Cadmium in the Mammary Gland and Neonatal Intestine

Transport Pathways and Interactions with Calcium and Iron

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Abstract

Cadmium is a ubiquitous toxic metal known to cause several adverse health effects in humans. Newborns have an increased gastrointestinal absorption of cadmium compared to adults and seem to be more sensitive to cadmium toxicity. The well-established association in adults between increased uptake of dietary cadmium and low iron status has not been investigated in newborns. The aim of this thesis was to study cadmium during the neonatal period, focusing on intracellular transport pathways and interactions of cadmium with calcium and iron.

Cadmium-induced effects on the lactating mammary gland were investigated in mice and in murine mammary epithelial HC11 cells. Cadmium reduced total intracellular calcium levels, expression of secretory pathway calcium-ATPase (SPCA) and β-casein gene expression both *in vivo* and *in vitro*. An involution-like remodeling of the mammary tissue, including increased fat content, was observed following cadmium exposure. The results indicate that cadmium disturbs the function of the mammary gland by reducing calcium, SPCA and β-casein levels in secreting mammary cells.

The impact of iron status on cadmium transport across the neonatal intestine was investigated in suckling piglets and in human immature intestinal epithelial Caco-2 cells. High iron status did not restrict cadmium absorption in the piglets; instead increased cadmium absorption was detected. Similar results were obtained in immature Caco-2 cells. Gene and protein expressions and localizations of the iron transporters DMT1, DMT1-IRE and FPN1 were not affected by iron status, indicating that the mechanism regulating iron absorption is age-dependent. Gene expression of multidrug resistance associated protein 1 (MRP1) was increased in the immature intestinal cells treated with cadmium and correlated to increased transport of cadmium across the cells.

In conclusion, cadmium exposure may decrease both calcium and protein levels in milk with potential negative developmental effects in the neonate. Furthermore, iron supplementation does not restrict, but rather increases cadmium absorption in newborns, which should be considered in risk assessment of cadmium.

Keywords: cadmium, newborn, calcium, iron, SPCA, DMT1, FPN1, MRP1, mammary gland, neonatal intestine

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To you with interest in the whole mighty cadmium puzzle consisting of more than 29 000 scientific papers.

Science is not about control. It is about cultivating a perpetual condition of wonder in the face of something that forever grows one step richer and subtler than our latest theory about it. It is about reverence, not mastery.

Richard Powers (1991)

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List of Publications

This thesis is based on the work in the following papers, referred to by Roman numerals in the text:

I H. Öhrvik, M. Yoshioka, A. Oskarsson, J. Tallkvist (2006). Cadmium-induced disturbances in lactating mammary glands of mice. *Toxicology Letters* 164, 207-213.

II H. Öhrvik, E. Ullerås, A. Oskarsson, J. Tallkvist (2010). Effects of cadmium on calcium transporter SPCA, calcium homeostasis and β-casein expression in the murine mammary epithelium (submitted).

III H. Öhrvik, A. Oskarsson, T. Lundh, S. Skerfving, J. Tallkvist (2007). Impact of iron status on cadmium uptake in suckling piglets. *Toxicology* 240, 15-24.

IV H. Öhrvik, E. Tydén, P. Artursson, A. Oskarsson, J. Tallkvist (2010). Cadmium transport in neonatal intestinal epithelial cells is correlated to MRP1 expression and not to iron transporters (submitted).

Abbreviations

ABC	ATP-Binding Cassette
ATPase	Adenosine triphosphatase
BCRP	Breast cancer resistance protein
CFTR	Cystic fibrosis transmembrane conductance regulator
DMT1	Divalent metal transporter 1
EFSA	European Food Safety Authority
ER	Endoplasmic reticulum
FPN1	Ferroportin 1
GSH	Glutathione
HBSS	Hank´s balanced salt solution
LD	Lactation day
LDH	Lactate dehydrogenase
ICP-AES	Inductively coupled plasma atomic emission spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
IRE	Iron responsive element
MRP	Multidrug resistance associated protein
MT	Metallothionein
PMCA	Plasma membrane calcium ATPase
PND	Postnatal day
P-gp	Permeability glycoprotein
PTWI	Provisional tolerable weekly intake
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SPCA	Secretory pathway calcium ATPase
TRPV	Transient receptor potential vanilloid
TWI	Tolerable weekly intake
WAP	Whey acidic protein
ZnT	Zinc transporter

1 Introduction

Cadmium has since 1858 been known to cause toxic effects in humans (Nordberg, 2009; Sovet, 1858). Today the metal is widely spread in our environment, naturally occurring as well as due to human activities. No biological function of cadmium has been identified but several adverse health effects in humans are caused by the metal. Cadmium is a divalent metal and have the ability to mimic other divalent metals e.g. calcium and iron. The interference with the homeostasis of these essential metals is fundamental for the cellular toxicity of cadmium. Virtually all food contains cadmium in varying concentrations; consequently, diet is the main source of cadmium for the general population. A large part of the adult population in Europe has a current intake of cadmium that is close to the tolerable dietary intake (EFSA, 2009).

Newborns and infants have a different exposure pattern and an increased absorption of cadmium than adults (Eklund *et al.*, 2001; Crews *et al.*, 2000). In general, newborns are considered to be more susceptible than adults to adverse health effects caused by environmental pollutants (Landrigan, 2004). The neonatal period (newborn period) continues until the newborn is four weeks old and this period is characterized by rapid growth and development of the neonate. During the neonatal period the central nervous system is particularly vulnerable due to the great evolvement and cadmium has been demonstrated to affect it, resulting in neurochemical and neurobehavioral effects (Petersson Grawé *et al.*, 2004b; Andersson *et al.*, 1997). Recently, The European Food Safety Authority (EFSA) stated that the tolerable dietary intake of cadmium in European children might be exceeded about two-fold (EFSA, 2009).

In 1758 Carl von Linné named the group of animals able to produce milk for their offspring *Mammalia*. The great advantage in having the capacity to nourish the offspring in any environment has been crucial for the strong evolution of mammals. In all mammals there is a close relationship between lactating mammary and neonatal gastrointestinal functions. The mammary gland produces milk containing essential nutrients, as well as protective, trophic and digestive factors, all tightly regulated and efficiently transferred from the mother to the neonate (Figure 1). The neonatal intestine is dependent on milk to compensate for the immature digestive and barrier functions (Le Huerou-Luron *et al.*, 2010). There is an intimate connection and a close interplay between the mammary gland and neonatal intestine, which in turn is necessary for adaptation to extrauterine life as well as health and development of the neonate.

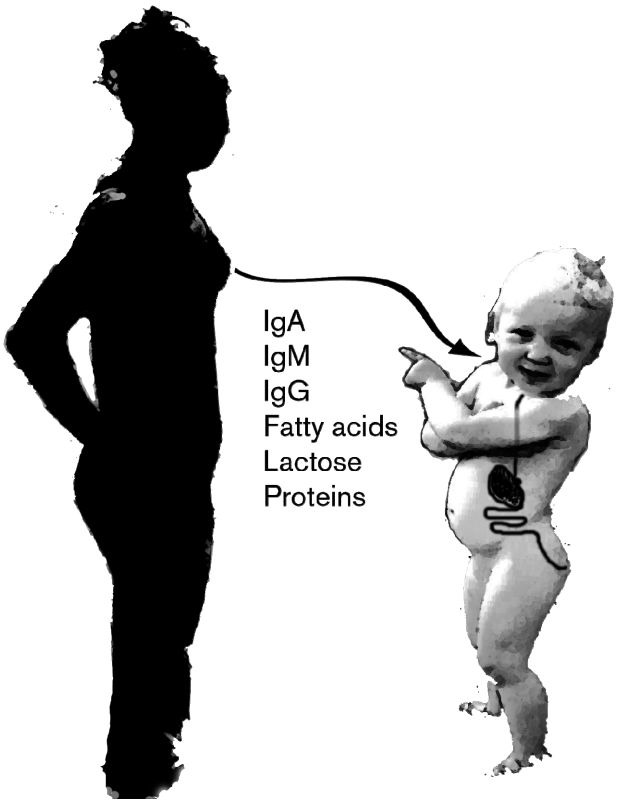

Figure 1. Interplay between mammary gland and neonatal intestine along with the transfer of important nutrients and immunoglobulins from the mother to the newborn.

2 Background

2.1 Cadmium exposure and adverse health effects

Cadmium is a toxic metal naturally occurring in soil, but is also spread in the environment due to human activities. The metal is persistent in the environment and the industrial use of cadmium, mainly in nickel-cadmium batteries and metallurgy of zinc but also in new nano-materials, results in an increasing risk of human exposure (Fowler, 2009).

2.1.1 Adults

Food constitutes the predominant source of cadmium exposure for non-smokers and non-occupationally exposed individuals (EFSA, 2009). Exposure to cadmium originates mainly from intake of vegetable food since cadmium in the soil is readily taken up by plants (Clemens, 2006). In Europe more than 80% of the cadmium intake derives from cereals, vegetables and potatoes (Olsson *et al.*, 2002), although the highest cadmium concentrations are found in oysters, chephalopods and crabs. Cadmium in drinking water and ambient air contribute minimally to the total exposure (Olsson *et al.*, 2002; Vahter *et al.*, 1991).

Already in 1972 the joint FAO/WHO Expert Committee on Food Additives and Contaminations established a Provisional Tolerable Weekly Intake (PTWI) for cadmium of 7-8 μg/kg body weight. Since then, several risk assessments concerning cadmium exposure have been conducted, but not until 2009 EFSA reduced the Tolerable Weekly dietary Intake (TWI) of cadmium to 2.52 μg/kg body weight (EFSA, 2009). In Europe the exposure of cadmium in adults is close to the TWI, and EFSA conclude that subgroups such as vegetarians, children, smokers and people living in

contaminated areas, may have an intake as high as twice the TWI (EFSA, 2009).

The critical adverse health effect of cadmium is nephrotoxicity (Åkesson *et al.*, 2005; Järup *et al.*, 2000; Friberg, 1948). The renal toxicity results from an accumulation of cadmium in the proximal tubules causing impaired reabsorption of nutrients, vitamins and minerals. Cadmium is retained in the kidney with a biological half-life of 10-30 years (Järup *et al.*, 1998). Recently it was observed that negative effects of cadmium on bone mineral density occur at similar low levels of exposure as for early renal effects (Åkesson *et al.*, 2006; Alfvén *et al.*, 2000). Already since 1957, high cadmium exposure has been known to cause both osteomalacia and osteoporosis (Nordberg, 2009; Hagino, 1957). The itai-itai disease is characterized by bone fractures and severe pain caused by decalcification of the bones and renal damages. In 1993, the International Agency for Research on Cancer (IARC) classified cadmium as carcinogenic to humans based on an association between cadmium exposure and increased incidence of lung tumors (IARC, 1993). There are also indications that cadmium induces kidney and prostate tumors in humans (Vinceti *et al.*, 2007; Il'yasova & Schwartz, 2005). Furthermore, there is evidence that cadmium exposure is associated with hormone-related cancers (Åkesson *et al.*, 2008; McElroy *et al.*, 2006).

2.1.2 Neonates

In neonates the exposure route of cadmium is via breast milk, formula or complementary food. Although cadmium levels in milk are low (Floris *et al.*, 2000; Petersson Grawé & Oskarsson, 2000; Hallén *et al.*, 1995; Smith *et al.*, 1991), neurodevelopmental effects have been seen in suckling rodent pups of exposed mothers. However, it is unclear whether these negative effects are caused by low levels of cadmium transferred via milk or by cadmium-induced alterations in milk composition due to effects in the mammary gland (Petersson Grawé *et al.*, 2004a). Observed effects are behavioral disturbances (Petersson Grawé *et al.*, 2004b), altered levels of neurotransmitters (Andersson *et al.*, 1997) and changes in fatty acid composition in the brain (Petersson Grawé *et al.*, 2004a). These effects have been demonstrated at doses well below the doses that cause renal dysfunction in the mothers (WHO, 1992), indicating that the developing offspring might be more sensitive to the toxic effects of cadmium and that the toxic response is age-dependent. Generally, neonates are considered more vulnerable than adults to chemical exposure (Landrigan, 2004), mainly because of rapid growth and development of organs and functions.

2.2 Cellular cadmium metabolism

2.2.1 Ionic mimicry by cadmium

The chemical and physical properties of cadmium is similar to iron, zinc, manganese and calcium. These divalent essential metals have rigorously controlled uptake and metabolism, because they cannot be synthesized or destroyed by the cells. To enable biological actions cadmium has to use transport pathways for essential metals by "ionic and molecular mimicry" (Bridges & Zalups, 2005).

Low status of both calcium and iron increases cadmium absorption in adult animals (Min *et al.*, 2008a; Reeves & Chaney, 2002; Kello *et al.*, 1979) and in adult humans (Kippler *et al.*, 2009a; Olsson *et al.*, 2002; Berglund *et al.*, 1994). This supports the notion that the molecular toxicity of cadmium involves interactions with the transport pathways of these essential metals and is in concordance with the findings that intestinal cadmium uptake in mice is mediated via several different pathways for essential metals (Min *et al.*, 2008a; Min *et al.*, 2008b). There are two separate ways for absorption across the intestinal epithelium; the paracellular- and the transcellular route. The paracellular transport pathway is passive diffusion across the tight junctions between cells and the transcellular pathway is regularly the energy-dependent transport across the two cellular membranes of the enterocyte, involving transport proteins.

Calcium transporters and channels

Cadmium is known to interfere with the tightly regulated levels of intracellular calcium by modifying the function of specific calcium transporters and calcium channels (Bridges & Zalups, 2005). Calcium can be transported paracellularly and transcellularly, mainly depending on calcium status (Khanal & Nemere, 2008). In individuals with normal calcium status the paracellular calcium transport dominates. However, during periods of particularly high calcium demand, e.g. lactation, or calcium deficiency, the absorption predominantly occurs via the transcellular pathway regulated by 1,25-dihydroxyvitamin D and the calcium uptake transporter, transient receptor potential vanilloid 6 (TRPV6, former CaT1) (Khanal & Nemere, 2008). TRPV6 is the main calcium transporter across the apical membrane of the intestine but is also expressed in e.g. mammary tissue (Bolanz *et al.*, 2008; Wissenbach & Niemeyer, 2007).

