DOCTORAATSPROEFSCHRIFT

2007 | School voor Levenswetenschappen

Cd stress in mice after chronic exposure to low doses: A multidisciplinary approach with main focus on the kidney

Proefschrift voorgelegd tot het behalen van de graad van
Doctor in de Biomedische Wetenschappen, te verdedigen door:

Sandy THIJSSEN

Promotor: prof. dr. Emmy Van Kerkhove
Copromotor: dr. Ann Cuypers

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List of abbreviations

α1-m	alfa 1 microglobulin
α-SMA	alfa smooth muscle actin
ALP	alkaline phosphatase
ANOVA	analysis of variance
AsA	ascorbate (reduced)
β2m	beta 2 microglobulin
BB	brush border
BBM	brush border membrane
B-Cd	blood cadmium
bw	body weight
CadmiBel	cadmium in Belgium study
CAT	catalase
Cd	cadmium
$CdCl_2$	cadmium chloride
CdMT	cadmium-metallothionein
cDNA	complementary DNA
CdO	cadmium oxide
Cys	cysteine
DAB	diaminobenzidine
DHA	dehydroascorbate
DMT1	divalent metal transporter 1
DNA	deoxyribonucleic acid
ds	double stranded
DTT	dithiothreitol
e^-	electron
ECM	extracellular matrix
EMT	epithelial-to mesenchymal transition
FAM	5-carboxyfluorescein
GF-AAS	graphite furnace atomic absorption spectroscopy
GFR	glomerular filtration rate
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
H_2O_2	hydrogen peroxide
Hmox1	heme oxygenase 1 gene
HMWP	high molecular weight protein
HO	heme oxygenase
HPLC	high performance liquid chromography
Hprt1	hypoxanthine phosphoribosyltransferase 1
HRP	horseradish peroxidase
HSP	heat shock protein
IARC	International Agency for Research on Cancer
Ig	immunoglobulin
IHC	immunohistochemistry
K-Cd	kidney cortex cadmium
kDa	kiloDalton
L-Cd	liver cadmium
LMW	low molecular weight

LMWP	low molecular weight protein
MDA	malondialdehyde
MRI	magnetic resonance imaging
MT	metallothionein
MTF-1	metal transcription factor 1
MTP1	metal transport protein 1
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
NAG	N-acetyl-β-D-glucosaminidase
NGS	normal goat serum
Ni-Cd	nickel-cadmium
NO	nitric oxide
NOS	nitric oxide synthase
Nox	NADPH-oxidase
O_2	oxygen
$O_2^{\cdot-}$	superoxide anion
O_2^{2-}	peroxide ion
$^{\cdot}OH$	hydroxyl radical
$ONOO^{-}$	peroxynitrite
OSCAR	Osteoporosis- Cadmium As a Risk factor
PAS	Periodic Acid Schiff
PCR	polymerase chain reaction
PCT	proximal convoluted tubule
PheeCad	Public Health and Environmental Exposure to Cadmium study
PHGPx	phospholipid hydroperoxide glutathione peroxidase
ppm	parts per million
Prdx2	peroxiredoxin 2 gene
Prx	peroxiredoxin
PT	proximal tubule(s)
PTC	proximal tubular cell(s)
PUFA	polyunsaturated fatty acid
$R^{\cdot}$	alkyl radical
RBP	retinol binding protein
RNA	ribonucleic acid
RNase	ribonuclease
$RO^{\cdot}$	alkoxyl radical
$ROO^{\cdot}$	peroxyl radical
ROS	reactive oxygen species
RT	reverse transcription
SAC	stretch-activated cation channel
SEM	standard error of the mean
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TEM	transmission electron microscopy
TGF-β	transforming growth factor-beta
ThR	thioredoxin reductase
Trx	thioredoxin
U-Cd	urinary cadmium
V-ATPase	vacuolar H^+-ATPase
WHO	World Health Organization
XO	xanthine oxidase
ZnT1	zinc transporter 1

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"All truths are easy to understand once they are discovered; the point is to discover them"

-**Galileo Galilei**-

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V-ATPase	vacuolar H^+-ATPase
WHO	World Health Organization
XO	xanthine oxidase
ZnT1	zinc transporter 1

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Chapter 1

Introduction

1.1 Introduction

New technologies are rapidly growing in our industrialized society. The amount of goods that are produced, the production processes and the ever-increasing consumption are threatening the environment and the people living in it. Contaminating agents can undermine our health status and interfere with processes in our body, influence organ functioning and be the cause of severe illnesses or death.

This research project deals with one particular contaminating agent, cadmium (Cd), which mainly affects occupationally exposed workers, smokers and humans living in contaminated areas. The objective of the present work is to create an animal model that is chronically exposed to relatively low Cd concentrations. The most important target organ for Cd is the kidney, where it will accumulate for many years. Therefore we want to investigate the kidney's 'health status' in a functional, histological and biochemical way. Furthermore we will focus on the defence systems in the kidney in their attempt to minimize Cd damage. We hope it may provide new insights in the early events of chronic Cd intoxication.

1.2 Cadmium

1.2.1 Occurrence and sources of Cd in the environment

Cd is a soft, ductile, silver-white metal that belongs together with zinc and mercury to group IIb in the periodic table and has an atomic number of 48 (WHO, 2000). It is categorized as a heavy metal due to its specific density of more than 5 g/cm^3 (8.64 g/cm^3) and has been used in many different areas over the past centuries (Järup, 2003; WHO, 2004). It is a bivalent cation and has a hexagonal crystal structure and a molecular weight of 112.4. Cd^{2+} has an ionic radius of 0.97 Å, which is very close to that of Ca^{2+} (0.99 Å). This enables Cd^{2+} to substitute for Ca^{2+} and interfere with Ca^{2+}- dependent signaling processes (also see 1.2.4). Furthermore it strongly interacts with sulfur and in this way can disrupt the structure and function of a number of thiol-containing proteins and enzymes. Together with other toxic elements such as lead, mercury and arsenic, it is considered to be a main threat to human health (Apostolova et al., 2006; Järup, 2003; WHO, 2000).

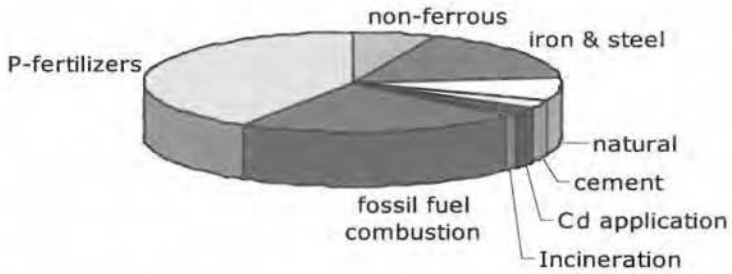


Fig. 1.1 *The relative importance of various cadmium sources to human exposure (Van Assche, 1998).*

Cd is a relatively rare element and is not found in its pure state in nature. It occurs naturally in ores together with zinc, lead and copper, or is emitted into the air through the process of volcanic emissions. It became commercial in the 20th century and approximately 85-90 % of total airborne Cd emissions nowadays arise from anthropogenic sources (Fig. 1.1). Cd is a by-product of the zinc industry and it also arises from fossil fuel combustion and municipal waste

incineration (WHO, 2000). Cd compounds are used as colour pigment, stabilizers in PVC products and most commonly in re-chargeable nickel-cadmium (Ni-Cd) batteries (Järup, 2003). Another important source of contamination is the use of commercial fertilizers derived from rock phosphate and sewage sludge. Soil contamination, which leads to food contamination (vegetables grown on contaminated soils), comes to a large extent from atmospheric Cd as a result of foliar absorption or root uptake of Cd deposited on soils (WHO, 2000).

1.2.2 Cd levels in air, water and soil

Cd levels in the environment vary widely and are depending on the presence of (former) industrial sites, extensive agricultural activities or dump sites. Emissions to the environment are normally transported continually between air, water and soil (International Cadmium Association, Brussels, Belgium).

Air

Ambient air Cd concentrations range from 0.1 to 5 ng/m^3 in rural areas, from 2 to 15 ng/m^3 in urban areas and from 15 to 150 ng/m^3 in industrialized areas (International Cadmium Association, Brussels, Belgium). For the general population not living in industrialized areas, Cd intake from air is maximal 0.01 µg per day (rural areas) and 0.2 µg per day (urban areas) (WHO, 1996; WHO 2000).

Cd in an occupational environment is usually inhaled in the form of either small particles of fume or larger particles of dust.

An additional source of Cd is cigarette smoking. As tobacco leaves accumulate and concentrate relatively high levels, Cd concentrations vary between 0.5 and 2 µg/g (dry weight) of tobacco, which leads to an estimated exposure of 2 to 4 µg of Cd per day from cigarette smoking (20 cigarettes a day) (WHO, 1996; WHO, 2000). Biological monitoring of Cd in the general population has shown that cigarette smoking significantly increases blood Cd (B-Cd) concentrations to levels that are 4-5 times higher than B-Cd levels of non-smokers (Järup, 2003).

Water

Levels of Cd in drinking water are generally very low, varying from 0.01 to 1 µg/l. However, contamination may occur as a result of the presence of Cd as an impurity in the zinc of galvanized pipes or Cd-containing solders in fittings, water heaters, water coolers and taps. Cd levels in unpolluted natural waters do not exceed a concentration of 1 µg/l, but polluted areas may easily contain more than 25 µg/l (WHO, 2004).

Soil and food

Cd in the soil is derived from both natural and anthropogenic sources. Through agricultural or industrial pollution, crops and animals will take up this Cd and transfer it finally to humans. For non-smoking, non-occupationally exposed people, this is the main route of exposure.

Cd levels vary widely in various types of foodstuffs. The concentration of Cd is in the range of 1-50 µg/kg in meat, fish and fruit and 10-300 µg/kg in staple fruits such as wheat, rice, potatoes and leafy vegetables (lettuce, spinach). Even higher concentrations are found in internal organs of animals (liver, kidney) and in certain mussels, scallops and oysters (10-1000 µg/kg). When grown on a Cd-polluted soil (in Japan for example, see 1.2.3), some crops such as rice can accumulate more than 1000 µg/kg.

The average daily Cd intake via food is 15-25 µg in Europe and North America with variations depending on age and dietary habits. In Japan these values are much higher and amount to a general daily intake of 40-50 µg and more in Cd-polluted areas (WHO, 2000).

House dust (indoor pollution) is another potential route of exposure in areas with contaminated soils and should be incorporated in the assessment of health risks (Hogervorst et al., 2007). Furthermore small children playing in soil and their 'pica-behaviour', hand-to-mouth contact, is an important endangered group that often is neglected as well (Hennighausen, 2004).

1.2.3 Cd effects on health: a worldwide problem

Population studies worldwide have elucidated that not only occupationally exposed workers but also environmentally exposed humans, especially the ones living near (heavily) contaminated sites, and smokers can be affected by Cd. Several studies conducted on environmentally or occupationally exposed humans such as the OSCAR study, CadmiBel and PheeCad study or studies concerning Itai-itai disease showed the health risks of Cd pollution. They are described briefly below. It should be said, to be complete, that Cd contamination and toxicity is, of course, not limited to the countries listed below.

Japan

One of the most studied and heavily contaminated countries in the world is Japan. It has a long history of mining activities. Many mines have been in operation since the sixteenth century, and during the late 1800s and early 1900s focus was on extraction of zinc, copper and lead. Around 1910 reports of an unusual disease in the Jinzu River basin in the Toyama Prefecture first appeared (Kaji, 2004). This disease, called Itai-itai (Fig. 1.2 A), which means "ouch-ouch", was endemic in the Jinzu River area between 1940-1965. Only in 1968 the disease was recognized to be caused by Cd (Nomiyama, 1980). Most patients were elderly females who suffered from a severe pain all over the body (Nomiyama and Nomiyama, 1998). Elderly women, often deficient in iron when in the menopause, tend to take up more Cd and have a faster bone demineralization compared to men. In combination with their quality of life (multiparous, war, little and monotonous food), they were more sensitive to Cd-induced damage (Horiguchi et al., 2004; Kazantzis, 2004; also see later 1.2.4).

The site from where Cd arose, the Kamioka Mine of Mitsui Capital, was located in the upper stream of the Jinzu River and produced mostly zinc and lead (Kaji, 2004). Cd had always been discarded into the rivers as an impurity during zinc mining activities. Cd reached the irrigation channels used for the region's rice fields, and the Jinzu River was also used for drinking, washing, fishing, etc. Now it is known that this Itai-itai disease that brings along bone (osteomalacia and osteoporosis) and renal damage (atrophy and degeneration of tubules) is caused

by eating primarily Cd-polluted rice (responsible for 30-40 % of Cd intake), along with nutritional deficiencies for iron, zinc and other minerals (Tsukahara et al., 2003).

Fig 1.2 *Cadmium intoxication in Japan. A. Person suffering from Itai-itai disease, characterized by severe malformations of the bones, osteoporosis, osteomalacia and renal dysfunction. B. Most heavily contaminated areas in Japan, mostly due to mining activities. Pref.= Prefecture.*

The estimated daily intake via food (reported in the late 60s) reached very high levels in the whole of Japan (Fig. 1.2 B), with the highest levels (600 µg/day) found in the Jinzu river basin, where Itai-itai disease was endemic, followed by the Sasu-Shiine river basin (490 µg/day), the Usui-Yanase river basin (400 µg/day) and the Namari-Nihazama river basin (320 µg/day). Also, direct human consumption of the river water might have taken place in the Jinzu river basin area, which could increase the (individual) estimated Cd intake to values as high as 1600 to 2000 µg/day. A reduction in the daily Cd intake has been reported since remediation work in the river basins started (Ikeda et al., 2004). Intake of Cd (40-50 µg/day) via food from non-polluted areas in Japan was higher than the intake in the general world population (15-25 µg/day in Europe and North-America) as well.

Belgium

Belgium is the principal producer of Cd in Europe. This has led to a severe environmental contamination by this metal (Staessen et al., 1995). Emissions from the non-ferrous metal industry have contaminated the Meuse valley near Liège and the rural northern part of the Kempen during the past two centuries (Bernard et al., 1992). The severity of the problem is clear when we see that Belgium is –by far- the leading country among 30 countries concerning Cd in soil (Sillanpää and Jansson, 1992). Studies in the early 1970s have shown that chronic Cd poisoning in Belgium has led to renal dysfunction with proteinuria in occupationally exposed workers (Lauwerys et al., 1974).

In the Northern Kempen an area of 280 km^2 has been contaminated with heavy metals: Balen, Mol, Hamont-Achel, Lommel, Neerpelt and Overpelt (Fig. 1.3), with soil Cd concentrations higher than 1 mg/kg soil, while normally concentrations vary between 0.1 and 0.8 mg/kg in non-polluted soil. Furthermore, 2 heavily contaminated areas with concentrations higher than 3 mg/kg arise in Balen-Wezel/Lommel (52 km^2) and in Overpelt (16 km^2).

Studies involving the general population were set up: the CadmiBel (Cd in Belgium; baseline) study, conducted from 1985 until 1989, and the PheeCad (Public Health and Environmental Exposure to Cd; follow-up) study, conducted from 1991-1995. Over 2300 men and women of 4 Belgian districts (Liege, Charleroi, Noorderkempen and Hechtel-Eksel) that were defined by urbanization grade and environmental Cd pollution level, participated in the first study. Follow-up studies were conducted in the Noorderkempen (Fig. 1.3) with the aim to examine the progression of kidney dysfunction (Hotz et al., 1999), the risk of bone fractures (Staessen et al., 1999) and the appearance of lung cancer (Nawrot et al., 2006) in this area.

In these studies, environmental characteristics were measured in the polluted area, with Hechtel-Eksel as a reference area. Soil concentrations were 6 times higher in the contaminated regions, and also Cd levels in vegetables (from home gardens) differed significantly, for example beans (0.42 parts per million (ppm) compared to 0.15 ppm) and celery (2.43 ppm compared to 0.68 ppm) (Staessen

et al., 1995). Furthermore the CadmiBel study showed that the Cd values in urine (U-Cd) were 30 % higher among the population living in the contaminated areas. It was shown that sub-clinical changes in tubular function such as tubular proteinuria (excretion of β2-microglobulin (β2-m), aminoacids, calcium and retinol-binding protein (RBP)) and the appearance of proximal tubular enzymes (N-acetyl-β-D-glucosaminidase (NAG)) in urine occurred in the general population above a threshold of U-Cd as low as 2 µg/24h (Buchet et al., 1990; Hotz et al., 1999). Cd also increased the risk of bone fractures, especially in women, even at a low degree of environmental exposure (Staessen et al., 1999).

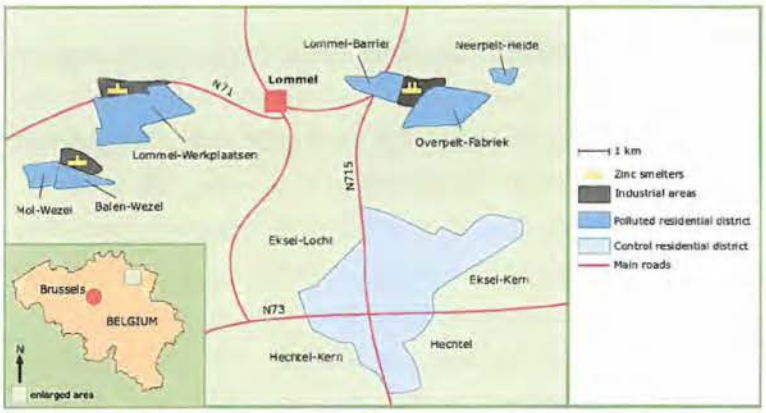


Fig. 1.3 *Map of six polluted and four control districts in Belgium, used in the CadmiBel and PheeCad study (Staessen et al., 1999).*

Recent studies showed significantly higher Cd levels in bean, potato, carrot, lettuce, leek and celery in the Noorderkempen. Cd levels in house dust were 3 times higher in the contaminated areas as well. Cd levels in these vegetables and in dust were all significantly correlated with U-Cd levels in the contaminated area. A significant association between the risk of lung cancer and environmental exposure to Cd has been described as well (Nawrot et al., 2006; Hogervorst et al., 2007).

Sweden

Swedish studies focused on environmental and occupational exposure to Cd. Similar to the Belgian studies, the OSCAR (Osteoporosis-Cd As a Risk factor) study was performed during the 90s in the south of Sweden, a region in the vicinity of Ni-Cd battery factories. This was a cross-sectional study performed on more than 1000 people, who had lived at least 5 years and were still living in an area close to a nickel battery plant between 1910 and 1992; some of them were also occupationally exposed, which resulted in a large range of Cd exposures. This study showed that there was a threefold increased risk of low bone mineral density and an increased risk of forearm fractures in people over 50 years of age (Alfvén et al., 2000, 2004; Järup et al., 2000).

The WHILA (Women's Health in the Lund Area) study, which was performed in southern Sweden on 820 women from 1999 through early 2000, also examined the association between low-level Cd exposure and osteoporosis, and Cd exposure in relation to tubular and glomerular function. The results suggested a negative effect of low-level Cd exposure on bone, possibly exerted via increased bone resorption, which was intensified after menopause (Akesson et al., 2006). Also impaired tubular reabsorption (increased urinary α1-microglobulin (α1-m) levels), increased general turnover of tubular cells (increased urinary NAG levels) and a decreased glomerular filtration rate (GFR) was observed at lower Cd levels (U-Cd of 0.8 µg Cd/g creatinine, corresponding to approximately 20 µg Cd/g kidney cortex) than in previous studies that observed effects at low-level Cd exposure (Akesson et al., 2005).

1.2.4 Cd enters the human body: uptake routes, target organs and health consequences

Ingestion versus inhalation

The general non-smoking human population is exposed to Cd via dietary sources: 98 % of the ingested Cd comes from terrestrial foods, while only 1 % comes from aquatic foods (fish, shellfish) and 1 % arises from Cd in drinking water (International Cadmium Association, Brussels, Belgium). Only 2 to 6 % of this ingested Cd will be taken up into the bloodstream, so more than 90 % of

ingested Cd will be excreted immediately via the faeces. However, iron deficient people tend to take up more Cd, i.e. up to 15 to 20 %, and factors as Cd species or type of diet can influence uptake rates as well (Järup et al., 1998; Zalups and Ahmad, 2003).

The second important route of exposure is inhalation, which will mostly affect occupationally exposed workers and smokers in the form of Cd oxide (CdO). Between 10 to 50 % of Cd in the lung will be taken up into the bloodstream, which makes it much more efficient than ingestion. Because of that, smokers have about 4 to 5 times higher B-Cd levels and twice as high kidney cortex Cd (K-Cd) concentrations as non-smokers (Järup et al., 1998).

A third but negligible route is dermal exposure, which is not regarded to be of significance although small amounts can be absorbed percutaneously during long periods of exposure (Wester et al., 1992).

Uptake and transport to target organs

The two main routes of exposure are ingestion and inhalation. The Cd transport throughout the body is shown in Fig. 1.4.

In the **gastrointestinal system**, the divalent metal transporter 1 (DMT1) is probably responsible for the uptake of Cd into enterocytes. DMT1 is a transmembrane, proton-coupled metal ion cotransporter, being expressed in enterocytes and cells in the liver, kidney, brain, lung, heart and testis. It is known for its ability to transport a broad range of bivalent metal cations including Fe^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+} and Pb^{2+} (Andrews, 1999; Gunshin et al., 1997). Upon iron deficiency the expression of intestinal DMT1 is increased, explaining the higher Cd absorption in iron deficient people (Satarug and Moore, 2004; Tallkvist et al., 2001). Other possible uptake routes include Ca^{2+}-channels and/or uptake of Cd as S-conjugates of cysteine (Cys) or Cys-containing oligopeptides (Zalups and Ahmad, 2003). A potential transporter responsible for the basolateral transport of Cd out of enterocytes is the metal transport protein 1 (MTP1), which is an Fe^{2+} transporter homologous to DMT1 and which has been identified in mouse (Abboud and Haile, 2000).

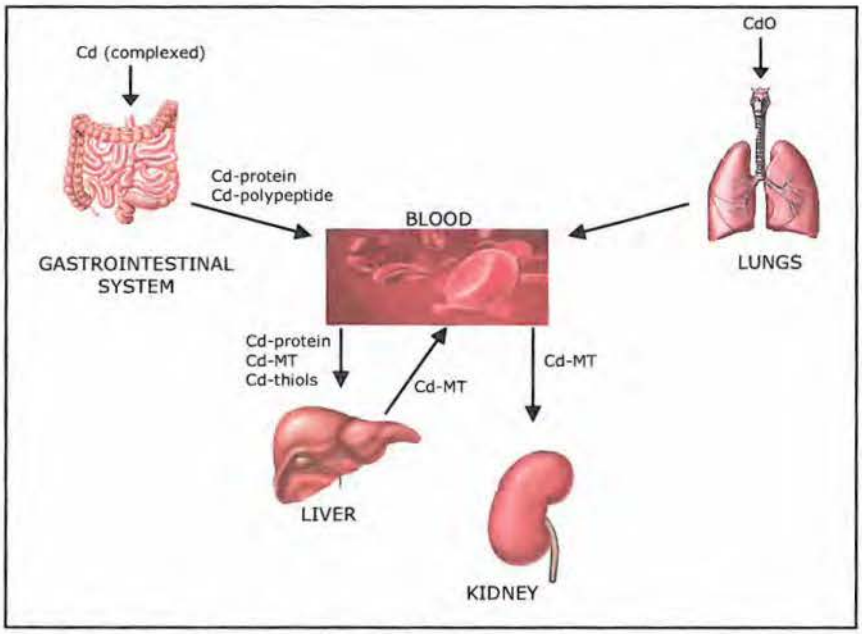


Fig. 1.4 *Cd transport throughout the body.*

Cd absorption and **lung** deposition vary with the inhaled particle size; particle deposition in the alveoli remains significant for diameters smaller than 10 μm, whereas particles of larger diameters deposit preferentially in the nasopharyngeal and thoracic region of the respiratory tract (Jumarie, 2002). CdO generated during the burning of cigarettes is highly bioavailable. Approximately 10 % of the inhaled CdO is deposited in lung tissues, and another 30-40 % is absorbed into the systemic blood circulation (Satarug and Moore, 2004). Particle absorption is considerably less for insoluble salts such as Cd-sulphide (WHO, 2000).

The absorbed Cd is transported in the **blood**, bound mainly to the high-molecular weight protein (HMWP) albumin (Nordberg et al., 1971).

The blood will transport these complexes to the **liver**, where Cd^{2+} may be taken up by hepatocytes by means of receptor-mediated endocytosis through protein binding (Bridges and Zalups, 2005) or via Ca^{2+} channels (Souza et al., 1997). Cd will induce the synthesis of metallothionein (MT), a low molecular weight

(LMW), Cys-rich, metal-binding protein which can bind up to 7 metal ions. This MT enables the liver to detoxify and protect itself from Cd (or other metals) and CdMT can accumulate in the liver (Klassen et al., 2004). Some CdMT is released when hepatocytes die due to Cd injury or CdMT leaks into the blood or might even be transported out of the hepatocytes back into the bloodstream.

The CdMT complex is transported in the **blood** to the second and most important target organ: the **kidney**. Being a LMW-complex, it can be freely filtered through the glomerulus and taken up by the kidney. The proximal tubule (PT) (Fig. 1.5) is responsible for the bulk uptake of this CdMT (Zalups and Ahmad, 2003; Bridges and Zalups, 2005). Megalin/cubulin, responsible for the uptake of many ligands with very different properties, appears to be the predominant receptor for uptake of MT and its conjugated heavy metals in the kidney (Klassen et al., 2004). Furthermore it is also thought that DMT1, ZnT1 (zinc transporter 1), stretch-activated cation channels (SAC) or the sodium-amino acid cotransporter (Cd complexed with Cys or histidine) might be involved in the toxic metal transport (Barbier et al., 2005). Another possible uptake route is via Ca^{2+} channels. Although it is very unlikely that Cd^{2+} exists as an unbound cation in the proximal tubular lumen, bound Cd^{2+} ions could undergo a ligand-exchange reaction and bind to the entry site of the Ca^{2+} channel and gain access to the intracellular milieu (Zalups and Ahmad, 2003). The latter uptake routes may proceed by ionic and/ or molecular homology or 'mimicry'; the free Cd^{2+} ions will predominantly enter in the straight (S3) portion of the PT, reflecting the segment-specific localization of the transporters (Bridges and Zalups, 2005).

As described before, Cd will predominantly enter the proximal tubular cell (PTC; especially S1 and S2 segment) in the form of CdMT via megalin/cubulin receptor mediated endocytosis (Klassen, 2004; Sabolic, 2006). When CdMT binds to these multi-ligand receptors at the apical membrane, ligands (CdMT or others) are internalized into clathrin-coated vesicles and delivered to early and late endosomes. Whereas the receptors are recycled via dense apical tubules to the apical membrane after pH-dependent release from its ligand, the CdMT-containing late endosomes fuse with lysosomes in order to further degrade the endocytosed ligand. In the endosomal/lysosomal compartments, the vacuolar

H^+-ATPase (V-ATPase) is responsible for the acidification of the vesicle, leading to the degradation of CdMT. The MT fraction is subsequently degraded further. The DMT1, which is expressed in late endo/lysosomal compartments and functions as an H^+-coupled transporter of a range of divalent transition metal ions, probably contributes to the transport of Cd^{2+} out of these acidic compartments into the cytosol (Abouhamed et al., 2006; Christensen et al., 1998; Gekle et al., 2001; Gunshin et al., 1997; Thévenod, 2003; Wolff et al., 2006).

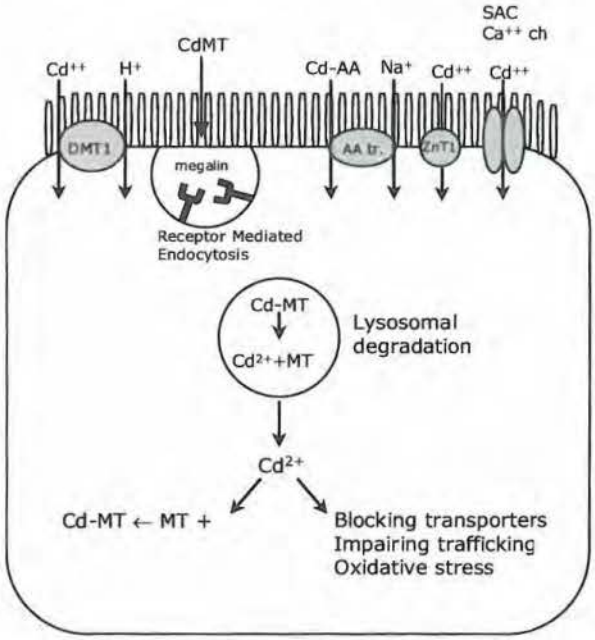


Fig 1.5 *Uptake and handling of Cd in proximal tubular epithelial cells. DMT1= divalent metal transporter 1; AA tr.= sodium- amino acid transporter; ZnT1= zinc transporter 1; SAC= stretch-activated cation channel; Ca^{2+} ch.= calcium channel;* ***Y****= megalin receptor.*

The cytosolic free Cd^{2+} will bind to preformed MT in the kidney (Klaassen et al., 1999). But when CdMT influx into the lysosomal compartment is high and the *de novo* synthesis of renal MT is insufficient or deficient to detoxify the excess free Cd^{2+}, it may cause damage to the cells (Nordberg and Nordberg, 2000).

Health consequences: acute exposure

Oral intake via food or drink of a single high dose of Cd can give rise to severe symptoms within 15 to 30 minutes (Nordberg, 1999). These include: abdominal pain, vomiting, salivation, muscle cramps, vertigo, shock, loss of consciousness and convulsions. Acute oral exposure of doses as low as 100 mg can already be lethal in humans (Hung and Chung, 2004).

Accidental acute **inhalation** of Cd fumes often causes chemical pneumonitis or metal fume fever. Symptoms of both diseases begin several hours after exposure and closely mimic each other, but it is essential to differentiate between the two since chemical pneumonitis can be life threatening (Ando et al., 1996; Hung and Chung, 2004; Leduc et al., 1993; Seidal et al., 1993).

Health consequences: chronic exposure

Exposure to Cd has been linked to pathological changes in a variety of tissues and organs. These include osteoporosis, increased bone fragility and lung cancer (Nawrot et al., 2006; Satarug et al., 2003; Staessen et al., 1999; Verougstraete et al., 2003). Cd also seems to potentiate the development of diabetic nephropathy (Bernard et al., 1991). Effects on the most important target organs are described briefly below.

The **kidney** is the primary target organ for Cd. A tubular proteinuria is the first sign of Cd-induced proximal tubular dysfunction and it is usually detected as an increased urinary excretion of LMW proteins (LMWP), which generally is irreversible (Bernard, 2004; Järup, 2002). However a reversible tubulotoxic effect has been reported. When reduction or cessation of Cd exposure (in workers) took place while urinary β2-m excretion did not exceed the upper reference limit of 300 µg/g creatinine, the risk of developing tubular dysfunction at a later stage was likely to be low, even in cases with U-Cd values higher than 10 µg/g creatinine but always below 20 µg/g creatinine (Roels et al., 1997).

Continued exposure may evolve to glomerular damage with a decreased GFR. A five times greater decrease in GFR than that accounted for by aging, was detected in occupationally exposed workers in a 5 year follow-up study after

removal from exposure (Roels et al., 1989). Severe nephrotoxicity is also characterized by renal glucosuria, aminoaciduria, hyperphosphaturia, hypercalciuria and polyuria (Järup et al., 1998; Järup, 2002; WHO 2000). The critical Cd concentration in the renal cortex associated with these changes is around 150 to 200 µg/g (WHO, 2000) in male Cd-exposed workers (Bernard et al., 1979; Roels et al., 1981, 1983, 1989). In the general population such changes have already been observed with K-Cd levels around 50 µg/g (Buchet et al., 1990; Satarug and Moore, 2004).

Long-term exposure to high doses of Cd may cause **skeletal** damage. This was first reported in Japan, where the Itai-itai disease was characterized by a combination of osteomalacia and osteoporosis (Nomiyama, 1980; Tsuchiya, 1969). The Belgian PheeCad study suggests that low-level Cd exposure may also be a risk factor for osteoporotic fractures (Staessen et al., 1999).

Prolonged urinary calcium loss caused by Cd is sufficient to promote skeletal demineralization, which may lead to increased bone fragility and risk of fractures (Satarug and Moore, 2004). Furthermore disturbances in vitamin-D metabolism play a role in bone lesions as well (WHO, 2000). Vitamin D is, together with parathyroid hormone and calcitonin, a major regulator of Ca^{2+} metabolism. The active form of vitamin D ($1,25\text{-}(OH)_2\text{-}D_3$) is formed in the kidneys and this activation may be reduced because of renal tubular dysfunction. This leads to a decreased Ca^{2+} reabsorption from the gut and impaired bone mineralization (Berglund et al., 2000). Besides this indirectly triggered skeletal damage, via renal damage, Cd also directly acts on bone. Animal studies have shown that Cd stimulates the formation and activity of osteoclasts, breaking down the collagen matrix in bone and decreasing bone mineralization (Kazantzis, 2004; Miyahara et al., 1988).

Lung toxicity due to Cd inhalation includes chronic edema, bronchitis and impaired lung function (Environment Agency, 2002; Jumarie, 2002). Mainly occupationally exposed workers suffer from these lung diseases, and an increase in the relative risk of lung cancer is found as well (Verougstraete et al., 2003).

Recently data have been published that significantly associate the risk of lung cancer with environmental exposure to Cd (Nawrot et al., 2006).

Liver damage has been associated with exposure to only very high Cd levels but data remain conflicting (Environment Agency, 2002). Animal studies have shown that liver dysfunction might be caused by free Cd^{2+} that induces free radicals in the liver (Nomiyama and Nomiyama, 1998). Rats exposed to 0.5 mg/kg and 1 mg/kg as $CdCl_2$ suffered from severe hepatic injury such as hydropic degeneration of hepatocytes, granulation and bile duct proliferation (Gubrelay et al., 2004). Periportal liver cell necrosis was apparent in animals exposed to 200 ppm of cadmium chloride ($CdCl_2$, via diet) after 2 months (Mitsumori et al., 1998).

In 1993 the International Agency for Research and Cancer (IARC) classified Cd as a group I human **carcinogen** (IARC, 1993). Cd has been associated with lung cancer (Nawrot et al., 2006; Verougstraete et al., 2003). Studies with respect to prostate and kidney cancer are conflicting (Järup, 2003; Satoh et al., 2002; Waalkes, 2003).

It has been shown in various cancer cells that a redox imbalance is related to oncogenic stimulation, and this could also be true for Cd (Valko et al., 2006). Furthermore the inhibition of DNA repair processes by Cd may contribute to the tumour initiation as well. Cd adversely affects all four major pathways of DNA repair: base-excision, nucleotide-excision, double-strand-break and mismatch repair (Il-yasova and Schwartz, 2005; Waisberg et al., 2003).

1.2.5 Markers for Cd toxicity

The use of reliable markers to assess early kidney damage in population and animal studies is of great importance. Several urinary markers as well as Cd concentrations in blood, urine and target organs have been studied intensively in order to find out whether a dose-response relationship could be established between Cd exposure, markers and (human) health.

Urinary markers

The tubular reabsorption of LMWP (i.e. molecular weight < 40 kDa) in healthy subjects is almost complete (more than 99.9%). Therefore even a small decrement in the tubular reabsorption capacity can be detected via a marked increase in the urinary excretion of microproteins (Bernard, 2004). A decreased tubular reabsorption of LMWP is seen as the most sensitive and specific indicator of Cd-induced renal dysfunction, leading to tubular proteinuria. β_2-m, RBP and α1-m are the major constituents of LMW proteinuria. The urinary excretion of β_2-m (11.8 kDa) is the most widely used method, but the drawback is the instability in acidic urine (pH < 5.6). RBP (14 kDa) is much more stable in acid urine and equally sensitive. Another very stable protein in urine is α1-m (26 kDa), but conflicting studies have been published concerning its sensitivity (Bernard et al., 1979; Bernard and Lauwerys, 1990; Bernard, 2004; Moriguchi et al., 2004). Any change in excretion rates of kidney enzymes can also be used as an indicator of Cd-induced kidney damage. Kidney-derived products originating from the brush border (BB) of PTC include alkaline phosphatase (ALP) and γ-glutamyltransferase; lysosomal enzymes include NAG, acid phosphatase, lysozyme and β-galactosidase (Price et al., 1997). More progressed proximal tubular failure is characterized by glucosuria, aminoaciduria, hypercalciuria and tubular necrosis (WHO, 2000).

