancreas and more pronounced after PBDE exposure.

Table 4  Gene expression of CYP1A1 in pancreas (  = expressed  = not expressed) among the individuals in the different test groups. Each circle represents one individual.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>BDE99</th>
<th>Bromkal</th>
<th>CVB3</th>
<th>CVB3 + BDE99</th>
<th>CVB3 + Bromkal</th>
</tr>
</thead>
</table>

However, there was no detectable gene expression of CYP2B in the pancreas in any group. For confirmation three randomly selected pancreas samples were run together with 3 randomly selected liver samples in a RT-PCR with CYP2B primers and probe (figure 4). Gene expression of CYP2B in the liver samples were detected, but not in the pancreas samples.
Fig. 4 Data analysis graph after RT-PCR. Liver samples clearly express CYP2B while pancreas samples do not. Fluorescence indicates successful amplification. The cycle number where the fluorescence of the probe is first detected is the CT-value.

The lungs
CYP1A1 was expressed in 21 of 28 individuals, (table 5). The infection in untreated mice did not affect CYP1A1 gene expression. Both BDE99 and Bromkal tended to increase gene expression in non-infected mice, but to decrease it in infected mice. Moreover, the non-infected and non-exposed group had a tendency, although not significant, of higher expression than the infected groups treated with BDE99 and Bromkal, (figure 5).

Table 5  Gene expression of CYP1A1 in the lungs ( = expressed = not expressed) among the individuals in the different test groups. Each circle represents one individual.

<table>
<thead>
<tr>
<th>Control</th>
<th>BDE99</th>
<th>Bromkal</th>
<th>CVB3</th>
<th>CVB3 + BDE99</th>
<th>CVB3 + Bromkal</th>
</tr>
</thead>
</table>
CYP1A1 expression in the lungs

Gene expression level (2^-ΔΔCT)

Control (n=4)

BDE99 (n=5)

Bromkal (n=5)

CVB3 (n=2)

CVB3 + BDE99 (n=2)

CVB3 + Bromkal (n=3)

Fig. 5 Gene expression of CYP1A1 in the lungs of untreated mice and mice treated with BDE99 or Bromkal, respectively, with or without CVB3 infection. The bar charts shows the mean value + SD of 2^-ΔΔCT in each group.

Similariy, BDE99 and Bromkal treatment did not affect the infection-induced increase in CYP2B. In mice not treated with PBDE there was significantly higher (p=0.0195) CYP2B expression in the infected group compared to the non-infected group. However, neither BDE99 nor Bromkal affected CYP2B gene expression in non-infected mice. Thus the infected mice treated with Bromkal showed a significantly higher (p=0.0200) CYP2B expression than non-infected mice treated with the same substance. Infected mice treated with BDE99 showed a numerically higher gene expression value than non-infected mice exposed to BDE99, but this difference was not significant. The infected group exposed to Bromkal had a significantly higher (p=0.0200) expression than the non-infected untreated group. The infected and non-exposed group had a significantly higher (p=0.0195) expression than the non-infected group exposed to Bromkal. The infected group exposed to BDE99 had a significantly higher (p=0.0160) expression than the non-infected group exposed to Bromkal and the non-infected untreated group (p=0.0122). There were no significant differences within the infected groups or within the non-infected groups. (Figure 6).

EROD

Infected groups showed a significantly lower EROD activity than non-infected groups treated with BDE99 (p=0.0122) or Bromkal (p=0.0369). Untreated groups showed the same tendency, although the decrease was not significant. Moreover, infected mice treated with Bromkal showed a lower EROD activity (p=0.0369) than non-infected mice treated with BDE99. Infected mice treated with BDE99 showed a lower EROD activity (p=0.0122) than non-infected mice treated with Bromkal. EROD activity was significantly induced (p=0.0122) by BDE99 in the non-infected mice and the same tendency, although not significant, was observed for non-infected mice treated with Bromkal. However, in the infected mice treated with BDE99 and Bromkal induction of EROD activity do not seem to occur. Untreated infected mice showed a lower EROD activity compared with non-infected mice treated with BDE99 (p=0.0200) or Bromkal (p=0.0200) (figure 7). The results are not in line with CYP1A1 gene expression data.
PROD activity in the liver of untreated mice and mice treated with BDE99 or Bromkal, respectively, with or without CVB3 infection. The bar charts shows the mean value + SD of EROD activity in each group.