Once inside the cell calcium binds to calcium binding proteins, such as calbindin and calmodulin to reduce levels of free calcium, since high concentrations of the unbound mineral is toxic to the cell. Calcium is,

besides being necessary for strong bones, a very important intracellular second messenger that controls a wide range of essential biological processes, including many signaling pathways and apoptosis in the cells. The endoplasmic reticulum (ER) is the main storage of intracellular calcium for signaling purposes. Calcium is transported into ER by sarco/endoplasmic reticulum calcium ATPases (SERCAs) and effluxed via inositol-1,4,5-triphosphate receptors and ryanodine receptors (Berridge *et al.*, 2003).

Secretory pathway Ca-ATPase (SPCA) transports calcium into the Golgi apparatus where calcium is required for translation and maturation of secretory proteins (Wuytack *et al.*, 2003). In the lactating mammary gland, calcium in Golgi is needed for phosphorylation of milk proteins (Faddy *et al.*, 2008; Neville, 2005). Plasma membrane calcium ATPase (PMCA) mediates efflux of cytosolic calcium across the basolateral membrane of the intestine into the blood and across the apical membrane of the mammary cell into the milk (VanHouten *et al.*, 2007; Hoenderop *et al.*, 2000).

Cadmium is suggested to affect calcium homeostasis by inhibiting SERCAs, PMCAs and voltage dependent calcium channels (Thévenod, 2009). However, there are studies showing that cadmium stimulates, instead of inhibits, calcium pathways in the cell, indicating that cadmium effects on calcium- transporters and channels are dose-dependent.

Iron transporters

Epidemiological and experimental studies have shown that iron deficiency in adults increases the gastrointestinal absorption of cadmium (Meltzer *et al.*, 2010; Kippler *et al.*, 2007; Bárány *et al.*, 2005; Berglund *et al.*, 1994; Flanagan *et al.*, 1978). Iron absorption occurs predominantly by the transcellular route in the duodenum, independently of iron status. However, the amount of iron absorbed across the intestinal epithelium is dependent on iron status. Two iron transporters, divalent metal transporter 1 (DMT1) and ferroportin 1 (FPN1), are critical for gastrointestinal absorption of non-heme iron (Donovan *et al.*, 2000; McKie *et al.*, 2000; Fleming *et al.*, 1998; Fleming *et al.*, 1997). Ferric iron is converted to ferrous by the duodenal cytochrome reductase b (DCYTB) and then transported across the apical membrane of the enterocyte by the proton-coupled DMT1 (Andrews & Schmidt, 2007). Once inside the cell iron is bound to ferritin and either stored in the enterocyte organelles or transported across the basolateral membrane into the blood by FPN1. Ferrous iron is converted back to ferric by the protein hephaestin that is colocalized with FPN1. Ferric iron is then bound to transferrin, which is responsible for transporting iron in the circulation. The expressions of DMT1, the isoform DMT1-IRE (containing

an Iron Responsive Element) and FPN1 are induced by iron deficiency in adult humans and repressed by iron sufficiency (Nelson *et al.*, 2010; Zoller *et al.*, 2001).

In the mammary epithelial cells both DMT1 and FPN1 are localized to the cytoplasm and are not present at the plasma membranes, indicating a different function than in the intestinal epithelial cells (Leong & Lönnerdal, 2005). Furthermore, the study by Leong & Lönnerdal (2005) indicates that the regulation of DMT1 and FPN1 in the lactating mammary gland appears to be different from that in the intestine. The iron uptake across the basolateral membrane of mammary epithelial cells has instead been suggested to involve the transferrin receptor. Transferrin bound iron and the receptor undergoes endocytosis into endosomes and DMT1 localized at the endosomal membrane efflux iron into the cytosol. Iron can then be utilized by the cell or bind to iron-binding proteins, such as casein, lactoferrin and transferrin and then secreted by exocytosis across the apical membrane (Lönnerdal, 2007).

DMT1 has been shown to transport a broad range of divalent metals *in vitro*, including cadmium, across the apical membrane of the enterocyte (Bannon *et al.*, 2003; Tallkvist *et al.*, 2001; Picard *et al.*, 2000). There are also indications that FPN1 is involved in cadmium absorption in adults (Kim *et al.*, 2007; Ryu *et al.*, 2004). Taken together with the relation between low iron status and high body burden of cadmium, these findings implicate that DMT1 and FPN1 play important roles in cadmium absorption in adults.

2.2.2 Cellular protection against cadmium

Two major systems of cellular protection against cadmium are known; one involving metallothionein (MT) and the other glutathione (GSH) (Klaassen *et al.*, 2009; Liu *et al.*, 2009; Wimmer *et al.*, 2005). Once absorbed into the circulation cadmium is predominantly bound to albumin, but other proteins, peptides and amino acids e.g. MT, transferrin and GSH can also bind the metal and act as carriers (Zalups & Ahmad, 2003). Cadmium is known to bind with high affinity to thiol (SH) groups in various peptides and proteins (Leverrier *et al.*, 2007; Diaz-Cruz, 1997). However, the binding of cadmium to MT has higher affinity than to GSH, due to differences in molecular structure (Vesey, 2010). This indicates that cadmium bound to GSH more readily can interact with other cellular ligands and transporters than cadmium bound to MT.

Metallothionein

Cadmium induces cellular MT, a low-molecular weight cystein-rich protein involved in the binding and detoxification of excessive intracellular levels of cadmium, zinc and copper (Klaassen *et al.*, 2009; Nordberg & Nordberg, 2000; Klaassen *et al.*, 1999; Kagi & Valee, 1960). Four major isoforms of MTs have been identified, MT1-4. MT1 and MT2 are the ones induced following cadmium exposure and are highly expressed in intestine, liver, kidney and pancreas. There are indications that the gene expression and protein level of MTs often show discrepancies (Bourdineaud *et al.*, 2006). MT sequesters cadmium in the cytosol and reduces the level of free cadmium, inhibiting the metal to damage biological proteins vital for the cell (Liu *et al.*, 1993). The importance of MT in limiting cadmium toxicity has been proven in MT-null mice that are hypersensitive to cadmium-induced effects in the kidney, although they had lower cadmium concentrations in their tissues (Liu *et al.*, 2000). Cadmium-MT complexes are transferred from the liver to the kidney where they are filtered through the glomerulus and reabsorbed in the proximal tubules. There are indications that cadmium-MT complexes enter lysosomes in the tubular cells, where they are degraded and free cadmium is released into the cytosol (Dorian *et al.*, 1992; Min *et al.*, 1992a).

It has been proposed that intestinal cadmium absorption may be limited by MT, which is synthesized in the intestinal epithelium following oral cadmium exposure (Kimura *et al.*, 1998; Min *et al.*, 1992b). However, studies with MT-null mice have not supported this hypothesis (Liu *et al.*, 2000; Liu & Klaassen, 1996), rather concluded that the impact of intestinal MT on cadmium absorption is of minor importance (Klaassen *et al.*, 2009). MT is also induced by oxidative stress and plays an important role in the cellular protection against free reactive oxygen species (ROS) (Chiaverini & De Ley, 2010; Min *et al.*, 2005; Klaassen *et al.*, 1999; Thornalley & Vasak, 1985).

Glutathione

The antioxidant GSH plays a fundamental role in defending the cell against oxidative stress and detoxification of xenobiotics. Oxidative stress or imbalance between generation of ROS and cellular antioxidant production may cause lipid peroxidation, protein modifications and DNA damage. Formation of ROS can also activate several intracellular signaling pathways, in conformity with calcium.

Acute cadmium exposure results in a rapid reduction in GSH levels due to the formation of cadmium-GSH complexes. This is believed to be the

first protection against free cadmium in the cytosol (Singhal *et al.*, 1987; Dudley & Klaassen, 1984). However, chronic exposure to the metal often results in elevated tissue levels of GSH (Kamiyama *et al.*, 1995), which will reduce the oxidative damage caused by cadmium. Despite the fact that cadmium is not a redox metal, there are numerous *in vivo* and *in vitro* studies suggesting that the cadmium indirectly generates ROS in mammalian cells (Pourahmad *et al.*, 2003; Yamano *et al.*, 2000; Thévenod & Friedmann, 1999). Cadmium has been suggested to induce ROS in three different ways: 1) by displacement of redox metals bound to MT (Dorta *et al.*, 2003); 2) by damaging critical cellular organelles, e.g. mitochondria (Wang *et al.*, 2004); 3) by a reduction and depletion of intracellular radical scavengers, such as GSH (L'Hoste *et al.*, 2009; Nigam *et al.*, 1999). Furthermore, cadmium exposed MT-null mice show stronger response in ROS-related gene expressions than wild type mice (Liu *et al.*, 2002), supporting the hypothesis that MT and GSH play complementary roles in the cellular protection against cadmium toxicity. Cadmium may also affect antioxidant enzymes as cytochrome oxidase complex (COX) catalase and superoxid dismutase (SOD) (Cannino *et al.*, 2009; Joseph, 2009; Badisa *et al.*, 2008). In a recent long-term low dose study in mice, cadmium exposure resulted in a biphasic oxidative stress response showing acute induction of MTs and the thioredoxin gene Prdx2 (Thijssen *et al.*, 2007). The late response included increase in the gene expression of several enzymes involved in eliminating ROS and indicated that the cells were able to adapt to the cadmium exposure. The thioredoxins play an important role in reducing ROS, together with e.g. GSH and SOD.

ABC transporters

The ATP-binding cassette (ABC) transporter superfamily is responsible for the active transport of a wide variety of compounds across biological membranes, including endo- and xenobiotics (Higgins, 2007). The family consists of seven subfamilies, including the three families: multi drug resistance protein permeability glycoprotein (P-gp), breast cancer related protein (BCRP) and multidrug resistance associated protein (MRP1-13) (Leslie *et al.*, 2005). In eukaryotes all ABC-transporters are efflux proteins involved in cellular- and tissue protection. In addition, to induce drug resistance (as implicated by the name), multidrug resistance associated proteins and P-gp reduce the accumulation of toxic substances and are thereby involved in the cellular defense. MRP1 is ubiquitously expressed in the whole body and located at the basolateral side of most cells, where it transports substrates into the blood (Cole & Deeley, 2006; Cole, 1992). In

the intestinal epithelium the overall gene and protein expression of MRP1 is low (Berggren *et al.*, 2007; Prime-Chapman *et al.*, 2004). Despite this, MRP1 has been demonstrated to play a critical role in protecting the small intestine from toxic substances (Kato *et al.*, 2009). MRP2 and P-gp are mostly expressed in tissues important for xenobiotic protection, such as intestine, liver blood-brain barrier and placenta, where they are located at the apical membrane effluxing their substrates (Haimeur *et al.*, 2004; Kimura *et al.*, 2004). MRP1 and MRP2 can actively transport GSH-complexes (Cole & Deeley, 2006; Dietrich *et al.*, 2001; Mao *et al.*, 2000) and have, in accordance with P-gp, broad substrate specificity to neutral and positively charged hydrophobic compounds (Klaassen & Aleksunes, 2010; del Amo *et al.*, 2009; Zaman *et al.*, 1995). The formation of ROS as well as altered levels of intracellular GSH have been suggested to regulate the gene expression of MRP1 (Tatebe *et al.*, 2002; Yamane *et al.*, 1998).

Several ABC efflux proteins have been proposed to be implicated in cadmium transport and MRP1 is suggested to be the most probable candidate (Thévenod, 2010). It has been shown *in vitro* that cadmium-GSH complexes are transported across the basolateral membrane of enterocytes by MRP1 (Li *et al.*, 1997; Tommasini *et al.*, 1996). A recent study found that cells with higher cadmium resistance show increased mRNA and protein levels of MRP1, as well as increased activity of the transporter, indicating that MRP1 protects the cell against cadmium toxicity (Oh *et al.*, 2009). The apical efflux proteins MRP2 and P-gp have also been demonstrated to be upregulated by cadmium (Huynh-Delerme *et al.*, 2005; Terlouw *et al.*, 2002), suggesting that these ABC transporters may reduce cadmium uptake from the intestinal lumen. Furthermore, the ABC efflux protein CFTR (cystic fibrosis transmembrane conductance regulator), which is an apical chloride channel, has recently been implicated in cadmium efflux in the renal tubule cells (L'Hoste *et al.*, 2009). CFTR is also localized in the apical membrane of enterocytes and, although not investigated, may therefore limit cadmium uptake from the intestinal lumen (Jakab *et al.*, 2010).

2.3 Lactating mammary gland

The mammary gland is a highly specialized secretory organ that is tightly regulated by a complex interplay between lactogenic and steroid hormones, e.g. prolactin and estrogen (Lamote *et al.*, 2004; McManaman & Neville, 2003). Milk production and secretion is highly conserved between species, even though the composition of milk differs. There are five main pathways for secretion of milk components: 1) Exocrine pathway responsible for the

major milk proteins (caseins and whey) and lactose; 2) Milk lipid pathway secreting triglycerides in large droplets called milk fat globuli; 3) Passive diffusion pathway of small molecules as sodium, potassium and chloride across membranes into the milk; 4) Transcytotic pathway transfers immunoglobulins via receptors, pinocytosis and exocytosis into milk; 5) Paracellular pathway, though the tight junctions is closed during full lactation and only accessible during pregnancy or mastitis.

Lactating mammary epithelial cells synthesize 80-95% of the proteins present in milk, including caseins, α-lactalbumin and whey acidic protein (WAP). Milk proteins are secreted into the milk alveolar lumen via the exocrine pathway (Burgoyne & Duncan, 1998; Jensen, 1995). Lactose, calcium and phosphate are also partially secreted by this pathway. Milk proteins are implicated in numerous physiological functions of significant importance for the development of the suckling neonate (Lönnerdal, 2003).