An enhanced glomerular permeability to proteins gives rise to an increased urinary excretion of HMWP such as albumin, IgG (immunoglobulin G) and transferrin. This proteinuria of glomerular origin may occur separately or in association with an increased excretion of LMWP, but will only appear in progressed kidney damage (Bernard and Lauwerys, 1990, 1991; Bernard et al., 1979).

The usefulness of various urinary and bloodborne analytes as potential biomarkers of Cd toxicity has been assessed in a European Collaborative research study. Three main groups of U-Cd thresholds were identified on the basis of various urinary biomarkers in male Cd workers. Biochemical markers (sialic acids and 6-keto-PGF1α) appeared with U-Cd levels around 2 µg Cd/g creatinine, markers such as ALP and NAG, indicating cytotoxic effects, appeared

around 4 µg Cd/g creatinine and around 10 µg Cd/g creatinine markers associated with dysfunction of the tubular reabsorption (microproteinuria; RBP and β2-m) appeared in urine (Roels et al., 1993).

Cd measurements in organs, blood and urine

Cd measurements in different target organs such as the liver and the kidney, as well as in blood and in urine, can also give information on the course of Cd intoxication. Because a direct measurement of K-Cd levels by means of a non-invasive technique such as neutron activation is not easily feasible in humans, although it has been done in the past (Roels et al. 1981), most studies have used the concentrations of Cd in urine or in blood as indicators of Cd body burden (Bernard, 2004). In animal studies, it is possible to expand this dose-response study with measurements in different target organs such as the liver and the kidney.

It is generally agreed that the B-Cd level is mainly a reflection of the metal uptake during the previous few months, i.e. an indication of a current exposure. On the other hand, the urinary excretion of Cd (per gram creatinine or 24 h) is a reliable indicator of the Cd body burden as 0.005 % of the total Cd body burden is daily excreted. U-Cd also seems to be better correlated with duration of exposure than B-Cd (Bernard et al., 1992; Lauwerys et al., 1976; WHO, 2000). It was confirmed that the threshold for the occurrence of LMW proteinuria was 10 µg Cd/g creatinine in the urine (corresponding to 200 µg Cd/g renal cortex) in occupationally exposed men (also see above; Bernard et al., 1979; Roels et al., 1981, 1983, 1999). For individuals in the general population a U-Cd of 2 µg/g creatinine should be regarded as a measure of the maximum tolerable internal dose of Cd; an increased prevalence of hypercalciuria was observed when U-Cd exceeded 2 µg Cd/g creatinine and microproteinuria started with U-Cd levels of approximately 3 µg Cd/g creatinine (Buchet et al., 1990; Staessen et al., 1991).

1.3 The primary target organ for cadmium: the kidney

1.3.1 Functional anatomy of the kidney

Kidneys are paired, bean shaped and retroperitoneal organs containing a complex mixture of vascular and epithelial elements. The kidney contains two basic layers, the cortex (granular, pale outer region) and the medulla (darker inner region) (Fig. 1.6). In humans, the medulla is subdivided in conically shaped renal pyramids, while the kidney of many laboratory animals such as the rat and the mouse, have a single renal pyramid (unipapillate). A kidney consists of nephrons (Fig. 1.6), the functional unit of the kidney that is repeated approximately 1 million times within each kidney in man. The PT, the target site for Cd, begins abruptly at the urinary pole of the glomerulus and consists of an initial convoluted portion, the *pars convoluta*, and a straight portion, the *pars recta*. Based on ultrastructure, the PT can alternatively be subdivided into three segments: S1, S2 and S3.

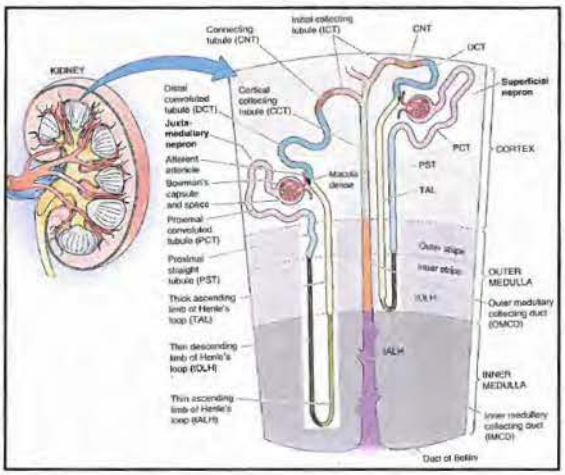


Fig. 1.6 *Structure of the nephron. The proximal tubule, the target site for cadmium, consists of a proximal convoluted tubule (PCT) and a proximal straight tubule. Mainly the PCT is targeted (picture from Giebisch and Windhager, Fig. 32-2 p. 739, from Boron and Boulpaep, Medical Physiology, 2003; with permission of Elsevier).*

The S1 segment is the initial portion of the PT, which begins at the glomerulus and constitutes approximately two thirds of the pars convoluta. A tall BB, a prominent endocytic-lysosomal apparatus and extensive invaginations of the

basolateral plasma membrane characterize the S1 cells. The S2 segment starts within the second half of the convoluted tubule and continues into the first half of the straight tubule. The BB is less prominent than in the S1 segment. Finally, the S3 segment represents the remainder of the PT and extends into the medulla. The BB in this segment is tall, but the endocytic-lysosomal apparatus is less prominent, correlating with a gradual decrease of reabsorptive rates along the tubule (Giebisch and Windhager, 2003; Tisher and Madsen, 2000).

Epithelia have the unique capacity to transport solutes and water from one surface to another. The PT is responsible for the retrieval of the largest fraction (50-60 %) of the solutes and water filtered at the glomerulus. The PTC reabsorb NaCl and $NaHCO_3$, as well as an osmotically obligated volume of water, divalent ions such as Ca^{2+}, HPO_4^{2-} and SO_4^{2-}, and organic solutes (glucose, amino acids). Furthermore, they play a role in the regulation of the acid-base balance by reabsorbing the bulk of filtered HCO_3^- and by secreting NH_4^+ and finally, several organic anion and cation transporters secrete a variety of endogenous and exogenous solutes into the PT lumen (Giebisch and Windhager, 2003; Moe et al., 2000).

1.3.2 Cd and proximal tubular damage

As Cd is reabsorbed mostly by the PTC, it will interfere with important and crucial processes in the kidney. This may lead to irreversible kidney damage and renal failure. The target site for Cd is especially the S1 and S2 segment, and to a smaller extent the S3 segment (Thévenod, 2003). Cd can induce a general transport defect of the PT, similar to the De Toni-Debré-Fanconi syndrome, where an impairment in proximal tubular function leads to urinary loss of proteins, amino acids, uric acid, glucose and phosphate (Jacquillet et al., 2006).

Several *in vivo* and *in vitro* studies have been conducted to elucidate the mechanisms involving kidney damage due to Cd. It should be stressed that the studies summarized below were conducted with high Cd concentrations: animals were injected with high concentrations and cells or organelles were exposed to concentrations up to 200 µM Cd.

In *in vitro* models the deleterious effects of free cytosolic Cd^{2+} have been described on several Na^+-dependent brush-border membrane (BBM) transporters for phosphate (NaPi-2a), glucose and amino acids (Ahn et al., 1999; Barbier et al., 2004; Blumenthal et al., 1990; Kim et al., 1990; Kim and Park, 1995; Lee et al., 1991; Park et al., 1997; Sabolic et al., 2002). Direct exposure of normal renal BBM vesicles to free Cd^{2+} (50-200 µM Cd) induced alterations in transport activities but exposure of vesicles to CdMT had no such effect. (Ahn et al., 1999). Such a reduction in transport capacity may be attributed, in part, to a loss of carrier units in the membrane due to inhibition of the *de novo* protein synthesis (Herak-Kramberger et al., 1996). Free Cd^{2+} may also directly interact with BBM transporters (Ahn et al., 1999; Sabolic et al., 2001).

The inhibition may also be due to an indirect effect and evidence for this theory is favoured by some *in vivo* studies, where rats were subcutaneously injected with 2 mg Cd/kg body weight (bw)/day as $CdCl_2$ during 14 days. K-Cd values in these Cd-treated rats were 265 µg/g kidney cortex. An impairment of the acidification in cell organelles, causing a loss of the V-ATPase and inhibiting the intrinsic V-ATPase activity, as well as dissipating the transmembrane pH gradient were found to inhibit endocytosis of filtered proteins and impair vesicle-mediated recycling of some membrane transporters (Herak-Kramberger et al., 1998; Sabolic et al., 2002). In time this will lead to a loss of megalin, V-ATPase, aquaporin-1 and type 3 Na^+/H^+ exchanger from the BBM (Sabolic et al., 2002). Furthermore the integrity of PT plasma membranes might be damaged, resulting in shortening and loss of microvilli and basolateral invaginations (Herak-Kramberger et al., 2001). The actin and tubulin cytoskeleton might be targeted as well, resulting in an impaired intracellular vesicle trafficking, loss of cell polarity and diminished clathrin-mediated recycling of BB transporters. The loss of basolateral invaginations and the associated Na^+/K^+-ATPase in the cortical PT may also contribute to the loss of PT function. (Sabolic et al., 2006).

Another target organel for Cd^{2+} is the mitochondrion. A nephrotoxic dose of CdMT caused respiratory dysfunction in rat renal cortical mitochondria, indicating an inhibition of electron transfer and oxidative phosphorylation (Tang

and Shaikh, 2001). An increase in the inner mitochondrial membrane permeability and osmotic swelling was observed in PTC exposed to > 2 μM Cd^{2+}, leading to an increase in cytochrome c release from the intermembrane space and ultimately to apoptosis (Lee et al., 2005). Furthermore a perturbation in the Ca^{2+} homeostasis caused by Cd is thought to lead to apoptosis; Cd^{2+} ions have been shown to interfere at various levels with Ca^{2+} homeostasis and Ca^{2+}-mediated signal transduction (Biagioli et al., 2005).

Another theory postulates the generation of reactive oxygen species (ROS) by Cd^{2+} in an indirect way, by depleting endogenous intracellular radical scavengers (Stohs and Bagchi, 1995) or by impairing electron transfer in mitochondria (Wang et al., 2004). This results in an imbalance between generation of ROS and cellular antioxidant production, causing lipid peroxidation, protein modification and DNA damage (Leonard et al., 2004). Oxidative stress, antioxidants and defence mechanisms will be described in more detail in section 1.4.

Unfortunately, so far, there is still no clear evidence concerning the precise mechanisms by which Cd induces these changes and these questions remain to be elucidated.

1.3.3 Histopathological features of Cd-induced nephrotoxicity

Ultrastructural changes

Not that much is known about the effects of a long-term low-level Cd exposure on the ultrastructure of the kidney. In many reports animals were injected with high Cd concentrations to study (often acute) histopathological changes. Cd administration produced damage to the entire kidney, including PTC degeneration, interstitial inflammation and fibrosis, glomerular swelling, atrophic and pyknotic nuclei, interstitial edema, glomerular basement membrane swelling, mitochondrial swelling, clear vacuoles, apoptosis, necrosis, occasional segmental sclerosis and mesangial expansion in the glomeruli (Kauer et al., 2006; Liu et al., 1998; Liu et al, 2000a; Tanimoto et al., 1999; Uriu et al.,

1998). The Cd content in the renal cortex of these Cd-injected animals was extremely high (up to 215 µg/g) and might explain the wide variety and severity of the histopathological changes.

Recently a greater interest is going towards the effects of a chronic low-level exposure via ingestion. Histopathological changes are often not that dramatic when compared to the injection-based effects and are confined to the PT (Asar et al., 2004; Gallien et al., 2001; Uriu et al., 2000). Morphological studies showed alterations in PT that were mainly characterized by vacuolization, numerous lysosomes and pinocytic vesicles, condensed mitochondria and tubular lumina filled with cytoplasmic debris (Asar et al., 2004; Gallien et al., 2001). Sometimes the BB was destroyed, the glomerular basement membrane was thickened and some nuclei were pyknotic or hypertrophic (Asar et al., 2004; Uriu et al., 2000). The K-Cd content was markedly lower in these ingestion-based exposure studies compared to the ones described above (injection-based).

The involvement of Cd in renal interstitial fibrosis

The extracellular matrix (ECM) consists of glycoproteins and glycosaminoglycans that are embedded within a fibrillar reticulum (collagen fibres I, III and VI and nonbanded microfilaments) (Asar et al., 2004). Excessive accumulation and deposition of ECM components in the kidney may lead to renal fibrosis. Simply speaking it is a failed wound-healing process of the kidney tissue after chronic, sustained injury; a process that may lead to loss of functional tissue and possibly to organ failure in the end (Liu, 2006).

When a kidney is injured, glomerular or interstitial infiltrated inflammatory cells become activated and produce ROS, fibrogenic and inflammatory cytokines. These, in turn, stimulate several cellular pathways, including mesangial and fibroblast (myofibroblastic) activation as well as tubular epithelial-to-mesenchymal transition (EMT) leading to the production of a large amount of ECM components. Characteristic for mesangial and fibroblast activation, as well as tubular EMT, are the *de novo* expression of α-SMA, a contractile protein normally restricted to perivascular smooth muscle cells, and overproduction of

interstitial matrix components such as type I and type III collagen and fibronectin. Myofibroblastic activation of mesangial cells and fibroblasts is an early fibrogenic response after injury, whereas tubular EMT often occurs in a delayed fashion. The excessive accumulation and deposition in the kidney, as well as a defect in its degradation, may lead to renal fibrosis.

Among many fibrogenic factors, transforming growth factor-β (TGF-β) is the one that plays a central role in fibrogenic cell activation. Some others, such as angiotensin II and high glucose, act as upstream TGF-β inducers.

Both an acute, transient renal injury and a chronic kidney disease trigger similar responses, but the former will result in damage repair while the latter will lead to maladaptation. This maladaptation leads to overproduction of matrix components and defects in its degradation and finally results in fibrosis. The processes described above then result in widespread tissue scarring that leads to the complete destruction of kidney parenchyma and to end-stage renal failure (Desmoulière et al., 2005; Groma, 1998; Liu, 2006; O'Donnell, 2000).

It is known that Cd exposure may lead to renal interstistial fibrosis: chronic renal injury, which may be caused by Cd, disrupts the integrity of tubular epithelial cells that then may undergo an EMT and convert into myofibroblasts. Other characteristics of tubulointerstitial fibrosis are tubular cell and nuclear hypertrophy (Kasper et al., 2004; Wolf and Neilson, 1995).

1.3.4 Protection against Cd toxicity

Cd has a long biological half-life, ranging from 17 to 30 years in man (WHO, 2004). The danger following exposure comes from the fact that Cd is poorly excreted in the urine (0,005 % of total body burden daily) and stays accumulated, especially in renal tissue (Barbier et al., 2005). In time, this accumulation can lead to renal impairment.

First of all, it has been shown that a marginal nutritional status of zinc, iron and calcium increases the rate of Cd absorption from various food sources 7- to 10-

fold in experimental animals (Reeves and Chaney, 2002). This could be due to the increased Cd uptake into enterocytes and the longer turnover time for Cd in these cells. This would lead to higher Cd absorption rates and a greater accumulation in the internal organs (Flanagan et al., 1978; Reeves and Chaney, 2004; Reeves et al., 2005). This has been a critical factor in humans suffering from Itai-itai disease; due to their monotonous eating behaviour (rice-based diets) and marginal life status, they were at higher risk of developing nephrotoxicity and bone diseases.

Heavy metal chelators such as the non-sulphured conjugate EDTA (ethylenediaminetetraacetate) or the sulphured conjugates DMSA (meso-2,3-dimercaptosuccinic acid) and DMPS (2,3-dimercapto-1-propanesulfonic acid) show real effectiveness in increasing metal elimination through urine. With such chelators the toxic effects of metals such as Pb, Hg and Cu have been diminished but unfortunately the elimination of Cd with such compounds has failed so far (Barbier et al., 2005; Blanusa et al., 2005, Burckhardt et al., 2002).

The natural D-Cys on the other hand can be used as a chelating substance without secondary effects (Barbier et al., 2005). Furthermore several studies have shown that supplementation with selenium (Nehru and Bansal, 1997), vitamin E (Choi and Rhee, 2003), zinc (Jacquillet et al., 2006; Tang et al., 1998), magnesium (Boujelben et al., 2006), N-acetyl Cys (Shaikh et al., 1999a) and caloric restriction (Shaikh et al., 1999b) prevented Cd-induced nephrotoxicity.

1.4 Oxidative stress and antioxidant defence systems

Oxygen is the most prevalent element in the earth's crust and exists in air as the diatomic molecule O_2 (Magder, 2006). It is bi-radical, or in other words, it has two unpaired electrons with parallel spin. This makes oxygen non-reactive to most organic molecules unless it is 'activated'. According to Pauli's exclusion principle, this is only possible with a divalent molecule that has two unpaired electrons with parallel spin opposite to that of oxygen. Due to this spin restriction, the most common mechanisms of oxygen reduction in biochemical reactions are those involving transfer of only a single electron (monovalent reduction) (Turrens, 2003).

In living organisms, except for some anaerobic bacteria, such reactions are necessary for energy production (Magder, 2006). The electron transport chain in the inner mitochondrial membrane generates these ROS as a part of normal cellular function (Leonard et al., 2004). Potent protective mechanisms have evolved to allow cells to cope with these dangerous species. Furthermore ROS play a role in the regulation of many intracellular signaling pathways as signal transduction messengers or as activators of signal transduction pathways (Leonard et al., 2004; Magder, 2006). This may lead to proliferation or apoptosis, immunity, inflammatory responses and defence against micro-organisms (Magder, 2006; Matés and Sànchez-Jiménez, 1999). Nevertheless high doses or inadequate removal of ROS can result in oxidative stress, which may cause severe malfunctions, cell injury and death (Matés and Sànchez-Jiménez, 1999).

1.4.1 Oxidative stress

Under normal conditions antioxidant systems of the cell minimize the perturbations caused by ROS (Matés, 2000). The effects of ROS are balanced by the antioxidant action of non-enzymatic antioxidants as well as antioxidant enzymes (Valko et al., 2004).

When ROS generation is increased to an extent that overcomes the cellular antioxidants, the result is oxidative stress (Matés, 2000). This implies that pro-oxidant systems outbalance the antioxidants, potentially producing oxidative damage to lipids, proteins, carbohydrates and nucleic acids. This may ultimately lead to cell death in severe oxidative stress. Besides an increased ROS production, oxidative stress may also be caused by a decrease in antioxidants because of depletion or mutations affecting antioxidant defence enzymes (Halliwell and Gutteridge, 2002).

A disturbance in pro-oxidant/antioxidant status may result from many different oxidative challenges such as radiation, metabolism of environmental pollutants and administered drugs and immune system responses to disease or infection (Thomas, 1999). ROS formation and antioxidant defence systems will be discussed in further detail below.

1.4.2 Reactive oxygen species

Formation of ROS

ROS, either beneficial and functional or deleterious and damaging, can be produced from both endogenous and exogenous substances (Valko et al., 2006). The term includes free radicals (chemical species with one unpaired electron) as well as related reactive species that can take part in radical type reactions but do not have unpaired electrons (Magder, 2006; Turrens, 2003).

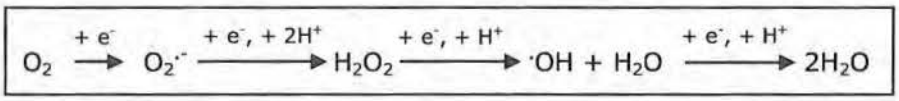


Fig. 1.7 *Univalent reduction of O_2 and formation of ROS. The total reaction: $O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$, occurs in the 'inner mitochondrial membrane' during the electron chain reactions.*

The reduction of oxygen (Fig. 1.7) by one electron produces the superoxide anion, $O_2^{\cdot -}$, which is the precursor of most ROS and a mediator in oxidative chain reactions (Turrens, 2003). Addition of an electron will give rise to the peroxide ion (O_2^{2-}), which is not a radical and is present as hydrogen peroxide (H_2O_2) in

biological samples. It is more stable than $O_2^{\cdot-}$ and can diffuse across membranes (Magder, 2006). Addition of another electron will give rise to the very harmful hydroxyl radical, ·OH. In the presence of a transition cation such as iron or copper, superoxide anions may give rise to this highly reactive species by the Haber-Weiss reaction (see below + Fig. 1.8) (Thomas, 1999). Addition of a fourth electron gives rise to water.

Besides H_2O_2, other examples of non-radical ROS are hypochlorous acid (HOCl), ozone (O_3) and singlet oxygen ($^1\Delta gO_2$) (Halliwell and Gutteridge, 2002; Magder, 2006). Besides oxygen-based radicals, there are also reactive nitrogen species such as nitric oxide ($NO^{\cdot}$) and nitrogen dioxide (NO_2), sulphur based molecules and carbon-centered molecules (Magder, 2006).

Sources and reactions of ROS

ROS can be produced from both endogenous and exogenous substances. The most important source of $O_2^{\cdot-}$ *in vivo* is the mitochondrial electron transport chain, which leaks some electrons directly onto O_2. Under physiological conditions about 1 to 3 % of the oxygen molecules in the mitochondria are converted into superoxide. Other cellular sources of $O_2^{\cdot-}$ are cytochrome P-450, xanthine oxidase (XO), microsomes and peroxisomes. The cytochrome P-450 is the terminal component of the monoxygenase system. It is responsible for the detoxification of foreign compounds into less toxic compounds by oxidation or hydroxylation reactions. During these reactions, electrons may be leaked onto oxygen molecules, forming H_2O_2 or $O_2^{\cdot-}$. XO catalyses the hydroxylation of purines. In particular XO oxidises hypoxanthine to xanthine and xanthine to uric acid. In both steps molecular oxygen is reduced, forming a superoxide anion and hydrogen peroxide (Valko et al., 2004; Valko et al., 2006). An extracellular source of ROS are the membrane NAD(P)H oxidases (NOX). The most studied one is the leukocyte NADPH oxidase with its ability of recognizing a foreign particle and undergoing a series of reactions called the respiratory burst. This enables the cell to provide oxidising agents for the destruction of the target cell (Valko et al., 2004). NADPH oxidase can also be found in non-inflammatory cells, where they may have a role in intracellular signaling processes (Pallone,

2006). A member of the NOX family, NOX 4, is widely expressed in kidney, vascular cells, osteoclasts etc.

Exogenous sources capable of producing ROS are various xenobiotics such as chlorinated compounds, metal (redox and non-redox) ions, radiation and barbiturates (Valko et al., 2006). More specifically, in the presence of a transition cation such as iron or copper, the superoxide anion may give rise to the hydroxyl radical species ·OH in the Fenton or Haber-Weiss reaction (Thomas, 1999; Valko et al., 2006). In the Fenton reaction a transition metal ion reacts with H_2O_2 to generate an ·OH radical and an oxidized metal ion (Fig. 1.8). In the Haber-Weiss reaction an oxidized metal ion is reduced by $O_2^{\cdot-}$ and then reacts with H_2O_2 to generate an ·OH radical (Leonard et al., 2004). The involvement of metal ions in ROS production will be discussed further in 1.4.3.

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + {\cdot}OH + OH^- \text{ (Fenton reaction)}$$

$$M^{(n+1)+} + O_2^{\cdot-} \rightarrow M^{n+} + O_2$$

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + {\cdot}OH + OH^-$$

$$O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + {\cdot}OH + OH^- \text{ (Haber-Weiss reaction)}$$

Fig. 1.8 *Fenton reaction and Haber-Weiss reaction. Both can be catalysed by redox-active metal ions (M).*

ROS can be involved in several damaging reactions in the cells. When a free radical reacts with a non-radical, a new radical may be generated and chain reactions may be set up. Examples of such damage are lipid peroxidation or damage to DNA and proteins (Valko et al., 2004).

1.4.3 Heavy metals and the formation of ROS

Metals such as iron, copper, zinc, cadmium, chromium, lead, mercury, nickel and vanadium can induce the production of ROS. Metal-mediated formation of free radicals causes DNA damage, enhanced lipid peroxidation and changes in calcium and sulphydryl homeostasis (Stohs and Bagchi, 1995; Valko et al., 2006). Cell signaling pathways involving redox-sensitive transcription factors

such as AP-1, nuclear factor κB and p53 can also alter due to metal ion-induced oxygen radicals (Kalyanaraman, 2004).

Redox-active metals such as iron, copper, chromium and nickel have the ability to generate free radicals via a Fenton-type or Haber-Weiss type reaction. These reactions generate the very reactive hydroxyl radical ·OH (Fig. 1.8) (Stohs and Bagchi, 1995). On the other hand, redox-inactive metals such as lead, cadmium and mercury deplete cells' major antioxidants (Ercal et al., 2001).

Cadmium itself can only generate free radicals indirectly (Fig. 1.9). It is thought that Cd can replace redox active metals such as iron and copper. The free or chelated copper and iron ions then participate in oxidative stress via Fenton reactions (Valko et al., 2006). ROS formation may also be due to depletion of endogenous intracellular radical scavengers (Ercal et al., 2001; Thévenod, 2003). Furthermore it has been reported that Cd inhibits DNA repair pathways (Giaginis et al., 2006; Hossain and Huq, 2002a) and binds to DNA (Hossain and Huq, 2002a, 2002b; Kasprzak, 2002). Cd also induces genes with protective functions, heat shock proteins and protooncogenes.

To counteract these ROS, the cells have various defence (antioxidant) systems in an attempt to prevent these deleterious effects. Antioxidants are substances that either directly or indirectly protect cells against adverse effects of xenobiotics, drugs, carcinogens and toxic radical reactions (Halliwell, 1995; Halliwell et al., 1995). A schematic overview of above described events is given in Fig. 1.9. The major antioxidants will be discussed in the next section.

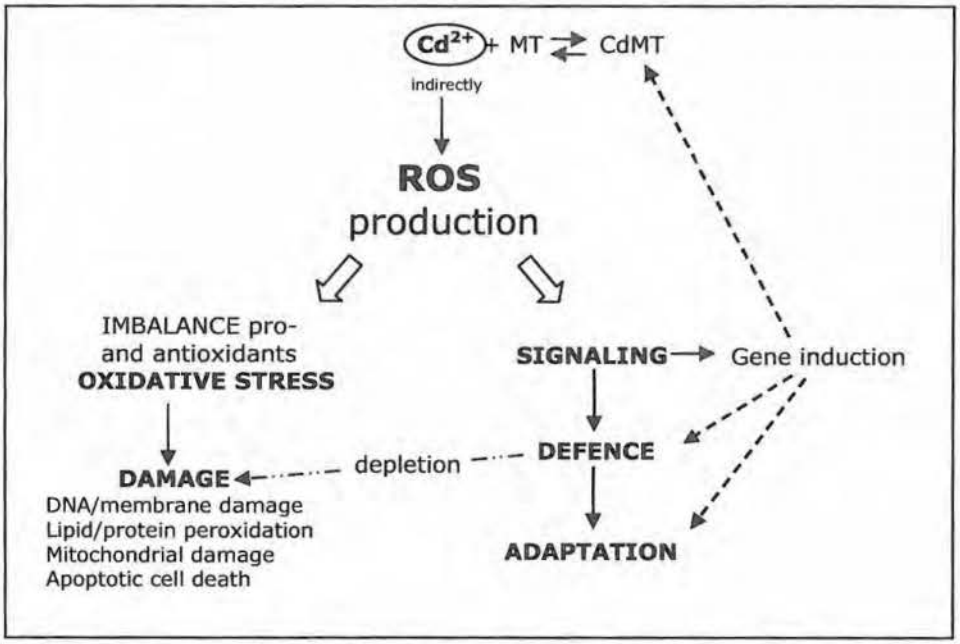


Fig. 1.9 *Schematic overview of the (indirect) formation of ROS by free Cd^{2+} in the kidney, which can lead to oxidative stress or a defence reaction. When Cd^{2+} enters the PTC, it will complex with MT to form the harmless CdMT complex. When MT is not available, free Cd^{2+} can indirectly induce the formation of ROS. These ROS can lead to two pathways, one of damage and one of adaptation/survival. If an imbalance between pro- and antioxidants arises, this may ultimately lead to damage. Whenever via signalling the defence system of the kidney is activated and can cope with the ROS (by for example inducing gene expression of defence proteins), the cell might survive. The depletion of radical scavengers such as GSH on the other hand, may also lead to cell damage.*

1.4.4 Antioxidant defence

To be able to overcome the problems associated with (increased) ROS production, cells have several sophisticated defence mechanisms. In general, antioxidant defence mechanisms can be divided into 4 major groups. Cells have **antioxidant molecules** such as GSH, MT and thioredoxin (Trx); **antioxidant enzymes** such as superoxide dismutase (SOD) and catalase (CAT); **anti-apoptotic molecules** such as Bcl-2 and various **stress proteins** such as heat

shock protein (HSP) 70, HSP 90 and heme oxygenase (HO) (Cabell et al., 2004; Valko et al., 2004).

Functionally antioxidant defences can be divided into four classes as well. Some antioxidants (SOD, CAT, thiol-specific antioxidants) catalytically remove free radicals and other reactive species. Others minimize the availability of pro-oxidants such as iron ions, copper ions and heme (MT, transferrins). Furthermore proteins such as heat shock proteins protect biomolecules against damage. And finally, some LMW agents such as GSH, α-tocopherol, ascorbic acid (AsA) and uric acid can scavenge ROS (Halliwell and Gutteridge, 2002). The most important enzymes and molecules will be discussed in further detail. Special attention is given to the biomolecules and enzymes that we have studied by means of real time RT-PCR or that have been quantified in our study; they are underlined in the following text. Figure 1.15 (on page 44) gives a schematic overview of these pro- and antioxidants present in the cell. Of course much more defence systems than presented in this graph are present in cells.

Antioxidant molecules

GSH is a multifunctional, intracellular, non-enzymatic antioxidant (γ-Glu-Cys-Gly). It is highly abundant in cytosol (1-11 mM), nuclei (3-15 mM) and mitochondria (5-11 mM) (Valko et al., 2006). Glutathione disulfide (GSSG), formed upon oxidation, consists of two GSH molecules joined by a disulfide bond. GSH functions as an antioxidant itself, as a hydrogen donor and is a cofactor of several detoxifying enzymes against oxidative stress such as glutathione peroxidase (GPx) (Valencia et al., 2001). GSH reacts with partially oxidized products and scavenges hydroxyl radicals and singlet oxygen directly (Fig. 1.10 (1)). Furthermore GSH is able to regenerate vitamins C and E back to their active forms (Valko et al., 2006). The reduction of GSSG back to GSH is catalyzed by glutathione reductase (GR), with NADPH as a co-factor (Fig. 1.10 (2)). The GSH/GSSG ratio is often considered as an indicator of the intracellular redox state, with decreased values of GSH/GSSG pointing towards oxidative stress (Lange et al., 2002).

(1) $2GSH + prot\text{-}S_2 \rightarrow GSSG + prot\text{-}(SH)_2$

(2) $GR\text{-}S_2 + NADPH + H^+ \rightarrow GR\text{-}(SH)_2 + NADP^+$

$GSSG + GR\text{-}(SH)_2 \rightarrow 2GSH + GR\text{-}S_2$

$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$

Fig. 1.10 *The glutathione redox system. (1) Reaction of glutathione (GSH) with oxidized proteins. (2) The reduction of GSSG to GSH with glutathione reductase (GR).*

MT is a ubiquitous, LMW (6.6 kDa), Cys-rich (30 %) metal-binding protein (Klaassen et al., 1999; Sato and Kondoh, 2002). The 20 Cys provide the basis for high-affinity binding of many transition metals (Wolff et al., 2006). The dominating metals are zinc, cadmium, mercury and copper with increasing stability of binding in the order mentioned (Nordberg and Nordberg, 2000). The ability of MT to capture hydroxyl radicals is also more than 300 times greater than that of GSH (Sato and Kondoh, 2002).

The MTs consist of four major groups. **MT-1** and **MT-2** are the two most dominant forms. They are present in almost all tissues. MT-3 is present in brain and MT-4 is specific for squamous epithelium and keratinocytes (Nordberg and Nordberg, 2000). MT is a 'storehouse' for zinc, a free-radical scavenger and protects against Cd toxicity (Klaassen et al., 1999). Under heavy metal load and oxidative stress, MT expression is strongly induced at the level of transcription. The induction is mediated by the metal regulatory transcription factor-1 (MTF-1), an essential protein for liver development and cell stress response (Lichtlen and Schaffner, 2001b). MTF-1 is a ubiquitously expressed zinc finger protein that is essential for basal and heavy metal-induced expression of MTs. MTF-1 requires elevated zinc concentrations for binding to the metal-responsive elements (MRE's), located in the promoter regions of MT genes (Lichtlen and Schaffner, 2001a, 2001b). The antioxidant response elements (ARE), the glucocorticoid response elements (GREs) and the elements activated by STAT (signal transducers and activators of transcription) are also involved in the

expression of MT and are induced by H_2O_2 and electrophiles, glucocorticoids and cytokines respectively (Sato and Kondoh, 2002).

Ascorbate (AsA) or **vitamin C** is an important and powerful antioxidant that can only be obtained from our diet (Halliwell and Gutteridge, 2002). Many other animals including the mouse are able to make it themselves in the liver. Ascorbate directly scavenges oxygen free radicals with and without enzyme catalysts and indirectly scavenges them by recycling tocopherol to the reduced form. It also protects membranes against oxidation. Ascorbate converts ROS into the poorly reactive semidehydroascorbate radical ($Asc^{\cdot-}$). It can either be converted back to ascorbate by NADH-dependent enzymes or undergo disproportionation to form dehydroascorbate (DHA) (Halliwell and Gutteridge, 2002; Valko et al, 2004; Valko et al, 2006).

Antioxidant enzymes

Superoxide dismutase (SOD) catalyzes the dismutation of the highly reactive superoxide radical to the less reactive H_2O_2 (Fig. 1.11). SOD also protects dehydratases against inactivation by the free radical $O_2^{\cdot-}$ (Benov and Fridovich, 1998). Four classes of SOD have been identified, containing either a dinuclear Cu/Zn or mononuclear Fe, Mn or Ni cofactor (Whittaker and Whittaker, 1998; Matés, 2000). The overall disproportionation is accomplished by Fe-, Mn-, and Cu/Zn-SOD in two steps whereby the redox property of the metals is used for reducing the superoxide radical (Fig. 1.11) (Miller, 2004).

SOD 1 or Cu/Zn-SOD exists as a homodimer of about 32 kDa each (Forsberg et al., 2001; Matés and Sanchez-Jiménez, 1999). It is mainly present in the cytosol, a smaller amount in the lysosomes, nucleus and the space between the inner and outer membrane of mitochondria (Halliwell and Gutteridge, 2002). The SOD activity is inhibited by H_2O_2 (Yan and Harding, 1997). It is believed to play a major role in the first line of defence and appears to be a very important enzyme for the prevention of aging and mutation by oxidative stress and hazardous effects from environmental factors (Matés, 2000). Knockout studies revealed that it is not essential for life, although female mice showed a reduced

fertility, neurological damage and hearing loss. Cancers can also develop at an accelerated rate as they age (Halliwell, 2006; Ho et al., 1998).