**PROD activity in the liver**

All CVB3 infected groups showed a numerically lower PROD activity than the non-infected groups, (figure 8). The PROD activity in non-infected mice treated with BDE99 was significantly higher (p=0.0120) than in non-treated and non-infected mice. A similar increase was observed in non-infected mice treated with Bromkal although the increase was not significant. It was interesting to note that the PROD activity in CVB3-infected mice was lower (p=0.0195) after exposure to Bromkal than after exposure to BDE99. The activity in the infected BDE99 treated group was comparable to that in the non-infected untreated groups. Thus, BDE99, but not Bromkal, seems to increase PROD activity regardless of infection. The results are in line with CYP2B gene expression data.

**Discussion**

**Gene expression**

The aim of this study was to investigate the possible effects of PBDE exposure on gene expression and associated enzyme activities in infected and non-infected mice in a well-known animal infection model. The effects of infection on the studied endpoints differ between organs and genes as do the effect of PBDE exposure and their combined effects.

**Gene expression in the liver**

In the liver the CYP2B expression was generally lower, although not significantly, in infected than in non-infected groups. Suppression of protein synthesis and gene expression as an effect of infection has previously been shown and the mechanism behind this also discussed by Darnerud et al. (2005) and Ilbäck (2005). A single exposure to BDE99 or Bromkal induces, although not significantly, CYP2B expression in the non-infected groups. Previous studies have shown that CYP2B is an inducible enzyme that is important for the metabolism of PBDEs (Meerts et al. 2000, Klaassen et al. 2001 a). Among the infected groups the mice exposed to BDE99 showed a significant (p=0.0200) induction of CYP2B expression compared both to the infected untreated mice and the infected mice treated with Bromkal. Notably, the induction of gene expression that
occurred in the non-infected group after exposure to BDE99 did not occur after Bromkal treatment to non-infected mice.

Gene expression of CYP1A1 showed a somewhat different pattern than CYP2B. Infection in untreated and BDE99 treated mice seemed to decrease gene expression, although not significantly. However, this response was not observed after exposure to Bromkal where no difference in expression could be detected between infected and non-infected groups. Thus exposure to PBDE does not seem to induce CYP1A1 expression. On the contrary, the gene expression was lower both after exposure to BDE99 and Bromkal, although not significantly, for both infected and non-infected groups. Studies have shown that other PBDEs, not studied here, may induced CYP1A1 expression and that this induction is Ah-receptor mediated. However, among the PBDE congeners it seems that BDE99 is one of the least dioxin like with the result that BDE99 induced CYP1A1 expression is almost negligible (Chen & Bunce 2003).

**Gene expression in pancreas**

CYP2B expression in pancreas could not be detected in any of the groups. This was confirmed by running pancreas samples and liver samples together in a RT-PCR with CYP2B primers and probe (figure 4). This finding is supported by studies of the human equivalent to the murin CYP2B enzyme, CYP2B6. RT-PCR investigations of CYP2B6 expression in human pancreas showed no detectable levels of CYP2B6 (Standop et al. 2002). However, the same study found CYP2B6 expression when looking at the Islet of Langerhans alone. This seems to be the case also for mouse thus immunohistochemistry using antibody staining comparing enzyme location in different tissues of mouse and man showed a similar pattern for CYP2B/CYP2B6 in the Islet cells (Ulrich et al. 2002). This indicates that although CYP2B expression was not detectable in pancreas there might be a certain expression in the Islet of Langerhans alone but not enough for detection in pancreas as a whole. Notably, immunostaining of pancreas ductal and acinar cells showed a weak to moderate amount of CYP2B/CYP2B6 in both mouse and human. This effect could not be showed or confirmed by using the RT-PCR technique (Standop et al. 2002, Ulrich et al. 2002).