The milk protein β-casein, one of the major constituents of the casein family, significantly contributes to the overall nutrition of the suckling offspring (Lönnerdal, 2003; Jensen, 1995; Greenberg *et al.*, 1984). Besides providing a source of amino acids to the developing neonate β-casein increases the bioavailability of calcium by forming micelles that keep the mineral soluble (Holt & Sawyer, 1988). The gene expression of β-casein in mice increases during late gestation and peaks during lactation (Robinson *et al.*, 1995). Furthermore, it has been shown that calcium is required for synthesis and phosphorylation of β-casein (Burgoyne & Duncan, 1998; Duncan & Burgoyne, 1996).

The lactating mammary gland transports as much as 400 mg of calcium across the mammary epithelial cells every day (Prentice, 2000). It is an impressive work load that the secretory mammary epithelial cells are coping with when mediating unidirectional transport of millimolar amounts of calcium from the blood ([Ca2+] = 1-3 mM) into milk ([Ca2+] = 3-8 mM) and at the same time maintaining cellular calcium homeostasis. The massive transport of calcium is tightly regulated by a range of calcium transporters and channels to protect the cell from calcium cytotoxicity as well as disturbances in cellular signaling (Shennan, 2008). The Golgi apparatus handles a large intracellular calcium pool and therefore it is necessary to have active transporters that efficiently transfer calcium into the Golgi. In an experimental model of mammary gland involution, Reinhardt and Lippolis (2009) found decreased calcium levels together with reduced expression of the calcium transporter SPCA, which is located in the Golgi membranes of mammary epithelial cells.

Iron transport across the mammary epithelial cells is believed to be mainly mediated by the transferrin receptor and released into the milk by exocytosis, as described above in the section about iron transporters.

Besides accumulating in the kidney cortex cadmium has also been demonstrated to be retained in mammary glands of rodents (Petersson Grawé & Oskarsson, 2000; Bhattacharyya *et al.*, 1982; Bhattacharyya *et al.*, 1981). The mammary gland retention is in line with the observation that the levels of cadmium in human, ruminant and rodent milk is low, indicating that the mammary gland partially acts as a barrier protecting the suckling offspring from exposure to the toxic metal (Floris *et al.*, 2000; Petersson Grawé & Oskarsson, 2000; Hallén *et al.*, 1995; Smith *et al.*, 1991). Since cadmium sequesters in the mammary gland there is a risk that the metal disturbs the normal function of this tissue and interferes with milk synthesis. In accordance with this, cadmium may be a contributing factor to the reduced milk protein levels from mothers with high cadmium exposure (smokers) compared to levels detected in milk from mothers with low cadmium exposure (non-smokers) (Milnerowicz & Chmarek, 2005). Furthermore, in epidemiologic studies a negative association between cadmium and calcium in milk has been found (Kippler *et al.*, 2009b; Honda *et al.*, 2003).

2.4 Neonatal intestinal epithelium

The intestine has digestive and absorptive functions and is one of the most important physiological barriers protecting the neonate against exposure to toxic substances. During the immediate neonatal period the gastrointestinal tract undergoes intense growth, morphological changes and functional maturation. These changes are closely related to the first days ingestion of colostrum, which is the breast milk rich in immunoglobulins, enzymes, hormones, growth factors and neuroendocrine peptides (Brandtzaeg, 2010; Sheard & Walker, 1988).

The crypt stem cell of the intestine differentiates into four cell types; enterocytes that constitutes more than 80% of the total cells, goblet cells that produce mucins, enteroendocrine cells and Paneth cells that secrete antimicrobal-, digestive- and growth factors. The enterocyte, goblet and enteroendocrine cells differentiate as they migrate from the crypt up to the villous tip, where they exfoliate from the tip into the intestinal lumen, within five days in the adult. The migration rate from the crypt to the villous tip has been demonstrated to be slower in newborn animals than in adult (Pacha, 2000). In newborns the gastric pH is high (6-8) compared to

adults (about 2) and the gastric emptying is slower reaching adult levels not until the age of six months. Maturation of the intestinal barrier occurs around gestational week 38, but continues in the neonatal period, which enables the newborn to absorb macromolecules (e.g. IgGs) from the milk (Newburg & Walker, 2007). Together with low levels of plasma proteins, high relative total body water and immature kidney function these age-dependent changes generally result in increased absorption, higher amount of unbound substances in plasma and decreased excretion in the newborn (Yokoi, 2009).

The oral absorption of cadmium is estimated to be 3-5% in the adult population (EFSA, 2009). However, the absorption rate of cadmium depends on several factors as mentioned above, including age, status of iron and calcium as well as body burden of cadmium (Flaig *et al.*, 2003). Oral absorption rates of 20-40% have been observed in young individuals and infants (Horiguchi *et al.*, 2004; Kikuchi *et al.*, 2003; Crews *et al.*, 2000). The high absorption in infants is probably due to the immaturity of the intestinal epithelium. In addition to a high absorption rate, infants may also a higher intake of cadmium per kg body weight due to specific exposure pattern.

In neonates calcium is predominately absorbed via the paracellular pathway as in adults with normal calcium status (Bass & Chan, 2006). Several calcium transporters, as mentioned above, rigorously control the calcium level in breast milk and therefore the neonate is breast fed with sufficient amounts of calcium. In accordance, the intestinal calcium transporters involved in transcellular calcium transport in adults with low calcium status have so far not been identified in the neonatal intestine (Figure 2).

Developmental changes in the intestinal absorption of iron in neonates have been suggested (Leong *et al.*, 2003b; Domellöf *et al.*, 2001). Thus, an epidemiologic study on breast-fed infants indicated that intestinal iron absorption is not regulated until 6-9 months of age (Domellöf *et al.*, 2001), and studies on suckling rat pups demonstrated that iron absorption and expressions of DMT1 and FPN1 are not regulated until the time of weaning (Leong *et al.*, 2003a; Leong *et al.*, 2003b) (Figure 2). Instead, it is believed that iron absorption from milk in newborns predominantly occurs via receptor-mediated endocytosis of lactoferrin bound iron (Suzuki *et al.*, 2001).

The expression of a majority of the ABC transporters in the neonatal intestine is at present not investigated. However, the expression of P-gp in mice has been demonstrated to be low at birth and continuously increasing until weaning when the P-gp expression reaches adult levels (Mahmood *et*

al., 2001). Peptide transporter 1 and apical sodium dependent bile acid transporter have been found to have a biphasic expression pattern with elevated levels at birth and again at weaning (Klaassen & Aleksunes, 2010).

In addition, neonatal tissues generally express higher levels of MT than adults (Yoshida *et al.*, 1998; Bakka & Webb, 1981). In newborn rats the concentration of MT is more than 10 times higher than in adult animals (Goering & Klaassen, 1984). Despite this, MT had no limiting effect on the intestinal absorption of cadmium in the newborn offspring to MT-null mice (Brako *et al.*, 2003).

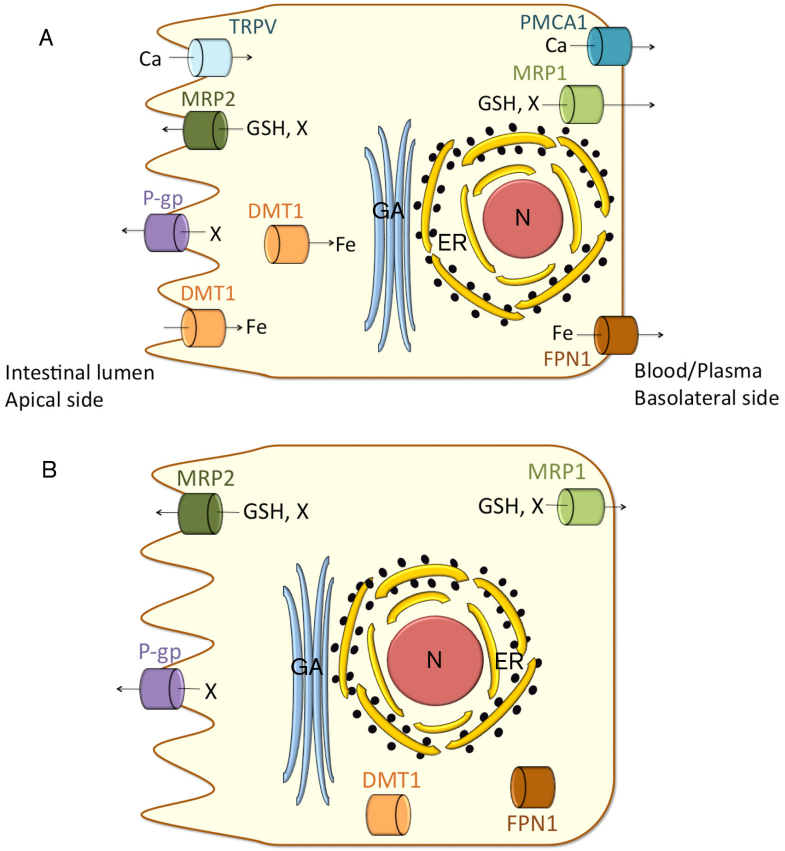

Figure 2. Calcium, iron and ABC-transporters in enterocytes of adult (mature) (A) and neonatal (immature) (B) intestine. TRPV, transient receptor potential vanilloid; PMCA1, plasma membrane calcium ATPase 1; DMT1, divalent metal transporter 1; FPN1, ferroportin 1; MRP2, multidrug resistance associated protein 2; P-gp, plasma glycoprotein; MRP1, multidrug resistance associated protein 1; GSH, glutathione; X, endogenous or exogenous substrate; N, nucleus; ER, endoplasmic reticulum; GA, Golgi apparatus.

3 Aims of the thesis

The overall aim was to study cadmium during the neonatal period, focusing on intracellular transport pathways and interactions of cadmium with calcium and iron. *In vivo* and *in vitro* models were used to study cadmium in epithelial cells of two critical tissues during early life: the lactating mammary gland and the neonatal intestine (Table 1).

The specific aims were to:

1. Investigate effects of cadmium in the lactating mammary glands and examine possible mechanisms for induced disturbances in mammary epithelial cells (paper I & II).

2. Determine the impact of iron status on cadmium absorption in neonatal intestine and examine transporters involved in cadmium transport across immature enterocytes (paper III & IV).

The long-term aim was to provide new insights in cadmium toxicity during early life and elucidate differences in the effects of cadmium between newborns and adults.

Table 1. *Overview of studies included in this thesis*

Model	Lactating mice	Secreting mammary epithelial HC11 cells	Suckling piglets	Immature intestinal epithelial Caco-2 cells
Paper	I & II	II	III & IV	IV
Aim	Study effects of cadmium on lactating mammary glands during peak lactation.	Study effects of cadmium on calcium homeostasis in mammary epithelial cells.	Study cadmium uptake in suckling piglets with different iron status.	Study transporters involved in cadmium absorption in neonatal intestine and impact of iron.
Cadmium exposure	5, 100 or 2000 μg/kg bw/day subcutaneously for 3 days	0.006, 0.13 or 2.5 μM for 3 days	20 μg/kg bw/day orally for 6 days	1 μM for 7 days
Toxicity parameters	Kidney histopathology	Intracellular ATP	Piglet weight	^{14}C-mannitol transport
		LDH leakage	Hemoglobin value	
Element analysis	Calcium, zinc, copper & iron	Calcium	Cadmium	-
Gene expression	β-casein & α-lactalbumin	β-casein, WAP & SPCA	-	DMT1, DMT1-IRE, FPN1, MRP1, MRP2, P-gp & MT1
Protein expression and/or localization	SPCA	-	DMT1, FPN1, MT & MRP1	DMT1, FPN1 & MRP1
N	6 mice/group; 4 groups	3-7 wells/group; 4 groups	4 piglets/block; 8 blocks	3-6 monolayers/group; 4 groups
Experimental design	Dose response:	Dose response:	2 x 2 factorial	2 x 2 factorial
	Control & 3 dose levels	Control & 3 dose levels	iron & cadmium	iron & cadmium

4 Materials and methods

This section is an overview of the methods used in this thesis. For further details, the reader is referred to the individual papers (I-IV).

4.1 Experimental models

To gain mechanistic insights into the cellular effects of cadmium in the lactating mammary gland and the neonatal intestine this thesis consists of a combination of *in vivo* and *in vitro* studies.

4.1.1 Lactating mice (paper I & II)

Cadmium-induced effects in the lactating mammary gland were studied by using lactating mice. NMRI-mice were given a standard pellet diet and tap water ad libitum. Litter sizes were normalized to eight on lactation day (LD) 2 and the dams with litters were kept in individual cages (the day of parturition = LD 0). On LD 8 the dams and their litters were randomized into four dose groups, each containing six animals. On LD 8, 9, and 10 the dams were given subcutaneous injections of cadmium (5, 100, 2000 μg Cd^{2+}/kg body weight as $CdCl_2$ dissolved in NaCl), whereas controls were given only saline. The lowest cadmium dose used is in the same magnitude as the PTWI value of 2.5 μg Cd^{2+}/kg body weight (EFSA, 2009). The two higher cadmium doses were chosen to examine possible dose-response effects. In paper II only the highest dose were used.

Animals were euthanized in random order by CO_2-asphyxiation on LD 11 and samples of the mammary glands were dissected and stored at -70°C pending isolation of total RNA, proteins and calcium analyses. Samples of the mammary glands and kidneys were also taken and fixed in paraformaldehyde/glutaraldehyde for histological examination. To minimize possible individual variations left inguinal mammary glands (4 and 5) were

dissected from all the 24 dams. All animal experiments were approved by the Local Ethics Committee of Animal Research (permit no. C 159/2).

4.1.2 Murine mammary epithelial HC11 cells (paper II)

The HC11 cells are non-tumorigenic and originally derived from murine mammary tissue taken at mid-gestation (Danielson *et al.*, 1984). When treated with prolactin, cortisol and insulin the HC11 cells adopt a secretory phenotype resembling mammary epithelial cells in the lactating mammary gland, including increased β-casein gene and protein expression (Desrivieres *et al.*, 2003; Ball *et al.*, 1988).