SOD 2 or Mn-SOD is a mitochondrial homotetrameric (96 kDa) SOD containing one manganese atom per subunit that cycles from Mn(III)-Mn(II) and back to Mn(III) during the two step dismutation of superoxide (Matés, 2000). It is extremely important because this enzyme is responsible for the elimination of $O_2^{\cdot-}$ radicals formed during the respiratory chain reactions. It also effectively suppresses apoptosis induced by various stimuli (Imai and Nakagawa, 2003). The expression of Mn-SOD is essential for the survival of aerobic life as dramatically illustrated by the neonatal lethality of mice that are deficient in Mn-SOD (Macmillan-Crow and Cruthirds, 2001). Furthermore the SOD family includes SOD 3 or extracellular SOD (= Cu,Zn-SOD) (Forsberg et al., 2001; Matés, 2000), the prokaryotic Ni-SOD (Miller, 2004) and Fe-SOD (prokaryotes and chloroplasts of eukaryotic algae and plants) (Grace, 1990).

SOD

$$M^{(n+1)+}\text{-enzyme} + O_2^{\cdot-} \rightarrow M^{n+}\text{-enzyme} + O_2$$
$$M^{n+}\text{-enzyme} + O_2^{\cdot-} + 2H^+ \rightarrow M^{(n+1)+}\text{-enzyme} + H_2O_2$$
$$\mathbf{2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2}$$

CAT

$$2H_2O_2 \rightarrow 2\,H_2O + O_2$$

GPx

$$GPx\text{-}(SH)_2 + ROOH \rightarrow GPx\text{-}S_2 + ROH + H_2O$$
$$GPx\text{-}S_2 + 2GSH \rightarrow GPx\text{-}(SH)_2 + GSSG$$
$$\mathbf{2GSH + ROOH \rightarrow GSSG + ROH + H_2O}$$

Fig. 1.11 *Antioxidant reactions performed by superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). M = metal ion.*

Catalase (CAT) is a tetrameric heme-containing enzyme that catalyses the dismutation of hydrogen peroxide into water and oxygen (Fig. 1.11) (Matés, 2000). The reaction is a disproportionation (dismutation); one H_2O_2 is reduced

to H_2O and the other is oxidized to O_2. The enzyme is largely located in peroxisomes, which contain many of the cellular enzymes that generate H_2O_2 (Halliwell and Gutteridge, 2002). It is so efficient that it cannot be saturated by H_2O_2 at any concentration *in vivo* (Lledias et al., 1998). Catalase becomes more significant in protecting against severe oxidant stress (Matés, 2000).

The selenium-containing **GPx** is, together with CAT, responsible for H_2O_2 detoxification (Fig. 1.11). The enzyme reduces H_2O_2 or some organic hydroperoxides with the help of a reducing cofactor, GSH (Matés, 2000). There are four GPx isoenzymes found in mammals. GPx 1 (= cytosolic or cellular GPx), GPx 2 (= gastrointestinal GPx) and GPx 3 (= plasma GPx) are tetrameric enzymes containing one selenocysteine residue in each of the four identical subunits. **GPx 4 or phospholipid hydroperoxide glutathione peroxidase (PHGPx)** on the other hand, is a 20-23 kDa monomer protein that is synthesized as a short form (cytosol, nuclei, endoplasmic reticulum) and a long form (mitochondria) due to two initiation sites in exon Ia of PHGPx genomic DNA (Imai and Nakagawa, 2003). PHGPx remains stable in moderate selenium deficiency and only decreases upon prolonged and substantial selenium depletion. Such a slow responder ranks higher in the hierarchy of selenoproteins, which means they are believed to play a more important physiological role than the fast responders such as the cellular glutathione peroxidase (Brigelius-Flohé, 1999; Holben and Smith, 1999). PHGPx uses a wide range of reducing substrates in addition to GSH (Imai and Nakagawa, 2003). Moreover it is capable of reducing phospholipid hydroperoxides, fatty acid hydroperoxides and cholesterol hydroperoxides that are produced in peroxidized membranes and oxidized lipoproteins (Brigelius-Flohé, 1999; de Almeida et al., 2004; Imai and Nakagawa, 2003). In this way, it has a protective role against lipid peroxidation (de Almeida et al., 2004; Holben and Smith, 1999). Furthermore PHGPx can modulate the activity of cellular regulatory elements by catalyzing the specific oxidation of protein thiols (Kühn and Borchert, 2002; Ursini et al., 1997). It also effectively suppresses apoptosis by inhibiting the release of cytochrome c from mitochondria (Imai and Nakagawa, 2003; Nakagawa, 2004).

Thioredoxin (Trx) is a small (12 kDa) multifunctional disulphide-containing redox protein that undergoes redox reactions with multiple proteins. Trx complements the GSH system in protection against oxidative stress (Hansen et al., 2006). The reduction of the disulphide back to the dithiol form is catalyzed by thioredoxin reductase (ThR), with NADPH as the source of electrons (Fig. 1.12 a) (Valko et al., 2006). Trx is thought to have anti-apoptotic effects and has been found to modulate the DNA-binding activity of certain transcription factors such as NF-κB (Saitoh et al., 1998). Besides this redox system, Trx is also involved in detoxification of peroxides (H_2O_2, peroxynitrite, organic hydroperoxides) by peroxiredoxins (Wood et al., 2003). **Peroxiredoxins (Prx)** are a family of homodimeric, multifunctional antioxidant Trx-dependent peroxidases that, besides cellular protection against oxidative stress, also modulate intracellular signaling cascades that apply H_2O_2 as a second messenger molecule and regulate cell proliferation (Immenschuh and Baumgart-Vogt, 2005). Until recently, not much attention was given to this family of H_2O_2-removal systems. Now it is thought that the peroxiredoxins might be the most important H_2O_2-removing enzymes, more important than the glutathione peroxidases (Halliwell, 2006). The Prx family contains six known protein subtypes; Prx I to IV use two conserved Cys residues for the sites of antioxidation, while Prx V and VI only use one conserved Cys residue. Prxs use Trx as the electron donor except for PrxVI (Oberley et al., 2001; Rhee et al., 2005) (Fig. 1.12 b). **PrxII** is, together with PrxI, a cytosolic Prx. It is, together with GPx and CAT (in peroxisomes), responsible for the removal of H_2O_2 in cells (Rhee et al., 2005).

a. Thioredoxin redox

$Trx\text{-}(SH)_2 + protein\text{-}S_2 \rightarrow Trx\text{-}S_2 + protein\text{-}(SH)_2$

$ThR\text{-}S_2 + NADPH + H^+ \rightarrow ThR\text{-}(SH)_2 + NADP^+$

$Trx\text{-}S_2 + ThR\text{-}(SH)_2 \rightarrow Trx\text{-}(SH)_2 + ThR\text{-}S_2$

$Trx\text{-}S_2 + NADPH + H^+ \rightarrow Trx\text{-}(SH_2) + NADP^+$

b. Peroxiredoxin

$Prx\text{-}(SH)_2 + ROOH \rightarrow Prx\text{-}S_2 + ROH + H_2O$

$Prx\text{-}S_2 + Trx\text{-}(SH)_2 \rightarrow Prx\text{-}(SH)_2 + Trx\text{-}S_2$

$Trx\text{-}(SH)_2 + ROOH \rightarrow Trx\text{-}S_2 + ROH + H_2O$

Fig. 1.12 *a. The thioredoxin redox system and b. the reaction performed by peroxiredoxin. Trx = thioredoxin; ThR = thioredoxin reductase and Prx = peroxiredoxin.*

Anti-apoptotic molecules

The **Bcl-2 family** consists of the anti-apoptotic factors **Bcl-2** and Bcl-X_L and the pro-apoptotic factors Bax and Bad (Cuttle et al., 2001). Bcl-2 is located in the mitochondrial outer membrane, and when overexpressed, it protects cells from lipid peroxidation and from thiol oxidation by H_2O_2 (Cai and Jones, 1998). Furthermore the anti-apoptotic Bcl-2 family proteins function as gatekeepers to prevent the release of both cytochrome c and the apoptotic protein AIF (apoptosis inducing factor) (Cai et al., 1998). Upon oxidative stress, the opening of the permeability transition pore of mitochondria is favoured. As a result cytochrome c moves from the intermembrane space into the cell's cytoplasm unless anti-apoptotic factors (including Bcl-2) prevent the mitochondrial permeability transition (Turrens, 2003). Cytochrome c, if released, joins another factor (Afap-1), which activates a cascade of caspases, eventually leading to cell death (Li et al., 1997; Robertson and Orrenius, 2000). The anti-apoptotic action of Bcl-2 is eliminated when pro-apoptotic factors are translocated to the mitochondria and start apoptosis (Turrens, 2003). Bcl-2 transcription is induced by Cd *in vitro* and impairs the apoptotic actions of the metal (Ishido et al., 2002).

Stress proteins

HSPs are a distinctive class of proteins that provide cellular defence against a wide range of cell injuries. They play an important role in the assembly and folding of intracellular polypeptides and help in restoring the biological activities of abnormal proteins (Razzaque and Taguchi, 2005). **HO-1** or **HSP 32** is the stress-induced isoform of heme oxygenase (Akagi et al., 2005; Halliwell and Gutteridge, 2002). It is the rate-limiting enzyme in heme degradation (Fig. 1.13) as well as the 32-kDa HSP that participates in the defence against oxidative stress. Heme plays an essential role as the prosthetic group of hemeproteins (such as haemoglobin, myoglobin and cytochromes) and other enzymes involved in cellular oxidative metabolism (Akagi et al., 2005). On the other hand, excess (free) heme can be severely toxic because it has strong pro-oxidant properties and it can damage lipid bilayers and organelles as well as a number of enzyme proteins (Akagi et al., 2005; Immenschuh and Schröder, 2006). HO-1, responsible for heme degradation, is upregulated by its substrate heme and a host of oxidative stress stimuli such as H_2O_2, heavy metals, UV-light and endotoxin. It has potent cytoprotective and anti-inflammatory functions (Immenschuh and Schröder, 2006; Satarug et al., 2006). It catabolizes cellular heme to biliverdin, carbon monoxide and free iron, and is considered one of the most sensitive and reliable indicators of cellular oxidative stress (Poss and Tonegawa, 1997). Biliverdin is then rapidly converted into bilirubin; both are considered to have an antioxidant function (Ashino et al., 2003). HO-1 also increases the intracellular level of ferritin that limits the availability of Fe^{2+} to catalyze harmful reaction (Matés, 2000).

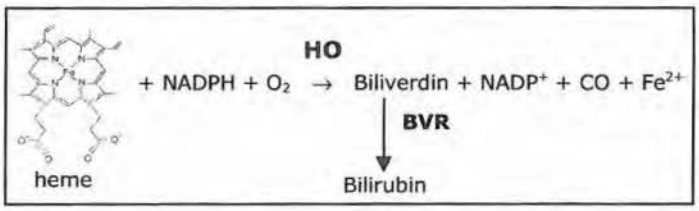


Fig. 1.13 *Oxidative catabolism reactions catalyzed by NADPH-cytochrome P450 reductase, heme oxygenase (HO) and biliverdin reductase (BVR). The electrons are provided by NADPH-cytochrome P450 reductase. Heme released from hemeproteins is cleaved by HO bound to the endoplasmatic reticulum, and yields an equimolar amount of iron, CO and biliverdin. Biliverdin is then reduced to bilirubin by biliverdin reductase.*

Other antioxidants

Many other biological compounds have an antioxidant function: vitamin E (α-tocopherol), vitamin A, carotenoids, polyamines, melatonin, NADPH, adenosine, coenzyme Q-10, urate, ubiquinol, flavonoids, phytoestrogens, cysteine, homocysteine, taurine, methionine, s-adenolyl-L-methionine, nitroxides, NOS (NO synthase), EPO (eosinophil peroxidase), lipoic acid.

A schematic overview of the pro- and antioxidants discussed above and possible damage, is given in Fig. 1.15 on page 44.

1.4.5 ROS-induced damage

A serious imbalance between the production of ROS and the antioxidant defence results in oxidative stress. Mild oxidative stress can usually be tolerated. By upregulating the synthesis of antioxidant defence systems, the cells try to restore the oxidant-antioxidant balance. When the imbalance reaches a level the cells cannot compensate for, oxidative stress will cause damage to all types of biomolecules, including **DNA**, **proteins** and **lipids** (Halliwell and Gutteridge, 2002). Radical-related damage has been proposed to play a key role in the development of age-dependent diseases such as cancer, arteriosclerosis, arthritis and neurodegenerative disorders (Valko et al., 2006). Furthermore antioxidant enzyme dysfunctions have been associated with amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's disease and Parkinson's disease (Matés, 2000).

Especially the hydroxyl radical ·OH is known to react with all components of the **DNA** molecule. The forms of DNA damage include the following: purine, pyrimidine or deoxyribose modifications, production of base-free sites, deletions, frame shifts, single- or double-stranded breaks, DNA-protein cross-links and chromosomal rearrangements. Permanent modifications through genetic alterations or modulation of gene expression are the first mechanisms by which oxidative damage may cause carcinogenesis or trigger apoptosis. However, there are specific and general repair mechanisms that repair for example DNA base modifications. Unfortunately, these repair mechanisms are often also

affected by ROS, resulting in a reduced capacity to repair damaged DNA (Magder, 2006; Valko et al, 2004; Valko et al., 2006). Furthermore Cd itself can inhibit DNA repair, when Cd^{2+} substitutes for Zn^{2+} in the zinc finger motifs of repair proteins (Filipic et al., 2006; Giaginis et al., 2006; Witkiewicz-Kucharczyk and Bal, 2006).

Protein oxidation disrupts receptors, enzyme function and signal transduction pathways and is associated with a number of age-related diseases and ageing (Magder et al., 2006; Valko et al., 2006). Potentially important functional sites for oxidation of proteins are the –SH groups. The formation of disulfide bonds may result in conformational changes in the protein and alter its function (Magder, 2006). Furthermore the oxidation of tyrosine residues to form –tyr-tyr- cross-links, the addition of lysine amino groups to the carbonyl group of an oxidized protein and the interaction of two carbon-centred radicals are also associated with the formation of many different kinds of inter- and intra-protein cross-links. The carbon-centred radicals are formed during the abstraction of a hydrogen atom from the polypeptide backbone. They also react with dioxygen to form peroxyl radicals. Other examples of protein damage include oxidative scission, loss of histidine residues and the formation of protein-centred alkyl ($R^{\cdot}$), alkoxyl ($RO^{\cdot}$) and peroxyl ($ROO^{\cdot}$) radicals (Halliwell et al., 1995; Valko et al., 2006).

One of the major toxic effects of excessive ROS is damage to cellular membranes by the process of **lipid peroxidation** (Magder, 2006). Polyunsaturated fatty acid (PUFA) residues of phospholipids are extremely sensitive to oxidation (Fig. 1.14). As a result the membrane may lose its fluidity and barrier function, which puts the integrity of subcellular organelles and the entire cell in danger (Kühn and Borchert, 2002).

The initiation of lipid peroxidation is caused by attack upon a lipid of any species, especially by the hydroxyl radical that can abstract a hydrogen atom (H) from a methylene ($-CH_2$) group (Hogg and Kalyanaraman, 1999; Halliwell and Gutteridge, 2002). This lipid radical subsequently reacts with oxygen to generate a peroxyl ($ROO^{\cdot}$) radical. Transition metal ions such as Cu or Fe may

increase the concentration of lipid radicals exponentially through the formation of alkoxyl (RO·) radicals and newly formed ·OH (Hogg and Kalyanaraman, 1999). The formed lipid radical is able to abstract H from another lipid molecule, thus causing an autocatalytic chain reaction. The radical combines with H to form a lipid hydroperoxide and a second lipid radical that again regenerates to a peroxyl radical. These reactions are the chain-propagation steps of lipid peroxidation (Halliwell and Gutteridge, 2002). Peroxyl radicals can be rearranged via a cyclisation reaction; the final product being malondialdehyde (MDA). This end-product itself is mutagenic in bacterial and mammalian cells and carcinogenic in rats (Valko et al., 2004). The lipid peroxidation chain reaction will terminate when two lipid radicals react to form non-radical products. Termination is also achieved by reaction of a peroxyl radical with chain-breaking antioxidants such as α-tocopherol (Halliwell and Gutteridge, 2002; Hogg and Kalyanaraman, 1999).

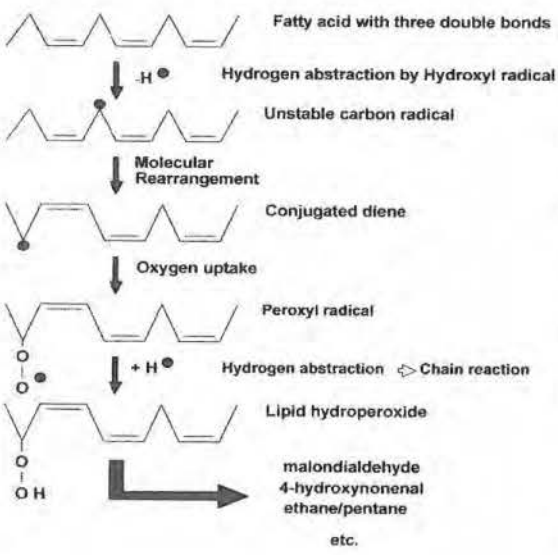


Fig. 1.14 *Oxidative stress-induced lipid peroxidation. The reaction is initiated by attack upon a lipid by -most often- the hydroxyl radical. This gives rise to the peroxyl radical. It is able to propagate the reaction by abstracting H from another lipid molecule. The chain reaction can be terminated by rearrangement via a cyclisation, by formation of a non-radical out of two radicals or by chain-breaking antioxidants (figure from Young and McEneny, 2001).*

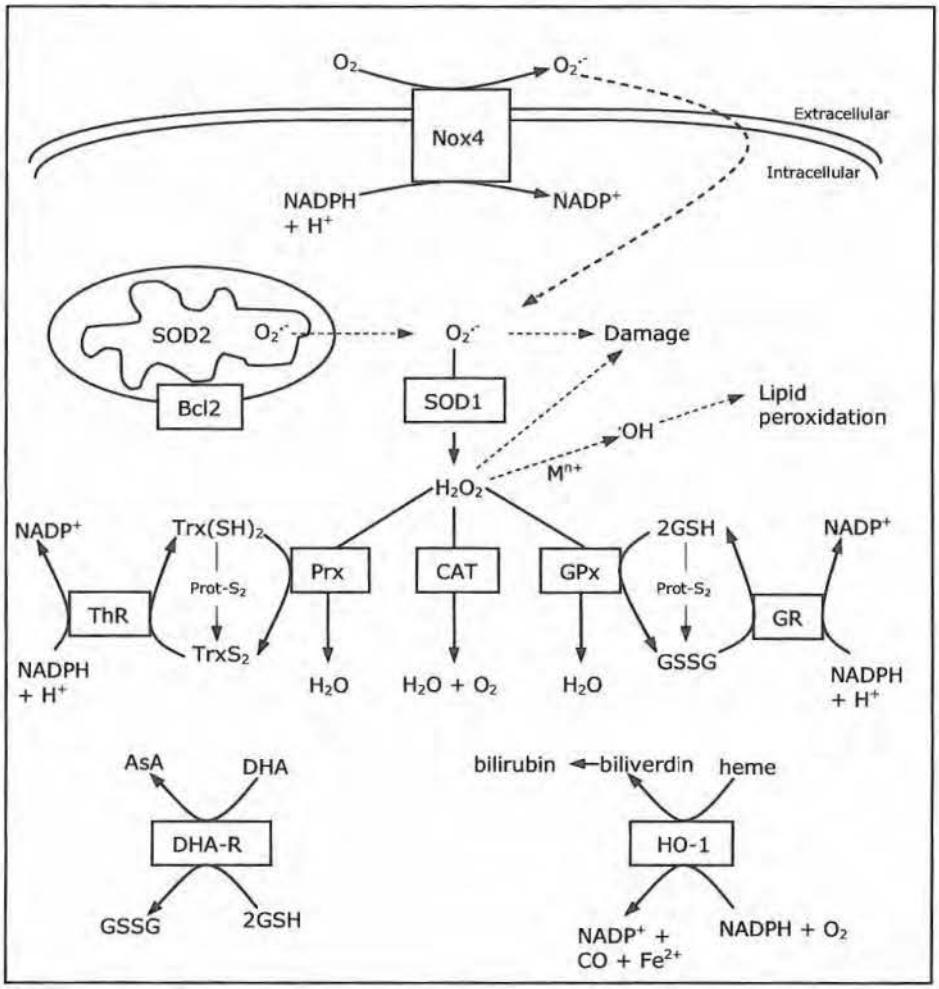


Fig. 1.15 *Schematic overview of ROS and defence systems in a living cell, with focus on the pro-and antioxidants studied and described in this thesis. Much more defence systems are of course present in a cell. Nox4 = NADPH oxidase 4; SOD = superoxide dismutase; CAT = catalase; Prx = peroxiredoxin; Trx(SH)$_2$ = reduced thioredoxin; TrxS$_2$ = oxidized thioredoxin; ThR = thioredoxin reductase; GPx = glutathione peroxidase; GSH = reduced glutathione; GSSG = oxidized glutathione; GR = glutathione reductase; AsA = ascorbate; DHA = dehydroascorbate; DHA-R = dehydroascorbate reductase; HO-1 = heme oxygenase 1; M^{n+} = metal ion; $0_2^{\cdot-}$ = superoxide anion; H_2O_2 = hydrogen peroxide; $^{\cdot}OH$ = hydroxyl radical; prot-S$_2$ = oxidized protein.*

1.4.6 Oxidative stress in renal pathophysiology

Oxidative stress has an important role in several renal diseases. Overproduction of ROS, reactive nitrogen species and modulation of cellular antioxidant enzymes are thought to be involved in the early phase of renal injury. This can result in activation of certain transcription factors, synthesis and release of inflammatory cytokines, chemokines, growth factors and ECM proteins, alter the balance in the microenvironment of the kidney and may activate signaling cascades that induce and propagate renal injuries (Abid et al., 2005). ROS generation in the renal cortex or medulla for example can both independently cause hypertension. Nitric oxide (NO), which, under normal conditions, regulates afferent arteriolar vasoactivity, salt reabsorption and the setpoint of tubulo-glomerular feedback in the renal cortex, may react with $O_2^{\cdot -}$ during oxidative stress, forming peroxynitrite ($ONOO^-$). This may lead to hypoperfusion and hypertension (Pallone, 2006; Ward and Croft, 2006).

Oxidative stress also appears to play a significant role in renal ischemia and reperfusion injury, a major cause of acute renal failure (Dubose et al., 1997). Renal ischemia not only increases $O_2^{\cdot -}$ and its two reaction products $OH^{\cdot}$ and $ONOO^-$, but also depletes antioxidant enzymes including SOD, GPX and CAT (Davies et al., 1995). On the other hand HO-1 has a protective role in the ischemic acute renal failure. In the reperfusion phase, when NO production increases through NO synthase (NOS) activation, reperfusion injury is markedly reduced by inhibiting NO biosynthesis, because the generation of peroxynitrite is prevented in this way. Induction of HO-1 is responsible for NOS inhibition: it degrades the essential prosthetic group (heme) for NOS (Akagi et al., 2005; Yu et al., 1994). HO-1 deficiency is characterized by advanced tubulointerstitial injury, indicating that intrinsic HO-1 production is essential for renal tubular survival under oxidative stress (Yang et al., 2003).

Besides the involvement in renal cell injury, oxidative stress is also believed to be associated with activation of various immunoinflammatory and fibrogenic molecules, leading to renal fibroproliferative diseases (Cochrane and Ricardo,

2003). Exogenous H_2O_2 as well as TGF-β1 induce EMT in tubular epithelial cells; antioxidants can effectively inhibit TGF- β1-induced EMT.

Furthermore the role of oxidants have also been shown in proliferative glomerulonephritis, membranous nephropathy, uremia, diabetic nephropathy, progressive kidney disease, etc. (Lahera et al., 2006; Shah, 2006; Rutkowski et al., 2006). All of this indicates that oxidative stress and damage participates in the progression and complications of acute and chronic renal diseases (El-Far et al., 2005).

1.4.7 *In vivo* and *in vitro* models used to study Cd-induced (renal) oxidative stress

Oxidative stress is believed to be a key player in early Cd-induced tubular damage. Most *in vivo* and *in vitro* studies have focused mainly on acute effects of Cd intoxication on the redox status in the liver and the kidney. Animals and cells were exposed to high Cd doses to assess the oxidative stress status. *In vivo* acute Cd toxicity resulted in multiple facets including oxidative damage (for example lipid peroxidation) and an increased expression of genes encoding HSPs and genes in response to DNA damage/repair (Bartosiewicz et al., 2001; Boujelben et al., 2006; Liu et al., 2002). Mice intraperitoneally injected with 40 μmol/kg as $CdCl_2$ for 3 hours showed a markedly increased expression of HSP, HO-1 and genes in response to DNA damage/repair in the liver, while genes encoding cytochrome P450 enzymes, Mn-SOD and CAT were suppressed by Cd (Liu et al., 2002). DNA arrays containing genes involved in heat shock, DNA repair, inflammation and transcription were used to examine gene expression patterns in the liver and the kidney of mice intraperitoneally injected with $CdCl_2$ (5 mg/kg bw) (Bartosiewicz et al., 2001). $CdCl_2$ injections (2.5 mg/day/ kg bw, 10 days) resulted in lipid peroxidation followed by the activation of antioxidant defence in the liver, kidneys and testes of adult male rats (Boujelben et al., 2006). Furthermore an increase in lipid peroxidation and a decrease in GSH levels were observed in the liver and kidney of rats receiving a single injection of 0.5 or 2 mg/ kg as $CdCl_2$ (El-Maraghy et al., 2001). On the other hand, chronically exposed animals receiving daily repeated doses of 0.5 or 2 mg

$CdCl_2$/kg bw during 2 weeks showed increased renal and hepatic GSH levels with decreasing MDA levels (El-Maraghy et al., 2001). In contrast to this, Shaikh et al. (1999c) noticed an increase in lipid peroxidation in the liver and the kidney, accompanied with an increase in GSH levels when injecting rats with 5 µmol $CdCl_2$/kg bw/day (5x/week) up to 22 weeks. So several *in vivo* and *in vitro* studies designate a direct and major role for oxidative stress as inducer of hepatic and renal toxicity upon (high) Cd intoxication.

1.5 The use of a mouse model in order to study Cd-induced nephrotoxicity

Many papers are available that describe the use of an animal model to study Cd toxicity. Research has been conducted towards dose-response relationships between Cd-intoxication and damage in the kidney, but also in the lungs, liver, heart and testes. The underlying mechanisms and the histopathological changes in Cd-induced nephrotoxicity, which are far from being understood, are examined in this way as well. Previously many studies were conducted by injecting animals with high Cd concentrations, in order to evaluate the effects of Cd in animal tissues. Nowadays more attention is going towards a more realistic administration; injection-based studies are often replaced by ingestion-based studies. In the study described in this thesis we administered Cd via the drinking water.

Most papers deal with Cd intoxication in rats (for example Aoyagi et al., 2003; Brzoska et al., 2003a, 2003b; Casalino et al., 2002; Herak-Kramberger et al., 1998; Matsuura et al., 1991; Shaikh et al., 1999a, 1999b, 1999c). Other animals that have been used are mice, frogs, fish, *Xenopus laevis* larvae and dolphins (for example Bonda et al., 2004; Gallien et al., 2001; Lange et al., 2002; Leffel et al., 2003; Liu et al., 2002; Loumbourdis, 2005; Mouchet et al., 2006; Sheader et al., 2006).

For several reasons we have chosen an inbred mouse model (C57BL/6) to study Cd-induced nephrotoxicity. The most important reason to choose mice (above for example rats) is the existence of very interesting transgenic and knockout mice. This allows the researcher to study the importance of proteins/enzymes in for example Cd-induced (nephro)toxicity. In the past the use of a (knockout) mouse model has already proven to be useful and successful in this research domain. In several studies the sensitivity towards Cd-induced nephrotoxicity and stress-related gene expression of wild type and MT-I/II-null and *Mtf1* knockout mice has been examined (Liu et al., 2000a, 2000b, 2002; Wimmer et al., 2005). The beneficial use of for example zinc has been studied in wild type and MTI/II-

null mice as well (Tang et al., 1998). Other interesting knockouts for the future are for sure the megalin-knockouts; megalin seems to be the most important CdMT transporter in the PTC. Also a knockout model with respect to for example endo/lysosomal acidification or trafficking should contribute to unravel underlying mechanisms of toxicity. Of course, before one can work with knockouts, an elaborate characterization of a wild type mouse model towards low-level Cd intoxication has to be set up first.

Another reason to choose a mouse model is the availability of a primary culture of PTC of this inbred mouse strain. The optimalization and characterization of this primary culture have been conducted in our laboratory (Terryn et al., submitted). PTC are cultured on Costar-Transwell filters, which allows the study of basolateral and apical uptake processes. The exposure to Cd of this primary culture and the *in vivo* exposure can complement each other in the study of Cd toxicity.

1.6 Aims of the present work

Contamination by heavy metals, and more specifically Cd, is a worldwide problem. A severely contaminated region of 180 km^2 is situated only a few miles away from our university. Although Cd has caused severe health problems in the past and the present in the occupationally exposed population or in Japan, it has become evident that the general human population might be affected more severely than previously anticipated.

(1) The present study describes the development of a **mouse model** that is **chronically exposed** to fairly **low** Cd concentrations through a realistic exposure route. Different parameters have been measured (general animal health status, urinary analyses, Cd determinations) to be able to obtain a **dose-response relationship** that gives us a realistic view upon Cd exposure and toxicity. By exposing to relatively low Cd concentrations, it is possible to find out if and from what moment on injury takes place. The exposure study will be described in **chapter 2**.

(2) We are interested in the **toxicokinetics** related to **detoxification** in our mouse model. Because oxidative stress is believed to be involved in early proximal tubular damage due to Cd stress, defence systems that were (de-) activated upon low Cd exposure will be examined. Gene expression studies will be performed to quantify **pro- and antioxidant** genes-of-interest. Furthermore the primary defence proteins GSH and AsA as well as MDA levels in kidney tissue will be measured. All of this will be discussed in **chapter 3**.

(3) Thirdly the **toxicodynamics** will be examined in the mouse model. Interest is going towards the changes in the **morphology** in the kidney itself due to Cd intoxication. At the light and electron microscopical level an extensive histopathological analysis will give an indication of the

nephrotoxic characteristics. Morphometrical analyses will add to this. Finally, immunohistochemical staining concerning the fibrotic markers fibronectin, collagen I and α-SMA will give an indication to what extent Cd induces **fibrosis** in our model. This will complete the histopathological study of the kidney **(chapter 4 and 5)**.

Chapter 2

In vivo exposure of mice to Cd stress

The results of this chapter are described in:

Chronic exposure of mice to environmentally relevant, low doses of cadmium leads to early renal damage, not predicted by blood or urine cadmium levels.

Sandy Thijssen, John Maringwa, Christel Faes, Ivo Lambrichts and Emmy Van Kerkhove (2007). Toxicology 229: 145-56.

2.1 Introduction

In exposure studies so far, animals were often subjected to high Cd concentrations, leading to severe kidney damage. In the present study, our main goal is to develop a mouse model in which animals were exposed to more realistic concentrations. The mouse is an interesting species to use because of the availability of knockouts for further studies. The relationship between low Cd exposure and the health status of the animals is assessed on various levels. Therefore several urine parameters and Cd determinations in blood, urine, liver and kidney cortex will be measured and kidney damage assessed. Furthermore general parameters will give an indication of the general health status of our animals.

2.2 Materials and methods

2.2.1 Experimental setup

C57black/6 mice were purchased from B&K Universal Ltd. (East Yorkshire, UK). They were housed under conventional conditions at a room temperature of 21-24°C, with a 12 h light/dark cycle and humidity of 50-60 %. The study was carried out on 126 mice that were bred in our facility. They had unlimited access to tap water pH 2.5 and mice feed (breeding chow; Carfill, Oud-Turnhout, Belgium). Male mice, aged 8-10 weeks, were chosen randomly and subdivided into 6 groups of 4 to 8 mice. They were exposed to 0, 1, 5, 10, 20 or 100 mg $CdCl_2$/l in the drinking water ($CdCl_2$: 99+%, A.C.S. Aldrich) during 1, 4, 8, 16 and 23 weeks.

Mice were weighed before the exposure started and placed overnight (16 hours) in a metabolic cage (Tecniplast metabolic cage, code 3700M022, Tecnilab-BMI, Someren, the Netherlands) for urine collection (Fig. 2.1 A, B and C). In the metabolic cage they received drinking water containing 2 % of sucrose. In literature, it has been described that urine samples are qualitatively indistinguishable if collected with or without sucrose load (with 10% sucrose; Leheste et al., 1999). This procedure increased the amount of water taken in by

the animals, so that the amount of urine produced was sufficient for the analyses that needed to be done. Afterwards, mice returned to their cages and were provided with drinking water containing the appropriate Cd concentration. Weekly measurements of body weight, food and water consumption were performed, and the drinking water was changed twice a week. During the exposure period, mice were placed in metabolic cages regularly (every 2- 4 weeks) during 16 hours, receiving water containing 2 % sucrose and the appropriate amount of Cd. To prevent degradation of the urinary proteins and enzymes, collection tubes contained protease-inhibitor (Complete Mini, Roche). Urine samples were collected on ice and aliquots were stored at -20°C and -80°C. After the exposure period, mice were killed by decapitation, and whole blood was collected in a tube with heparin (250U; Sigma-Aldrich, H4784). Liver and kidneys were isolated, immediately frozen in liquid nitrogen and stored at -80°C until further use.

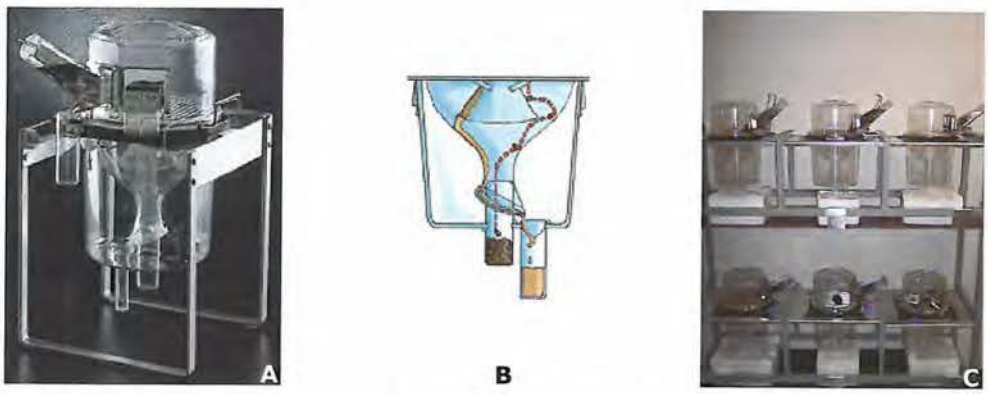


Fig. 2.1 *Metabolic cage. A. Standard metabolic cage used to collect urine of mice. B. Principle of metabolic cage. Urine and faeces are separated upon collection. C: Set-up of metabolic cages in our laboratory. Collection tubes were placed in ice (white boxes) for better urine preservation.*

2.2.2 Urinary analyses

Osmolality was measured immediately upon collection (Knauer Halbmikro-Osmometer, Germany). Total protein content was determined using the Bradford Assay (Bio-Rad Protein Assay, Bio-Rad), using bovine serum albumin

(Standard II, Bio-Rad) as a standard. This dye-binding assay is based on the binding of Coomassie Brilliant Blue G-250 to proteins that can be detected as an absorbance shift from 465 nm to 595 nm. A specific assay measuring LMWP such as RBP or β2-m would have been more appropriate, but specific assays for mouse were not available. Creatinine determination (Kreatinine kit, ref Dias 117119990314, VWR) was done spectrophotometrically according to the Jaffé method by which creatinine forms a coloured orange-red complex in an alkaline picrate solution. The difference in absorbance at fixed time periods during conversion is proportional to the concentration of creatinine in the sample. A commercial NAG Test kit (PPR Diagnostics LTD, London) was used according to the manufacturers guidelines which is based on the study of Yuen et al. (1982). The intensity is measured at 505 nm and bovine kidney NAG is used as a calibrant. Glucose was determined with the Glucose RTU kit (Biomérieux). Alkaline phosphatase (ALP- EC 3.1.3.1) activity was measured colourimetrically according to the two-point method. This method is based on the formation of p-nitrophenol out of p-nitrofenylphosphate. ALP activity was measured at 420 nm and the amount of p-nitrophenol liberated per time unit is a measure of the phosphatase activity (Walter and Schütt, 1974).