CYP1A1 expression could be detected only in one of the non-infected mice, treated and untreated combined. This corresponds well with the study by Ulrich et al. (2002) where only small amounts of CYP1A1 were detectable in the Islet cells. One should also be aware of possible species differences in this case between the mouse and humans. Thus, a comparison using immunostaining techniques between human and murin pancreas showed higher amounts of CYP1A1 in the human pancreas (Standop et al. 2002, Ulrich et al. 2002). When using RT-PCR technique the human pancreatic CYP1A1 expression is very poor, as show also for mouse in this study. Surprisingly expression was detected in three of the infected mice exposed to BDE99 (n=5) and in three of the infected mice exposed to Bromkal (n=4) (table 4). It is well known that CYP1A1 expression is inducible (Klaassen et al. 2001 a) but in the present study this induction only occurred in the infected groups. This finding might propose a possible explanation to the fact that infection target organ specifically changes the distribution of BDE99. This may explain the previous observed decrease in pancreatic concentrations of BDE99 in infected mice compared to non-infected mice (Darnerud et al. 2005). Two possible explanations for this could be decreased uptake or affected metabolic pathways of BDE99 due to infection. The results from this study points at the later possibility.
Gene expression in the lungs
Infection induced significantly CYP2B gene expression in the lungs. Furthermore, the infected group exposed to Bromkal showed a similar and significantly higher expression than the non-infected group. The pattern was similar, although not significant, after exposure to BDE99. In general there were significantly higher expression in the infected groups (figure 6). Darnerud et al. (2005) showed that the concentration of BDE99 was greatly decreased in the lungs of CVB3 infected mice. The results from this study suggest that the lungs, as well as the pancreas, are involved in the detoxification of BDE99 and that this ability is induced by infection per se. The mechanisms behind this are not known and are important to further evaluate. However, PBDE exposure does not seem to effect the CYP2B expression and no significant differences were found within the non-infected groups or the infected groups.

Infection seems to greatly suppress CYP1A1 expression and this suppression counteracts the normal induction observed in healthy animals treated with BDE99 or Bromkal. Moreover, six individuals in the infected groups did not even express measurable levels of CYP1A1, (table 5). Those that expressed CYP1A1 showed a lower expression than non-infected mice that received the corresponding PBDE treatment, (figure 5). Protein synthesis and gene expression are down regulated by infection (Darnerud et al. 2005, Ilbäck 2005). However, the untreated groups do not show the same decrease in gene expression. Moreover, treatment with BDE99 and Bromkal tends to induce gene expression in the non-infected groups. It is well known that CYP1A1 expression is inducible (Klaassen et al. 2001 a), although in this case the induction is small and only observed in non-infected mice.

Standard deviation
The regulation of induced gene expression has a sensitive on and off switch mechanism, which probably was the reason for the high standard deviation in gene expression data. After genes have been induced, production of proteins starts within hours. When the sufficient amount of proteins have been synthesised, or when the inducer is no longer present, the gene expression is shut down. This is a sensitive regulation that differs between individuals and depends on the phase of the disease. Thus it is not likely that all individuals within the different test groups show the same gene expression at the same point in time. This might explain the large difference in standard deviation for both CYP2A and CYP1A1 in the studied organs. It is also interesting to note that the difference in standard deviation seems to be smaller in the infected groups compared with the non-infected groups. As previously mentioned infection suppresses protein synthesis and changes the metabolism. This may explain the general tendency of smaller difference in standard deviation within these groups (Ilbäck 2005). Moreover, the standard deviation is lower for both the EROD and PROD measurements.

Enzymatic activities
PROD activity
The PROD activity corresponded well to the gene expression of CYP2B. The general effect of infection seems to be a decrease in PROD activity. Groups exposed to BDE99, Bromkal and non-exposed groups showed all numerically lower PROD activity in the infected mice compared to the non-infected. This general decrease in PROD activity has previously been showed and discussed by Darnerud et al. (2005). Exposure to BDE99 in non-infected mice significantly increases the PROD activity and the same tendency was observed among the infected groups. This corresponds to the gene expression where BDE99 significantly increases CYP2B expression
among the infected groups and the same tendency was observed in the non-infected groups. The observed tendency that PBDEs induces PROD activity in rodents is supported by Hallgren et al. (2002) in showing a dose-dependent increase in PROD activity in rats after exposure to 2,2',4,4'-tetra-BDE (BDE47).

**EROD activity**
The EROD activity corresponded to the gene expression of CYP1A1 considering the infected mice. The untreated mice and the mice treated BDE99 and Bromkal showed no differences in either gene expression or enzyme activity. However, the EROD activity was induced by PBDE exposure while induction of CYP1A1 gene expression did not occur. Higher EROD activity has been confirmed after oral exposure of penta-BDE in rodents (Fowles et al. 1994, Zhou et al. 2001). The differences between the gene expression and enzyme activity may be a result of the sensitive gene regulation of CYP1A1. The gene expression shuts down within hours when sufficient amounts of the enzyme are produced (Ilbäck 2005). There is a possibility that the amount of RNA decreases more rapid compared to the amount of enzyme. Another explanation might be that the efficiency of the enzyme is decreased due to infection.