HC11 cells were cultured in RPMI 1640 media supplemented with fetal calf serum, insulin, epidermal growth factor (EGF) and gentamycin in polycarbonate flasks. Medium was changed every 2 or 3 days and cells sub cultured every 3 or 4 days. HC11 cells were seeded at a density of 1.5×10^5 cells/cm^2 and two days postconfluency, the medium was replaced by serum-free medium without EGF containing prolactin and cortisol to promote differentiation of the cells into a secretory phenotype and incubated for 72 h. HC11 cells were exposed to nontoxic cadmium and /or nifedipine concentrations that neither affected intracellular ATP levels nor LDH leakage into the medium. Cells were treated with medium containing 0.006, 0.13, or 2.5 μM cadmium; 1 or 10 μM nifedipine; 1 μM nifedipine and 2.5 μM cadmium during the differentiation. Cells treated with medium without cadmium or nifedipine supplementation were used as controls.

Cytotoxicity was assessed by the determination of intracellular ATP and medium lactate dehydrogenase (LDH) levels. The ATP assay is based on the firefly luciferase enzyme's requirement for ATP in producing light. The cytosolic enzyme LDH is released in cell media upon cell lysis and is based on the enzyme's capacity to convert a tetrazolium salt into a red formazan product. HC11 cells were differentiated in the presence of cadmium (0.8 pM - 1 mM) (vehicle NaCl), nifedipine (125 nM - 10 μM) (vehicle DMSO) or cadmium+nifedipine (0.125 - 2.5 μM + 0.1 - 10 μM) for 72 h. Luminescence, as an indicator of intracellular ATP-levels, and absorbance at 490 nm, as indirect LDH measurement were, measured in a multiplate reader.

4.1.3 Suckling piglets (paper III & IV)

Pigs are omnivorous with anatomical and physiological conditions of the gastrointestinal tract that resemble those of humans, in particular the duodenum is similar, but also the diet composition, gastric pH and transit time for food (Miller & Ullrey, 1987).

Purebred Yorkshire piglets (n = 32, same boar) were held indoors together with their respective sow. All piglets were individually tattooed with a number in the ear and no routine medical treatment was given. The animal experiments were approved by the Local Ethics Committee of Animal Research (permit no. C 29/5).

Piglets in paper III were divided into eight similar blocks (four piglets per block) on postnatal day (PND; day of birth = 0) 2 according to the following criteria: litter, gender and weight. Each animal of a block was randomly assigned to one of the four treatments: iron deficient not given cadmium (C), iron supplemented not given cadmium (Fe), iron deficient given cadmium (Cd) and iron supplemented given cadmium (Fe+Cd). In paper IV one control (C) and one iron supplemented (Fe) piglets were used. Iron supplementation of the piglets was performed by administration of iron dextran intramuscularly on PNDs 3 and 14. This routine iron deficiency prophylaxis treatment is recommended by The Swedish Animal Health Service and performed on piglets in pig production. If not supplemented with iron, piglets become iron deficient a few days after birth, due to low levels of iron in the sow's milk, in combination with the rapid growth and expansion of hemoglobin mass (Szabo & Bilkei, 2002).

Cadmium exposed piglets (Cd, Fe+Cd) were orally given cadmium on the basis of the daily weight on PNDs 10-15 by a blunt syringe extended with a plastic tube directly in the pharynx. At each administration the piglets were given 20 μg cadmium/kg body weights solved in glycerol and piglets not given cadmium (C, Fe) were given equal volumes of glycerol orally. In between administrations, all piglets were kept with their respective sow and allowed to suckle. On PND 16, piglets were killed in random order by an intraperitoneal overdose of pentobarbital. Blood, kidney cortex, and duodenum samples were collected and stored at -70°C prior to cadmium analyses and protein isolation. Samples of the duodenum were dissected and fixed in formalin for immunohistochemistry. To minimize possible individual variations all piglets were dissected and samples taken in the same sequences each time.

4.1.4 Immature human intestinal epithelial Caco-2 cells (paper IV)

In paper IV, 7 days postconfluent human intestinal Caco-2 cells, which are not fully differentiated, were used as an experimental model of neonatal intestine (referred to as "immature Caco-2 cells"). To validate the immature Caco-2 cell model, barrier function, size and localizations of the nuclei and of iron transporters were assessed (Bravo *et al.*, 2004; Anderle *et al.*, 2003; Ma *et al.*, 2002; McKie *et al.*, 2000; Chantret *et al.*, 1988). Twenty-one days

postconfluent Caco-2 cells, which are fully differentiated (mature), have been extensively used as an in vitro model of the adult human intestinal epithelium (Artursson *et al.*, 2001). Under normal cell culture conditions, Caco-2 cells spontaneously differentiate into a phenotype that resembles polarized absorptive enterocytes (Artursson, 1990; Chantret *et al.*, 1988; Pinto M, 1983). The Caco-2 model has been widely used to investigate cadmium absorption and effects (Cardin *et al.*, 2009; Boveri *et al.*, 2004; Bannon *et al.*, 2003; Tallkvist *et al.*, 2001; Jumarie *et al.*, 1997). Mature Caco-2 cells treated with iron have reduced uptake and transport of cadmium that correlates to lower DMT1 levels (Tallkvist *et al.*, 2001).

The Caco-2 cells were expanded in normal tissue culture flasks in DMEM supplemented with fetal calf serum and gentamycin and seeded onto permeable polycarbonate filters at a density of 2.5 x 10^5 cells/filter support (Ø 12 mm, pore radius 0.4 μm). The Caco-2 cells were then allowed to differentiate for 7 days postconfluency prior to transport and uptake experiments, analyses of DMT1, DMT1-IRE, FPN1, MRP1, MRP2, P-gp and MT1 gene expressions, and DMT1, FPN1 and MRP1 protein localizations. The medium was changed every second day and the cells were daily examined by reverse phase microscopy for confluency, shape of cells and dome formations.

Each cell monolayer was randomly assigned to differentiate for 7 days postconfluency in one of the four experimental media: control (C) media or media supplemented with: 65 μM iron ($Fe(NO_3)_3$ x $9H_2O$ dissolved in a fourfold molar excess of nitrilotriacetic acid (NTA) (Fe), 1 μM cadmium ($CdCl_2$ x H_2O) (Cd) and both 65 μM Fe and 1 μM Cd (Fe+Cd).

Transport and uptake experiments were performed at 37°C, pH 7.4, in HBSS buffered with HEPES. Three monolayers for each experimental media and each radiolabeled metal were transferred to new wells where pre-warmed HBSS was added to the apical (0.5 ml, pH 6.3, supplemented with L-ascorbic acid) and basolateral (1.5 ml, pH 7.4, supplemented with human apo-transferrin) chambers. The apical solution was either supplemented with 30 nM ^{109}Cd or 20 μM ^{59}Fe. To determine the continuous transfer of ^{109}Cd or ^{59}Fe across the monolayers samples were taken from the basolateral chambers at 30 min intervals for 3 h and the cell monolayers were at the same time transferred to new wells containing pre-warmed HBSS. The radioactivity was measured by γ-spectrometry. To determine the apical uptake of ^{109}Cd or ^{59}Fe into the cells following the 3 h incubation, the filters with the monolayers were rinsed in ice cold HBSS and dissolved in 0.5 M NaOH, followed by γ-spectrometry of an aliquote of the lysate.

The integrity of the monolayers was controlled by determining of the permeability of the hydrophilic marker ^{14}C-mannitol, to ensure that none of the pretreatments used in the transport studies impaired the monolayer integrity.

4.2 Experimental techniques

Unless otherwise stated, the preparation and analyses of samples were performed at the Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden.

4.2.1 Mineral and cadmium analyses (paper I-III)

Total intracellular calcium, zinc, copper and iron

Total cellular calcium concentrations in the mouse mammary glands (paper I) and HC11 cells (paper II) were determined using inductively coupled plasma–atomic emission spectrometry. In addition, total concentrations of zinc, copper and iron were measured in the mammary glands. Tissues and cells were digested by a mixture of 65% HNO_3, 70% $HClO_4$ and 95% H_2SO_4 and diluted with 1 M HNO_3. The method used is a modification of an accredited (SWEDAC, Swedish Board for Accreditation and Conformity Assessment) in-house method. The analytical accuracy was checked against reference material. The sample preparation and analyses of total calcium concentrations were performed at SVA, National Veterinary Institute in Uppsala, Sweden.

Short-term calcium fluxes

Effects of cadmium on calcium transporters and/or channels were investigated in ionomycin stimulated HC11 cells using nifedipin as a positive control (paper II). The calcium ionophore ionomycin induces a rapid elevation of the intracellular calcium levels, allowing studies in short-term calcium fluxes. Cells were seeded in 96 well plates and two days postconfluency the media were removed and the cells were incubated with a fluorescent calcium indicator (Fluo-4). Probenecid was used to inhibit efflux of the indicator out of the cell during the pretreatment with Cd (2.5 μM), nifedipine (10 μM) or Cd + nifedipine (2.5 μM + 10 μM) for 10 min. Ionomycin (10 μM) was added at time 0. Fluorescence was measured at 485 nm (excitation) and at 535 nm (emission) with a multiplate reader at time 0 (background fluorescence measurement F_0) and then at 20 sec intervals for 250 sec. The changes in fluorescence where analyzed as a function of time

and expressed as $(F-F_0)/F_0$ x 100 (%), where $F-F_0$ denotes the change in fluorescence occurring during ionomycin stimulation and F_0 represents the fluorescence level of cells before stimulation (time 0).

Cadmium concentrations

The concentrations of cadmium in piglet whole blood, kidney and iron supplements were determined by inductively coupled plasma-mass spectrometry (paper III). The blood samples were prepared and analyzed according to Bárány *et al.* (1997). The kidney samples were acid digested in concentrated nitric acid and diluted with deionized water. The cadmium concentration in the iron dextran supplement (Pigeron vet) was below the detection limit (30 ng cadmium/l). The analytical accuracy was checked against reference material. The sample preparation and analyses were performed at Lund University Hospital, Sweden.

4.2.2 Gene expression (paper I, II & IV)

Total RNA from lactating mammary glands was isolated by the use of TRIzol reagent (paper I) and from HC11 and Caco-2 cells by NucleoSpin RNA II kit containing Dnase I (paper II & IV). The integrity of the RNA was confirmed by agarose gel electrophoresis and quantification of the RNA was performed with the RNA specific Quant-iT RiboGreen protocol. Gene expressions were normalized to total RNA.

Quantification of gene expressions of the proteins presented in Table 2 were performed by real-time RT-PCR using Mastermix for SYBR Green I (paper I) and QuantiTect SYBR Green RT-PCR (paper II & IV) reagents. Primer concentration was 0.4 μM and 200 or 500 ng total RNA was used as template. Gene-specific primers for mouse β-casein, α-lactalbumin, WAP and SPCA2 (paper I & II) and human DMT1, DMT1-IRE, FPN1, MRP1, MRP2, P-gp and MT1 (paper IV) were designed by using NCBI primer designing tool or Primer3 and all PCR products were sequenced and blasted against the mouse or human genome (Table 2). In the analyses five known concentrations of the primer-specific cDNA, and a non-template control served as internal controls. Melt curve analysis was performed for each sample to check the specificity of the obtained PCR products. Relative quantification of mRNA expressions was performed by comparing the threshold cycle (Ct) between controls and treated cells according to the $2^{-(\Delta Ct)}$-method (Livak & Schmittgen, 2001). Fold differences were calculated setting untreated control to one.

Table 2. Primer sequences used for quantitative real-time PCR

Primer	Oligo sequence	NCBI GenBank Accession No.	Product size (bp)
β-casein forward	5′-CTTAACCCCACCGTCCAAT-3′	NM_009972	143
β-casein reverse	5′-AGCATGATCCAAAGGTGAAAA-3		
α-lactalbumin forward	5′-TGATGCATTTCGTTCCTTTG-3	NM_010679	148
α-lactalbumin reverse	5′-AAAACACAGGCCCATTCAAG-3		
WAP forward	5′-TATCATCTGCCAAACCAACG-3′	NM_011709	185
WAP reverse	5′-GGTCGCTGGAGCATTCTATC-3		
SPCA2 forward	5′-ACTCCGGCACATGCTCGCAC-3′	NM_026922	149
SPCA2 reverse	5′- CGCATGGCTCCACTTCGCCT-3′		
DMT1 forward	5′-CGTGGCGGATTGCAGGAGGA-3′	NM_001174126	124
DMT1 reverse	5′-ACGCTGACCACAGCAGCCAC-3′		
DMT1-IRE forward	5′-GCCATCAGAGCCAGTGTGTTTCT-3′	NM_001174125	198
DMT1-IRE reverse	5′-TGTCAGCTTTTCAAAGATCCCACC-3′		
FPN1 forward	5′-CGAGATGGATGGGTCTCCTA-3′	NM_014585	219
FPN1 reverse	5′-GGCTACGTCGAAAATGTGGT-3′		
MRP1 forward	5′-GCAAATCCAGGAGACAGCTC-3′	NM_004996	113
MRP1 reverse	5′-TGATGTGCCTGAGAACGAAG-3′		
MRP2 forward	5′-CTGGTTGGGAACCTGACTGT-3′	NM_000392	172
MRP2 reverse	5′-CAACAGCCACAATGTTGGTC -3′		
P-gp forward	5′-GCTGTTAAGGAAGCCAATGC-3′	NM_000927	120
P-gp reverse	5′-AGCAATGGCGATTCTCTGTT-3′		
MT1 forward	5′-GCAAATGCAAAGAGTGCAAA -3′	NM_005946	213
MT1 reverse	5′-ATGGGTCAGGGTTGTATGGA -3′		

4.2.3 Protein expression and localization (paper II, III & IV)

Western Blot

Microsomal proteins were isolated from mouse mammary gland and total proteins from piglet duodenum. Frozen tissues were homogenized in Tris-HCL (mammary) and Ripa lysis buffer (duodenum) containing protease inhibitors. The mammary homogenate was sedimented at 6 500 x g for 20 min to remove intact cells, nuclei and mitochondria. To obtain the microsomal fraction the resulting supernatants were centrifuged at 140 000 x g for 90 min. Duodenum homogenate was incubated 30 minutes on ice and centrifuged at 16 000 x g for 30 minutes to remove insoluble materials. Microsomal fraction and total protein concentrations were quantified using the bicinchoninic acid (BCA) protein assay.