2.2.3 Cd determination in blood, liver, kidney cortex and urine

Cd concentrations were measured in blood, liver, kidney cortex and urine using graphite furnace atomic absorption spectrometry (GF-AAS). Glass tubes were washed with 10 % HCl before use. Five hundred µl of whole blood, 1 ml of urine or a weighed amount of tissue (liver or kidney cortex) was wet-digested with 500 µl HNO_3 s.p. (J.T. Baker, 70% HNO_3 for trace metal analysis). After partial evaporation, samples were cooled down, 500 µl H_2O_2 (J.T. Baker, 36.5- 38.0 % for trace metal, analysis) was added and the solution was totally evaporated. The precipitate was dissolved in 1 ml HNO_3 and analysis was performed with the GF-AAS Perkin Elmer 5100 HGA-600 with autosampler AS-60 and Zeeman 5100 background correction. Concentrations were measured using a standard calibration curve. The quantification limit was set at 0.1 µg/l.

2.2.4 Statistical analyses

The general parameters (food, water and weight) as well as the urine parameters were analysed using a linear mixed model (Verbeke and Molenberghs, 2000) with the mean structure being formulated as a piecewise linear function (Ruppert et al., 2003).

2.3 Results

2.3.1 Consumption of drinking water and food

The addition of Cd to the drinking water did not result in significant differences in water consumption between the control and experimental groups, but the consumption decreased slowly in time. The average daily Cd intake, calculated on the basis of water consumption, is shown in Table 2.1.

Table 2.1 *Average daily Cd intake per concentration group*

Group (mg $CdCl_2$/l)	Cd intake (µg/24h)
0	/
1	3
5	16
10	30
20	60
100	270

Average daily Cd intake per concentration group (µg/24 h), calculated from the amounts of water taken in multiplied with the applied Cd concentration. Data represent means ± SEM (n= 16-24). Plotting the average daily Cd intake against the exposure concentration resulted in linearity with p = 0.999, which proved that water consumption did not depend on the Cd concentration the animals were exposed to.

Food consumption was lowest in the control group at each point in time and seemed to be correlated with the Cd dose the animal was exposed to. Within each concentration level we observed a constant food consumption level up to 8 weeks, whereafter it changed depending on the concentration level. The animals exposed to higher Cd concentrations ate slightly more.

2.3.2 Body weight gain

During the experiment all mice increased in weight (Fig. 2.2). From 6 weeks on the mice exposed to higher Cd concentrations appeared to be growing faster than the ones exposed to a lower concentration, although the differences were not significant. However at 23 weeks, the weight of the group exposed to the highest concentration (100 mg $CdCl_2$/l) was significantly higher than that of the control ($p < 0.05$). The control group had the smallest weight overall, which is consistent with the smallest food intake.

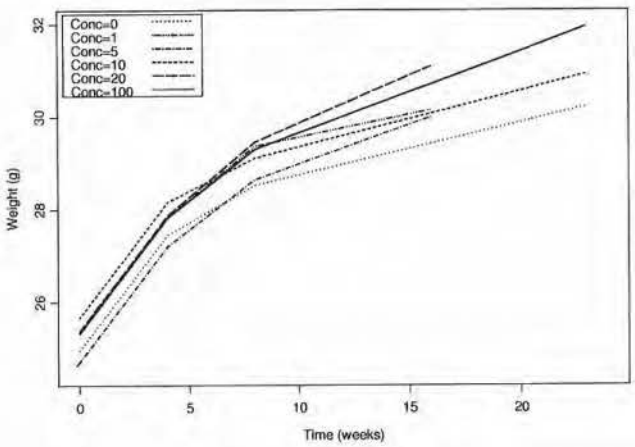


Fig. 2.2 *Linear mixed model describing the weight of the mice (in g) as a function of time (in weeks). The weight was measured weekly. At 23 weeks, the weight of the animals exposed to 100 mg $CdCl_2$/l was significantly different from the weight of the control group ($p = 0.03$).*

2.3.3 Cd measurements in blood, liver, kidney cortex and urine

The B-Cd levels (Fig. 2.3 A) increased linearly in a dose- and time-dependent manner up to 8 weeks of exposure. From that moment on, the group exposed to 100 mg $CdCl_2$/l showed a plateau phase with a maximal value of 56.3 ± 8.8 µg Cd/l ($n = 4$) whereafter the B-Cd level decreased. The B-Cd level of the other

groups kept increasing. Liver Cd (L-Cd) levels (Fig. 2.3 C) increased in a time and dose dependent manner except in mice exposed to 100 mg $CdCl_2$/l: a peak of 20.9 ± 2.3 µg Cd/g (n= 8) was reached after 16 weeks of exposure whereafter the L-Cd level decreased.

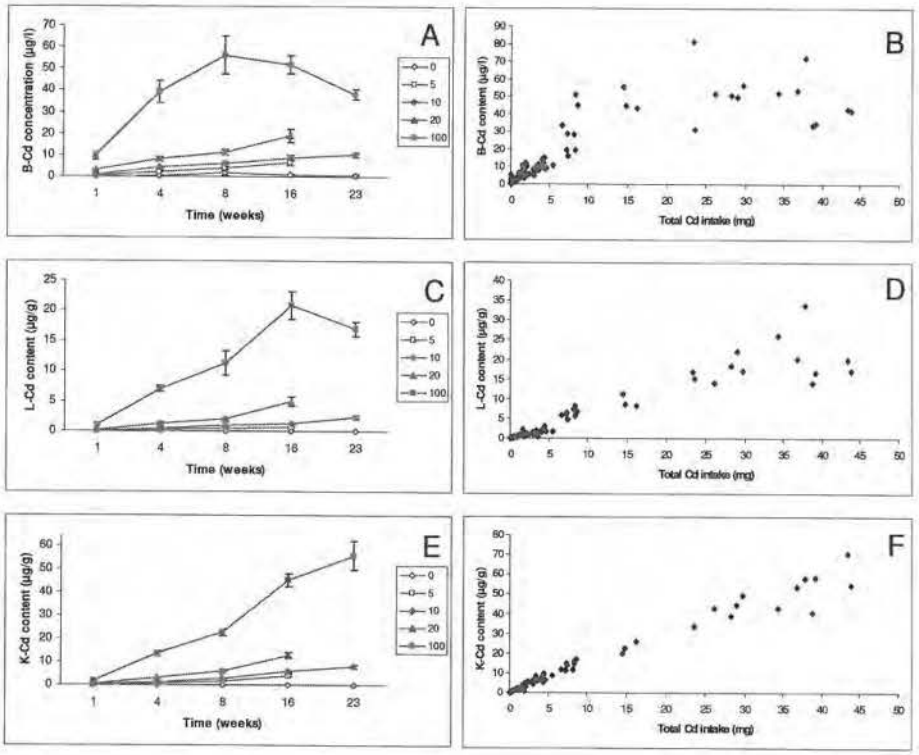


Fig. 2.3 *Cd concentration in A. blood (B-Cd; µg/l), C. liver (L-Cd; µg/g) and E. kidney cortex (K-Cd; µg/g) plotted in time (weeks) and its corresponding graph plotting B. B-Cd content, D. L-Cd content and F. K-Cd content plotted against the total Cd ingested (mg) by each animal. This resulted in following correlation coefficients: ρ = 0.73 (blood); ρ = 0.90 (liver) and ρ = 0.98 (kidney). A, C, E: each point represents mean ± SEM (n = 3-8). Mice were exposed up to 16 or 23 weeks to 0, 1, 5, 10, 20 or 100 mg $CdCl_2$/l in the drinking water.*

The administration of Cd resulted in a dose- and time-dependent increase in the K-Cd level (Fig. 2.3 E), leading to maximal values of 56 ± 6.1 µg Cd/g (n= 4) after 23 weeks of exposure in the highest concentration group. An overview of

the Cd levels found in blood, liver and kidney after 1, 4, 8, 16 and 23 weeks can be found in Table 2.2. High correlation coefficients were noted between Cd concentrations in blood and liver (ρ = 0.91), blood and kidney (ρ = 0.88) and kidney and liver (ρ = 0.95), indicating strong relationships amongst these variables.

A linear relationship (ρ=0.98) was found when plotting the K-Cd content against the total amount of Cd ingested (Fig. 2.3 F) that was calculated for each mouse individually, indicating that the K-Cd levels are a direct representation of the overall amount of Cd taken up. When plotting L-Cd levels against the total amount of Cd ingested (Fig 2.3 D), a correlation coefficient of 0.90 was found. The non-linear behaviour at the end of the exposure time was also seen in Fig. 2.3 C, where L-Cd values slightly decreased for the highest concentration group after 23 weeks. A correlation coefficient of 0.73 was obtained when plotting B-Cd levels against the total amount of Cd ingested (Fig. 2.3 B), indicating no linear relationship, consistent with the plot in Fig. 2.3 A.

The U-Cd levels (graph not shown) were lowest for the control group during the whole experiment, followed by the group exposed to 1 mg $CdCl_2$/l. Animals exposed to higher doses showed variable (rather low) amounts of Cd in urine. The values can be found in Table 2.2. No correlation between Cd concentrations in kidney and urine and in blood and urine could be found.

2.3.4 Urinary markers of renal toxicity

The administration of Cd had no effect on urinary osmolality (data not shown). The activities of the epithelial enzymes NAG (lysosomal enzyme; in µg/24h) and ALP (BB enzyme; in µg/24h) did not differ significantly among the different groups, nor did we find any signs of glucosuria (data not shown). On the other hand, exposure to Cd had an effect on the total protein content found in urine (Fig. 2.4 A). We fitted a longitudinal regression model for the urine protein content (Fig. 2.4 B). A significant increase was seen in animals exposed to the highest dose (100 mg $CdCl_2$/l) from 16 weeks on. The p-value at 16 weeks exposure was 0.027; at 23 weeks the p-value was 0.0001.

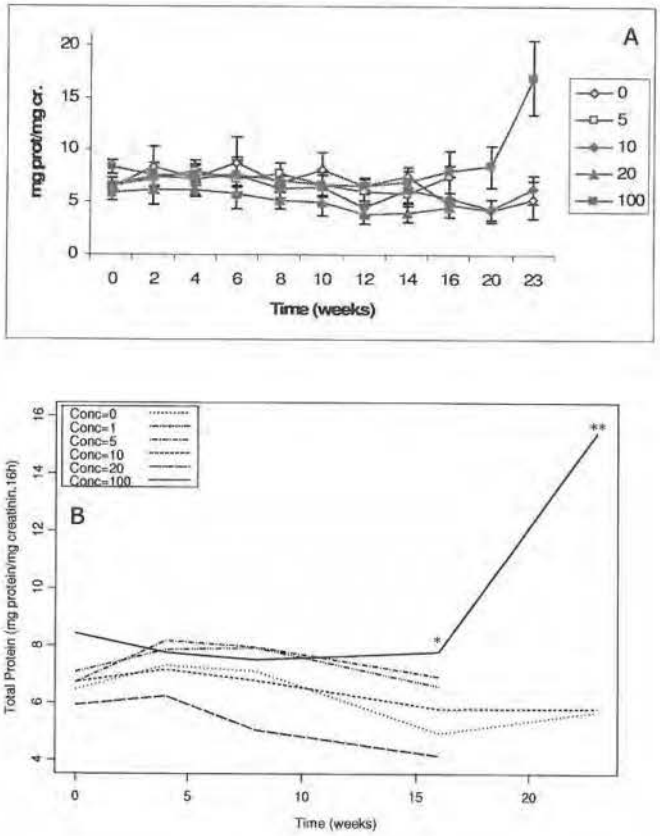


Fig. 2.4 *A. total protein content (mg protein/mg creatinine) in urine in the different experimental groups as a function of exposure time. Each point represents mean ± SEM (n= 3-11). The plot of the concentration group receiving 1 mg $CdCl_2$/l was omitted from the graphs for clarity; the urinary protein values were not (significantly) different from the other data. Inset: Cd concentrations in the drinking water in mg $CdCl_2$/l. B. Longitudinal regression model fitting the urinary protein content in function of time. Animals exposed to 100 mg $CdCl_2$/l showed signs of proteinuria from 16 weeks of exposure. *P-value at 16 weeks = 0.027; **p-value at 23 weeks = 0.001. Significances are calculated compared to the control group. Inset: Cd concentrations in the drinking water in mg $CdCl_2$/l.*

Table 2.2 *Average Cd content in blood, liver, kidney cortex and urine*

Cd conc (mg $CdCl_2$/l)	Exp.time (weeks)	n	B-Cd (µg/l)	L-Cd (µg/g)	K-Cd (µg/g)	U-Cd (µg/24 h)
0	1	4	0.3*	0.004 ± 0.001	0.02 ± 0.004	0.02 ± 0.008[b]
1	1	4	0.2 ± 0.11**	0.01 ± 0.001	0.04 ± 0.002	0.02 ± 0.005[b]
5	1	4	0.4 ± 0.04	0.03 ± 0.003	0.2 ± 0.08	0.02 ± 0.004[b]
10	1	4	0.9 ± 0.2	0.08 ± 0.02	0.2 ± 0.03	0.04 ± 0.02[b]
20	1	4	2.9 ± 0.3	0.2 ± 0.02	0.4 ± 0.01	0.04 ± 0.01[b]
100	1	4	9.5 ± 1.7	0.9 ± 0.1	1.6 ± 0.3	0.04 ± 0.008[b]
0	4	4	0.4 ± 0.02	0.008 ± 0.002	0.03 ± 0.001	0.02 ± 0.003
1	4	4	0.9 ± 0.1	0.04 ± 0.002	0.2 ± 0.009	0.07 ± 0.03
5	4	4	2.3 ± 0.2	0.3 ± 0.08	0.7 ± 0.04	0.2 ± 0.1
10	4	4	4.1 ± 0.7	0.5 ± 0.1	1.3 ± 0.1	0.2 ± 0.08
20	4	4	8.2 ± 1.1	1.3 ± 0.6	3.1 ± 0.1	0.3 ± 0.1
100	4	4	39.4 ± 5.2	7 ± 0.5	13.7 ± 1.3	0.2 ± 0.09
0	8	4	1.8 ± 1.3	0.008 ± 0.001	0.04 ± 0.01	0.04 ± 0.02
1	8	4	0.9 ± 0.4	0.06 ± 0.006	0.3 ± 0.04	0.04 ± 0.01
5	8	4	3.9 ± 0.9	0.5 ± 0.08	1.8 ± 0.2	0.2 ± 0.1
10	8	4	6.2 ± 0.0.7	0.9 ± 0.08	2.7 ± 0.2	0.1 ± 0.05
20	8	4	11.2 ± 1.4	1.9 ± 0.3	6.1 ± 0.7	0.1 ± 0.06
100	8	4	56.3 ± 8.8	11.3 ± 2	22.7 ± 1.5	0.2 ± 0.06
0	16	8	1 ± 0.3	0.02 ± 0.008	0.06 ± 0.01	0.01 ± 0.002
1	16	7[a]	1.4 ± 0.2	0.09 ± 0.01	0.09 ± 0.07	0.02 ± 0.004
5	16	5	6.3 ± 1.3	0.7 ± 0.08	4.2 ± 0.5	0.2 ± 0.1
10	16	7[a]	8.5 ± 1.2	1.3 ± 0.2	5.8 ± 0.4	0.06 ± 0.01
20	16	5	18.9 ± 3	4.9 ± 0.8	12.9 ± 1.3	0.1 ± 0.08
100	16	8	52.1 ± 4	20.9 ± 2.3	45.5 ± 2.8	0.08 ± 0.03
0	23	4	0.6 ± 0.5	0.03 ± 0.02	0.04 ± 0.003	0.006 ± 0.001
10	23	3[a]	10.2 ± 1.1	2.3 ± 0.4	8.1 ± 0.8	0.08 ± 0.05
100	23	4	38.2 ± 2.4	16.9 ± 1.2	56 ± 6.1	0.3 ± 0.2

Average blood Cd content (B-Cd in µg/l), average liver Cd content (L-Cd in µg/g wet weight), average kidney cortex Cd content (K-Cd in µg/g wet weight) and average urine Cd content (U-Cd in µg/24 h) of the animals included in the study. The mice chow contained small amounts of Cd as well (0.05 µg/g; 4 samples) which led to the small amounts of Cd in the blood, the liver, the kidney cortex and the urine of the control group. n = number of animals included in each concentration- and time- group. [a] *one animal died in this group and is not included in the study.* [b] *These U-Cd values were measured at week 0, so at the start of the experiments, and not after 1 week of exposure. *B-Cd value of one sample; the other three measurements were below the detection limit.** Mean B-Cd value of two samples; the other two measurements were below the detection limit.*

2.4 Discussion

2.4.1 Animal model

The present study was conducted to elucidate the effects of low doses of Cd in a chronically exposed animal model and to clarify the relationship between the development of renal damage and the accumulation of Cd in the kidney. This is one of the first studies that describes the effects of a chronic exposure to low levels of Cd in mice. We preferred mice above rats as test species because of the existence of very interesting knockouts for further studies (such as megalin knock-outs).

As ingestion is the most important route of human exposure, we chose for an exposure via the drinking water. The doses of 1, 5, 10, 20 and 100 mg $CdCl_2$/l drinking water were chosen according to average human intake data, soil Cd concentrations from contaminated sites (Blanusa et al., 2002; Satarug and Moore, 2004; Staessen et al., 1995; WHO, 2000) and Cd levels from animals caught in polluted areas (Damek-Poprawa and Sawicka-Kapusta, 2003; Damek-Poprawa and Sawicka-Kapusta, 2004). Cd levels in whole kidneys of bank voles trapped in contaminated areas in Poland for example amount to 33 µg/g dry weight. The Cd concentrations we applied were markedly lower (Liu et al., 2000a; Mitsumori et al., 1998; Noel et al., 2004; Shaikh et al., 1999b; Zeng et al., 2003) or in the range of previous studies (Brzoska et al., 2003a; Choi and Rhee, 2003; Leffel et al., 2003). Our B-Cd levels ranged from 0.2 to 56 µg/l. In the general population averages of 1.1 to 9.6 µg/l were found in Belgium (Staessen et al., 2000), concentrations up to 22.2 µg/l were found in Belgian occupationally exposed workers (Roels et al., 1989) and values up to 6 times higher in Japan (Ikeda et al., 2004).

In human studies, the weight of individuals living in a high exposure area was not different from the weight of people living in low exposure areas (Hogervorst et al., 2007). In previous animal studies the data may have been biased because of significant differences in water or food intake or the significantly smaller body weight increase and even weight loss of Cd-exposed animals

compared to the controls (Aoyagi et al., 2003; Brzoska et al., 2003a; Mitsumori et al., 1998). The Cd doses we applied did not decrease the weight of the mice, and therefore may reflect the human situation more closely. Water consumption decreased in all experimental groups in a concentration-independent manner. Food consumption was lowest in the control group at each time point and consumption tended to increase with increasing dose, resulting in a slightly higher weight for the higher concentration groups. A partial explanation might be that this group of animals already had a slightly higher weight at the start of the exposure. It should also be mentioned that a comparable finding (higher food intake by mice exposed to Cd) has been described earlier (Lind et al., 1997). These results indicate that Cd exposure did not affect water consumption, nor did it inhibit growth.

2.4.2 Blood, liver and kidney cortex Cd content

B-Cd levels showed a dose- and time-dependent increase for all groups except the group receiving 100 mg $CdCl_2$/l: those B-Cd values reached a peak value after 8 weeks of exposure, after which the values started to decrease. This showed that B-Cd levels will reach a plateau at a certain concentration in time whereupon it will set at a certain equilibrium point (steady state). Previously it has been demonstrated in humans that B-Cd levels increased linearly upon Cd exposure, to reach a plateau after about 4 months. In rats it has been demonstrated that the height of this plateau was a function of the intensity of exposure. Therefore B-Cd levels reflect a current Cd exposure; this statement relies on a substantial body of evidence derived from numerous human and experimental studies (Bernard et al., 1980a, 1980b, 1992; Lauwerys et al., 1979) and is also supported by the non-linear relationship (ρ = 0.73) we found between B-Cd levels and total Cd ingested. Our B-Cd data from mice exposed up to 10 µg Cd/l can be considered 'environmentally relevant'; the higher B-Cd data do not reflect a realistic (human) situation anymore (Staessen et al., 2000). Our L-Cd data showed a similar behaviour. All but the group receiving 100 mg $CdCl_2$/l showed a dose- and time-dependent increase, while L-Cd values of animals exposed to the highest concentration (100 mg $CdCl_2$/l) reached a maximum value at 16 weeks after which values decreased. The non-linearity

when plotting L-Cd content against total Cd ingested, which was seen when total Cd intake values increased, are a reflection of this finding as well. The death and loss of damaged hepatocytes, filled with Cd, might explain the decreased L-Cd level at 23 weeks. L-Cd values, even for the group receiving 100 mg $CdCl_2$/l, remained relatively low compared to other animal studies, where values of up to 200 µg/g have been measured (Groten et al., 1994; Shaikh et al., 1999b). K-Cd content on the other hand showed a time- and concentration-dependent increase for all experimental groups. Furthermore the linear relationship between K-Cd content and total Cd intake indicates that K-Cd content is a reliable indicator of the total body burden. K-Cd content is a representation of the overall Cd taken up and it is an indication that the kidney indeed is the final storage space.

2.4.3 Cd content in the urine

Several human and animal studies have suggested that U-Cd might be a suitable marker for total Cd body burden. About 0.005 to 0.01 % of total Cd burden is excreted via the urine each day. An increase in U-Cd is thought to be caused by leakage and later on by an increase in proximal tubular cell death, resulting in the release of the metal in urine. Population studies (non-occupationally exposed individuals) have shown that subclinical changes in tubular function occur at U-Cd levels around 2-3 µg Cd/g creatinine or above a threshold of 2 µg/24h (Buchet et al., 1990; Roels et al., 1993).

In rats chronically exposed to Cd the urinary excretion of Cd also increased in parallel with the body burden (Bernard et al., 1980a). Furthermore a relationship between U-Cd levels and kidney dysfunction was demonstrated after already some weeks of exposure in recent animal studies (Aoyagi et al., 2003; Brzoska et al., 2003a; Liu et al., 1998). During our exposure experiment we could not detect such an increase in U-Cd levels related to blood or kidney Cd content. The control animals had the lowest U-Cd level throughout the whole experiment, followed by the group exposed to 1 mg $CdCl_2$/l. Groups receiving higher Cd concentrations had variable amounts of Cd in their urine. These U-Cd levels may be considered environmentally relevant; the threshold of

2 µg Cd/24 h above which subclinical renal changes occur in the general population, are far from being reached in our experiments (Buchet et al, 1990).

So in our study we cannot confirm that U-Cd levels are a reliable indicator of K-Cd levels. The experimental design (exposure period) used in our study does not allow us to corroborate this statement. Significantly increased U-Cd levels, which correlate with K-Cd levels, are expected when increasing the exposure time. On the other hand in comparable studies, elevated U-Cd levels were observed in rats early in time (Brzoska et al., 2003a; Liu et al., 1998).

2.4.4 Renal damage

Another important goal was to find out whether kidney damage could be detected and from what moment on. The nephrotoxic action of Cd can be detected on the basis of several urinary markers that reflect the integrity of the PT or the interference of Cd with the glomerular function. LMW proteinuria and enzymuria (BB enzymes/ lysosomal enzymes) result from proximal tubular damage (Bernard and Lauwerys, 1990; Price et al., 1997). A severe proximal tubular failure is characterized by glucosuria, aminoaciduria, hypercalciuria, excessive loss of major ions and tubular necrosis (Barbier et al., 2005; WHO, 2000). These manifestations have previously been detected in the human population starting from U-Cd concentrations of 2-3 µg/g creatinine or above a threshold of 2 µg/24h, which corresponds to K-Cd values around 50 µg/g kidney cortex (Buchet et al., 1990; Roels et al., 1993). As a cause of this proximal tubular dysfunction a loss of BB transporters due to the impairment of cell organelles or oxidative stress is proposed, though further studies have to be performed to clarify this (Sabolic et al., 2002; Thévenod, 2003).

Our results indicated the existence of early renal damage. Proteinuria emerged from 16 weeks on in the group exposed to 100 mg $CdCl_2$/l. Urine osmolality was not changed nor did we find increased levels of NAG or ALP. This is in contrast with the study of Brzoska et al. (2003a), where increased urinary activities of NAG and ALP were evident after 6 weeks in rats exposed to 50 mg Cd/l in the drinking water with a K-Cd concentration of 24.1 ± 1.7 µg/g.

The appearance of tubular or mixed-type proteinuria suggests that Cd can affect kidneys when chronically exposed to reasonably low concentrations. In our study it was not possible to perform assays measuring LMWP, although a specific assay (for LMWP such as RBP or β2-m) would be more conclusive to determine the existence of a tubular or mixed-type proteinuria. But those assays are based on the use of antibodies, and because suitable antibodies were not available for our tests (all antibodies are generated in mouse and therefore are not applicable for mouse urine), these measurements could not be done. Nevertheless the measurement of total protein content in urine has been used many times in the past in comparable studies (Brzoska et al., 2003a, 2003b; Kauer et al., 2006; Liu et al., 1998, 2000a, 2000b; Tang et al., 1998).

2.5 Conclusions

The present study demonstrates that chronic exposure of mice to (relatively) low Cd concentrations induced early kidney damage. Cd affects the kidney at relatively low accumulation levels and functional effects can be observed (for histological effects see chapter 4). This corroborates the findings previously made in rats. Furthermore our model has proven again that only K-Cd content is a reliable marker for chronic exposure, while blood is indicative for a current exposure. Although many studies focus on U-Cd levels as an indicator of total body burden, this could not be confirmed in our study, at least at the doses the animals were exposed to. On the other hand, our U-Cd levels may be considered 'environmentally relevant'. The present model seems to be a good model for examining a long-term low-level Cd exposure and will be used to clarify in more detail the mechanisms involved in a chronic exposure to low Cd concentrations.

Chapter 3

Oxidative stress and defence mechanisms in the kidneys of mice exposed to Cd

The results of this chapter are described in:

Low Cd exposure triggers a biphasic oxidative stress response in mice kidneys

Sandy Thijssen, Ann Cuypers, John Maringwa, Nele Horemans, Ivo Lambrichts and Emmy Van Kerkhove. *Submitted.*

3.1 Introduction

Oxidative stress plays an important role in several renal diseases. Overproduction of ROS, reactive nitrogen species and modulation of cellular antioxidant enzymes are thought to be involved in the early phase of renal injury (Pallone, 2006). Oxidative stress is also believed to participate in the early processes of Cd- induced proximal tubular kidney damage. The aim of our study is to get a better understanding of the cellular mechanisms involved in Cd nephrotoxicity and more specifically of the role of oxidative stress in proximal tubular kidney damage caused by a chronic, low-level Cd exposure. Gene expression studies of several pro- and antioxidants as well as glutathione, ascorbate and MDA levels will be measured in kidneys of mice exposed to different Cd concentrations up to 23 weeks. A schematic overview of these pro- and antioxidants is given in figure 1.15 on page 44.

3.2 Materials and methods

3.2.1 RNA isolation

Kidney samples of animals exposed during 1, 8 and 23 weeks to 0, 10 and 100 mg $CdCl_2$/l in the drinking water were used for RNA isolation. Total RNA was extracted with the SV Total RNA Isolation System (Z3100, Promega). Briefly the kidney tissue was homogenized using a mortar and pestle under liquid nitrogen. The ground tissue was transferred to a tube containing lysis buffer. After addition of dilution buffer, the mixture was heated at 70°C for 3 minutes and centrifuged for 10 minutes at maximum speed. The lysate was transferred to a Spin Basket from which the RNA could finally be eluted after several washing steps and a DNase treatment. Quality of RNA was assessed with the GeneQuant pro (Biochem Ltd, Cambridge, England). RNA was stored at –80°C until further analysis.

3.2.2 RT-PCR

One µg of RNA was used in a 50 µl reverse transcription (RT)-reaction. The RT was performed with the Taqman Reverse Transcription reagens (N808-0234, Applied Biosystems) and oligo-(dT) primer (N808-0128, Applied Biosystems) according to the manufacturers description. The RT-reaction consists of 4 steps: (1) 10 minutes at 25 °C (annealing), (2) 30 minutes at 48°C (reverse transcription), (3) 5 minutes at 95°C (reverse transcription inactivation), (4) 4°C until storage at -70°C

3.2.3 mRNA quantification by means of real-time PCR

The real-time PCR was run on the ABIprism 7000 (Applied Biosystems) using the Taqman chemistry in a final reaction volume of 25 µl. Primers and probes specific for mouse were ordered as 'assays by design' (Taqman gene expression assays, Part number 4331182, Applied Biosystems) as a 20x mixture containing the particular unlabeled forward and reverse primer and a Taqman MGB probe (6-FAM dye-labelled). Furthermore the Taqman® Universal PCR Master Mix (Applied Biosystems, cat. No. 4304437) containing AmpliTaq Gold® DNA Polymerase, AmpErase® UNG, dNTPs with dUTP, Passive Reference 1 and optimized buffer components was used to perform the PCR. The real-time PCR reaction consisted of 4 steps: 2 minutes at 50°C, 10 minutes at 95°C (activation of AmpliTaq Gold), 15 seconds at 95°C and 1 minute at 60 °C. Steps 3 and 4 are repeated 40 times. To be able to normalize the samples, two endogenous controls were run and 10 genes of interest were quantified (Table 3.1). An independent sample (a kidney sample from a random animal) was used to prepare standard curves. Standard curve samples were measured in triplicate, while the experimental samples were measured in duplicate. Four biological replicates were measured for each exposure condition (only 3 biological replicates were available for the group exposed to 10 mg $CdCl_2$/l during 23 weeks).

Table 3.1 *The housekeeping genes and genes of interest used in our study*

ActB	Mm 00607939_s1	*Housekeeping genes*
Hprt1	Mm 00446986_m1	
Sod1	Mm 01700393_g1	*Genes of interest*
Sod2	Mm 001449726_m1	
Gpx4	Mm 00515041_m1	
Cat	Mm 00437992_m1	
Mt1	Mm 00496660_g1	
Mt2	Mm 00809556_s1	
Prdx2	Mm 01705126_s1	
Nox4	Mm 00479246_m1	
Hmox1	Mm 00516004_m1	
Bcl2	Mm 00477631_m1	

For all genes the code for the ABI Taqman® Gene Expression Assays is given. ActB = β-actin; Hprt1 = hypoxanthine phosphoribosyltransferase 1; Sod = superoxide dismutase; Gpx = glutathione peroxidase; Cat = catalase; Mt = metallothionein; Prdx = peroxiredoxin; Nox = NADPH-oxidase; Hmox1 = heme oxygenase 1.

3.2.4 Real-time analyses

The standard curve method was used for analyzing the samples. The transcription levels of genes involved in pro- or antioxidant functioning were normalized to the geometric mean of 2 housekeeping genes, according to the principle of geNorm (Vandesompele et al., 2002). The calculation of the relative expression of the (normalized) experimental samples compared to the (normalized) controls was done by means of the Fieller method (Fieller, 1954). This method enabled us to construct confidence intervals for the ratio (experimental group/ control group). Whenever the confidence interval did not include '1' as relative expression level, that expression level was significantly different ($p < 0.05$) from the expression level of the control group (which is 1).

3.2.5 Lipid peroxidation assay

Lipid peroxidation is determined by measuring thiobarbituric acid reactive substances (TBARS) and is expressed in terms of MDA equivalents. A weighed amount of liver or kidney sample of mice exposed to 0, 10 and 100 mg $CdCl_2$/l during 1, 4, 8, 16 and 23 (only liver) weeks was homogenized in a mortar and pestle under liquid nitrogen. The ground tissue was then homogenized in PBS pH 7.4 to a final concentration of 50 mg/ml and disrupted by sonication during 15 seconds on ice. The uncentrifuged whole homogenates were used for analysis with the TBARS assay kit (OXItek, Zeptometrix Corporation, New York). Briefly, 25 µl homogenate, 25 µl SDS Solution and 625 µl TBA/Buffer Reagent were mixed and incubated at 95°C for 60 min. This mixture was cooled in an ice bath for 10 min and centrifuged at 1600*g* for 15 min. Supernatants were used for measurements on the PTI Felix 32 fluorometer (Photon Technology International, UK). Excitation was set at 530 nm and emission at 550 nm and. An MDA standard was used to construct a standard curve. TBARS values were normalized to the protein content of the homogenate.

3.2.6 Protein determination

The liver and kidney homogenates, described previously, were used for Bradford protein determination (Bio-Rad Protein Assay, Bio-Rad). Fifteen µl protease inhibitor (Complete Mini, Roche) was added to 100 µl homogenate and centrifuged at 10600*g* during 10 min at 4°C. Liver supernatants were diluted 6 times and kidney supernatants 4 times before measuring the protein content.

3.2.7 Determination of AsA and GSH content by High Performance Liquid Chromography (HPLC)

Extraction of AsA and GSH

A weighed amount of liver or kidney sample of animals exposed to 0, 10 and 100 mg $CdCl_2$/l during 1, 4, 8, 16 and 23 weeks was crushed with a mortar and pestle under liquid nitrogen. The ground tissue was mixed with 2 ml of ice-cold

6 % meta-phosphoric acid. The suspension was then centrifuged at 20000*g* for 10 min at 4°C (Eppendorf centrifuge 5804R). The supernatant was collected and kept on ice in the dark until use. Samples were diluted 1:3 in mobile phase (2 mM KCl; pH 2.5) before injection.

Reduction of DHA and GSSG

Total AsA (AsA + DHA) or total GSH (GSH + GSSG) was determined after reduction of DHA (or GSSG) to AsA (or GSH) with dithiothreitol (DTT). For this purpose, 50 µl of a 48 $mg.ml^{-1}$ Tris + 30 $mg.ml^{-1}$ DTT solution (pH not adjusted) was added to 100 µl of the sample, which brought the final pH of this mixture to pH 6.5. After 15 minutes of incubation the reduction was stopped by the mobile phase (2 mM KCl at pH 2.5), thus lowering the pH to 3. The DHA concentration was calculated as the difference between the reduced and total AsA concentration.

Determination of AsA and GSH

AsA/GSH determination was carried out on reverse phase HPLC. Separation occurred over a reverse-phase type C-18 column (3 µm particle diameter, Polaris 3 C18 column, Chromsep SS 100 x 4.6 mm, Varian Europe, Middelburg, The Netherlands), which was kept at a constant temperature of 40°C in a column oven (CTO-10AVP, Shimadzu, The Netherlands). The mobile phase consisted of 2 mM KCl, set at pH 2.5 by dropwise addition of concentrated *o*-phosphoric acid. The flow was driven by an isocratic pump (LC-10ADVP, Shimadzu, The Netherlands), set at 0.8 $ml.min^{-1}$. Oxygen was removed from the mobile phase by passing the flow through a degasser (DGU-14A, Shimadzu, The Netherlands). Injection was performed via an autosampling unit (SIL-10ADVP, Shimadzu, The Netherlands). Detection occurred between 190 nm and 250 nm via diode array (SPD-M10AVP, Shimadzu, The Netherlands), set in tandem with a home-made amperometric detection system (glassy carbon working electrode, calomel reference electrode, reference potential 1000 mV). The latter was kept at a constant temperature within the column oven. The electrochemical detector was connected to a computer, in conjunction with a separate system controlling unit (SCL-10AVP, Shimadzu, The Netherlands), via an SS420 board (Shimadzu, The Netherlands). Chromatogram analysis was performed with the ClassVP

software package (Shimadzu HPLC Class VP 612 SP5, Shimadzu, The Netherlands).