**The effect of BDE99 compared with the effect of Bromkal**
The gene expression levels are numerically higher, although not significantly, in the lungs of non-infected groups treated with BDE99 than in mice treated with Bromkal. There were significantly higher gene expression levels for CYP2B in the infected group exposed to BDE99 than in the groups exposed to Bromkal. This was also the case in terms of the corresponding protein level where the same effect was observed in PROD activity. It seems that Bromkal, compared with BDE99, in some cases have a tendency to inhibit induction of the gene expression and synthesis of the corresponding enzyme. The BDE99 used in this study has a purity of 99% whereas Bromkal is a technical mixture that not only contains a minor amount of BDE99 (35%) but also BDE47 (37%). Thus it can been speculated that there could be dioxin-like contaminants from the manufacturing process present as well that influence to effects (Siddiqi et al. 2003, Darnerud 2003). It has been shown that the environmentally prominent congeners BDE47 and BDE99 are among the least active with respect to a dioxin-like behaviour in terms of an Ah receptor (AhR) antagonists that possibly could inhibits TCDD-induced EROD activity. This present results indicate that BDE99 is in active in AhR signal transduction except from the observed weak CYP1A1 induction (Chen & Bunce 2003). This interference with AhR mechanisms might be a possible explanation for the observed differences in gene expression and enzyme activity. Another possible explanation might be a delay in response for one of the substances. However, it is important to remember that this effect is only significant when looking at CYP2B expression and PROD activity in the liver of infected mice. More studies and a chemical analysis of the Bromkal mixture have to be performed before it is possible to draw any further conclusions.

**Conclusions and summary**
Exposure to PBDEs seems to induce PROD and EROD activity in the liver in non-infected mice. A similar tendency was observed for CYP2B gene expression. However, this induction does not occur in infected mice with the exception of BDE99 treatment that induced CYP2B gene expression and corresponding PROD activity. Although CYP1A1 gene expression seemed to be unaffected by PBDE exposure one could draw the conclusion that infection in general decrease both gene expression and enzyme activity and that exposure to PBDE is of moderate importance
for this decrease. The only difference observed in the infected groups both regarding gene expression and enzyme activity could be the result of contaminants of the Bromkal mixture as previously discussed. PBDE exposure induces gene expression and enzyme activity in the liver of healthy animals but the same exposure does not cause a greater decrease in infected animal compared to unexposed animals.

The very poor gene expression of CYP1A1 and CYP2B in the pancreas of non-infected mice might increase the risk of PBDE bioaccumulation in the pancreas. PBDEs have a high level of lipophilicity (WHO 1994). Since distribution is governed by lipophilicity accumulation occur in lipid rich tissues. However, CYP1A1 is normally expressed in human islet cells (Standop et al. 2002). The pancreas has been observed as a target organ for CVB3 infection and apoptosis among the exocrine cells of pancreas has been confirmed (Henke et al. 2000). Moreover, a possible connection between CVB3 infection and idiopathic chronic pancreatitis and insulin-dependent type I diabetes mellitus (IDDM) has also been postulated (Huber & Ramsingh 2004, Whitton 2002, Trianaflou et al. 2004). In the present study is gene expression of CYP1A1 induced after PBDE exposure in CVB3 infected mice. This is interesting since the normal response of infection is deceased gene expression and protein synthesis in order to compensate the increased energy demands of the immune response (Ilbäck 2005). Could a disturbance in this process because of toxic damages due to bioaccumulation or increased energy demands due to PBDE induced gene expression influence the pathogenesis of CVB3 infection and the associated diseases? Mice exposed to TCDD shows a dose-responsive increase in mortality after infection with an otherwise non-lethal influenza virus infection (Warren et al. 2000). Moreover, TCDD has also shown to suppress both the humoral and adaptive (cell-mediated) immune responses, mainly by suppression of T-lymphocyte function resulting in suppression of proliferation, differentiation, T-cell dependent B-cell responses and cytokine production (e.g. of IFNγ and IL-2) (Kerkliet et al. 1996). However, it is not possible to draw any conclusions about this but it is indeed an important field for further research.

As in pancreas the lungs also showed an induction in gene expression in infected mice, but in this case regarding CYP2B expression. Interestingly the effect of PBDE seems to be of no importance for gene expression in the infected mice. The reasons for these two effects are unknown at the present.

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