The SPCA protein expression in mouse mammary tissue, and DMT1, FPN1 and MT protein expressions in piglet duodenum were quantified with Western blots. Aliquots of mammary microsomes and piglet duodenum proteins were separated by SDS-PAGE. Separated microsomes and proteins were transferred to nitrocellulose membranes, and equal loading and transfer was confirmed by Ponceau S staining. Membranes were blocked followed by primary antibody incubation SPCA, DMT1, FPN1 or MT. Horse-radish-peroxidase-conjugated secondary antibodies were applied to the membranes and detected by chemiluminecence. Intensities of obtained bands were quantified by the Chemi-Doc Gel quantification System and the background subtracted. The expression of SPCA, DMT1, FPN1 and MT was normalized to the protein expression of tubulin.

Immunohistochemistry

Mouse mammary gland tissues and kidneys (paper I) were dissected and fixed in paraformaldehyde containing glutaraldehyde. Fixated tissues were either stained with toluidine blue or hematoxylin–eosin. Mammary glands stained with toluidine blue were post-fixated in OsO_4. The mammary tissues were first dehydrated in ethanol and then in acetone prior to embedding in Agar 100 resin. Multiple 1 μm sections were taken from each mammary gland and then stained. The hematoxylin–eosin stained mammary glands and kidneys were paraffin embedded and multiple 5 μm sections were taken and stained.

Sections of piglet duodenum (paper III) and Caco-2 cell monolayers (paper IV) were fixed in formalin and stored in ethanol before dehydration and embedding in paraffin. Multiple sections <5 μm were taken of the

duodenum and cell monolayers and used for immunohistochemistry of DMT1, FPN1, MT (paper III) and MRP1 (paper IV). Unstained paraffin sections of piglet duodenum and cell monolayers were deparaffinized in xylen and antigen retrieval was performed in citrate buffer and endogenous peroxidase activity in tissues was blocked with H_2O_2. Sections were blocked in normal goat serum and incubated with primary antibodies. The DMT1 antibodies used recognize both DMT1 and the isoform DMT1-IRE (Leong *et al.*, 2003b). Sections were then incubated with secondary antibodies and avidin-biotin peroxidase complex was applied and immunoreactivity was detected with 3,3-diaminobenzidine staining.

4.3 Statistical analysis

Non-parametric methods were used in paper I & II, since the data showed evidence of not being normally distributed. Kruskal-Wallis test was applied for detecting differences between the experimental groups and Wilcoxon Mann-Whitney Rank-sum test for verifying differences between pair of groups. Jonckheere-Terpstra's test (Lehmann, 1975) was applied for examining possible dose dependent relationships between the four different dose groups. Spearman Rank Correlation was used to assess the monotone correlation between β-casein expression and calcium levels.

Parametric methods were used in paper III & IV. We tested data for homogeneity of variances by Bartlett's test. In accordance with the test results the cadmium levels in blood and kidney (paper III) and transport values on cadmium and iron were log transformed before analysis. A general linear model (GLM) was used to investigate the effects of cadmium and iron and their interaction on the dependent variables followed by Tukey's test to determine pairwise differences between means. Pearson's correlation was used to assess the linear correlation between cadmium concentration in kidneys and blood.

5 Results and discussion

This section contains the main results obtained in this thesis, including unpublished figures comparing *in vivo* and *in vitro* results. For further details, the reader is referred to the individual papers I-IV.

5.1 Cadmium effects in the mammary epithelium (paper I & II)

Little is known about the effects of cadmium on the physiological function of the mammary gland. However, there has previously been reported that cadmium affects fatty acid composition of rat milk (Petersson Grawé *et al.*, 2004a) indicating that cadmium retained in the lactating mammary tissue may interfere in various intracellular processes.

The aim was to investigate possible effects of cadmium on the lactating mammary gland by using a model of lactating mice (paper I). To further examine the mechanism behind the cadmium-induced disturbances in the mammary gland an *in vitro* model of murine mammary epithelial HC11 cells was used (paper II).

5.1.1 Histology (paper I)

Cadmium exposure to mice during peak lactation resulted in histological changes in the mammary gland. The histological examination of the control mammary glands taken at LD 11 showed features characteristic of peak lactation. Thus, the alveolar epithelium had a flattened morphology and milk fat globules present in the alveolar epithelial cells and in the alveolar milk lumen (Figure 3A). Large quantities of fat were observed in the mammary glands of the dams exposed to 2000 μg cadmium/kg body weight compared to controls where only minute amounts was observed (Figures 3A and 3D). The fat content in mammary tissues tended to be increased in the dams exposed to 5 and 100 μg cadmium/kg body weight as compared

to controls at LD 11 (Figures 3A–C). The structure of the cadmium-exposed mammary glands was generally denser and the alveolar milk lumen appeared reduced. Thus, a remodeling of the tissue with massive quantities of fat and degenerated alveolar epithelial cells was seen, typical of non-lactating mammary glands. Similar morphological changes normally occur at the time of weaning when the mammary gland involutes from a lactating to a resting phenotype. Although this effect was most prominent in the animals exposed to the highest cadmium dose similar morphological alterations were indicated at the low and intermediate doses (Figures 3B–D). No histological alterations in the kidneys of cadmium-exposed dams were observed.

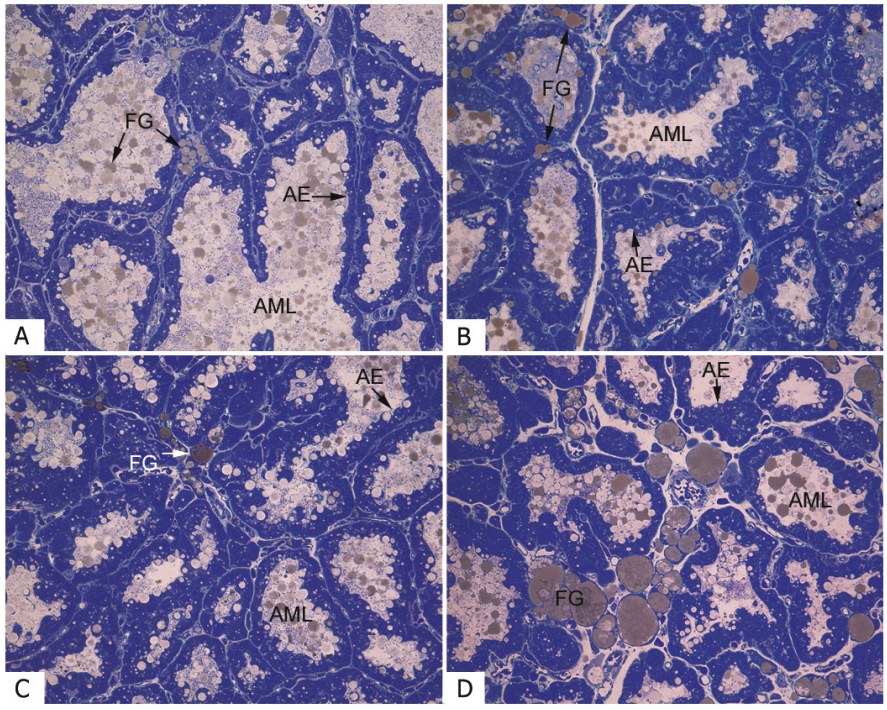

Figure 3. Representative histological sections of mouse mammary tissue at LD 11 of dams exposed to cadmium on LD 8, 9 and 10. (A) 0 (control); (B) 5; (C) 100; (D) 2000 µg Cd/kg body weight. AE, alveolar lumen; AML, alveolar milk lumen; FG, fat globule. Magnification: 400X.

5.1.2 Mineral levels and short-term calcium fluxes (paper I & II)

The total calcium concentration in the mammary glands of the dams exposed to 2000 µg cadmium/kg body weight was reduced with about 50%

compared to the control ($p = 0.01$) (Figure 4A). No other significant differences in the total concentrations of calcium, zinc, iron and copper in the mammary tissue were detected among the cadmium dose groups. Total intracellular calcium levels were also decreased approximately 20% in the HC11 cells following cadmium treatment (Figure 4B).

To further study if cadmium affects calcium transporters and/or channels in secreting mammary epithelial cells, short-term intracellular calcium fluxes were investigated in the HC11 cells. The calcium ionophore ionomycin was used to induce a rapid elevation in intracellular calcium levels to be able to study cadmium effects on short-term calcium fluxes. Cells were pretreated with cadmium and/or the antagonist of L-type voltage-dependent calcium channels nifedipine (used as a positive control) and then stimulated with ionomycin. Pretreatment of the HC11 cells for 10 min with 2.5 μM cadmium resulted in a 20% reduction of the ionomycin stimulated cellular calcium elevation during the entire measurement interval (0-250 sec). Pretreatment with 10 μM nifedipine reduced the ionomycin stimulated calcium elevation initially by about 80% during the first 120 sec and then by about 50% during the remaining of the measurement interval. Combined pretreatment of the HC11 cells with 2.5 μM cadmium and 10 μM nifedipine resulted in an 80% reduction of the ionomycin stimulated calcium elevation that lasted throughout the measurement period.

Thus, the results demonstrate that cadmium inhibits ionomycin stimulated calcium elevation in the HC11 cells and that this inhibition is augmented by nifedipine. Whereas nifedipine blocks L-type voltage-dependent calcium channels in the plasma membrane, cadmium affects cellular calcium availability by unspecific blocking/binding of calcium transporters and channels in the plasma membrane as well as at intracellular sites (Thévenod, 2009; Bridges & Zalups, 2005; Palade *et al.*, 1989). In a study by Palade *et al.* (1989) it was shown that cadmium but not nifedipine inhibits the inositol-triphosphate receptor induced calcium release from the ER. This suggests that the combined effect by cadmium and nifedipine to decrease the stimulated intracellular calcium elevation in the HC11 cells may in part be due to differences in sites of action.

5.1.3 β-casein (paper I and II)

Cadmium exposure reduced β-casein expression in a concentration dependent manner in the lactating mammary gland at LD 11 ($p = 0.009$) (Figure 4C). Based on the observations that neither of the cadmium doses altered the α-lactalbumin gene expression or the histology of the maternal

kidneys, the reduced β-casein expression cannot be explained by a mechanism that involves general toxicity of cadmium in the dam.

A negative concentration dependent relationship was also detected in the HC11 cells between cadmium concentrations and β-casein mRNA levels, demonstrating that cadmium affects β-casein expression at the cellular level in the mammary epithelial cells and not via cadmium-induced alterations in the levels of systemic mediators (Figure 4D). None of the cadmium concentrations used affected the intracellular ATP levels, LDH leakage or WAP mRNA levels in the HC11 cells, and the reduced β-casein expression can therefore not be explained by general toxicity of cadmium. To examine if the cadmium-mediated reduction in β-casein expression was related to an effect on cellular calcium homeostasis, nifedipine was applied. Similar to the effect of cadmium a significant concentration dependent reduction in β-casein gene expression was observed following treatment with nifedipine. In addition, a potentiation of the reduction of β-casein gene expression was detected as a result of co-treatment with cadmium and nifedipine. Interestingly, it can be noted that the β-casein mRNA levels correlated well to the effects on short term calcium fluxes caused by cadmium and nifedipine individually as well as in combination. Consequently, one may speculate that the initial decrease in cell calcium concentrations is related to the reduced β-casein gene expression 72 h later.

A positive association was demonstrated between β-casein expression and total calcium levels in the mammary glands of mice exposed to cadmium during lactation ($p = 0.011$). Similar correlation has earlier been reported in milk from various species, the highest casein content being present in milk from species with the highest concentrations of total calcium (Jenness, 1979). A possible explanation for the reduced β-casein expression might be that cadmium interferes with the entry of calcium into the mammary cells or intracellular stores. Previous investigations have indicated that the synthesis of β-casein is calcium-dependent (Burgoyne & Duncan, 1998), and it is well known that cadmium is able to disturb the tightly regulated levels of intracellular calcium by blocking calcium-channels and/or binding to calcium transporters in various tissues (Bridges & Zalups, 2005).

5.1.4 Calcium transporter SPCA (paper II)

The protein expression of the calcium transporter SPCA was reduced by about 55% in the mammary glands of the lactating dams given cadmium (Figure 4E). Calcium secretion into milk by mammary epithelial cells is of fundamental importance and secretory pathway Ca-ATPase, SPCA, is a key protein in this process. SPCA protein is upregulated in the mammary gland

during late gestation and the abundance of this calcium transporter increases as lactation progresses, indicating its importance in milk synthesis (Reinhardt & Horst, 1999). SPCA, which is located at the membranes of the Golgi, mediates active transport of calcium into the organelle, where it is essential for synthesis of caseins, including β-casein (Burgoyne & Duncan, 1998; Duncan & Burgoyne, 1996). We also observed a 20% reduction of SPCA gene expression in secreting mammary epithelial HC11 cells treated with cadmium for 72 h, demonstrating that cadmium affects SPCA expression at the transcriptional level in the mammary epithelial cells, either directly or indirectly via a decreased calcium level (Figure 4F).