3.2.8 Statistical analyses

The mRNA levels of the genes of interest in the experimental groups versus the control groups were analyzed by means of the Fieller method (Fieller, 1954). P-values less than 0.05 were considered significant.

The lipid peroxidation data as well as the AsA and GSH data were expressed as mean ± SEM. Statistical significance of differences between means was determined by means of ANOVA. P-values less than 0.05 were considered significant.

3.3 Results

3.3.1 Relative quantification of gene expression levels

Metallothioneins

Mt1 and *Mt2* (Fig. 3.1 A and B) are both transcriptionally induced after 1 week of exposure in the group exposed to the highest concentration (100 mg $CdCl_2$/l). Induction factors were 2 and 1.7 for *Mt1* and *Mt2,* respectively. After 8 weeks of exposure, the expression level of the group exposed to 10 mg $CdCl_2$/l was only significantly increased for *Mt1* (factor 1.41), while *Mt2* was increased with a factor of 2.3 but the confidence interval was [0.94; 4.11]. The relative expression levels for the group exposed to 100 mg $CdCl_2$/l increased with a factor 5.0 for *Mt1* and 5.6 for *Mt2* as compared to the control group after 8 weeks. At 23 weeks of exposure, *Mt1* and *Mt2* expression levels were significantly upregulated for both experimental groups. The relative expression level of the group receiving 10 mg $CdCl_2$/l increased with a factor 3.8 (*Mt1*) and 2.6 (*Mt2*) while the level of the group exposed to 100 mg $CdCl_2$/l levels increased with a factor 10.8 (*Mt1*) and 7.9 (*Mt2*).

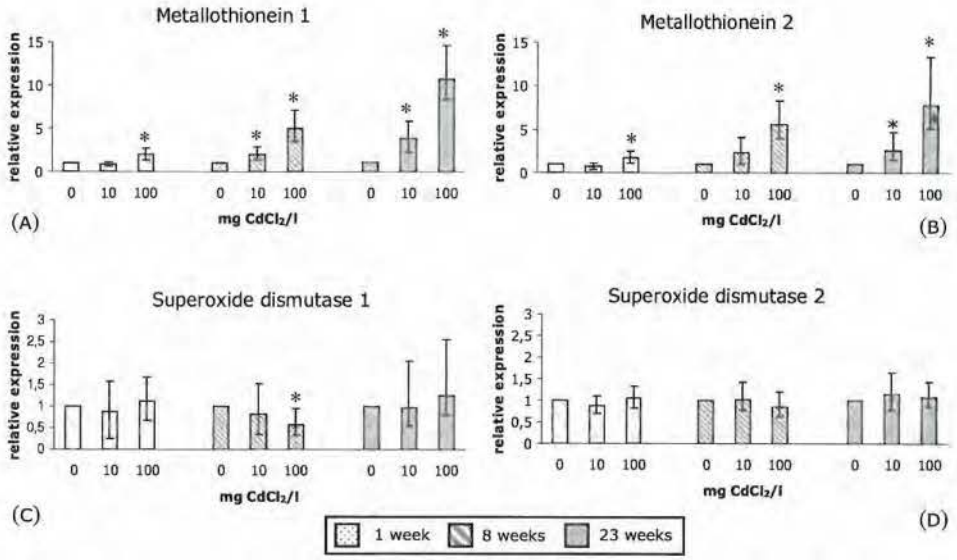


Fig. 3.1 *Quantification of antioxidant genes by means of real-time PCR. The graphs represent the relative transcription levels of the genes of interest measured in kidneys of mice exposed to 10 and 100 mg $CdCl_2$/l during 1, 8 and 23 weeks. The transcription levels are quantified in a relative way to the control group, whose expression level is set at 1 for each exposure time. Gene expression profile of (A) metallothionein 1, (B) metallothionein 2, (C) superoxide dismutase 1 and (D) superoxide dismutase 2. Data represent mean ± confidence interval determined by the Fieller method (* $p < 0.05$). ($n=4$; $n=3$ for the group exposed to 10 mg $CdCl_2$/l during 23 weeks).*

Superoxide dismutase

After 1 week of exposure there is no difference in the expression level of the cytosolic *Sod1* between the experimental groups and the control (Fig. 3.1 C). After 8 weeks of exposure, the experimental group receiving 10 mg $CdCl_2$/l still had a transcriptional level similar to the control, but the expression level of the group exposed to 100 mg $CdCl_2$/l was significantly reduced to 0.6. The decline in transcriptional level was restored at 23 weeks of exposure.

The mRNA levels of the mitochondrial *Sod2* of the experimental groups remained unaltered at the different exposure times (1, 8 and 23 weeks) (Fig. 3.1 D).

Enzymes scavenging H_2O_2

The relative expression level of *Cat* (Fig. 3.2 A) did not change after 1 and 8 weeks of exposure in the experimental groups 10 and 100 mg $CdCl_2$/l when compared to the control. After 23 weeks of exposure, the transcriptional level of the group exposed to 100 mg $CdCl_2$/l was significantly increased with a factor of 1.8. The expression level of the group exposed to 10 mg $CdCl_2$/l was not significantly increased; mean expression levels were 1.3 compared to the control group.

For *Gpx4* no difference in relative expression level of both experimental groups was noticed after 1 and 8 weeks of exposure (Fig. 3.2 B). After 23 weeks of exposure, both experimental groups were transcriptionally upregulated with a factor 2.9 and 2.5 in the group exposed to 10 and 100 mg $CdCl_2$/l, respectively.

The relative expression level of *Prdx2* (Fig. 3.2 C) showed a mean increase with a factor 1.5 for the experimental group receiving 100 mg $CdCl_2$/l after 1 week of exposure. The confidence interval for this condition was [0.95; 2.27], with the lower limit being just below 1, indicating a borderline case of nonsignificance. After 8 weeks of exposure, no change in expression level was observed of the group exposed to 10 mg $CdCl_2$/l compared to the control. A decreasing trend was seen for the group exposed to 100 mg $CdCl_2$/l. The relative expression level declined to 0.6 as compared to the control group (= 1), although this was not significant since the confidence interval [0.34; 1.04] had an upper limit just above 1. After 23 weeks of exposure, this decreasing trend sustained; the expression level was 0.7 but again this decrease was on the border of significance ([0.46; 1.09]). The group exposed to 10 mg $CdCl_2$/l showed a significant decrease in expression level to 0.5 compared to the control.

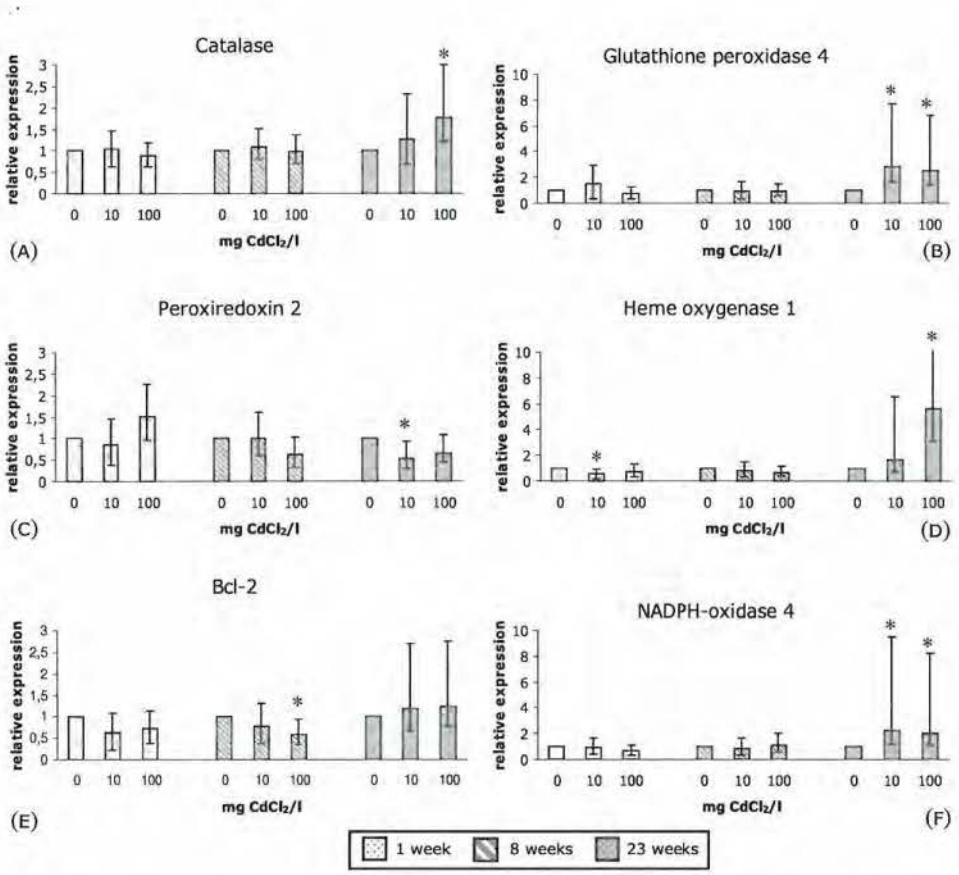


Fig. 3.2 *Quantification of pro-and antioxidant genes by means of real-time PCR. The graphs represent the relative transcription levels of the genes of interest measured in kidneys of mice exposed to 10 and 100 mg $CdCl_2$/l during 1, 8 and 23 weeks. The transcription levels are quantified in a relative way to the control group, whose expression level is set at 1 for each exposure time. Gene expression profile of (A) catalase, (B) glutathione peroxidase 4, (C) peroxiredoxin 2, (D) heme oxygenase 1, (E) Bcl-2 and (F) NADPH oxidase 4. Data represent mean ± confidence interval determined by the Fieller method (* $p < 0.05$). (n=4; n=3 for the group exposed to 10 mg $CdCl_2$/l during 23 weeks).*

Stress proteins: Heme oxygenase 1 (Hmox1)

The expression level of *Hmox1* or heat-shock protein 32 (*Hsp32*) was significantly reduced to 0.6 after 1 week of exposure (Fig. 3.2 D), although only for the group exposed to 10 mg $CdCl_2$/l. After 8 weeks of exposure, the expression level of the group (10 mg $CdCl_2$/l) was normal again, with a mean expression level of 0.8 and a confidence interval of [0.32; 1.47]. The group exposed to 100 mg $CdCl_2$/l had a mean expression level of 0.7 and a confidence interval of [0.39; 1.16]. After 23 weeks of exposure, a mean expression level of 1.7 was measured for the concentration group 10 mg $CdCl_2$/l with a confidence interval [0.78; 6.57]. The group exposed to 100 mg $CdCl_2$/l had a significantly elevated transcription level (confidence interval [3.13; 21.72]) with a mean induction factor of 5.6.

Anti-apoptotic factor Bcl2

A decreased expression level of *Bcl2* was seen after 1 week of exposure for both experimental groups (10 and 100 mg $CdCl_2$/l) (Fig. 3.2 E). Mean expression levels were down to 0.6 and 0.7 respectively, but significance was just not reached (confidence intervals: [0.22; 1.09] and [0.39; 1.15]). After 8 weeks of exposure the mean expression level of the group exposed to 10 mg $CdCl_2$/l was 0.8 but not significantly different (confidence interval [0.37; 1.32]) from the control group. A significant decrease in mRNA level was observed after 8 weeks for the group exposed to 100 mg $CdCl_2$/l with an expression of 0.6 as compared to the control group (confidence interval [0.35; 0.93]). After 23 weeks of exposure, mRNA levels of both experimental groups were back to normal and comparable to the control group.

Pro-oxidant enzyme NADPH oxidase 4

After 1 and 8 weeks of exposure there was no difference in gene expression level of *Nox4* between the experimental groups and the control (Fig. 3.2 F). After 23 weeks a significant increase was measured with a mean induction factor of 2.3 and 2.0 for the group receiving 10 and 100 mg $CdCl_2$/l, respectively.

3.3.2 Lipid peroxidation

There was no significant change in TBARS level in the kidney or the liver of the experimental groups compared to the control group at any exposure time (Table 3.2).

Table 3.2 *Lipid peroxidation assay of kidney and liver samples, expressed in µmol MDA/ mg protein*

Exp. time (weeks)	KIDNEY Control	10	100	LIVER Control	10	100
1	1.86 ± 0.31	1.66 ± 0.16	1.72 ± 0.22	0.74± 0.09	0.85 ± 0.12	0.85 ± 0.10
4	1.83 ± 0.33	2.03 ± 0.12	1.78 ± 0.23	1.26 ± 0.05	1.00 ± 0.13	1.86 ± 0.29
8	1.46 ± 0.28	2.18 ± 0.09	2.10 ± 0.48	2.02 ± 0.79	1.55 ± 0.28	2.06 ± 0.92
16	2.02 ± 0.14	2.32 ± 0.15	2.41 ± 0.39	1.72 ± 0.42	1.97 ± 0.39	2.85 ± 0.90
23				1.95 ± 0.22	1.24 and 0.98	1.38 ± 0.34

Lipid peroxidation assay of kidney and liver samples of mice exposed to 0 (control), 10 mg $CdCl_2$/l (10) and 100 mg $CdCl_2$/l (100). Values are expressed as µmol malondialdehyde (MDA) per mg protein. Exposure periods (exp. time) were 1, 4, 8, 16 and 23 weeks. Data represent mean ± SEM; n = 3-7. Statistical significance was determined by means of ANOVA; $p < 0.05$. No significance was found between experimental and control groups of kidney and liver samples at the different exposure times.

3.3.3 Glutathione and ascorbate levels

The GSH levels in the liver and the kidney did not change in the experimental groups compared to the control: there was no effect of concentration or exposure time ($p > 0.05$). Reduced AsA levels in the liver did not result in significant changes either among the experimental and control groups. In the kidney a time dependent but no concentration dependent decrease was observed in the AsA levels: average values were significantly lower after 16 weeks of exposure compared to values after 1, 4 and 8 weeks. At 23 weeks, the AsA levels in the kidney were below the detection limit. The GSSG and DHA levels in liver and kidney did not differ significantly. Average values are given in Table 3.3.

Table 3.3 *Average AsA, DHA, GSH and GSSG values (µmol/g wet weight) measured in the liver and the kidney of mice after Cd exposure*

Exp. time (weeks)	AsA (µmol/g)	DHA (µmol/g)	GSH (µmol/g)	GSSG (µmol/g)
Liver	0.56 ± 0.016	0.14 ± 0.023	4.66 ± 0.13	1.04 ± 0.21
Kidney				
1	0.38 ± 0.073			
4	0.36 ± 0.028			
8	0.36 ± 0.016	0.19 ± 0.035	2.29 ± 0.090	0.43 ± 0.070
16	0.26 ± 0.017*			
23	BDL			

Liver and kidneys were derived from animals exposed to 0, 10 and 100 mg $CdCl_2$/l during 1, 4, 8, 16 and 23 weeks. Data were analyzed by means of a two-way ANOVA. There was a time dependent decrease of AsA levels in the kidney (p-value < 0.05; n = 8-21). This decrease was similar, whether mice were exposed to Cd or not. The average renal AsA value for each exposure time, irrespective of the applied Cd concentration, was calculated and is expressed as mean ± SEM. No time- or dose-dependent changes in AsA were observed in the liver. DHA, GSH and GSSG did not change with time or with Cd exposure in kidney or liver. Therefore all data were pooled. Values are given as mean ± SEM (n = 50-72). Exp. time = exposure time; BDL = below detection limit.*

3.4 Discussion

The cellular processes underlying Cd hepato- and nephrotoxicity are poorly understood. Growing attention is going towards the oxidative status of cells and the Cd-mediated production of ROS, which is considered an important key player in Cd-induced toxicity. Previous studies described acute or chronic toxicity in the liver or the kidney after an exposure to high Cd concentrations, often applied via injections (El-Maraghy et al., 2001; Boujelben et al., 2006; Shaikh et al., 1999c; Zirong and Shijun, 2006). Our study describes the redox status and the antioxidant defence response in kidneys of mice chronically (up to 23 weeks) exposed to relatively low Cd concentrations (10 and 100 mg $CdCl_2$/l). As ingestion is the most important route of human exposure, we chose for an exposure via the drinking water. We described (in chapter 2 and 4) that

proteinuria accompanied with an increase in vacuolization level and increasing amounts of lysosomes in the PTC, was the only functional or histological damage detected in mice chronically exposed to low Cd concentrations. Signs of oxidative stress-related damage such as apoptosis or swollen or degenerated mitochondria were not detected (see chapter 4 and Thijssen et al., 2007). In the present study a first stress response was observed after 1 week of exposure: early antioxidants were upregulated. However, after 8 weeks of exposure the kidneys seemed to lose control over the Cd-induced oxidative stress. Cd elicited a downregulation of some major antioxidant and anti-apoptotic genes. Unlike in previous studies, this situation did not result in lipid peroxidation or GSH depletion. A second major cellular Cd response was noticed in the kidney after 23 weeks of exposure, which probably indicates adaptation to Cd-stress. Furthermore the redox balance was stable throughout the whole exposure period. Our study provided evidence that an exposure to (reasonably) low Cd concentrations causes changes in the anti and pro-oxidative systems in the cell that lead to adaptation and survival.

3.4.1 Early antioxidant defence response to Cd-stress

The first induction of antioxidant genes was observed after 1 week of exposure. *Mt1* and *Mt2*, but also *Prdx2* were upregulated when mice were exposed to 100 mg $CdCl_2$/l. MT1 and MT2, the two most common MT's in animals, are considered an important and early adaptive mechanism decreasing $CdCl_2$ nephrotoxicity (Wimmer et al., 2005). Under heavy metal and radical load, their expression is strongly induced at the transcriptional level by the induction of the transcription factor MTF-1 (Klaassen and Liu, 1998; Lichtlen and Schaffner, 2001b). From our results it was clear that MT indeed had the most important role in the early Cd responses. Furthermore a time and dose dependent increase in mRNA content for both experimental groups was seen throughout the whole exposure experiment. On the protein level this dose and time dependent increase was observed as well (see chapter 5).

Besides the early response observed for MT, an increasing trend in transcriptional level was seen for the gene *Prdx2*, which is part of the

thioredoxin redox system. After 1 week of exposure to 100 mg $CdCl_2$/l the transcription level had increased 1.52 times. Until recently, not much attention was given to this family of H_2O_2-removal systems. Now it is thought that the Prxs might be the most important H_2O_2-removing enzymes, even more important than the glutathione peroxidases and catalase (Halliwell, 2006). Its major function consists in cellular protection against oxidative stress, modulation of intracellular signaling cascades and regulation of cell proliferation (Immenschuh and Baumgart-Vogt, 2005). The increase observed in our study suggests that Prx2, an enzyme located in the cytosol of proximal and distal tubules (Oberley at al., 2001), might be important as a first line of defence against Cd induced oxidative stress. Another reason for the early increase might be that the enzymes of the thioredoxin system are inhibited by Cd (Radyuk et al., 2003) or partially inactivated (by excess H_2O_2) to allow signaling (Halliwell, 2006; Rhee et al., 2005). By upregulating its transcription level, the kidney may attempt to restore the thioredoxin system. An upregulation of a *Prdx* was also observed in the European flounder using microarray analysis 3 days after a single $CdCl_2$-injection (Sheader et al., 2006).

3.4.2 Toxicity due to Cd-stress after 8 weeks?

After an early induction after 1 week, *Prdx2* exhibited a downregulating trend at 8 and 23 weeks of exposure for the group exposed to 100 mg $CdCl_2$/l and a significant decline for the group exposed to 10 mg $CdCl_2$/l after 23 weeks. This enzyme probably serves as an early defence response, and its later downregulation might be important in cell signaling towards adaptation. Besides the mRNA decline for *Prdx2*, a significant downregulation was seen after 8 weeks for the antioxidant enzymes *Sod1*, the cytosolic *Sod*-isoform, and for the anti-apoptotic factor *Bcl2*, but only in the group exposed to 100 mg $CdCl_2$/l. This decrease apparently represents a temporary impairment since both transcriptional levels were back to normal after 23 weeks. A significant decrease in expression level of *Bcl2* in mouse testes after 72 h and an induction at the transcriptional and expression level in kidney cells (that was earlier than the *Mt* induction) were described in the literature, after administration of a single and high Cd dose (single injection of 5 µmol $CdCl_2$/kg bw and 10 µM, respectively)

(Ishido et al., 2002; Zhou et al., 2004). These conflicting results only indicated an early effect of high Cd concentrations and cannot be compared with the findings in our study. The temporary suppression of *Sod1* expression in our study and the complete recovery later in time was also seen by Casalino et al. (2002) at the level of protein expression, but other studies often described a significant decrease of total SOD activity (Yalin et al., 2005) and sometimes an increase (Jurczuk et al., 2004). The impairment at 8 weeks in the kidney was not reflected in differences in MDA levels, which are an indication of lipid peroxidation, in the liver and the kidney of control and exposed animals.

Although the downregulation of above described antioxidants suggested a weakening of the defence in the kidney, several other parameters, besides the MDA levels, proved that the overall redox balance remained stable throughout the whole exposure period. The normal expression level of *Sod2* suggested that the free radical generation in the respiratory chain in the mitochondria, the major site of superoxide production (Adam-Vizi, 2005; Inoue et al., 2003), was not increased upon Cd load in our experiment, and therefore *Sod2* expression did not change, as often stated in literature (Liu et al., 2002). We also did not observe significant mitochondrial damage (loss of cristae or swelling) at the ultrastructural level in these animals (see chapter 4 and Thijssen et al., 2007). Furthermore depletion (El-Maraghy et al., 2001; Zirong and Shijun, 2006) or an increase of reduced GSH (El-Maraghy et al., 2001; Shaikh et al., 1999c) that was described in several acute and chronic exposure studies where high Cd concentrations were applied (injections up to 2 mg/kg as $CdCl_2$ or fish exposed to 3 mg/l), was not observed in the kidney or the liver in our study. GSH is considered the most important thiol-containing antioxidant that can scavenge radicals. This results in the oxidation of GSH to GSSG. The stable GSH levels in our study were an additional indication for a normal redox state in the kidney. Also AsA (= vitamin C), an antioxidant that can be produced in mice but not in man, was not depleted or increased in the experimental groups when compared to the control in response to Cd stress. An overall decrease in time was observed for AsA in the kidney, which might be age-dependent.

3.4.3 Signaling pathways leading to adaptation?

As stated previously the defence activation in our study consisted of a biphasic response, with the second gene induction measured after 23 weeks of exposure. Whereas the transcriptional upregulation after 1 week of exposure was only observed for *Mt* and *Prdx2* in the group exposed to 100 mg $CdCl_2$/l, an upregulation of the genes *Cat*, *Gpx4*, *Hmox1* and *Nox4* was only observed after 23 weeks of exposure for the group exposed to 100 mg $CdCl_2$/l and the genes *Gpx4* and *Nox4* were also upregulated in the concentration group 10 mg $CdCl_2$/l. They may be categorized as late defence responders, because their transcription levels changed only after 8 weeks (except for *Hmox1*). While CAT only eliminates H_2O_2, GPx4 reduces, besides H_2O_2, phospholipid hydroperoxides, fatty acid hydroperoxides and cholesterol hydroperoxides that are produced in peroxidized membranes and oxidized lipoproteins (Brigelius-Flohé, 1999; de Almeida et al., 2004; Imai and Nakagawa, 2003). These enzymes probably take over the role of the Prx2. This upregulation contradicts what is generally hypothesized. It is thought that Cd produces oxidative stress by disturbing the antioxidant defence systems through the depletion of GSH or by decreasing CAT, GPx and SOD activity. The reason for the discrepancies of the findings in these studies with our results might be explained by the different experimental approach. The use of low Cd concentrations via the drinking water may cause another defence pattern than the one observed in other studies, in which high Cd concentrations were applied, often via injections. Surprisingly the induction of the *Hmox1* gene was only observed after 23 weeks of exposure, while it was previously suggested to be a general response to oxidative stress and one of the most sensitive biomarkers for acute Cd exposure (Liu et al., 2002). The administration of high Cd concentrations in acute exposure studies up to 20 µM and 100 µmol $CdCl_2$/kg (*in vivo* and *in vitro*; Alam et al., 1989; Gong and Hart, 1997; Liu et al., 2002; Ossola and Tomaro, 1995) might explain this early upregulation: the *Hmox1* gene appears most inducible at higher Cd concentrations (Gong and Hart, 1997). We even measured a downregulation of the transcriptional level with a factor of 0.55 after a short exposure time (1 week) that was restored to normal after 8 weeks.

Furthermore the induction of the gene expression of the pro-oxidant enzyme Nox4, as a reaction to chronic exposure to low Cd concentrations, has not been studied so far. The best-known member of the NADPH oxidase family, the phagocytic NADPH oxidase or Nox2, produces superoxide anions (oxidative burst) and plays a crucial role in host defence (Quinn et al., 2006). Nox4 on the other hand is constitutively expressed in a wide variety of tissues including the renal cortex and vascular smooth muscle and endothelium but its precise physiological function is unknown (Krause, 2004; Quinn et al., 2006). It may be linked to the pathogenesis of angiotensin II-induced glomerular fibrosis and hypertension. Other Nox4 roles have also been reported, i.e. cardiac hypertrophy, endothelial resorption, cardiac hypertrophy, endothelial dysfunction due to atherosclerosis and insulin signal transduction in adipocytes (Lee et al., 2006a). In melanoma cells the overexpression of Nox4 enhanced proliferation while inhibition blocked the proliferation and even resulted in apoptosis (Mochizuki et al., 2006; Quinn et al., 2006). In our study, a significant Nox4 induction was observed at 23 weeks of exposure to both 10 and 100 mg $CdCl_2$/l. Its role in Cd toxicity has not been described yet; it might produce radicals for signal transduction or cell proliferation by activating or up-regulating antioxidants or it might produce an excess of ROS and in this way promote the pathological condition of Cd-induced nephrotoxicity.

3.5 Conclusions

Our study provides evidence for an early and a late oxidative stress induced Cd-response in kidneys of mice chronically exposed to reasonably low Cd concentrations via the drinking water. At 8 weeks of exposure, Cd seemed to impair antioxidant expression (*Bcl2*, *Prdx2* and *Sod1*), but this situation was restored later (for *Bcl2* and *Sod1*) and other parameters proved that the overall redox balance was stable. A second stress response noticed after 23 weeks indicates that activation of ROS in cell signaling leads to adaptation. In general the kidney and liver seemed to cope well with the Cd-induced oxidative stress and controlled the situation. *Prdx2* seemed to be involved in the early Cd response, while *Nox4* was upregulated at 23 weeks. These findings are in contrast with previously published experiments, where high Cd concentrations were applied and Cd-induced oxidative stress was detrimental. Exposure to low Cd concentrations probably causes changes in the anti- and pro-oxidative systems in the cell that lead to adaptation and survival.

Chapter 4

Histological analyses of kidneys of mice exposed to Cd

Part of this chapter is described in:

Changes in expression of fibrotic markers and histopathological alterations in kidneys of mice chronically exposed to low and high Cd doses

Sandy Thijssen, John Maringwa, Ivo Lambrichts and Emmy Van Kerkhove. *In preparation.*

4.1 Introduction

In humans it is not easy to obtain biopsies to assess the histological state of the kidney in case of Cd nephrotoxicity, but in animals a thorough histological analysis is often not performed. In this chapter we aimed to describe the observations made in kidneys of mice exposed to reasonably low (up to 100 mg $CdCl_2$/l) and high (250 and 500 mg $CdCl_2$/l) Cd concentrations, with main focus on the PTC. Preliminary studies in kidneys of mice exposed up to 100 mg $CdCl_2$/l showed that rather minor changes were detected at the histological level; therefore we expanded this morphological part with an exposure experiment during 16 weeks to high (250 and 500 mg $CdCl_2$/l) Cd concentrations. Because the mouse species in relation with Cd intoxication has not been studied intensively, light microscopy, electron microscopy and morphometrical analyses were performed to picture the histopathological features as complete as possible.

4.2 Materials and methods

4.2.1 Sampling for histological analyses

Kidneys of mice exposed during 16 and 23 weeks to 0, 1 (only 16 weeks exposure), 10 and 100 mg $CdCl_2$/l in the drinking water, were used for microscopical analyses (exposure conditions described in detail in chapter 2). Additionally some animals (n = 3) were exposed to 250 and 500 mg $CdCl_2$/l during 16 weeks for electron microscopical analyses and Perl's iron staining.

4.2.2 Fixation procedures

Light microscopy

The kidneys were cut in half and fixed in 4 % formaldehyde (Unifix, Duiven, The Netherlands), embedded in paraffin and sectioned at 4 µm. Paraffin sections were mounted on poly-L-lysine coated glass slides. Light microscopical images were taken with the Nikon Coolscope.

Transmission electron microscopy (TEM)

The kidneys were dissected and the kidney cortex was cut into small pieces. They were fixed overnight in 2 % glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.3) at 4°C. Samples were post-fixed in 2 % OsO_4 in 0.05 M sodium cacodylate buffer (pH 7.3) for 1 h, stained with 2 % uranyl acetate in 10 % acetone for 20 min., dehydrated in graded concentrations of acetone and embedded in epoxy resin (Araldite). Ultra-thin sections (0.06 µm) were mounted on 0.7 % formvar coated grids, contrasted with uranyl acetate followed by lead citrate, and examined in a Philips EM 208 transmission electron microscope operated at 80 kV. Images were taken with the MORADA 10/12 camera (Olympus, Germany).

4.2.3 Staining procedures

Periodic acid-Schiff

Formalin fixed, paraffin-embedded tissue samples (0-10-100 mg $CdCl_2$/l; 16 and 23 weeks) were stained with periodic acid-Schiff (PAS). It is mainly used for staining structures containing a high proportion of carbohydrate macromolecules (glycogen, glycoprotein, proteoglycans) found in connective tissues and basal laminae.

Perl's iron stain

Formalin fixed, paraffin-embedded tissue samples of mice exposed to 0, 100 and 500 mg $CdCl_2$/l during 16 weeks were stained with Perl's iron stain. The Perl's iron stain is the classic method for demonstrating iron in tissues. After treatment with hydrochloric acid and potassium ferrocyanide an insoluble blue compound is produced (= Prussian blue reaction).

Toluidin-blue

Tissue samples prepared for electron microscopy (fixed in glutaraldehyde) were stained with toluidin-blue (except for kidneys of mice exposed to 250 and 500 mg $CdCl_2$/l). This enabled us to assess the morphology of the PT using light

microscopy. Toluidin-blue is a basic dye that stains basophilic structures. Slices (500 nm) were mounted on glass cover slips.

4.2.4 Morphometrical analyses

Semi-thin slices of animals exposed during 23 weeks (concentrations 0, 10 and 100 mg $CdCl_2$/l) were used for measuring the area and diameter of PT and glomeruli. From each animal (n= 4), 10 glomeruli were randomly picked throughout the kidney cortex and measured (40x magnification; 1 glomerulus/image). From each animal 7 areas throughout the kidney cortex were randomly picked (40x magnification) and all visible PT were measured (variable amounts of proximal tubules/image). Measurements were performed with Image J on the Nikon Coolscope.

Electronmicroscopical images at 36000x magnification were used for the other morphometrical analyses. Kidneys of mice exposed during 23 weeks were used. The thickness of the basement membrane of PT was measured. Ten to twenty images were captured in each animal, with 10 measurements per image. The glomerular basement membrane was measured in the same way. Concerning the filtration slit width, 10 to 20 images were randomly taken in each animal, with a variable number of measurements in each image, depending on the amount of filtration slits. The thickness of microvilli was measured on 10 to 15 pictures per animal, with 15 measurements per picture. The area and perimeter of 14 to 16 nuclei was measured in each animal. Measurement were performed using Analysis software (SIS-Soft-Imaging, Munster, Germany).

A linear mixed model was applied to analyze the data. A random intercept model was included to account for the within subject correlations that existed due to the measurement of several images from one mouse.

4.3 Results

4.3.1 General features of the kidney tubules- PAS staining

The PAS staining in control kidneys and in kidneys of mice exposed to Cd was similar: different staining patterns of basal membranes or the BB were not observed. The kidney cortex showed a variable amount of PAS positive staining (Fig. 4.1 A). The glomerular and the tubular basement membranes (basal laminae) were stained positively for PAS. Furthermore there was a clear difference between the proximal and distal tubules; the PT displayed its BBM as a pink region at the apical side, while the distal tubules did not have this feature. In the medulla, the limb of Henle's loop, collecting ducts and the vasa recta can be distinguished (Fig. 4.1 B). Other important structures that can be distinguished on the PAS-stained samples are the macula densa in the cortex (Fig. 4.1 C) and the kidney papilla with the ducts of Bellini in the inner medulla (Fig. 4.1 D).

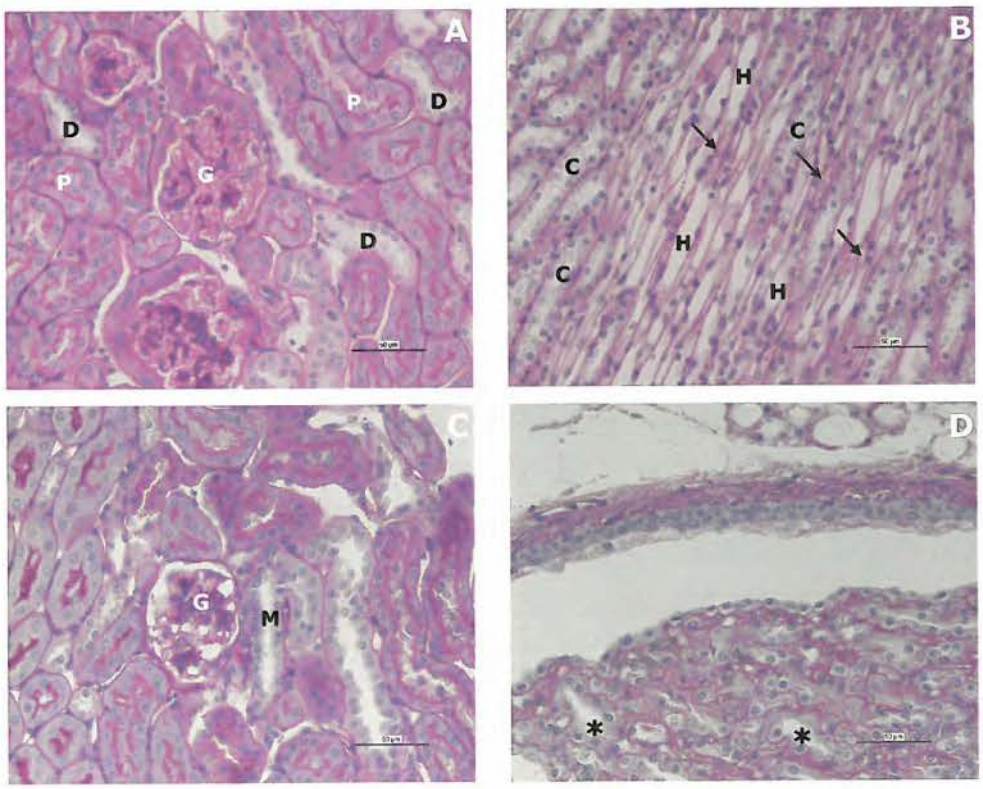


Fig. 4.1 *(A) PAS staining in the kidney cortex. The proximal tubules show dark staining of the brush border membrane/ glycocalyx, in contrast to the distal tubules. The basal lamina of the tubules and glomeruli show positive staining as well. (B) PAS staining of the outer medulla. Vasa recta (arrows), limbs of Henle's loop and collecting ducts can be distinguished. (C) The cortex displaying the macula densa, a region of specialized epithelial cells of the thick ascending limb where it contacts its glomerulus. (D) The inner medulla displaying the kidney papilla with the ducts of Bellini.*

*Scale bar = 50 µm. P = proximal tubule; D = distal tubule; G = glomerulus; C = collecting duct; H = limbs of Henle's loop; arrow = vasa recta; M = macula densa; * = ducts of Bellini*

4.3.2 Perl's iron stain

In control kidneys iron particles were observed in the PTC, especially in the ones close to the glomeruli (Fig. 4.2 A). The particles are located at the apical side of the cells (Fig. 4.2 B). In the kidneys of mice exposed to Cd, the amount of Prussian blue iron staining was rarely observed; the amount of particles had decreased drastically. This appeared concentration-dependent: the amount of iron in the kidneys of animals exposed to 500 mg $CdCl_2$/l was even less than in the kidneys of animals exposed to 100 mg $CdCl_2$/l (Fig. 4.2 C-D).

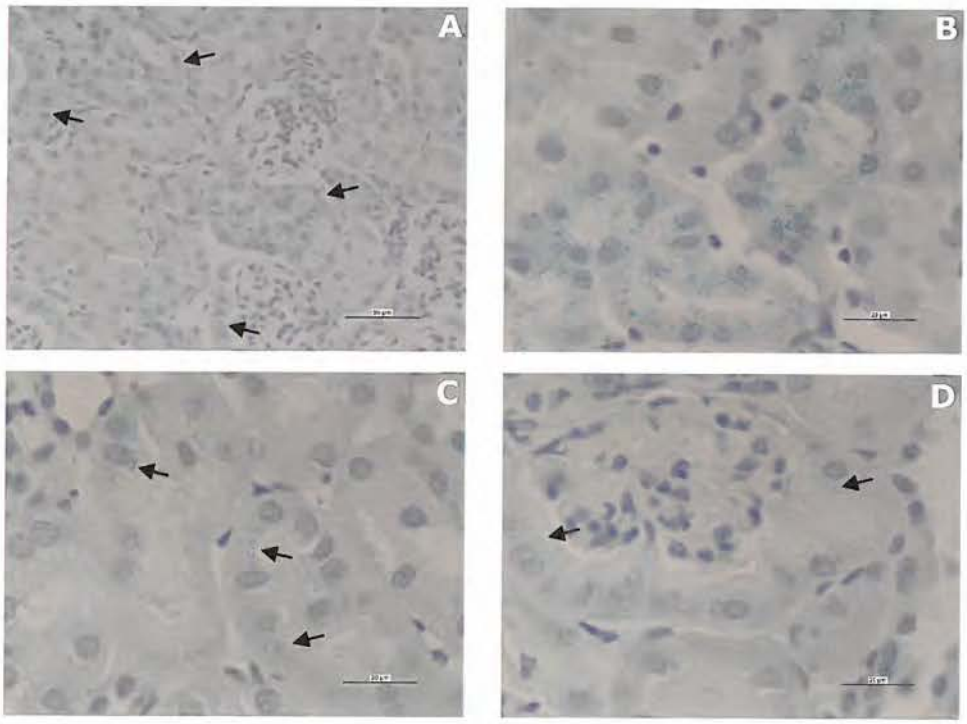
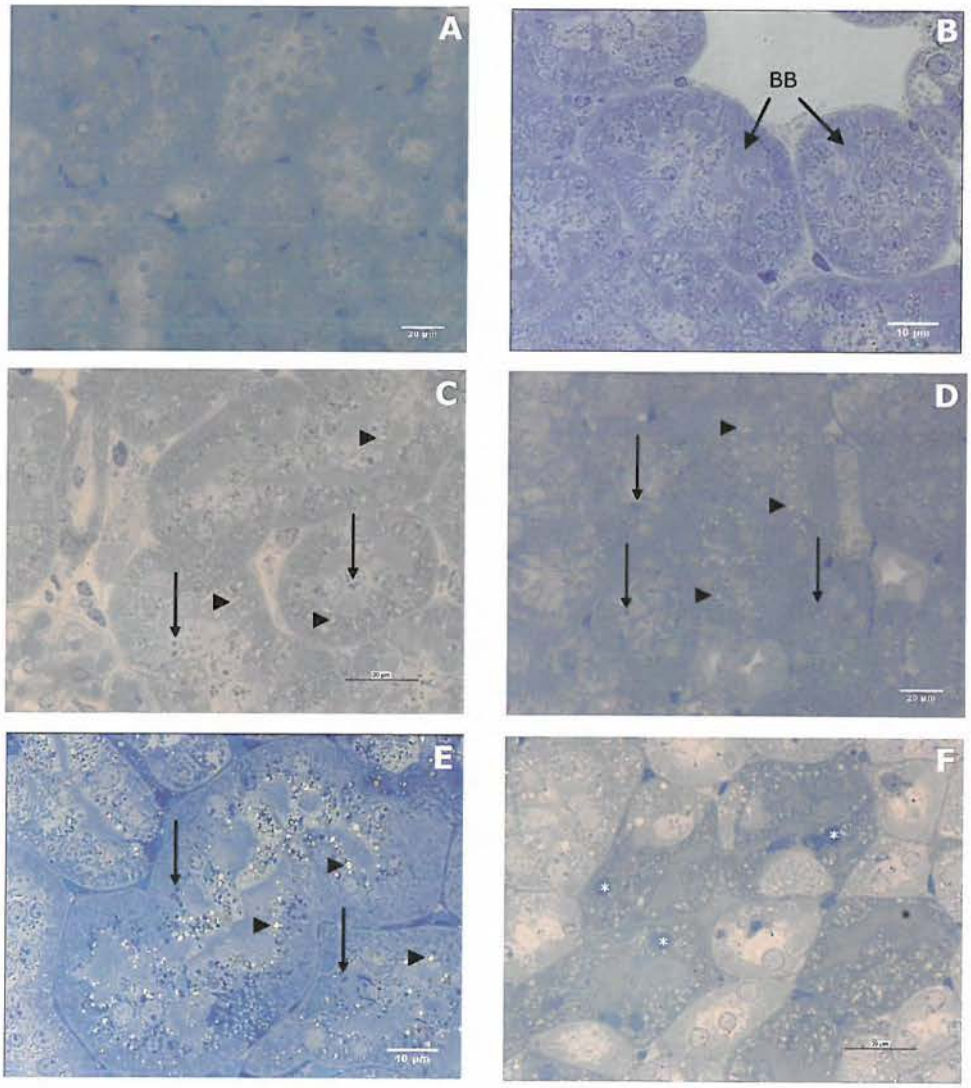


Fig. 4.2 *Images of kidneys stained with Perl's iron stain. (A-B) Kidneys of a control animal. The Prussian blue stain is clearly visible in some proximal tubules around the glomeruli. The iron is located at the apical side of the cells. (C) Kidney of an animal exposed to 100 mg $CdCl_2$/l during 16 weeks. The amount of iron in the tubules was limited. (D) Kidney of an animal exposed to 500 mg $CdCl_2$/l during 16 weeks. Hardly any iron particles were detected in the cortical tubules. Black arrow = iron particles. Scale bar = 50 µm (A); 20 µm (B, C, D).*

4.3.3 Light microscopical analyses of the kidney cortex

Light microscopical analyses of kidneys stained with toluidin-blue allowed us to obtain an idea about the general state of the PTC and the whole kidney. The PT of all control mice had a normal structure. Fig. 4.3 A shows a cross-section through the PT in the cortex. Each cell contains a nucleus, with its nucleolus. In Fig. 4.3 B the BB can clearly be seen as a darker area in the middle of the tubule. There were hardly signs of vacuolization and the lysosomal appearance was normal. Kidneys of mice exposed to 1 mg $CdCl_2$/l in the drinking water did not show any differences compared to the control groups (not shown). Vacuolization was visible in certain PTC in the group exposed to 10 mg $CdCl_2$/l, and more lysosomes were present in some PT tubules after 16 and 23 weeks of exposure (Fig. 4.3 C). The histological changes became more pronounced when the applied Cd concentration was higher (100 mg $CdCl_2$/l) (Fig. 4.3 D, E). A general vacuolization of cells was seen in many tubules and an increase in the number of lysosomes was now evident. Lysosomes were mainly present near the microvilli. Furthermore exposure time clearly played a role when comparing animals exposed to 100 mg $CdCl_2$/l during 16 and 23 weeks: the number of lysosomes and the vacuolization increased with time. Necrotic cells with pyknotic nuclei (Fig. 4.3 F) were occasionally observed in all groups (also in the control group), which indicates no increased amounts of necrotic cells in kidneys of mice exposed up to 100 mg $CdCl_2$/l.

Fig. 4.3 *Light photomicrographs of renal tissue in chronic Cd-intoxicated mice. Cross-section through the PT in the renal cortex. (A-B) Control group. The appearance of the PT is normal; BB = brush border. (C) Exposure to 10 mg $CdCl_2$/l during 23 weeks. Vacuoles (arrowheads) appear in some PT, as well as an increase in the number of lysosomes (black arrows; similar results after 16 weeks). (D) Exposure to 100 mg $CdCl_2$/l during 16 weeks. An increase in the number of vacuoles (arrowheads) and lysosomes (black arrows) is seen in several PT. (E) Exposure to 100 mg $CdCl_2$/l during 23 weeks. The number and the size of vacuoles (arrowheads) have even more increased compared to the kidneys after 16 weeks of exposure and are becoming larger. Lysosomes (black arrows) are present in all PT and seem to be clustered. (F) Necrotic tubule with pyknotic nuclei (*); occasionally seen in all groups. Scale bar = 20 µm (A, C, D, F); 10 µm (B, E).*


A
B
BB
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F

4.3.4 Ultrastructural observations in Cd-intoxicated kidneys

Each kidney cortex consisted of glomeruli and proximal and distal tubules. In Fig. 4.4 A a proximal and a distal tubule are shown. The densely packed microvilli (BB) in the PT and the lack of this in the distal tubule make it easy to distinguish them. This also makes the lumen of the distal tubule much larger. Because Cd-intoxication mainly damages the PT, we will focus on this nephron segment. The PT of controls occasionally contained some vacuoles and a normal amount of lysosomes (Fig. 4.4 A). A detail of the BB, with its endocytotic vesicles, is shown in Fig. 4.4 B. At the basolateral side, the basolateral plasma membrane forms extensive lateral invaginations (infoldings) of the basal membrane. In Fig. 4.4 C the cigar-shaped mitochondria are arranged in rows parallel with the infoldings; this basal labyrinth with its mitochondria is present in the convoluted part (especially S1) and is absent in the straight part (S3) of the PT. In the S3 segment mitochondria are small and distributed within the cell.

An increase in vacuolization and increasing amounts of lysosomes were observed in kidneys exposed to Cd. After 23 weeks of exposure the kidneys of mice exposed to 10 mg $CdCl_2$/l showed relatively small vacuoles in the PT, however this varied considerably from one tubule to the other. Also (occasionally) many lysosomes were observed, close to the BB (Fig. 4.4 D). In the kidneys of mice exposed to 100 mg $CdCl_2$/l during 23 weeks, the features became more conspicuous. Fig. 4.4 E shows a section of a PT, where vacuoles were prominent and different types of lysosomes appeared. Again these features were not present in all PT, but it was more common than in kidneys of mice exposed to 10 mg $CdCl_2$/l. An increase in endocytotic vesicles was commonly observed in Cd-intoxicated PTC (Fig. 4.4 F) and the lysosomes were often larger. Cd-intoxicated tubules contained especially many electron-dense bodies. The glomeruli had a normal appearance in the experimental groups and damaged mitochondria (swollen/ loss of cristae) could hardly be observed. Basal infoldings as well as microvilli were intact.

The PT of mice exposed to 250 and 500 mg $CdCl_2$/l during 16 weeks showed more pronounced damage. Besides the appearance of large vacuoles and many lysosomes, cytoplasmic edema was prominent (Fig. 4.5 A and C). Furthermore loss of cristae in swollen or degenerative mitochondria and poorly developed basal infoldings of the PTC were frequently seen (Fig. 4.5 B and D). The number of mitochondria increased as well; some PT were fully packed (Fig. 4.5 E). The glomeruli were intact. The degrees of these degenerative changes varied from cell to cell.

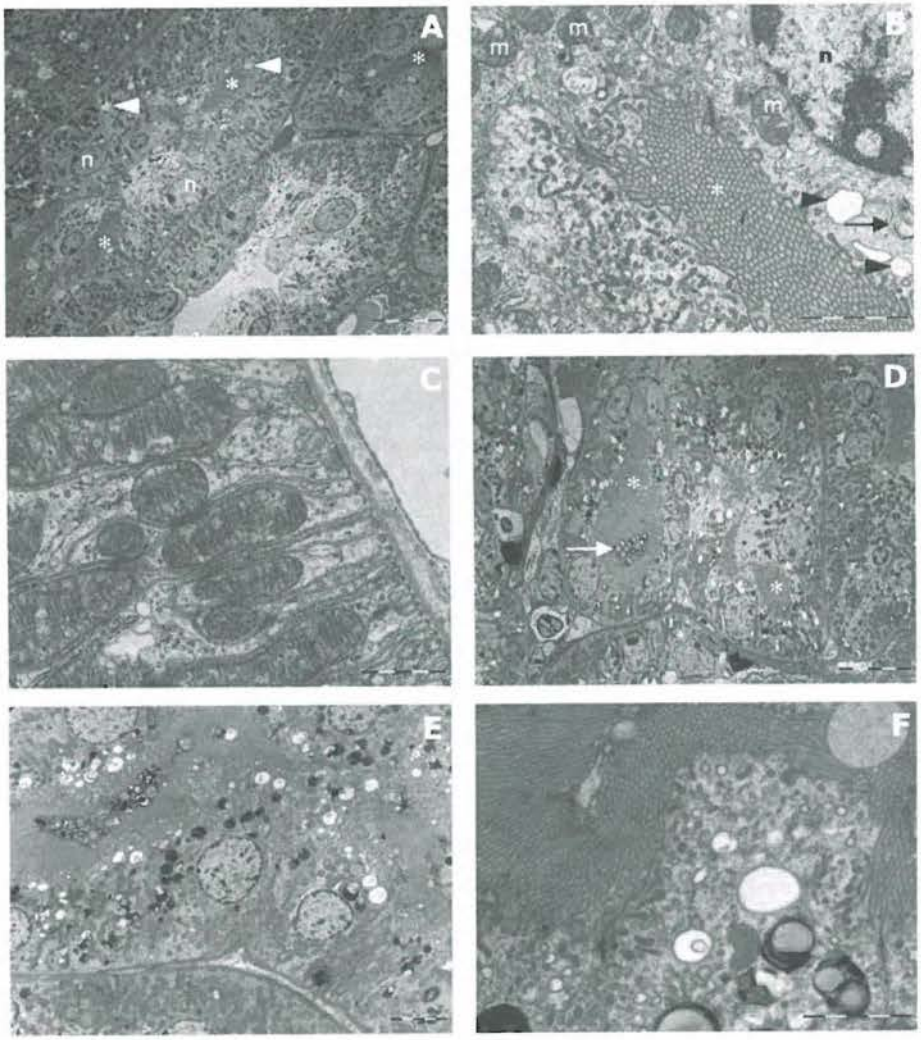
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Fig. 4.4 *TEM micrographs taken in the kidney. (A-C) Images of control kidneys, receiving normal tap water during 23 weeks. (A) Overview of a proximal (on the left) and a distal tubule (on the right) in a control kidney. In the PT the microvilli, the nucleus and some vacuoles can be seen. (B) The microvilli of the brush border with many endocytotic vesicles are shown. Mitochondria, vacuoles and a lysosome (autophagosome) can be observed as well. (C) The basolateral membrane of a PT with its basal infoldings. Elongated mitochrondria are located in the lateral cell processes in proximity to the plasma membrane. (D) Image taken in a kidney of an animal exposed to 10 mg $CdCl_2$/l during 23 weeks. Small vacuoles are spread throughout the PT; note the bulk accumulation of lysosomes near the microvilli. (E-F) Images taken in a kidney of an animal exposed to 100 mg $CdCl_2$/l during 23 weeks. (E) Part of a PT, showing a large amount of vacuoles and lysosomes around the brush border. (F) Higher magnification of the apical area in the PT, showing the brush border with endocytotic vesicles, enlarged vacuoles and lysosomes.*
** = microvilli; n = nucleus; arrowhead = vacuole; m = mitochondrion; arrow = lysosome. Scale bar= 10 µm (A, D); 5 µm (E); 2 µm (B, F); 1 µm (C).*

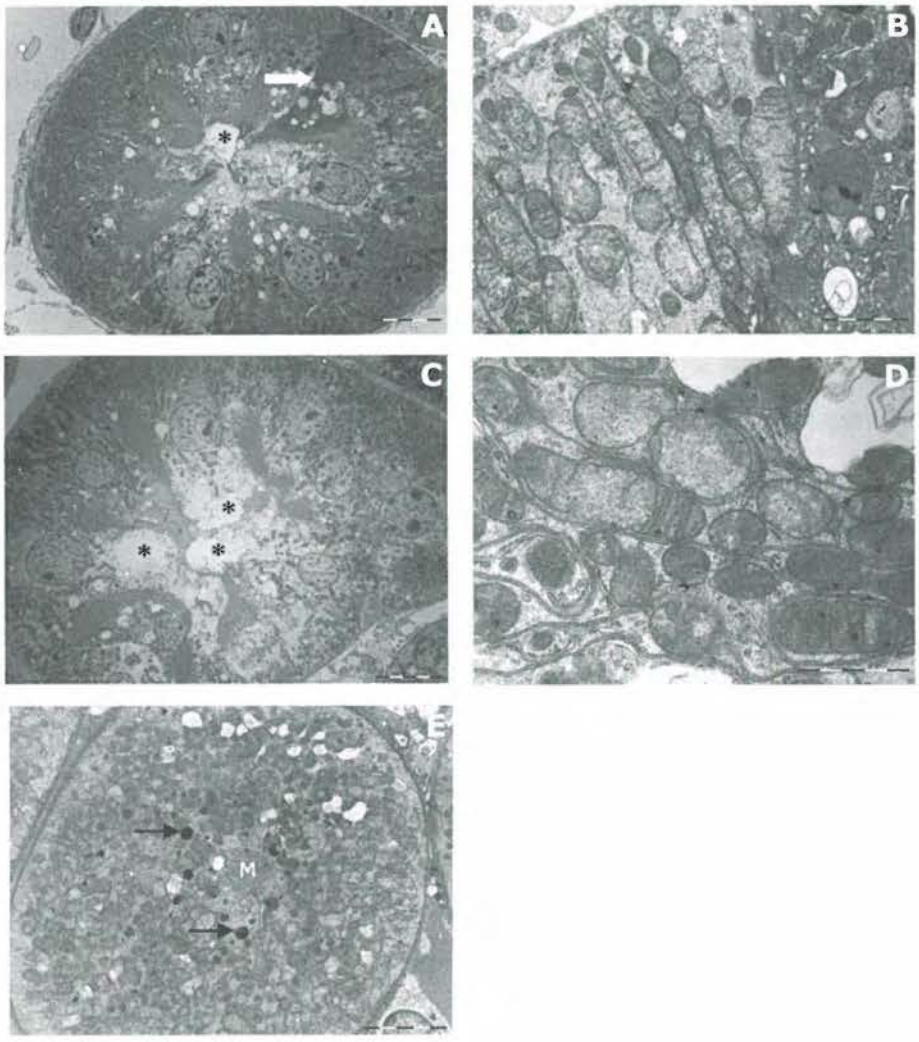
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Fig. 4.5 *TEM micrographs taken in the kidneys of mice exposed to 250 (A-B) and 500 (C-E) mg $CdCl_2$/l during 16 weeks. (A) PT showing, besides increased amounts of lysosomes and vacuoles, cytoplasmic edema. This tubule also contains a necrobiotic cell (block arrow). (B) Image of the basolateral side of a PT: basal invaginations are poorly developed; mitochondria are rounded or irregularly shaped and cristae are shortened or absent. (C) PT of a mouse exposed to 500 mg $CdCl_2$/l. Again cytoplasmic edema is largely present; the amount of vacuoles in this tubule is not that large, indicating the variable histopathological changes observed in the kidneys. (D) Many mitochondria are swollen and rounded, have lost their cristae and are spread all over. Also note the amount of mitochondria and the large vacuoles. (E) A PT with its microvilli and some large lysosomes and vacuoles. The tubule is packed with damaged mitochondria which are spread throughout the whole tubule.*

** = Cytoplasmic edema; M = microvilli; black arrow = lysosome. Scale bar= 10 µm (A, C); 5 µm (E); 2 µm (B); 1 µm (D).*

4.3.5 Morphometrical analyses

A log transformation was considered as a response for the area and perimeter measurements of glomeruli and tubules. The area and perimeter of glomeruli and tubules of animals exposed during 23 weeks to 10 or 100 mg $CdCl_2$/l were not significantly increased compared to the control. The electron-microscopical morphometrical analyses showed no significant differences between the experimental groups 10 and 100 mg $CdCl_2$/l and the control group after 23 weeks of exposure. Average values are given in Table 4.1.

Table 4.1 *Morphometrical measurements made in kidneys of mice exposed to 0, 10 and 100 mg $CdCl_2$/l during 23 weeks*

Glomerulus (area)	2706 ± 119 μm^2
Proximal tubulus (area)	1100 ± 41 μm^2
Nucleus (area)	23 ± 1 μm^2
Basement membrane *	184 ± 8 nm
Glomerular basement membrane *	200 ± 6 nm
Microvilli (cross-section)	64 ± 2 nm
Filtration slit *	38 ± 0.8 nm

Data were analysed by means of a linear mixed model (longitudinal regression model). Data are given as mean ± SEM. Because no differences were measured in kidneys of mice exposed to 0, 10 and 100 mg $CdCl_2$/l during 23 weeks, average values were calculated from the whole data set, irrespective of the Cd dose. Measurements were made in kidneys of 11 mice exposed to 0, 10 or 100 mg $CdCl_2$/l (9 mice).*

4.4 Discussion

Many studies have used animals that were injected with high Cd concentrations or that received high Cd doses orally in order to assess Cd-induced histopathological changes in the kidney. The substantial changes of renal injury in chronic Cd poisoning have not been fully established. In our study we aimed to describe the ultrastructure of the kidney and more specifically of the PTC of mice exposed to low Cd concentrations (up to 100 mg $CdCl_2$/l) and to high Cd concentrations (250 and 500 mg $CdCl_2$/l). The high Cd concentrations serve to complement the histological analyses in the mice exposed to low Cd concentrations.

4.4.1 Kidneys of mice exposed to low Cd concentrations

The earliest signs of Cd-induced changes at the ultrastructural level in the kidney and more specifically in the PTC that have been described in many histopathological studies are the increase in vacuolization and in the amount of lysosomes. This general feature has been described in both injection-based (Matsuura et al., 1991; Uriu et al., 1998) and ingestion-based (Asar et al., 2004; Gallien et al., 2001, Kauer et al., 2006; Thophon et al., 2004) studies. At the light microscopical level those characteristics of Cd-intoxication were evident in our study. In our experiments, kidneys of animals receiving 10 mg $CdCl_2$/l showed increasing vacuolization levels after 16 and 23 weeks of exposure as well as an increase in the number of lysosomes in some tubules. The vacuolization and the amount of lysosomes increased with time and Cd dose. This was clearly seen in the animals exposed to the highest concentration (100 mg $CdCl_2$/l), where the number of lysosomes was highly increased in a time-dependent manner. The ultrastructural electron microscopical analyses confirmed this dose- and time dependent increase. Predominantly secondary lysosomes were observed in Cd-intoxicated PTC. They are important for the degradation of the CdMT complexes that entered the cells via endocytosis. Together with the increase in the number of lysosomes, the amount of endocytotic vesicles has increased as well. The higher the Cd dose, the more

CdMT will reach the PTC and subsequently endocytotic vesicles increase in number to deal with the CdMT complexes.

Many studies reported hypertrophy of epithelial cells and their nuclei, as well as enlargement in the size of nucleoli in PTC with increasing Cd doses (Brzoska et al., 2003b; Damek-Poprawa and Sawicka-Kapusta, 2003; Matsuura et al., 1991; Tanimoto et al., 1999). Our morphometrical analyses of the size of nuclei and PT, as well as the electronmicroscopical observations showed that in the kidneys of mice exposed up to 100 mg $CdCl_2$/l during 23 weeks these features were not displayed. Glomerular damage was not observed either. This was in accordance with the study of Aoyagi et al. (2003), where no signs of glomerular damage were detected in rats injected with 0.6 mg Cd/kg per day for periods of 3, 5 and 8 weeks. However many other studies noted glomerular atrophy, thickening of the glomerular basement membrane, increased fusion of podocyte foot processes, closed filtration slits and mesangial expansion (Asar et al., 2004; Liu et al., 1998; Liu et al., 2000a; Uriu et al., 1998). Furthermore in our study no signs of nuclear atrophy, damaged mitochondria or an increase in apoptosis or necrosis were detected in the kidneys of mice exposed up to 100 mg $CdCl_2$/l up to 23 weeks.

4.4.2 Kidneys of mice exposed to high Cd concentrations

Besides the increase in the amount of lysosomes and vacuoles, which have clearly become larger in the kidneys of mice exposed to 250 and 500 mg $CdCl_2$/l during 16 weeks, some other features that were not observed in the mice exposed to low Cd concentrations, were noted. One striking observation was the appearance of cytosolic swelling that was seen in both experimental groups. Pronounced changes including swelling have been reported before in the study of Liu et al. (1998) in rats receiving multiple injections of either $CdCl_2$ (0.8- 1.2 mg Cd/kg) or CdMT (0.05 mg Cd/kg) daily, 6 days/week for 6 weeks. Also in the kidneys of the white sea bass, *Lates calcarifer*, that was exposed to 10 mg Cd/l for 96 h or to 0.8 and 3 mg Cd/l for 3 months, tubular cells exhibited swelling (Thophon et al., 2004). The latter conditions even caused a disorganisation of the BB; a phenomenon that was not observed in our experiments.

Another histopathological feature that was observed in the kidneys of mice exposed to high Cd concentrations but not in the animals exposed to low Cd concentrations, was the appearance of damaged mitochondria. In many tubules mitochondrial swelling with diminished matrix density and loss of cristae was observed. Because this was only noticed in our high exposure group, this phenomenon should be categorized as a feature seen in environmentally unrealistic situations. Many other studies have reported these features, together with substantial loss of basal infoldings (Asar et al., 2004; Matsuura et al., 1991; Thophon et al., 2004; Uriu et al., 1998). This latter characteristic was also observed in our study, which points to damage of the basolateral membrane. Although no morphometrical analyses were performed concerning the amount of mitochondria seen in the PT in the kidneys of our mice, it was apparent that much more mitochondria were observed in several tubules of mice exposed to 250 and 500 mg $CdCl_2$/l and often this correlated with severe mitochondrial damage. This mitochondrial fission, together with the remodeling of the cristae, is thought to happen at an early stage during apoptosis. This might indicate that in the kidneys of mice exposed to 250 and 500 mg $CdCl_2$/l many cells are undergoing apoptotic cell death (Bossy-Wetzel et al., 2003; Karbowski and Youle, 2003; Youle and Karbowski, 2005).

Because no morphometrical analyses were performed on the kidneys of mice exposed to 250 and 500 mg $CdCl_2$/l it was not possible to make any conclusions on thickening of basal membranes or swelling of glomeruli, nuclei and tubules. This was, as described earlier, often observed in other animal studies. The cytosolic swelling observed in many PT hint an enlargement of the tubules. Also an increased appearance of apoptosis or necrosis was not observed, although we only investigated this at the electron microscopical level, which makes it more difficult to get an overview and make quantification possible. However the massive fission of mitochondria might indicate an early stage of apoptosis.

4.4.3 Iron localization in kidneys of mice exposed to Cd

Interesting was the decrease in the number of iron particles in Cd-intoxicated kidneys. Under normal physiological conditions considerable amounts of transferrin (= iron-transporting protein) are filtered by the glomeruli, reabsorbed in the PT via megalin/cubilin-mediated endocytosis, and degraded in lysosomes. DMT1, which is present in late endosomes/lysosomes, may transport iron out of the vesicles and may constitute a physiological mechanism of iron acquisition by PTC (Abouhamed et al., 2006; Kozyraki et al., 2001). It is assumed that megalin and cubulin are also responsible for the endocytosis-mediated uptake of the CdMT complex in the PTC (Klassen et al., 2004). Transferrin and CdMT might compete for these endocytotic receptors to the advantage of CdMT.

4.5 Conclusions

From our study it is clear that the histopathological changes caused by Cd varied considerably from one animal to the other, and from one individual cell to another. An exposure to relatively low Cd concentrations elicited only minor changes that were restricted to increasing amounts of lysosomes and vacuolization. When high Cd concentrations were applied (250 and 500 mg $CdCl_2$/l), the changes became more pronounced and featured mitochondrial damage and fission, cellular swelling and loss of basal invaginations. It should be stressed that these features are only observed in extreme situations, which means that they probably will not be observed in kidneys of species exposed to environmentally relevant Cd concentrations. Through Perl's iron staining a decreased amount of iron particles was observed in Cd-intoxicated kidneys. A competition for megalin/cubulin between transferrin and CdMT should be confirmed by other experiments; a higher transferrin (iron) excretion in the urine might potentiate this hypothesis.

Chapter 5

Immunohistochemical staining for MT and markers for fibrosis in kidneys of mice exposed to Cd

Part of this chapter is described in:

Changes in expression of fibrotic markers and histopathological alterations in kidneys of mice chronically exposed to low and high Cd doses

Sandy Thijssen, John Maringwa, Ivo Lambrichts and Emmy Van Kerkhove. *In preparation.*

5.1 Introduction

The kidney has several mechanisms to cope with the threats of a heavy metal like Cd. MT is one of the most important early defences with respect to heavy metal toxicity and ROS. Besides the gene expression study described in chapter 3, we will investigate the expression of MT at the protein level by means of immunohistochemistry (IHC) in this chapter.

But whenever the attempts of the kidney to deal with a toxic metal like Cd are failing, chronic renal injury may occur. It is known that Cd exposure may lead to renal interstitial fibrosis, although this has hardly been studied upon a chronic low- and high-level exposure. Therefore the changes in expression and distribution of the ECM components collagen I and fibronectin and the fibrotic (EMT) marker α-SMA are examined in our model by means of IHC.

5.2 Materials and methods

5.2.1 Sampling for immunohistochemistry

Kidneys of mice, exposed during 4, 16 and 23 weeks to 0, 10 and 100 mg $CdCl_2$/l in the drinking water, were used for IHC. Additional series of animals exposed during 16 weeks to concentrations 250 and 500 mg $CdCl_2$/l were set up.

5.2.2 Fixation procedure

The kidneys were cut in half and fixed in 4 % formaldehyde (Unifix, Duiven, The Netherlands), embedded in paraffin and sectioned at 4 µm. Paraffin sections were mounted on poly-L-lysine coated glass slides.

5.2.3 Immunohistochemistry

Immunohistochemical analyses were performed on formalin-fixed, paraffin embedded kidney samples using the peroxidase-based EnVision+ System HRP for Rabbit Antibodies® (Cat.nr. K4010, DakoCytomation, Glostrup, Denmark). When dealing with mouse antibodies on mouse tissue, the mouse-to-mouse IHC detection system kit with DAB substrate (Cat.nr. 2700, Biognost, Chemicon International, Heule, Belgium) was used.

Paraffin-embedded slides with DAB EnVision kit

After deparaffinization of the slides, antigen-retrieval with Dako-citrate buffer (1:10 in PBS) was applied, followed by a marking step with PAP pen and by a Peroxidase Block to quench endogenous peroxidase activity. After 3 washing steps (5 min) with PBS, non-specific binding sites were blocked with 3 % normal goat serum (NGS) in PBS for 20 min. After washing, the tissue was incubated for 1 hr with the primary antibodies (Table 5.1), washed again, and incubated for 30 min with goat anti-rabbit horseradish peroxidase-conjugated (HRP) secondary antibodies. A high sensitivity diaminobenzidine (DAB) chromogenic substrate system and Mayer's hematoxylin were respectively used to visualize the peroxidase and for counter staining. After dehydration through graded concentrations of alcohol up to Histosol (Shandon, Cergy Pontoise, France), tissue samples were mounted with DPX (prod. nr. 361254D, BDH Laboratory Supplies, Poole, England).

Paraffin-embedded slides with mouse-to-mouse IHC detection system kit

After deparaffinization of the slides, antigen-retrieval with Dako-citrate buffer (1:10 in PBS) was applied, followed by a marking step with PAP pen and by a Peroxidase Block to quench endogenous peroxidase activity. After 3 washing steps (5') with PBS, non-specific binding sites were blocked with 10 % NGS in PBS for 20 min. After washing, the tissue was first incubated with a Pre-antibody blocking Solution during 1 hr, washed and incubated overnight with the primary antibodies (Table 5.1). Then the Post-antibody Blocking solution was added (10 min) followed by a washing step and incubated with the secondary antibody

Poly-HRP-Anti-Mouse IgG (AP181P; 1:300 in 10 % NGS) for 10 min. A high sensitivity DAB chromogenic substrate system and Mayer's hematoxylin were used to visualize the peroxidase and for counter staining, respectively. After dehydration through graded concentrations of alcohol up to Histosol (Shandon, Cergy Pontoise, France), tissue samples were mounted with DPX (prod. nr. 361254D, BDH Laboratory Supplies, Poole, England).

Table 5.1 *Antibodies used for immunohistochemistry*

Antibodies	Species	Dilution	Incubation cond.	Source
Collagen I	Rabbit IgG	1:500	1 hr, room temp.	BP1101, Acris, Germany
Fibronectin	Rabbit	1:1400	1 hr, room temp.	A0245 DakoCyt., Denmark
α-SMA	Mouse IgG2a	RTU	ON, room temp.	NCL-SMA, Novocastra, UK
MT	Mouse IgG1	1:200	ON, room temp.	12228; Abcam, UK

RTU = ready to use; ON = overnight; cond. = conditions.

5.3 Results

5.3.1 Metallothionein induction in kidneys of mice exposed to Cd

Control kidneys did not show any immunoreactivity towards MT (Table 5.2; Fig. 5.1 A; 5.2 A; 5.3 A). After 4 weeks of exposure, the kidneys of mice exposed to 10 mg $CdCl_2$/l showed no different reactivity compared to the control kidney (images not shown). An exposure to 100 mg $CdCl_2$/l during 4 weeks increased the MT immunostaining (Fig. 5.1 B). Some PT stained positive and were spread throughout the cortex. Distal tubules did not stain positive, neither did the medulla.

After 16 weeks of exposure, immunoreactivity was already evident in kidneys exposed to 10 mg $CdCl_2$/l (Fig. 5.2 B). The staining was comparable to the staining observed in the kidneys of mice exposed to 100 mg $CdCl_2$/l after 4 weeks. Again only cortical PT were stained. Exposure to higher concentrations (100, 250 and 500 mg $CdCl_2$/l; Fig. 5.2 C-H) provoked an increase in MT

expression in the cortex in a dose-dependent manner. Much more PT were stained and the staining was much more pronounced. Also distal tubules showed some moderate staining in kidneys exposed to 100 mg $CdCl_2$/l (Fig. 5.2 D) and more pronounced staining when exposed to 250 and 500 mg $CdCl_2$/l (Fig. 5.2 E, F). The difference in immunoreactivity between proximal and distal tubules was very obvious (Fig. 5.2 G). Furthermore the outer medulla was positive for MT as well after an exposure to 250 and 500 mg $CdCl_2$/l (Fig. 5.2 H).

MT expression did not only increase in a dose-dependent manner but also in a time-dependent way. When comparing kidneys exposed during 16 and 23 weeks, the immunoreactivity was more pronounced after 23 weeks of exposure to 10 (Fig. 5.3 B) and 100 mg $CdCl_2$/l (Fig. 5.3 C). The medulla was very weakly stained (Fig. 5.3 D) in the group exposed to the highest concentration (100 mg $CdCl_2$/l).

Table 5.2 *Comparisons of MT immunostaining in kidneys of mice exposed to 0-500 mg $CdCl_2$/l*

mg $CdCl_2$/l	**MT**		
	4 wks	16 wks	23 wks
0	-	-	-
10	-	+	+(+)
100	+	++	+++(+)
250		+++(+)	
500		++++	

- no staining; + mild; ++, moderate; +++ pronounced; ++++ pronounced in cortex and medulla. Empty spaces = no data available. Within control kidneys, no MT immunostaining was observed. In Cd-intoxicated kidneys, proximal tubules and later on distal tubules stained positive; when very high Cd concentrations were applied, medullary tubules became moderately positive as well.