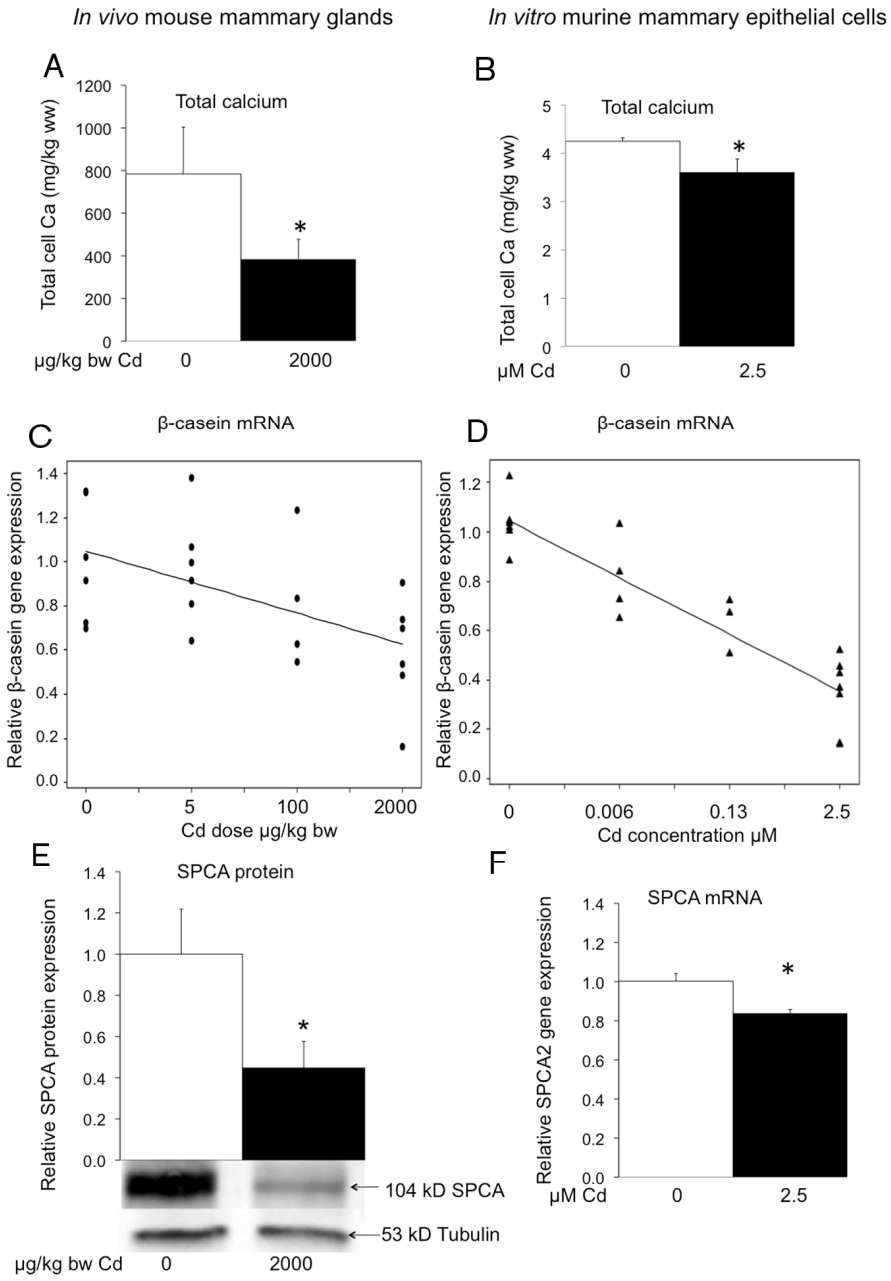

Figure 4. Comparison between *in vivo* (A, C & E) and *in vitro* (B, D & F) results on total intracellular calcium levels (A & B), cadmium concentration dependent reduction in β-casein expression ($p \leq 0.01$) (C & D) and SPCA expression (E & F). All data represents mean ± SD; n = 6 (A & C), n = 2 T25 culture flasks (B), n = 3-7 (D), n = 4 (E), n = 8 (F); ⋆$p \leq 0.05$.

5.1.5 Summary (paper I & II)

The combined *in vivo* and *in vitro* results demonstrate that cadmium disturbs the function of the lactating mammary gland by interfering with the tightly regulated calcium homeostasis in the mammary epithelial cells and that one of the mechanisms involves effects on SPCA (Figure 5).

Cessation of milk synthesis occurs naturally in the lactating glands of rodents at the time of weaning (~ LD 21) when the suckling pup turns to solid food. In a study where pups were separated from the lactating dams at LD 12, calcium concentrations were reduced by 50% and SPCA expression decreased, as a result of a cessation of milk synthesis caused by forced weaning (Reinhardt & Lippolis, 2009). The downregulation of SPCA may reflect the reduced requirement for calcium in the Golgi apparatus where the major secretory milk protein β-casein is formed. In line with this, our results show that calcium concentrations were reduced by 50% as well as the expression of SPCA in the lactating mammary glands of mice exposed to cadmium. A remodeling of the mammary tissue, including increased amount of fat and a more condensed appearance of the milk alveoli was observed in the mice following cadmium exposure. Similar histological alterations normally occur in the mammary gland during the involution at the time of weaning. Furthermore, decreased concentrations of total calcium and SPCA expression were detected in the HC11 cells treated with cadmium for 72 h. Hence, the cadmium-induced effects on calcium levels and SPCA expression in the mammary epithelium resemble the effects observed in the mammary glands as a result of weaning. Irrespective of whether the lactating dams are exposed to cadmium or separated from their pups during peak lactation similar alterations in mammary epithelial calcium levels can be observed that coincide with a reduced SPCA expression.

Our results provide evidence that the reduced SPCA and β-casein expression in mammary cells treated with cadmium is related to a reduced availability of cellular calcium. In addition to reducing total intracellular calcium levels, cadmium also disturbed the short-term calcium fluxes in ionomycin stimulated HC11 cells.

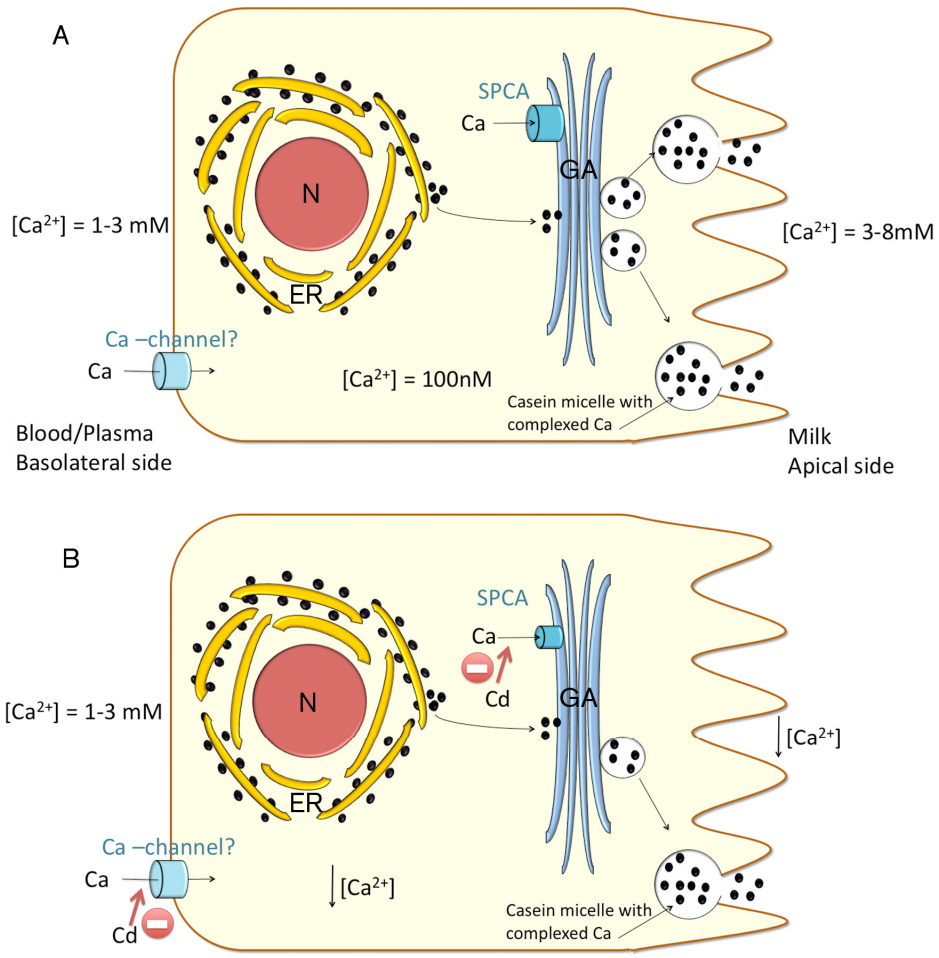

Figure 5. Proposed mechanism for the cadmium-induced disturbances in the mammary epithelial cell. Panel A showing a schematic normal milk producing mammary epithelial cell and panel B a schematic cadmium exposed mammary epithelial cell. SPCA, secretory pathway calcium ATPase; N, nucleus; ER, endoplasmic reticulum; GA, Golgi apparatus.

5.2 Cadmium effects in the neonatal intestinal epithelium (paper III & IV)

The gastrointestinal uptake of cadmium has been found to be higher in newborns than in adults, but the reason is not fully understood (Eklund *et al.*, 2001; Crews *et al.*, 2000). It is well known that there is a strong correlation between low iron stores and an increased absorption of cadmium (Meltzer *et al.*, 2010; Bárány *et al.*, 2005; Åkesson *et al.*, 2002; Olsson *et al.*, 2002; Berglund *et al.*, 1994; Flanagan *et al.*, 1978; Valberg *et al.*, 1976) and there are indications that the major iron transporters DMT1 and FPN1 are involved in this process (Kim *et al.*, 2007; Ryu *et al.*, 2004). However, these studies were performed in adults and adolescents and not in suckling newborns. Our aim was to investigate cadmium absorption in neonates with different iron status, by applying a model of suckling piglets (paper III). To further examine the mechanism behind the cadmium absorption in the neonatal intestine we used an *in vitro* model of immature human intestinal epithelium (immature Caco-2 cells) (paper IV).

5.2.1 Cadmium transport (paper III & IV)

The cadmium-exposed piglets in our study had a mean concentration of 108 ng Cd/l in whole blood. This concentration is lower than the cadmium levels in human infants (400 ng Cd/l) reported by Krachler *et al.* (1999). The cadmium dose used in the present study was thus realistic and did not affect the growth or health of the piglets. The hemoglobin levels in the iron-deficient piglets were lower than in the ones supplemented with iron from PND 7 and throughout the experimental period. Iron supplementation increased the serum iron concentrations at PND 15. Cadmium exposure did neither affect hemoglobin nor serum iron levels.

In the iron-supplemented piglets given cadmium (Fe+Cd), the blood concentrations of cadmium tended to be higher (127 ± 58 ng/l) (mean ± SD) than in the iron-deficient piglets given cadmium (Cd) (89 ± 25 ng/l), although the difference was not significant (Figure 6A). Cadmium levels in the blood were below the detection limit in piglets not given cadmium (C and Fe). In the kidneys, a significant two-fold increase in cadmium levels was observed in the iron-supplemented piglets given cadmium (Fe+Cd) (42 ± 25ng/g ww), as compared to the iron-deficient piglets given cadmium (Cd) (17 ± 9 ng/g ww) (Figure 6B). No difference in kidney cadmium

concentrations was detected in the two groups of piglets not given cadmium (C and Fe). A positive and statistically significant correlation between cadmium levels in kidneys and blood was observed ($r = 0.87$, $p < 0.001$). We found no evidence that iron deficiency increases the intestinal absorption of cadmium in suckling piglets. In contrast, there are indications that iron supplementation to suckling newborns leads to an increased absorption of cadmium, as measured by cadmium levels in kidneys, which reflects an accumulated effect of cadmium exposure. The reason for the discrepancy between our results in newborns and previous reports in adults probably involves developmental changes in the handling of iron and cadmium.

The transport of cadmium across the immature Caco-2 monolayers was increased by 170% in cells pretreated with Cd and by 270% after treatment with Fe+Cd compared to untreated control cell monolayers. Compared to the treatment with only Cd, the cadmium transport seemed to be higher in cells treated with Fe+Cd, although the difference was not statistically significant (Figure 6C). Cd pretreatment increased the transport of iron across the monolayers by 20%. Pretreatment with only Fe did neither influence cadmium nor iron transport across the cell monolayers. The passive diffusion of the paracellular marker mannitol was not increased, showing that the augmented cadmium transport was not due to disrupted tight junctions.

Cadmium uptake into the Caco-2 cells was reduced by approximately 20% compared to the controls in all pretreated groups. The uptake of iron into the cells was significantly affected both by Fe and Cd pretreatment. Fe pretreatment reduced iron uptake by 31% compared to controls, whereas Cd pretreatment increased iron uptake by 31%. Interestingly, we observed an increased iron uptake in immature cells pretreated with cadmium. The mechanisms behind this effect are not known. The pretreatment with Fe+Cd did not influence the iron uptake.

5.2.2 Iron transporters (paper III & IV)

No quantitative differences were observed in DMT1 or FPN1 protein expression due to iron status or cadmium exposure, although higher intestinal cadmium absorption was found in the iron-supplemented piglets. This indicates that another mechanism may be operative for the absorption of cadmium in the duodenum of the newborn. In human infants, there are indications that iron regulation is immature at young age (Domellöf *et al.*, 2001). Thus, between 4 and 6 months of age, iron supplementation increased hemoglobin in an unregulated manner, and not until 6–9 months

the effect of iron supplementation on hemoglobin was as expected (Domellöf *et al.*, 2001).

We were not able to detect any differences in localization of DMT1 and FPN1 among the four experimental groups and thus, the increased cadmium uptake in the piglets cannot be explained by differences in cellular localization of the iron transporters. The localizations of DMT1 and FPN1 in the duodenal villous epithelium were in concordance to what has been described previously in adults; DMT1 both in the apical membrane and in the cytosol and FPN1 in the basolateral membrane of the absorptive enterocytes (Zoller *et al.*, 2001; Canonne-Hergaux *et al.*, 1999). It has been suggested that iron absorption from milk in the newborn occurs via receptor-mediated endocytosis of lactoferrin bound iron (Suzuki *et al.*, 2001). However, because it has been demonstrated that lactoferrin does not affect the absorption of cadmium (Mata *et al.*, 1996), it is unlikely that the increased cadmium absorption observed in the iron-supplemented piglets involves this iron binding milk protein.