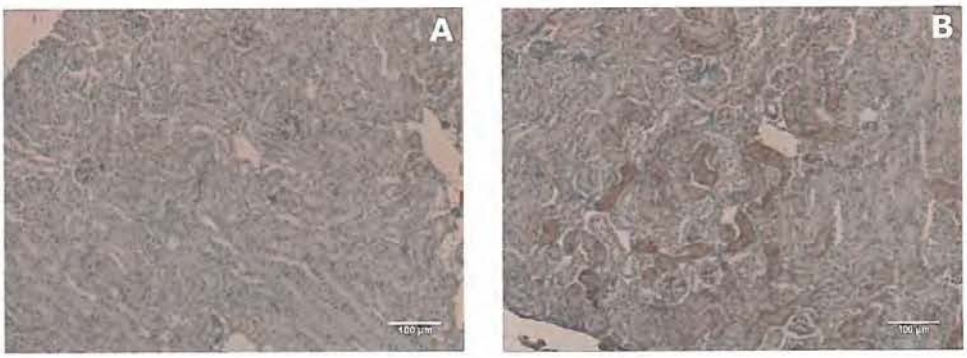
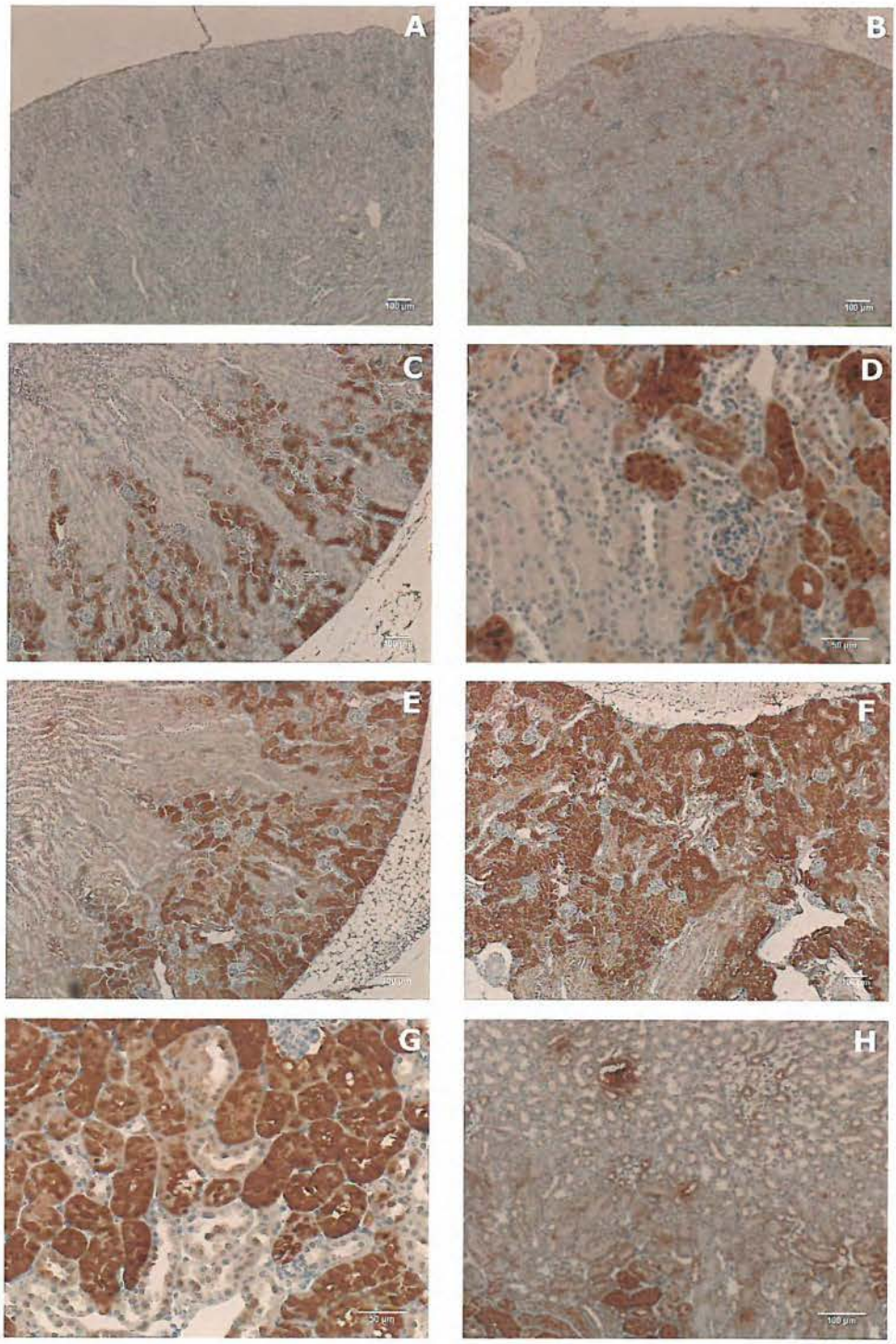


Fig. 5.1 *Immunohistochemical staining for MT. Animals were exposed during 4 weeks. (A) Overview of a control kidney of a mouse receiving normal tap water pH 2.5. No immunoreactivity is observed. (B) Animal receiving 100 mg $CdCl_2$/l. In the cortex some PT are positive for MT. Scale bar = 100 µm.*

Fig. 5.2 *Immunohistochemical staining for MT. Animals were exposed during 16 weeks. (A) Control kidney showing no immunoreactivity. (B) Kidneys of mice exposed to 10 mg $CdCl_2$/l. Positively stained PT are distributed throughout the cortex. (C, D) Kidneys of mice exposed to 100 mg $CdCl_2$/l. (C) More PT are stained compared to condition (B), showing a dose-dependent increase for MT. (D) Higher magnification of the kidney cortex. Distal tubules occasionally show staining as well. (E) Exposure to 250 mg $CdCl_2$/l increased immunoreactivity in proximal and distal tubules. The medulla stained positive as well. (F-H) Kidneys of mice exposed to 500 mg $CdCl_2$/l. (F) All PT are intensely stained; the distal tubules are moderately stained. (G) Higher magnification of the kidney cortex. (H) The medulla is positive for MT. Scale bar = 100 µm (A, B, C, E, F, H); 50 µm (D, G).*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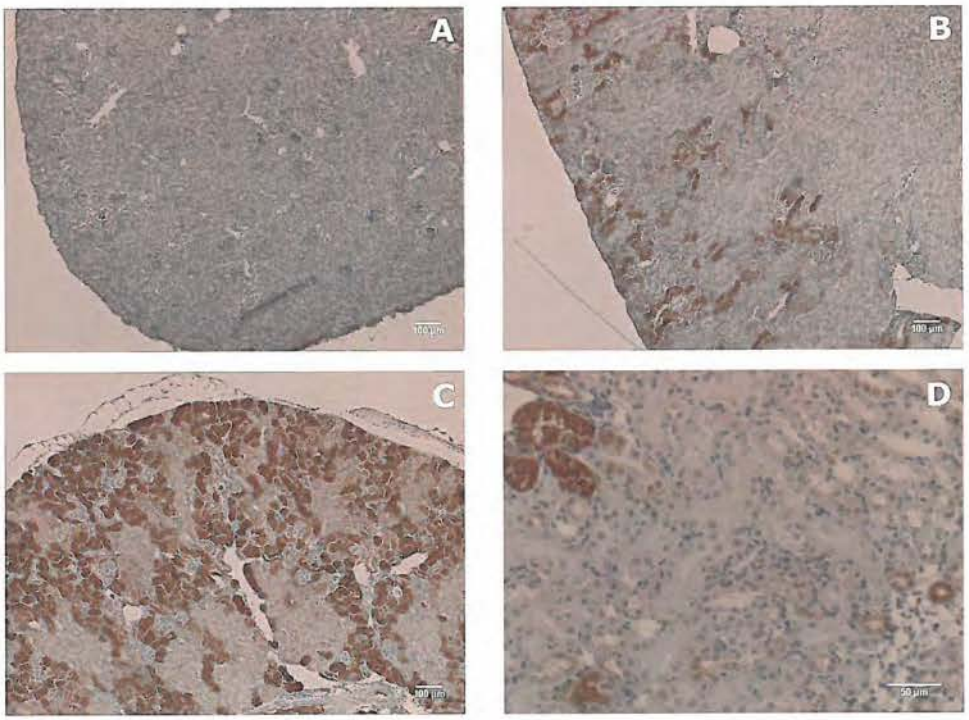

Fig. 5.3 *Immunohistochemical staining for MT. Animals were exposed during 23 weeks. (A) Overview of a control kidney. No staining is observed. (B) Immunostaining in a kidney of mice exposed to 10 mg $CdCl_2$/l showed more and more severe staining of PT compared to the kidney exposed during 16 weeks to this concentration (Fig. 5.2 B). (C, D) Kidney of mice exposed to 100 mg $CdCl_2$/l. (C) Dark brown staining of PT and lighter brown staining of distal tubules is observed. Comparing these images with the situation after 16 weeks of exposure (Fig. 5.2 C, D) clearly shows a time-dependent increase in MT. The comparison after 23 weeks between 10 and 100 mg $CdCl_2$/l shows the dose-dependent increase in MT. (D) The corticomedullary area shows some weak staining. Scale bar = 100 µm (A, B, C); 50 µm (D).*

5.3.2 Collagen I expression in kidneys of mice exposed to Cd

Immunohistological staining for collagen I (Table 5.3) in control kidneys and kidneys exposed to 10 and 100 mg $CdCl_2$/l during 4, 16 and 23 weeks did not result in any alteration (Fig. 5.5 A versus Fig. 5.5 B-C). In control kidneys collagen I staining was observed around blood vessels and around some tubules in the cortex and medulla (Fig. 5.4 A-B). After 16 weeks of exposure to 250 and 500 mg $CdCl_2$/l an increase in immunoreactivity was occasionally seen on the transition of cortex and medulla (Fig. 5.4 C-F). These observations might be not relevant because pronounced or clear differences were not observed.

Table 5.3 *Comparisons of immunostaining for markers for fibrosis in kidneys of mice exposed to 0-500 mg $CdCl_2$/l during 4, 16 and 23 weeks*

mg	**Collagen I**			**Fibronectin**			**α-SMA**		
$CdCl_2$/l	4 wks	16 wks	23 wks	4 wks	16 wks	23 wks	4 wks	16 wks	23 wks
0	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-
100	-	-	-	-	+	±	-	+	++
250		±			++			+++	
500		±			++			+++	

- no stain or staining comparable to the control; ± minimal; + mild; ++ moderate; +++ pronounced. Empty spaces = no data available. In control kidneys, collagen I staining is observed around blood vessels and around some tubules in cortex and medulla. In control kidneys, blood vessels are moderately stained for fibronectin in cortex and medulla. The corticomedullary region and the vasa recta showed more immunostaining after Cd exposure. Within control kidneys α-SMA immunoreactivity was observed in smooth muscle cells of blood vessels in the cortex and medulla. Occasionally peritubular staining was observed as well. In Cd-intoxicated kidneys an increased immunoreactivity was seen in the vasa recta; higher Cd concentrations also elicited more α-SMA in the cortex.

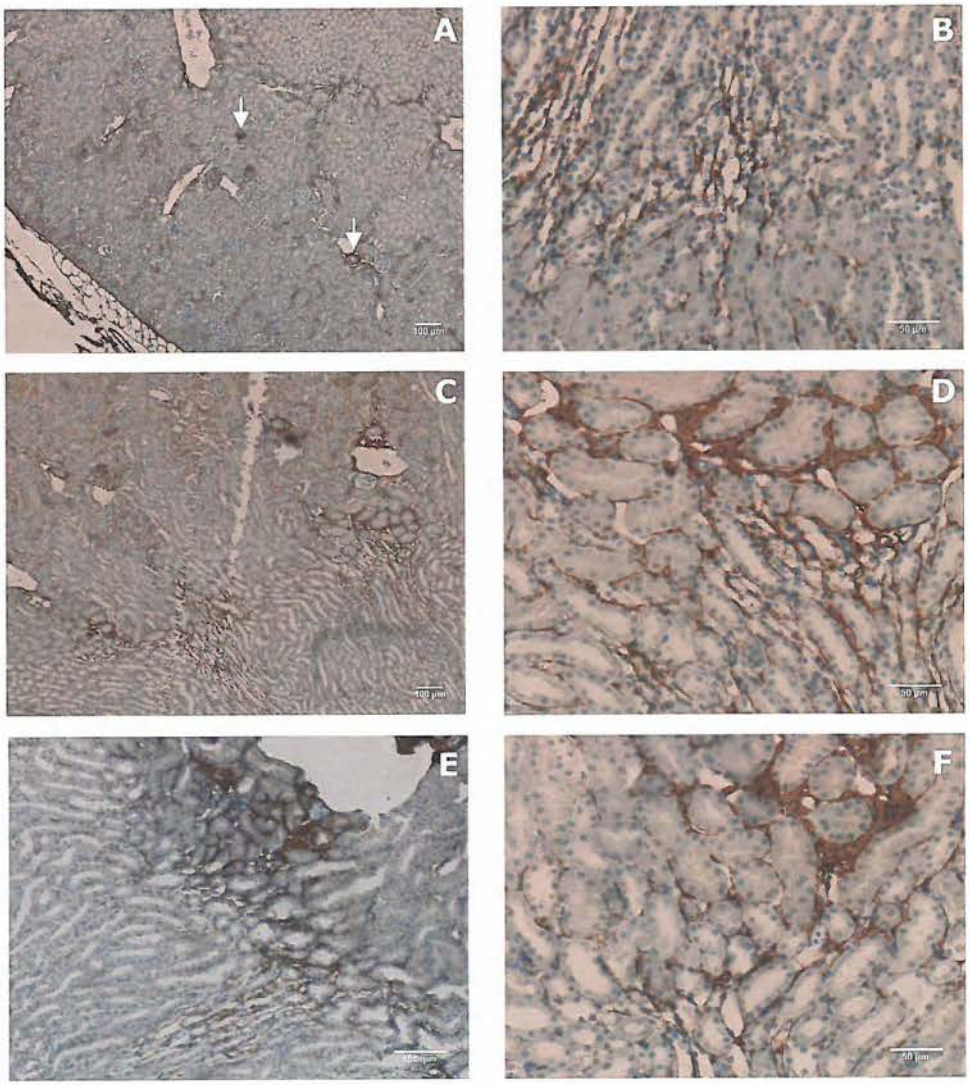
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Fig. 5.4 *Immunohistochemical staining for collagen I. Animals were exposed during 16 weeks. (A-B) Control kidney. (A) Overview of the cortex, showing some normal collagen I staining around blood vessels (arrows). (B) The medulla shows high immunoreactivity around the vasa recta. (C-D) Animal exposed to 250 mg $CdCl_2$/l. (C) A slight increase in immunoreactivity can be seen in the corticomedullary region. This is only observed occasionally and is not a general feature for the whole kidney. (D) Higher magnification of the darker stain around the vasa recta. (E-F) Animal exposed to 500 mg $CdCl_2$/l. (E) Findings were similar to C and D. Around some tubules in the neighbourhood of vasa recta, an increase in immunoreactivity is noted. (F) Higher magnification on the transition of cortex and medulla. Scale bar = 100 µm (A, C, E); 50 µm (B, D, F).*

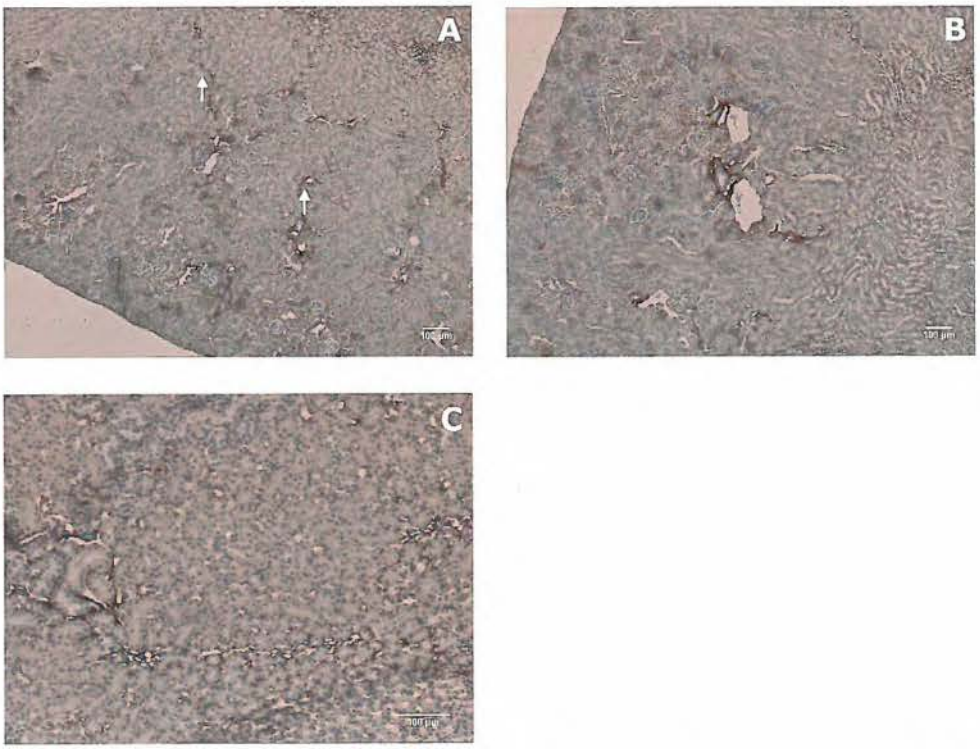

Fig. 5.5 *Immunohistochemical staining for collagen I. Animals were exposed during 23 weeks. (A) Control kidney. Overview of the cortex and part of the outer medulla. The cortex shows staining around blood vessels and around some tubules (arrows). (B-C) Animal exposed to 100 mg $CdCl_2$/l. (B) The cortex shows similar staining compared to the control kidney. (C) Transition of the cortex to the medulla. No change in immunoreactivity is detected. Scale bar = 100 µm.*

5.3.3 Fibronectin expression in kidneys of mice exposed to Cd

The immunoreactivity for fibronectin (Table 5.3) was very limited in control kidneys (Fig. 5.6 A and 5.8 A-B). Around blood vessels in the cortex and medulla a moderate staining could be observed (Fig. 5.6 B, C). After 16 weeks of exposure, the situation was the same when exposing to 10 mg $CdCl_2$/l (images not shown). Kidneys of mice exposed to 100 mg $CdCl_2$/l did not show any difference in immunoreactivity in the cortex (Fig. 5.6 D). On the other hand the tubules in the corticomedullary region were sometimes surrounded by fibronectin labeling (Fig. 5.6 E, F). In the medulla, the vasa recta and some tubules were surrounded by a more defined band of fibronectin compared to the control (Fig. 5.6 G, H). This feature was more pronounced when exposing mice to 250 and 500 mg $CdCl_2$/l. In the medulla, the tubules were bordered with a thin film of fibronectin labeling (Fig. 5.7 A-C and G-H). Around the vasa recta similar observations were made (Fig. 5.7 K, L). This phenomenon was more pronounced after an exposure to 250 and 500 mg $CdCl_2$/l than to 100 mg $CdCl_2$/l. The observed phenomena were not restricted anymore to the medulla but also appeared in the cortex (Fig. 5.7 D-F, I, J).

After 23 weeks no considerable differences were observed between control animals (Fig. 5.8 A-B) and animals exposed to 10 mg $CdCl_2$/l (images not shown). A higher concentration of Cd (100 mg $CdCl_2$/l) increased the proportion of fibronectin expression slightly around the vasa recta and sometimes in the interstitium (Fig. 5.8 C-E).

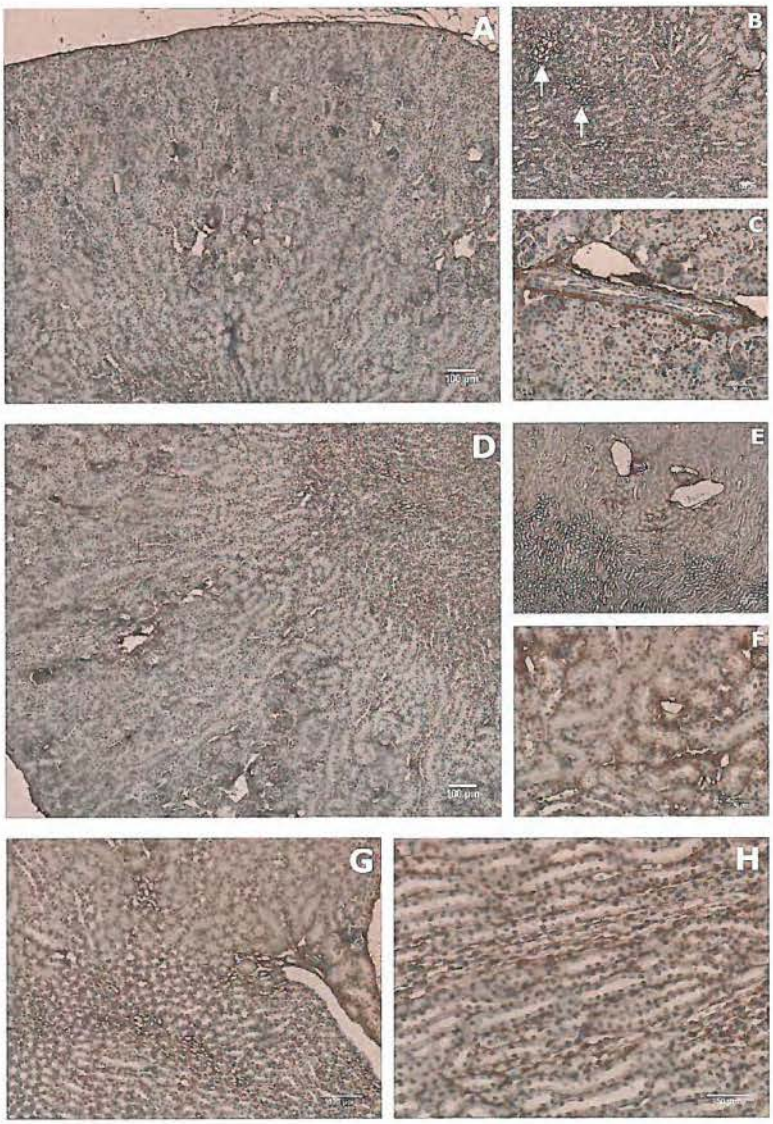
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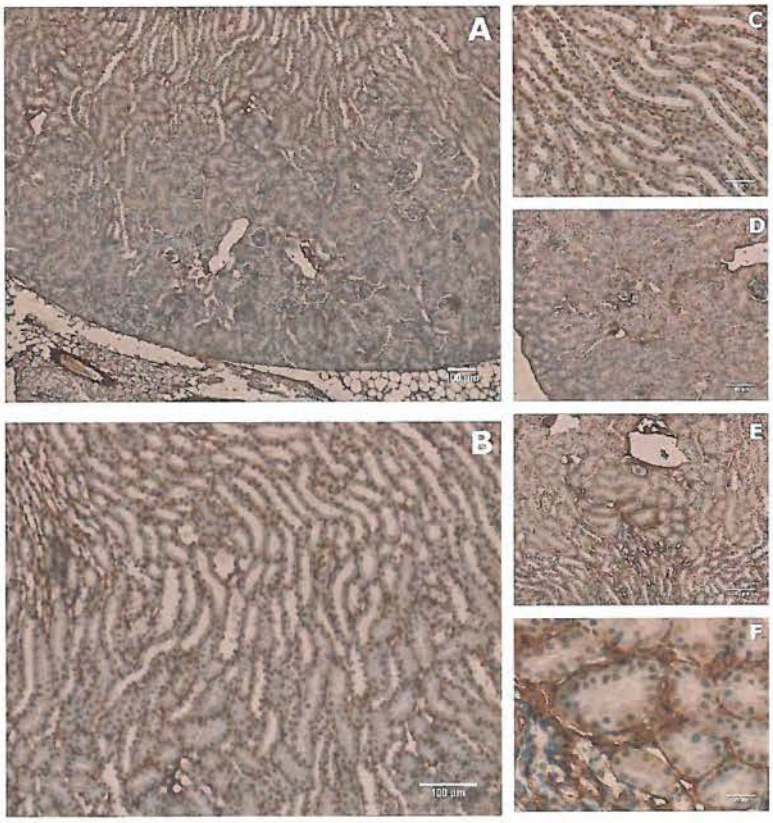
Fig. 5.6 *Immunohistochemical staining for fibronectin. Animals were exposed during 16 weeks. (A-C) Pictures of a control kidney. (A) Normal view of the kidney cortex; some immunoreactivity around blood vesssels is visible. (B) The kidney medulla shows moderate immunoreactivity around the vasa recta (arrows). (C) Blood vessel surrounded by fibronectin labeling. (D-H) Kidney of mouse exposed to 100 mg $CdCl_2$/l. (D) Overview of the cortex and outer medulla. The cortex looks similar to the control; the medulla shows some increased immunoreactivity around some tubules and around the vasa recta. (E-F) Higher magnification of positive staining for fibronectin on the transition of cortex and medulla. (G-H) Higher magnification of the medulla. The vasa recta are surrounded with a thicker band of fibronectin compared to the control. The tubules are more defined. Scale bar = 100 µm (A, B, D, E, G); 50 µm (C, F, H).*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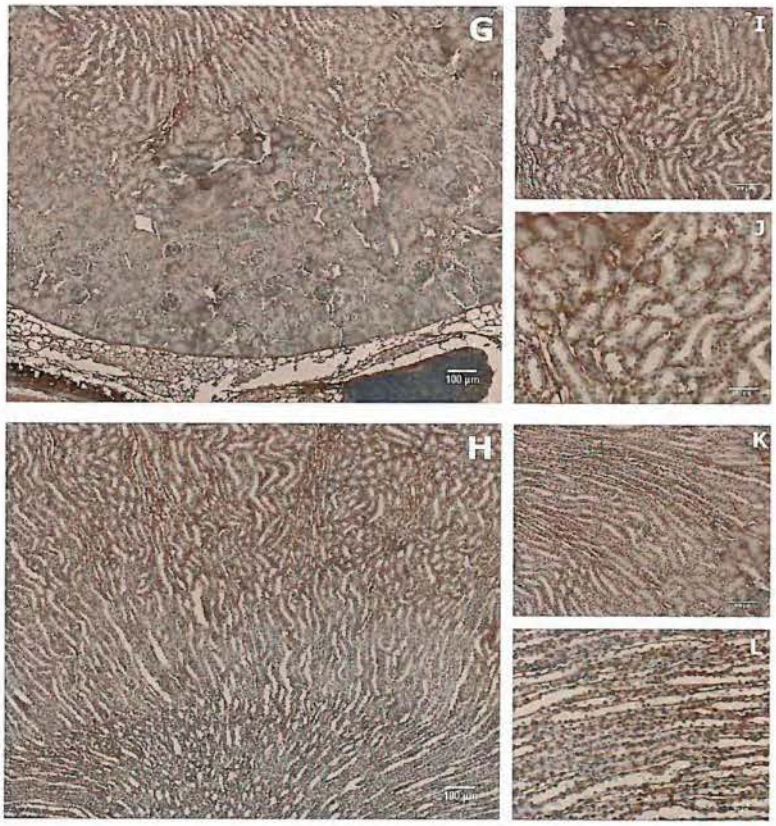


Fig. 5.7 *Immunohistochemical staining for fibronectin. Animals have been exposed during 16 weeks. (A-F) Pictures of a kidney exposed to 250 mg $CdCl_2$/l. (A) Overview of the cortex and outer medulla, showing increased immunoreactivity compared to the lower concentration groups. Immunoreactivity starts from the corticomedullary area. (B-C) Higher magnification of the proximal and distal tubules on the transition of cortex and medulla. Fibronectin accumulation around the tubules is clearly visible. (D-F) Also in the cortex an increase in immunoreactivity was observed occasionally; this was not seen throughout the whole cortex. (G-L) Pictures of a kidney exposed to 500 mg $CdCl_2$/l. (G) Overview of the cortex and outer medulla, which shows dark brown staining for fibronectin. It clearly shows that tubules in the cortex are also positive for fibronectin. (H) Overview of the medulla. Dark staining is visible around the vasa recta and the tubules in the outer medulla. The inner medulla shows only some weak staining. (I-J) Higher magnification of the proximal and distal tubules in the cortex. They are framed by a thin band of fibronectin. (K-L) Higher magnification of the tubules and vasa recta in the medulla. Scale bar = 100 µm (A, B, D, E, G, H, I, K); 50 µm (C, J, L); 25 µm (F).*

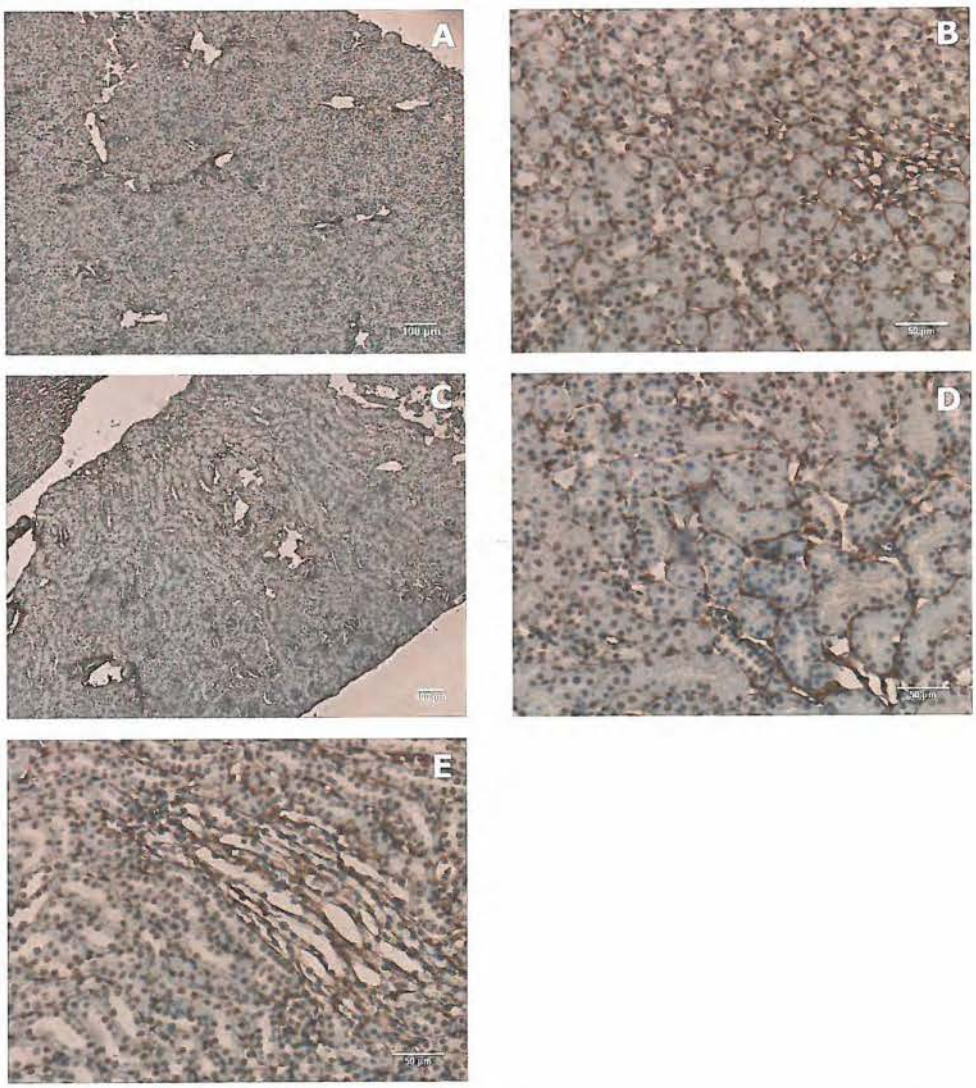


Fig. 5.8 *Immunohistochemical staining for fibronectin. Animals have been exposed during 23 weeks. (A-B) Control kidney. (A) Overview of the kidney cortex, comparable to the situation after 16 weeks (Fig. 5.6 A-C). (B) Medulla of a control kidney, showing moderate staining around the vasa recta. (C-E) Kidney of animals exposed to 100 mg $CdCl_2$/l. (C) Overview of the cortex showing some immunoreactivity around tubules. (D-E) Higher magnification of the cortex (D) and the medulla. Scale bar = 100 µm (A, C); 50 µm (B, D, E).*

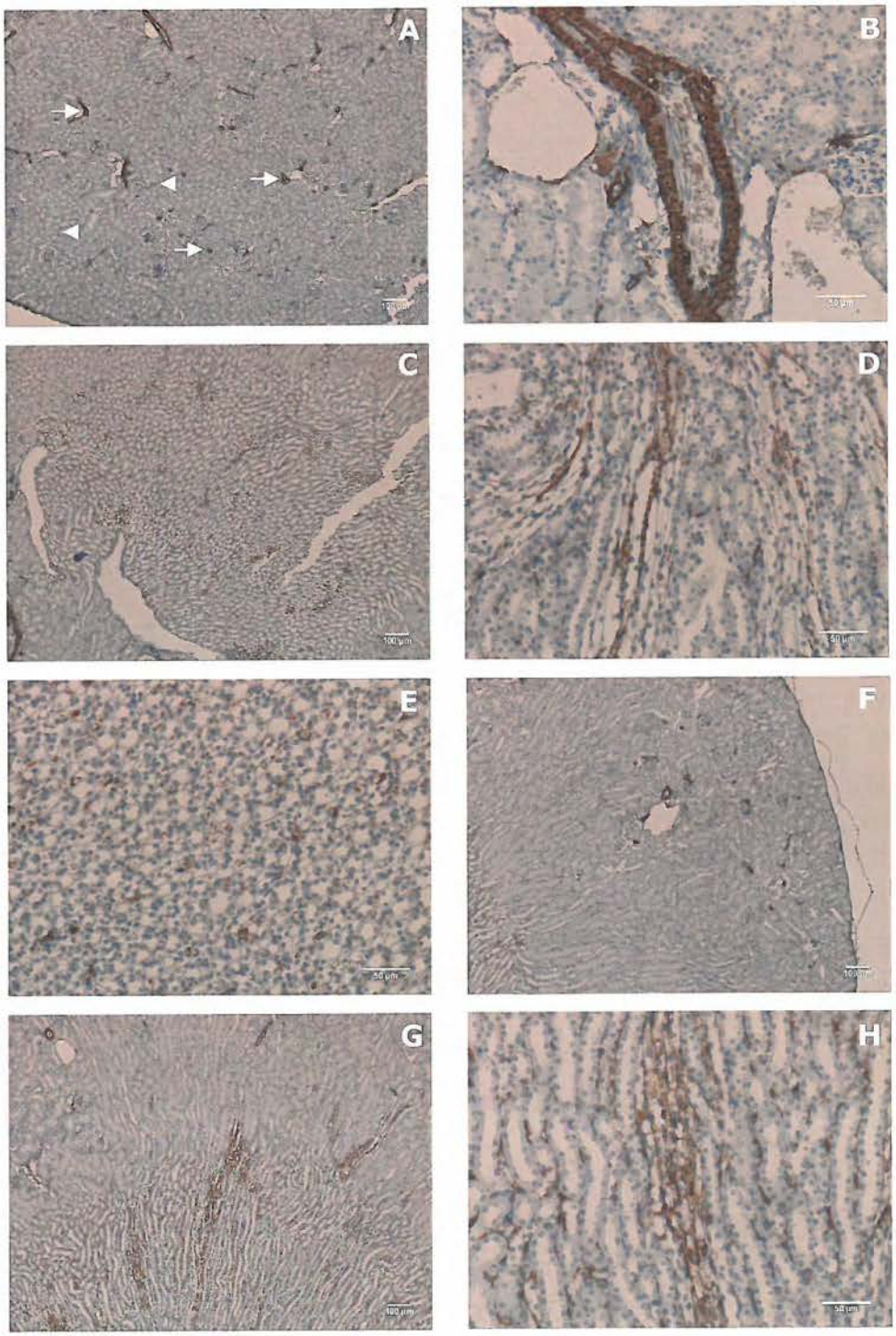
5.3.4 α-SMA expression in kidneys of mice exposed to Cd

Within control kidneys, α-SMA immunoreactivity was observed in smooth muscle cells of blood vessels in the cortex (Table 5.3; Fig. 5.9 A, B and 5.11 A). Occasionally peritubular staining was observed as well. In the medulla the vasa recta (smooth muscle cells) were positive for α-SMA (Fig. 5.9 C, D and Fig. 5.11 B, C). There was no difference perceptible after 4, 16 and 23 weeks. In the inner medulla, an occasional brown staining around the tubules was seen (Fig. 5.9 E). No staining in other cells was observed.