In the immature Caco-2 cells we also included the DMT1-IRE isoform of DMT1 containing an iron responsive element. The *in vivo* results were confirmed in the Caco-2 immature cells and no effects on mRNA levels of the iron transporters DMT1, DMT1-IRE and FPN1 were observed after iron treatment. The gene expressions of both DMT1 and DMT1-IRE were reduced in the Caco-2 cells pretreated with Fe+Cd.

The results demonstrate that the uptake of cadmium and iron into the iron-loaded immature Caco-2 cells does not correlate to the gene expression of DMT1 or DMT1-IRE. Nevertheless, iron pretreatment reduced iron uptake in the immature cells, indicating the presence of other iron regulated mechanisms. A reduced cadmium uptake into iron-loaded cells was also detected, which may reflect that cadmium and iron shares non-DMT1 mediated transport mechanisms across the apical membrane of immature enterocytes.

The cellular localization of DMT1 and FPN1 in the immature Caco-2 cell monolayers did not differ among the experimental groups. Both the DMT1 and FPN1 staining was only detected intracellularly and the staining was diffuse and homogeneous within the partially differentiated Caco-2 cells. This is in accordance with the localization in suckling rat pups at lactation day 10 (Leong *et al.*, 2003b). There are indications that the localization of DMT1 and FPN1 in the duodenum is age-dependent. Leong *et al.* (2003b) have demonstrated that DMT1 and FPN1 expressions in suckling rats were not affected by iron supplementation at lactation day 10. The expressions of the iron transporters were not significantly decreased in

the iron-supplemented pups until lactation day 20. This indicates that the regulation of iron homeostasis is immature during early infancy and that there may be other mechanisms for intestinal iron absorption in the newborn.

5.2.3 ABC transporters (paper IV)

The MRP1 gene expression in immature Caco-2 cells pretreated with Cd increased 55% and the expression in the cells pretreated with Fe+Cd increased 70%. Cells pretreated with Fe showed a tendency of increased MRP1 gene expression compared to controls, although the difference was not significant. Thus, pretreatment of immature Caco-2 cells with cadmium causes an upregulation in the gene expression of the basolateral transporter MRP1, which correlated to an increased cadmium transport across the monolayers (Figure 6D). Cadmium is known to bind with high affinity to the thiol (SH) groups in GSH (Diaz-Cruz, 1997) and the complex can be transported by MRP1 (Li *et al.*, 1997; Tommasini *et al.*, 1996). The gene expression of MRP1 is shown *in vitro* to be regulated by intracellular ROS and GSH levels (Yamane *et al.*, 1998). Therefore it is possible that cadmium-induced oxidative stress and altered GSH levels may induce MRP1 expression. Indeed, MRP1 has been demonstrated to be involved in the protection against cellular cadmium toxicity by mediating export of cadmium-GSH conjugates (Oh *et al.*, 2009). Cadmium treatment decreased the thiol content by 65% in Caco-2 cells differentiated for 7 days, while in mature Caco-2 cells no difference in thiol content was observed (Cardin *et al.*, 2009), indicating developmental changes in the cell defense against cadmium-induced oxidative stress. Immature enterocytes may be more sensitive than mature cells to cadmium-induced oxidative stress. We suggest that pretreatment of immature Caco-2 cells with cadmium or cadmium plus iron induces the formation of cadmium-GSH complexes, which will have a facilitated transport across the basolateral membrane by the upregulated MRP1.

Surprisingly also an increased iron transport across immature Caco-2 cells pretreated with cadmium or cadmium plus iron was observed, which did not correlate to FPN1 expression. Cadmium-induced MRP1 may explain also the increased iron transport. It has previously been reported that MRP1 is involved in the transport of iron complexes across cell membranes (Watts *et al.*, 2006).

MRP1 was detected in both control and iron supplemented piglet enterocytes with an intense and granular staining of MRP1 protein on the basolateral side of the nucleus. Iron supplementation did not affect cellular

localization of MRP1 protein. Similar localization of MRP1 protein has been demonstrated in human intestinal epithelium from adults (Blokzijl *et al.*, 2008). Even though MRP1 is not present at membranes, the granular staining indicates that this ABC transporter mediates influx into vesicles that in turn are exported across the basolateral membrane of the cells. Indeed, it has been shown that MRP1 is involved in vesicular transport of various substances, such as doxorubicin and cobalamin (Beedholm-Ebsen *et al.*, 2010; Rajagopal & Simon, 2003).

The cellular localization of MRP1 in the Caco-2 cell monolayers did not differ among the four experimental groups. MRP1 protein was found intracellularly and around the nuclei with no apparent plasma membrane staining. The MRP1 staining was granular and spotty compared to DMT1 and FPN1 staining. This is in accordance with previous findings in mature Caco-2 cells (Cummins *et al.*, 2001).

The MRP2 and P-gp mRNA levels were not significantly affected by any pretreatments, even if there was a tendency of upregulation of P-gp after pretreatment with Fe+Cd. Cadmium uptake into the Caco-2 cells was decreased by Cd pretreatment. This finding may be related to the tendency of increased gene expression of the apical efflux protein P-gp. The reduced cadmium uptake in the Cd treated immature cells did not correlate to the transport of the metal into the basolateral chamber. However, it should be noted that the amount of cadmium transported across the cells only constitutes a minor fraction of the total uptake of the metal. Hence, even if the intracellular cadmium levels are decreased there is still enough of the metal for e.g. MRP1 to efflux out of the cell.

5.2.4 Metallothionein (paper III & IV)

The Western blot analysis did not reveal any differences in quantitative MT expression in the duodenum of suckling piglets among any of the four experimental groups. However, the cellular localization of MT protein differed between piglets exposed to cadmium and none exposed piglets. In the cadmium-exposed piglets (Cd and Fe+Cd), MT was localized in the villous epithelium and the MT staining was most pronounced at the upper part of the villous. In addition, a diffuse MT staining was observed on the serosal side of the intestinal epithelium in the cadmium-exposed piglets. In the piglets not given cadmium, MT was mainly localized in the crypt epithelium, where it presumably functions to recruit various essential metals, such as zinc and copper, for biochemical processes in the differentiating cells. Thus, iron status did not affect the quantitative MT expression or cellular

localization of the protein. Apparently, the increased cadmium absorption in the iron-supplemented piglets cannot be explained by involvement of MT.

Gene expression of MT1 in immature Caco-2 cells was increased 30 times after cadmium pretreatment (C: 1.0 ± 0.1; Fe: 0.8 ± 0.02; Cd: 31.5 ± 2.1; FeCd: 28.3 ± 0.9; mean ± SEM; n = 3). Despite the strong induction, *in vivo* studies have demonstrated that MT only plays minimal roles in the gastrointestinal absorption of cadmium, though the retention of the metal in other tissues is MT-dependent (Klaassen *et al.*, 2009). Accordingly, uptake of cadmium in the immature Caco-2 cells pretreated with the metal was significantly reduced, indicating that MT is not involved in the retention of cadmium in neonatal enterocytes. On the other hand, the transport of cadmium across the immature cells was associated with increased MT expression following cadmium treatment. Although not investigated, it might be possible that cadmium-MT complexes are transported across the basolateral membrane. However, at present there is no consensus regarding the mechanism for the possible export of cadmium-MT complexes out of intestinal cells.

The strong increase in MT mRNA level after cadmium pretreatment of the immature Caco-2 cells, may be related to increased oxidative stress. Previously, a 5-fold increase in MT expression has been reported after long term cadmium treatment of mature Caco-2 cells (Blais *et al.*, 1999), indicating that the immature cell is more sensitive to the cadmium pretreatment and therefore induces MT expression more strongly. In concordance, Cardin *et al.* (2009) demonstrated a higher induction of MT in immature Caco-2 cells compared to mature ones.

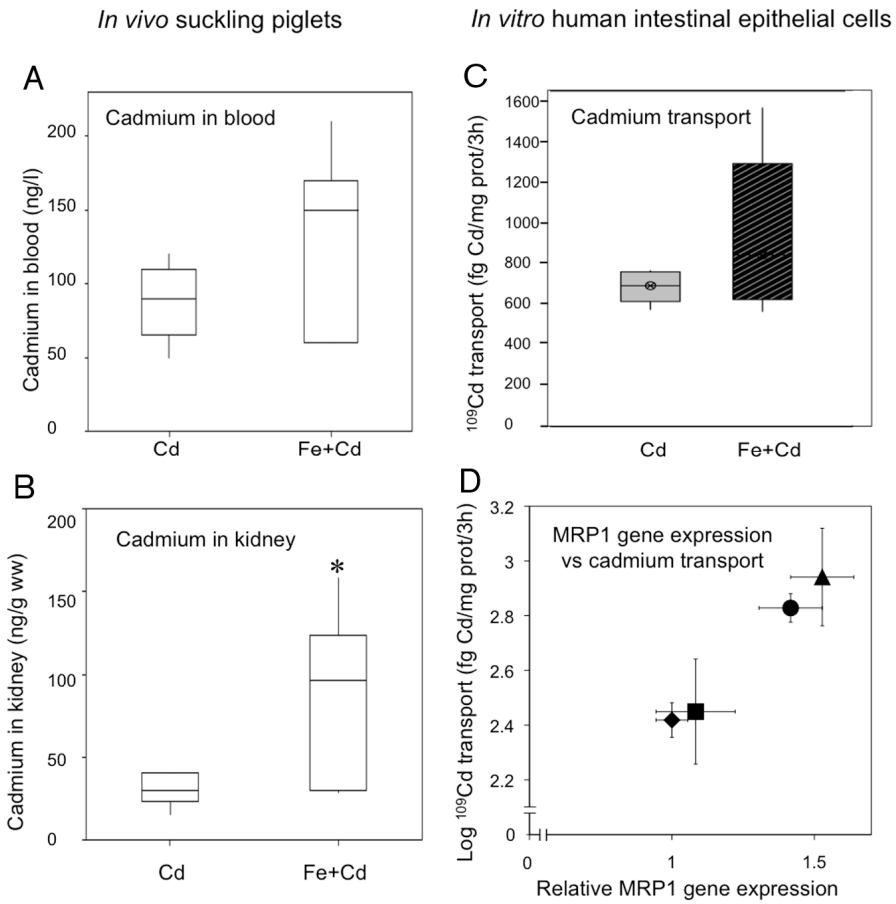

Figure 6. Cadmium absorption in suckling piglets (A & B) and cadmium transport across Caco-2 cell monolayers (C). The boxes are defined by the upper and lower quartiles and with the medians marked by a subdivision of the boxes. The highest and lowest observations are indicated by the whiskers that extend from the top and bottom of the boxes; n = 6; $^{\star}p \leq 0.05$ (A-C). Panel D show the positive association between MRP1 gene expression and cadmium transport across immature Caco-2 cells (Diamond: C; Square: Fe; Circle: Cd; Triangle: Fe+Cd). Data are presented as mean ± SEM; n = 3 (MRP1); n = 6 (^{109}Cd transport) (D).

5.2.5 Summary (paper III & IV)

The combined *in vivo* and *in vitro* results indicate that cadmium absorption in the neonatal intestine involves the basolateral efflux protein MRP1 and that iron supplementation does not restrict the absorption of the metal in newborns (Figure 7).

Neonates are more sensitive to cadmium than adults, due to the interference of the element in the development of the central nervous system (Petersson Grawé *et al.*, 2004b). Thus, it is of paramount importance to minimize cadmium exposure and uptake in newborns. Our hypothesis was that iron supplementation would be beneficial for the newborn to reduce the intestinal cadmium absorption. However, cadmium absorption in iron-deficient suckling piglets did not increase. In contrast, a higher cadmium uptake was detected in the iron-supplemented ones, suggesting that in newborns the cadmium absorption is more efficient following iron supplementation. The expressions and localizations of duodenal DMT1, FPN1 and MT in the piglets were not affected by iron status and could therefore not explain the increased cadmium uptake. In the immature Caco-2 cell model we demonstrated that cadmium treatment of the cells upregulates MRP1, which correlated to an increased transport of the metal across the basolateral membrane. Furthermore, cadmium transport across immature Caco-2 cells was not decreased, but instead tended to be increased by iron, and is not dependent on DMT1 or FPN1 expressions and localizations. The expression of MT was induced by cadmium treatment and correlated to the increased transport of the metal across the immature intestinal cells. However, it is not clarified if the efflux of cadmium-MT complexes occurs from the intestinal cells.

Supposedly, immature cells are more sensitive than mature cells to oxidative stress and therefore more prone to form cadmium-GSH complexes initiating upregulation of MRP1. As a result, neonates have a higher intestinal absorption of cadmium than adults. Our findings indicate that neonatal intestinal transport of cadmium is not restricted by iron; rather there is a tendency of an increased cadmium transport by treatment with cadmium plus iron. Our results also support the notion that intestinal iron homeostasis is not active at early age.

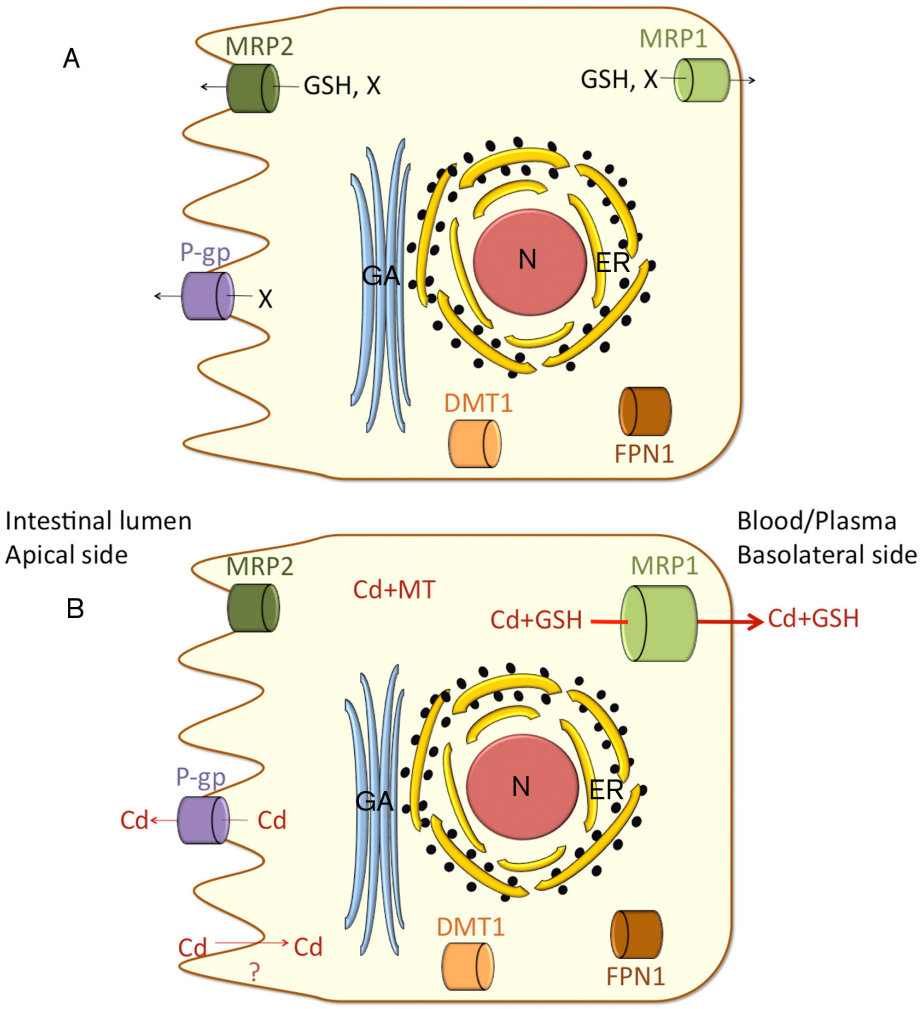

Figure 7. Proposed mechanism for cadmium transport in immature neonatal enterocytes. Panel A describes a schematic unexposed immature enterocyte and panel B describes a schematic cadmium exposed immature enterocyte. DMT1, divalent metal transporter 1; FPN1, ferroportin 1; MRP2, multidrug resistance associated protein 2; P-gp, plasma glycoprotein; MRP1, multidrug resistance associated protein 1; GSH, glutathione; X, endogenous or exogenous substrate; MT, metallothionein; N, nucleus; ER, endoplasmic reticulum; GA, Golgi apparatus.

6 Concluding remarks and future perspectives

In this thesis two transport pathways that cadmium interferes with during early life were identified. In the lactating mammary epithelium calcium homeostasis was affected by cadmium and in the neonatal intestine expression of the ABC-transporter MRP1 was affected.

Cell models

Mechanistic insights on cellular effects of cadmium in the mammary epithelium and neonatal intestine were obtained by combining *in vivo* and *in vitro* studies. The animal experiments were performed initially so that the cell models could be validated to distinguish relevant mechanisms.

The HC11 cells have previously been used mainly to study the hormonal regulation of mammary cell differentiation. It is concluded that the HC11 cell model is useful also to examine the impact of toxic compounds on the mammary epithelial cells.

The immature Caco-2 cells constitutes a promising system for detailed studies concerning developmental changes in the intestinal transport of both cadmium and iron in newborns based on similar results in suckling piglets and in the cells, as well as the intracellular localization of iron transporters. It is concluded that the immature Caco-2 cell model may be used to study age-dependent intestinal absorption.

Cadmium in the mammary epithelium

The results indicate that cadmium disturbs the tightly regulated calcium homeostasis in the mammary epithelial cells leading to reduced expressions of SPCA and β-casein. The histological changes are involution-like in the mammary glands exposed to cadmium and resemble the effects observed in the tissue due to forced weaning. In addition to mammary gland, SPCA

protein expression is high in brain, testis and kidney (Xiang *et al.*, 2005) and one could therefore suggest that the toxicity of cadmium may involve SPCA also in these tissues. Future research on SPCA expressions in these tissues following cadmium exposure is recommended. In conclusion, maternal cadmium exposure may disturb calcium regulation and decrease the levels of β-casein in milk with potential nutritional and developmental implications for the breast-fed newborn.

Further studies are required to examine if the cadmium-induced effects in the mammary epithelium also involve other calcium transporters as the apical PMCA2 and basolateral TRPV6, or zinc transporters, particularly the apical efflux proteins ZnT1, 2 and 4. Zinc is necessary for the development of the newborn and several zinc transporters rigorously regulate the level of zinc in milk. Cadmium is known to affect zinc levels and zinc transporters by ionic mimicry.

Generally, research on the mammary gland investigates breast cancer or lactational transfer of various compounds across the mammary epithelial cells. The focus in this thesis was effects in the mammary gland and its function following exposure to cadmium. The results demonstrate that cadmium disturbs the function of the mammary epithelial cells. Future studies should be performed to identify other chemicals having the potency to interfere with the function of mammary gland.

Cadmium in the neonatal intestinal epithelium

The results demonstrate that iron supplementation does not restrict cadmium absorption in neonates. The cadmium uptake was not higher in iron-deficient suckling piglets; rather, a higher cadmium uptake in the iron-supplemented ones was detected. The expression and localization of DMT1, FPN1 and MT were not affected by iron status and could therefore not explain the increased cadmium uptake. The results suggest that the handling of both iron and cadmium in the intestinal epithelium is age-dependent and differs between newborns and adults. This should be taken into consideration in iron intervention programs for infants. Further, it appears that MRP1 plays a significant role in the absorption of cadmium and possibly also iron in neonatal intestine.

Future studies applying specific MRP1 and P-gp inhibitors could give more detailed information about cadmium efflux from the immature enterocyte. Also the possible involvement of other ABC transporters as BCRP and CFTR in cadmium absorption remains to be investigated. The zinc transporters play important roles also in the intestine and there are studies suggesting that the regulation of zinc is immature in the neonatal

intestine (Jou *et al.*, 2010). Therefore, studies investigating the impact of cadmium on zinc transporters in the immature intestine, particularly the basolateral efflux protein ZnT1 are encouraged. Furthermore, studies that examine indicators of oxidative stress e.g. GSH levels and SOD system activity, following cadmium exposure in immature intestinal cells are needed to increase the understanding of age-dependent cadmium absorption.

In general, a more detailed understanding of mechanisms for cadmium-induced disturbances in cellular calcium and generation of ROS requires further investigations. It is possible that calcium and ROS play major roles in the cellular toxicity of cadmium and may constitute the signal molecules initially affected by cadmium in all target organs. Therefore, research efforts are needed to understand if cadmium-induced disturbances in calcium homeostasis and ROS generation may be involved in the higher susceptibility to cadmium in neonates compared to adults.

Conclusions

Newborns and children are vulnerable groups for toxic effects of chemicals. Cadmium is ubiquitous in our environment and it is impossible to avoid exposure to the metal. In this thesis two new aspects concerning adverse health effects of cadmium during early life have been investigated and novel findings have been revealed:

- Cadmium disturbs the milk synthesis in the mammary gland. The health consequences in neonates need to be studied as well as the potential of other toxic compounds to affect the mammary gland function.

- The well-known association in adults between low iron status and high body burden of cadmium does not apply for neonates. Iron supplementation does not restrict, but rather increases cadmium absorption in neonates, which should be considered in risk assessment of cadmium.

7 Populärvetenskaplig sammanfattning

Kadmium är en toxisk metall som vi främst får i oss via livsmedel och tobaksrökning på grund av att metallen effektivt tas upp av växter som rotfrukter, grönsaker, spannmål och tobak. I jorden finns kadmium naturligt i varierande grad, men frisätts också genom mänskliga aktiviteter som exempelvis brytning i zinkgruvor där kadmium är en biprodukt. Kadmium används som metallegering, i nickel-kadmium batterier, som pigment och stabiliserare i plaster, och även i nya nanomaterial. Kemiskt liknar kadmium ett antal för kroppen nödvändiga (essentiella) metaller som behövs till en mängd olika funktioner, bland annat i röda blodkroppar, skelettet och många enzymer. På grund av likheten med de essentiella metallerna kan kadmium transporteras in och runt i kroppen av proteiner avsedda för exempelvis järn och kalcium.

I över tvåhundra år har det varit känt att kadmium har toxiska effekter på människor. I vuxna är det främst njuren som skadas då kadmium ansamlas där och påverkar återupptaget av bland annat näringsämnen, mineraler och vitaminer. Negativa effekter av kadmium uppstår också i skelettet. Itai-itai-sjukan som innebär att skelettet urkalkas och att spontana benfrakturer kan uppkomma upptäcktes första gången i Japan på 1950-talet efter att ris bevattnats med kadmiumhaltigt vatten. Femtio år senare står det klart att benskörhet kan orsakas även av ett relativt lågt intag av kadmium. Upptaget av kadmium från tarmen hos vuxna är mellan 3-5%, men varierar med bland annat ålder och näringsstatus, främst nivåer av järn men också kalcium.

Hos nyfödda förefaller det centrala nervsystemet vara ett av de känsligaste organen för kadmiums skadliga effekter. Nyfödda har dessutom ett ökat upptag av kadmium från tarmen, vilket vi idag inte har någon förklaring till. Förutom att ansamlas i njuren har studier på gnagare visat att kadmium även ansamlas i mjölkproducerande bröstvävnad.

Carl von Linné döpte 1758 den grupp av djur som kan producera mjölk till sin avkomma för *Mammalia* (däggdjur, från ordet dägga, att ge di). Den stora fördelen att kunna ge di till sina ungar har varit avgörande för däggdjurens utveckling. I alla däggdjur finns det nära samband mellan den mjölkproducerande bröstvävnaden och avkommans tarmfunktion. Bröstet producerar mjölk som innehåller både näring och skydd, och avkommans tarm är beroende av mjölken för att kompensera för dess omogna funktioner samt för att mogna.

I den här avhandlingen har kadmiums effekter under nyföddhetsperioden studerats, det vill säga motsvarande livets fyra första veckor hos människor. Målet har varit att studera kadmiums effekter på de bröstceller som producerar mjölk åt den nyfödde och att studera upptaget av kadmium från den nyföddes tarmar. Vi har använt oss av fyra olika modeller för våra studier: 1) mjölkproducerande möss; 2) odlade bröstceller; 3) diande griskultingar; 4) odlade omogna tarmceller som liknar den nyföddes tarmar.

Resultaten i avhandlingen visar att kadmium har en förmåga att störa kalciumregleringen i den mjölkproducerande bröstvävnaden och att uttrycket av kalciumtransportören SPCA och mjölkproteinet β-kasein minskar. Kadmium orsakade också förändringar av bröstvävnaden liknande de som uppkommer normalt i vävnaden vid avvänjning, då avkomman börjar äta fast föda och mjölkproduktionen upphör. Konsekvensen av att kadmium ansamlas i bröstvävnad kan därför bli att den nyfödde får i sig lägre halter av kalcium och protein, vilket kan leda till en generellt sämre utveckling och tillväxt. Kalciumtransportören SPCA finns förutom i bröstvävnad även i hög grad i hjärnan och njuren. Den eventuella effekten av kadmium på uttrycket av SPCA i dessa vävnader är ett viktigt område för framtida studier.

Det har visat sig att vuxna människor som har låg järnstatus har förhöjda halter av kadmium i kroppen och mycket tyder på att det har med ett ökat uttryck i tarmen av de två järntransportörerna DMT1 och FPN1 att göra. I vår studie med diande griskultingar var resultatet mycket överraskande då upptaget av kadmium var högre hos grisar med hög järnstatus jämfört med låg järnstatus. Vidare visade det sig att järntransportörerna inte påverkades av järnstatus och de kunde därför inte vara inblandade i det ökade upptaget av kadmium. I cellmodellen med omogna tarmceller upptäcktes att den ökade kadmiumtransporten sammanföll med ett ökat uttryck av transportproteinet MRP1.

Sammanfattningsvis visar resultaten i avhandlingen att kadmium kan störa funktionen i de mjölkproducerande cellerna. Mjölkproduktionen påverkas negativt och det i sin tur ger mindre näring och kalcium till den nyfödde.

Tidigare studier har visat att nyfödda har ett högre upptag av kadmium och att även att kadmium kan lagras in i den omogna njuren. Då kadmium har en mycket lång halveringstid i njuren (10-30 år) är det av stor vikt att minimera kadmiumexponeringen särskilt vid unga år. Våra resultat visar att åldersberoende skillnader gör att järntillskott inte begränsar kadmium-upptaget från tarmen hos den nyfödde. Resultaten ger nya insikter om kadmiums effekter under nyföddhetsperioden och visar på viktiga skillnader mellan hur kadmium hanteras hos nyfödda jämfört med vuxna.

Slutsatser

Nyfödda och barn är känsliga grupper som vi behöver mer kunskap om när det gäller påverkan av miljöföroreningar. Kadmium finns överallt i vår natur och vi kan omöjligt undgå att få i oss metallen. I den här avhandlingen har kadmiums effekter under nyföddhetsperioden studerats ur två nya perspektiv. Resultaten visar att:

- Kadmium kan störa mjölkproduktionen i bröstvävnaden. Betydelsen av fyndet för den nyfödde kvarstår att undersöka. Det är också viktigt att studera om det finns andra miljöföroreningar som har förmågan att störa mjölkproduktionen i bröstet.

- Det välkända sambandet hos vuxna mellan låg järnstatus och högt upptag av kadmium gäller inte för nyfödda. Järntillskott begränsar inte, utan tycks istället öka kadmiumupptaget hos nyfödda, vilket bör beaktas vid riskbedömning av kadmium.

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