The immunoreactivity after 4 weeks of exposure to 10 and 100 mg $CdCl_2$/l and after 16 weeks to 10 mg $CdCl_2$/l was not different from the control (images not shown). An exposure for 16 weeks to 100 mg $CdCl_2$/l did not change the α-SMA staining in the cortex (Fig. 5.9 F) but an increase in α-SMA immunoreactivity of the vasa recta was observed compared to the control (Fig. 5.9 G, H). Around distal tubules and collecting ducts in this area moderate immunoreactivity was observed (Fig. 5.9 H). In the interstitium of the limbs of Henle's loop and collecting ducts in the inner medulla a marked increase in immunoreactivity was noticed as well (Fig. 5.9 I).

The above described alterations in immunoreactivity were more pronounced in the kidneys of mice exposed to 250 and 500 mg $CdCl_2$/l. In the interstitium of the proximal and distal tubules in the cortex an increase in immunoreactivity was observed (Fig. 5.10 A, E). An intense staining of the vasa recta and surrounding tubules was seen as well (Fig. 5.10 B-D and F-H).

After 23 weeks of exposure the kidneys exposed to 10 mg $CdCl_2$/l did not show any change in immunoreactivity in the cortex or medulla (images not shown). When exposing mice to 100 mg $CdCl_2$/l the cortex showed increased immunoreactivity in the cortical interstitium (Fig. 5.11 C-D). This was previously detected after 16 weeks when exposing to 250 and 500 but not to 100 mg $CdCl_2$/l. Consistent with the situation after 16 weeks, the vasa recta showed an increase in α-SMA immunoreactivity (Fig. 5.11 E).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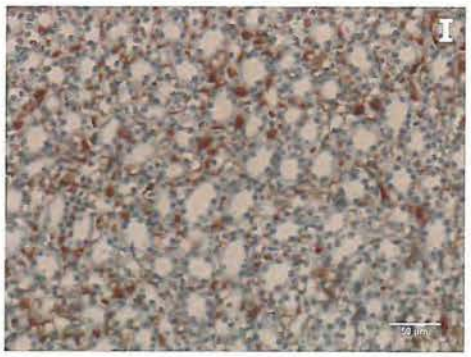

Fig. 5.9 *Immunohistochemical staining for α-SMA. Animals were exposed during 16 weeks. (A-E) Control kidney. (A) Overview of the α-SMA immunostaining in a normal kidney cortex, showing immunoreactivity in blood vessels (arrows). Occasionally peritubular staining was observed (arrowheads) (B) Picture at a higher magnification showing strong α-SMA immunoreactivity in vascular smooth muscle cells. (C) Overview of the immunostaining in the medulla. The vasa recta (smooth muscle cells) are positive for α-SMA. (D) The vasa recta at higher magnification. (E) Detail of the inner medulla, showing some reactivity in the interstitium (F-I) Kidney of an animal exposed to 100 mg* $CdCl_2$*/l. (F) Overview of the kidney cortex, where no change in immunoreactivity was detected compared to the control kidney. (G) Immunoreactivity in the medulla. (H) Picture at higher magnification showing an increase in α-SMA immunoreactivity of the vasa recta compared to the control. The interstitium shows some positive staining for α-SMA (I) The inner medulla shows higher immunoreactivity as well (peritubular). Scale bar = 100 μm (A, C, F, G); 50 μm (B, D, E, H, I).*

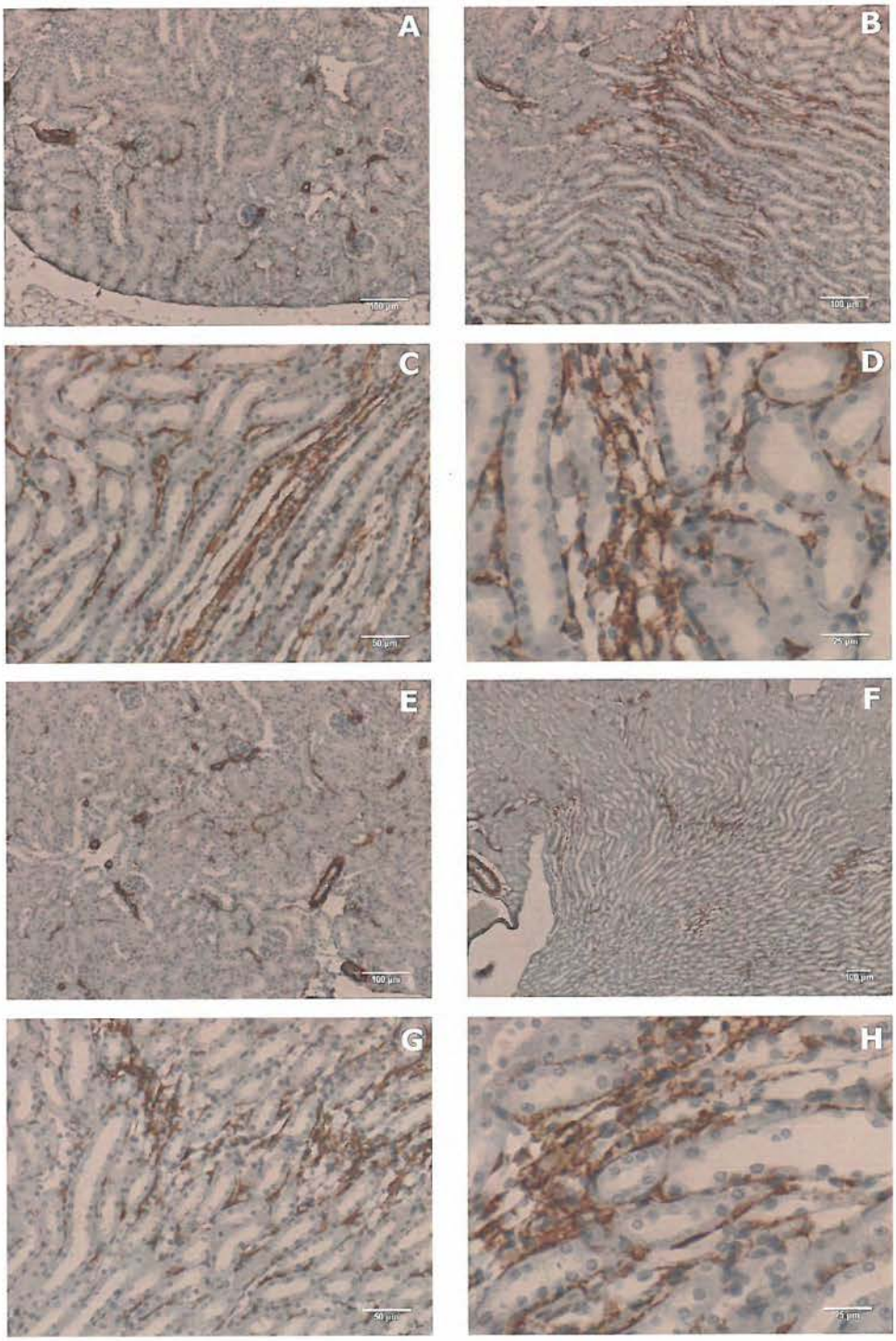
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Fig. 5.10 *Immunohistochemical staining for α-SMA. Animals have been exposed during 16 weeks. (A-D) Pictures of kidneys of mice exposed to 250 mg $CdCl_2$/l. (A) Cortex showing increased immunoreactivity around the PT. The immunoreactivity was more intense compared to the lower Cd and control groups. (B) Transition of the cortex and the outer medulla. Intense staining of the vasa recta and surrounding tubules. (C-D) Higher magnification of the vasa recta and the surrounding tubules. (E-H) Pictures of kidneys of mice exposed to 500 mg $CdCl_2$/l. (E) Cortex showing comparable immunoreactivity around the PT as above (250 mg $CdCl_2$/l). (F-G-H) The kidney medulla, and more specifically the vasa recta, are intensely stained, as well as the surrounding tubules. Scale bar = 100 μm (A, B, E, F); 50 μm (C, G); 25 μm (D, H).*

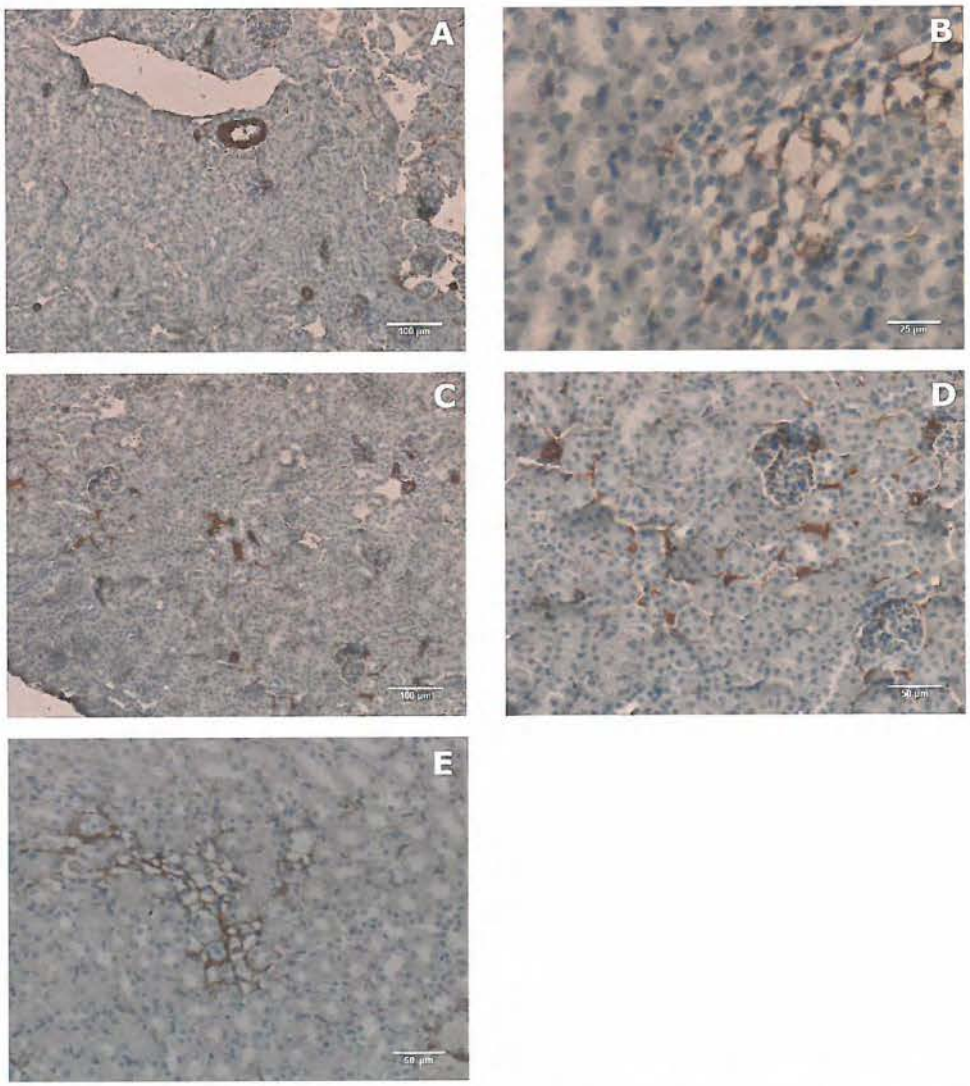

Fig. 5.11 *Immunohistochemical staining for α-SMA. Animals have been exposed during 23 weeks. (A-B) Control kidney. (A) Overview of the α-SMA immunostaining in a normal kidney cortex. (B) Immunostaining of the medulla. Some vasa recta are positively stained. (C-E) Kidney of animal exposed to 100 mg $CdCl_2$/l. (C) The cortex shows increased immunoreactivity for α-SMA (D) Higher magnification of the cortex. Note the intense staining around the tubules. (E) Medulla, and more specifically the vasa recta, showing a moderate increase in immunoreactivity. Scale bar = 100 µm (A, C); 50 µm (D, F); 25 µm (B, E).*

5.4 Discussion

5.4.1 Dose- and time-dependent MT expression

The mRNA level of MT 1 and 2 showed a time- and dose-dependent increase in the kidneys of mice exposed to Cd as described in Chapter 3. This was also observed on the protein level. After 4 weeks of exposure a considerable increase in MT immunoreactivity was already noticed in animals exposed to 100 mg $CdCl_2$/l, which demonstrated the early defensive role of the molecule. When exposing to 250 and 500 mg $CdCl_2$/l during 16 weeks and to 100 mg $CdCl_2$/l during 23 weeks, the tubules in the medulla stained (moderately) positive as well. All these observations in mice are therefore in accordance with the study of Tanimoto et al. (1999) in rats, where IHC staining with MT revealed a similar time-dependent increase in animals injected with 0.6 mg Cd/kg bw during a maximum of 4 weeks. An increase in MT content has also been observed in several other tissues (liver, kidney, heart, lung) in different species upon (acute or chronic) Cd exposure (Bobillier-Chaumont et al., 2006; Bonda et al., 2004; Lange et al., 2002; Marie et al., 2006).

5.4.2 Markers for fibrosis

Few studies have been published explaining the mechanisms that are triggered by Cd causing tubulointerstitial fibrosis. In the present study the expression of collagen I, fibronectin and α-SMA was evaluated within control and Cd-intoxicated kidneys. Furthermore it was interesting to assess the severity of fibrotic action in the kidneys of mice exposed to (relatively) low and high Cd doses. In normal murine kidneys vascular smooth muscle cells stained positive for α-SMA while fibronectin and collagen I immunoreactivity was mostly found surrounding blood vessels. These matrix macromolecules (fibronectin, collagen but also tenascin, osteopontin, thrombospondin) are secreted by tubulointerstitial fibroblasts, tubular epithelium and vascular endothelium. They stabilize the physical structure of cells (Kelly and Neilson, 2000).

As described in chapter 1, one of the unifying histopathological hallmarks of fibrotic disorders is the deposition of type I and type III collagen in the glomerulus and interstitium (Groma, 1998; Liu, 2006). Transcriptional activation of collagen gene expression is probably the most important regulatory mechanism leading to fibrosis (Schiller et al., 2004). In our study significant changes in the expression pattern of collagen I were not observed in the experimental groups. In the renal cortex, moderate immunoreactivity was found perivascularly and occasionally peritubularly. In the medulla the vasa recta stained highly positive for collagen I. Exposing the animals to high Cd concentrations (250 and 500 mg $CdCl_2$/l) during 16 weeks sometimes resulted in more peritubular immunoreactivity but this was not a general feature so it might not be a relevant observation yet. In other studies collagen I expression in Cd-induced renal fibrosis has not been investigated as such. One study exposed male rats to 15 ppm $CdCl_2$/l in their drinking water for one month and studied several ECM proteins immunohistochemically. A decrease in collagen type IV, one of the principal components of the basement membrane, was observed (Asar et al., 2004).

An increased accumulation of fibronectin, another important ECM protein, is also thought to be a fundamental process in the development of tubulointerstitial scarring. Moreover it is a mesenchymal marker for EMT as well (Lee et al., 2006b; Thiery and Sleeman, 2006). While in control kidneys in our study a moderate staining for fibronectin was observed around large blood vessels and the vasa recta in cortex and medulla, the medulla showed increased immunoreactivity around some tubules and around the vasa recta in mice exposed to 100 mg $CdCl_2$/l during 16 weeks. After 23 weeks of exposure to the same concentration, comparable staining was observed. An exposure to 250 and 500 mg $CdCl_2$/l during 16 weeks clearly demonstrated a dose-dependent increase in fibronectin in the medulla (perivascular and peritubular). Furthermore this positive staining was expanding into the cortical area in the kidney. Similar findings have been described in the study of Asar et al. (2004) where rats were exposed to 15 ppm $CdCl_2$ in their drinking water for one month. An increased fibronectin production in glomeruli and in the tubular interstitium was observed. Also in lead-treated rats, a strong labeling to fibronectin in all

renal basement membranes together with a decrease in their thickness was described (Sanchez et al., 2001). Furthermore in the lung, Cd-telluride treated rats suffered from pulmonary interstitial fibrosis with increased levels of fibronectin (Morgan et al., 1997).

The third protein that was studied, α-SMA, is a typical marker for myofibroblasts as well as for EMT. Depending on the activated cellular pathways, α-SMA expression can be observed in functional epithelial cells undergoing EMT or in the interstitial matrix, where myofibroblasts are residing upon attraction and activation (Badid et al., 2002; Liu et al., 2006).

Our study revealed that an increase in α-SMA expression was observed in Cd-intoxicated mice. After 16 weeks of exposure to 100 mg $CdCl_2$/l there was an increase in α-SMA around the vasa recta and in the interstitium in the medulla. When a higher concentration (250 and 500 mg $CdCl_2$/l) was applied, the medulla showed a higher peritubular and perivascular immunoreactivity. The interstitium of the cortical tubules was also positively stained. After 23 weeks of exposure to 100 mg $CdCl_2$/l comparable findings were observed. These findings are in agreement with other studies on kidney fibrosis, where an increased number of α-SMA-positive cells or positive peritubular staining were detected (Groma, 1998; Roberts et al., 1997).

The high perivascular immunoreactivity observed in Cd-intoxicated kidneys might be explained by the activation and differentiation of pericytes into myofibroblasts. Pericytes, relatively undifferentiated cells associated with the walls of small blood vessels, can differentiate into fibroblasts, which in its own turn will differentiate into myofibroblasts expressing α-SMA, upon activation. Those activated pericytes can also migrate to the interstitium, where also other α-SMA expressing cells might reside which were attracted. This explains the peritubular immunoreactivity towards α-SMA that was observed in Cd-intoxicated kidneys (Desmoulière et al., 2005; Liu et al., 2006). Furthermore we did not find any α-SMA immunoreactivity in epithelial cells (undergoing EMT), indicating that this process is a late fibrogenic response, while myofibroblastic activation (and accordingly interstitial α-SMA immunoreactivity) is an early

response (Liu et al., 2006). Additional immunogold labeling for α-SMA might be interesting to specify which cell types are expressing α-SMA in the renal interstitium.

In relation with Cd-toxicity, no other studies have been conducted that study α-SMA expression *in vivo*. In general fibrosis has only been described in several *in vivo* studies in the liver or the kidney of rats, frogs and mice by means of light microscopic examination (Damek-Poprawa and Sawicka-Kapusta, 2003; Liu et al., 1998; Liu et al., 2000a; Loumbourdis, 2005; Uriu et al., 1998).

5.5 Conclusions

In conclusion, the present study demonstrated that Cd-intoxicated kidneys showed increased immunoreactivity towards α-SMA and the ECM marker fibronectin. The changes became prominent after an exposure to 100 mg $CdCl_2$/l after 16 weeks and became more pronounced when increasing the Cd dose. On the other hand no change in expression of collagen type I was observed in Cd-intoxicated kidneys. Signs of EMT were also not found; α-SMA immunoreactivity was not found in epithelial cells but only in the interstitium. Furthermore the findings described in Chapter 3, where a dose- and time-dependent increase in MT-mRNA was observed, are confirmed in this chapter. On the protein level, MT expression also increased in a dose- and time-dependent manner.

Chapter 6

Summary and General Discussion

Summary

The study reported in this thesis described the effects of a long-term, low-level cadmium (Cd) exposure in mice and more specifically in the kidney, with focus on the proximal tubular cells (PTC). We did not only try to picture a dose-response relationship between Cd exposure and Cd-induced kidney damage, but we were also interested in the histolopathological changes in the kidneys. Furthermore we wanted to investigate if oxidative stress plays a major role in Cd-induced proximal tubular kidney damage.

In ***chapter 2*** we have described the set-up of the exposure study. Mice were exposed up to 23 weeks to Cd concentrations up to 100 mg $CdCl_2$/l. The results concerning the animal's weight, water and food intake proved that our exposure conditions corresponded to a chronic, (reasonably) low-level exposure. Proteinuria was observed in mice exposed to 100 mg $CdCl_2$/l starting from 16 weeks. Kidney cortex cadmium (K-Cd) content was confirmed to be a very reliable indicator of chronic exposure while blood cadmium (B-Cd) content is an indicator of current exposure.

In ***chapter 3*** the oxidative stress status of mice chronically exposed to Cd concentrations up to 100 mg $CdCl_2$/l (as described in chapter 2) is described, with main focus on the kidney. Gene expression studies of pro- and antioxidants as well as quantification of GSH, AsA and MDA levels were performed. Our study provided evidence that an exposure to low Cd concentrations causes changes in the anti and pro-oxidative systems in the cell that lead to adaptation and survival, instead of changes leading to damage and GSH depletion. A biphasic stress response (after 1 and 23 weeks) was observed. Major roles were designated for *Prdx2* and *Nox4*, which have not been studied in a chronic exposure experiment to relatively low Cd concentrations before.

In ***chapter 4*** the morphology of the kidneys and more specifically of the PTC of chronically exposed mice is described. Preliminary studies in kidneys of mice exposed up to 100 mg $CdCl_2$/l showed that rather minor changes were detected

at the histological level; therefore we expanded this morphological part with an exposure experiment during 16 weeks to high (250 and 500 mg $CdCl_2$/l) Cd concentrations. The kidneys of mice exposed to these high concentrations, showed substantial damage to mitochondria and basal infoldings.

In ***chapter 5*** the effects of Cd on the expression of metallothionein (MT) and fibrotic markers (fibronectin, collagen I and α-SMA) in the kidneys of mice exposed up to 500 mg $CdCl_2$/l are studied by means of immunohistochemistry. The expression of MT, a heavy metal and radical scavenger, was dose and time dependent. The Cd-intoxicated kidneys of mice exposed to 100 mg $CdCl_2$/l or higher showed increased immunoreactivity towards α-SMA and the extracellular matrix (ECM) marker fibronectin.

General discussion

New technologies are rapidly developing in our industrialized society. The amount of goods that are produced, the production processes and the ever-increasing consumption are threatening the environment and the people living in it. Contaminating agents can undermine our health status and interfere with processes in our body, influence organ functioning and be the cause of severe illnesses or death.

Cd, which was commercialized in the 20th century due to industrial applications, has been considered a main threat to human health, together with other toxic elements such as lead, mercury and arsenic (Järup, 2003; WHO, 2000). Moreover several population studies in Belgium, Sweden and Japan have shown that not only occupationally exposed workers but also environmentally exposed populations can experience moderate to severe health problems due to Cd toxicity. Exposure to Cd has been correlated with tubular kidney damage, osteoporosis (Äkesson et al., 2006; Järup, 2002; Nordberg, 2004), hepatic dysfunction (El-Demerdash et al., 2004; WHO, 2000) and lung cancer (Nawrot et al., 2006; Verougstraete et al., 2003). The most important target organ in chronic Cd exposure is, by far, the kidney. Therefore it is important to understand the mechanisms leading to Cd nephrotoxicity. This can be achieved by developing an experimental set-up to study chronic Cd intoxication in an animal model. In Table 6.1 an overview of the most important findings described in this thesis, is given.

Table 6.1 *Schematic overview of the most important results described in this thesis*

		Exposure time (weeks)				
Cd conc (mg $CdCl_2$/l)	Experiment	1	4	8	16	23
0						
10	Gene expr.	↓ *Hmox1* ↙ *Bcl2*		↑ *Mt1* ↗ *Mt2*		↑ *Mt1-Mt2-Gpx4-Nox4* ↓ *Prdx2*
	IHC				↑ MT	↑ MT
	Morph.				lys-vac	lys-vac
100	Gene expr.	↑ *Mt1-Mt2* ↙ *Bcl2* ↗ *Prdx2*		↑ *Mt1-Mt2* ↓ *Sod1-Bcl2* ↙ *Prdx2*		↑ *Mt1-Mt2-Gpx4-Hmox1-Nox4-Cat* ↙ *Prdx2*
	IHC		↑ MT		↑ MT-fibr- α-SMA	↑ MT-fibr- α-SMA
	Morph.				lys-vac ↓ Fe particles proteinuria	lys-vac proteinuria
250	IHC				↑ MT-fibr- α-SMA ↗ Coll I	
	Morph.				lys-vac- mitoch. + basol. damage-edema	
500	IHC				↑ MT-fibr- α-SMA ↗ Coll I	
	Morph.				lys-vac- mitoch. + basol. damage-edema ↓ Fe particles	

Gene expr. = gene expression; IHC = immunohistochemistry; Morph. = morphological features; fibr = fibronectin; coll I = collagen I, lys = increase in amount of vacuoles; vac = increase in vacuolization; mitoch. + basol. damage = mitochondrial and basolateral damage; ↑ increase (in expression level); ↓ decrease; ↗ small increase (borderline of significance); ↙ small decrease (borderline of significance). Sod2 expression levels remained stable, MDA, GSH, GSSG and DHA levels did not change and AsA levels decreased in a concentration-independent way. U-Cd levels can be considered environmentally relevant.

In this thesis we have described the set up of a Cd exposure experiment in mice, which is fairly new, as previous studies generally focused on exposure studies in rats. The advantage of the mouse model, namely the availability of knockouts, was the decisive argument to choose for this model. Different aspects of Cd intoxication in kidneys were pictured. The urinary Cd (U-Cd) levels that were measured, were environmentally relevant; B-Cd levels below 10 µg Cd/l, measured in mice exposed up to 10 mg $CdCl_2$/l, were environmentally relevant as well. Furthermore our model has proven again that only K-Cd content is a reliable marker for chronic exposure, while B-Cd is indicative of an ongoing exposure. Although many studies focus on U-Cd levels as an indicator of total body burden, this was not confirmed in our study, at least at the doses the animals were exposed to. The relatively short exposure time can explain this; when exposing to low Cd concentrations, it takes a time before U-Cd levels increase linearly. All of this makes the present model a good model to examine the mechanisms involved in a long-term low-level Cd exposure.

On the functional level the appearance of proteins in the urine of mice chronically exposed to Cd (100 mg $CdCl_2$/l; from 16 weeks on) was an indication of renal damage. At the moment that proteinuria was detected, K-Cd levels were only 45.5 ± 2.8 µg/g. This proteinuria can be of tubular or tubular/glomerular origin; because morphologically no signs of glomerular damage were detected, a tubular proteinuria seems most likely. However increased levels of other biomarkers (NAG, ALP, glucose) for Cd toxicity did not appear in the urine at any time.

Because oxidative stress is believed to participate in the early processes leading to renal damage, we investigated the possibility of that in our model. In contrast to what is found in literature, we did not find indications of oxidative stress as inducer of renal damage. Increased MDA levels, an indication of lipid peroxidation, were not detected in the kidneys of mice exposed up to 100 mg $CdCl_2$/l during 23 weeks. Depletion of GSH was also not measured, although upon defensive failure, this feature is characteristic for Cd intoxication. On the other hand our study provided evidence that the kidneys were able to deal with the Cd-induced oxidative stress and adapted to this stress situation. A chronic

exposure to Cd concentrations up to 100 mg $CdCl_2$/l triggered a biphasic defence response in the kidney. A first stress response was observed after 1 week of exposure, when early antioxidants were upregulated; a second response after 23 weeks, which probably indicates adaptation to Cd-stress. It is beyond doubt that MT plays a crucial role in the detoxification against Cd and that it stays important during the whole exposure period. This was seen at the gene and protein expression level. Interesting was the observation that *Prdx2* seemed to be involved in the early Cd response, while *Nox4* was upregulated at 23 weeks. These changes in pro- and anti-oxidative systems in the cells might lead to adaptation and survival.

Besides the appearance of proteinuria, which probably is not triggered by oxidative stress, morphologically minor changes (vacuolization, increased amounts of lysosomes) were observed in kidneys of mice exposed up to 100 mg $CdCl_2$/l during 23 weeks. It was clear that the effects were restricted to the PT and that the degree of changes varied from cell tot cell. On the other hand our study demonstrated that Cd-intoxicated kidneys of mice receiving 100 mg $CdCl_2$/l showed increased immunoreactivity towards the fibrotic markers fibronectin and α-SMA. Tubular cell and nuclear hypertrophy, which are also characteristic for tubulointerstitial fibrosis, was not detected. There were also no signs of EMT: epithelial cells were not positively stained with α-SMA. Mice receiving lower Cd concentrations did not display increased immunoreactivity towards these fibrotic markers in their kidneys.

Signs of more advanced PTC damage were detected in the kidneys of mice exposed to very high (250 and 500 mg $CdCl_2$/l) Cd concentrations. Mitochondria exhibited swelling and degeneration and basolateral infoldings were lost. An increase in the amount of mitochondria (=fission) in several tubules and remodeling of cristae are early signs of apoptosis. The immunoreactivity towards the fibrotic markers was also much more pronounced compared to the reactivity in kidneys of mice exposed to 100 mg $CdCl_2$/l. Still no indications of EMT were found. All of this indicates that exposing mice to 250 and 500 mg $CdCl_2$/l elicits severe PTC damage, which will -probably- never be found in humans exposed to environmentally relevant Cd concentrations.

Future perspectives

Finally other properties and aspects of Cd toxicity might be interesting to study in our model. Preliminary experiments were already performed concerning magnetic resonance imaging (MRI) of Cd-intoxicated (fixated) kidneys (0, 100 and 500 mg $CdCl_2$/l during 16 weeks; n=2). A darker region in the corticomedullary area of kidneys of mice exposed to 500 mg $CdCl_2$/l and edema in kidneys of mice exposed to 100 (and sometimes 500) mg $CdCl_2$/l have been observed by means of MRI. An elaborate study of fixated kidneys of mice exposed to low and high Cd concentrations during several months, as well as *in vivo* imaging of Cd-intoxicated mice might lead to the development of a non-invasive method to study Cd nephrotoxicity.

Some knockouts may be very interesting to study the underlying mechanisms of Cd toxicity. For sure the megalin knockouts will be useful; megalin seems to be the most important CdMT transporter in the PTC. Also a knockout model with respect to the endo/lysosomal acidification should contribute to unravel underlying mechanisms of toxicity, because it is thought that this could be the cause of nephrotoxicity.

Another interesting approach is the proteomic analysis of urine and kidneys of Cd-intoxicated animals. Proteomics is the technique for biomarker discovery, and has become more and more important for toxicological and epidemiological studies (Nordberg, 2004). This might result in the detection of new (early) markers or give indications for studying possible mechanisms for Cd toxicity in more detail.

Concluding remarks

The study of a metal exposure on the cellular and species level is of crucial importance to understand the underlying mechanisms that result in damage. Without this knowledge it is not possible to anticipate on these threats.

When dealing with an environmental problem such as Cd contamination, it is also of crucial importance to set up a reliable and relevant study. Previously investigators were interested in eliciting and examining severe effects resulting from Cd intoxication after applying high doses. In the present study we aimed to find a balance between administering reasonably low Cd concentrations that might evoke early signs of this intoxication in our animal model. We aimed to obtain a better understanding of the early effects of Cd on the kidney and of the cellular mechanisms involved in Cd nephrotoxicity.

Samenvatting en Algemene Discussie

Samenvatting

Cadmium (Cd) is een toxisch metaal dat onder natuurlijke omstandigheden in zeer lage concentraties voorkomt in de aardkorst maar in hoge concentraties kan gevonden worden in industriële gebieden (zoals in de Noorderkempen) en landbouwgronden. Cd wordt vanuit de omgeving opgenomen via inhalatie van bijvoorbeeld sigarettenrook dat Cd-oxide (CdO) bevat. Verder wordt Cd hoofdzakelijk opgenomen door het eten van met Cd verontreinigd voedsel (voornamelijk in de vorm van Cd-chloride; $CdCl_2$). Studies zoals CadmiBel en zijn opvolger PheeCad onderzochten de gevaren van Cd-vervuiling op de mens in de Noorderkempen in België (Bernard et al., 1992; Hotz et al., 1999; Staessen et al., 1999). Ook in het buitenland werden soortgelijke studies gedaan, o.a. in Zweden (Järup et al., 1995; Järup et al., 1997; Järup et al., 2000) en in Japan, waar halverwege de 20^e eeuw Itai-itai, een ziekte veroorzaakt door zware Cd pollutie, leidde tot zware tubulaire nierdysfunctie, osteoporose, osteomalacia en een verhoogd sterftecijfer (Nomiyama en Nomiyama, 1998).

Na opname door long- of darmepitheelcellen komt Cd in het bloed terecht waar het bindt aan grote transportproteïnen zoals albumine. Deze complexen worden naar de lever getransporteerd. In de lever zal de opname van Cd leiden tot een verhoogde productie van metallothioneïne (MT), een klein eiwit met hoge affiniteit voor divalente ionen zoals Cd^{2+}, Zn^{2+} en Pb^{2+}. In de hepatocyten zal vrij Cd^{2+} complexeren met dit MT waardoor de schadelijke effecten worden afgeremd. Een voldoende productie van MT is dus noodzakelijk om de cellen te beschermen tegen Cd. Gradueel wordt het CdMT-complex vrijgelaten door de hepatocyt en via de bloedbaan naar de nieren getransporteerd. Ter hoogte van de nier zal het CdMT-complex opgenomen worden door proximale tubulaire cellen. Na afbraak van het complex in de lysosomen wordt Cd^{2+} vrijgelaten in het cytosol en zal het binden aan MT dat *de novo* gevormd wordt in de niercellen. Wanneer de CdMT influx in het lysosomale compartiment echter té hoog is en de synthese van MT ontoereikend, dan kan het vrije Cd^{2+} schade aanrichten in de cellen (Nordberg en Nordberg, 2000; Zalups and Ahmad, 2003). Dit resulteert in proximaal tubulair nierfalen wat zich uit in het

verschijnen van proteïnen, glucose, fosfaat, calcium en allerlei enzymen in de urine.

Hoewel vele onderzoekers de effecten van een chronische (lage) Cd blootstelling op de nieren probeerden te ontrafelen, is het nog altijd onduidelijk op welke manier Cd cellulaire stress responsen veroorzaakt bij de gewone bevolking die in gecontamineerde en niet-gecontamineerde gebieden woont.

De studie in deze thesis beschrijft de effecten op de nieren van muizen die gedurende een lange tijd blootgesteld werden aan lage Cd concentraties. De meeste aandacht ging naar de proximale tubulaire cellen (PTC). We brachten niet enkel een dosis-respons relatie in beeld tussen de gebruikte Cd concentraties en de effecten ter hoogte van de nier, maar we bestudeerden ook de effecten op histologisch vlak. Verder gingen we na of oxidatieve stress een rol speelt in vroege proximaal tubulaire nierschade geïnduceerd door Cd.

In ***hoofdstuk 2*** wordt de set-up van de blootstellingsstudie beschreven. Dieren werden blootgesteld aan Cd concentraties tot 100 mg $CdCl_2$/l gedurende maximaal 23 weken. De resultaten met betrekking tot het gewicht van de muizen, water- en voedselconsumptie bewezen dat de dieren geen grote hinder ondervonden en dat onze blootstellingscondities geschikt waren om de effecten van een chronische blootstelling aan tamelijk lage Cd concentraties te bestuderen. Proteïnurie werd gedetecteerd in muizen blootgesteld aan 100 mg $CdCl_2$/l vanaf 16 weken. Cd concentraties in de nier bleken een goede indicator van een chronische blootstelling terwijl bloed Cd waarden enkel kunnen gebruikt worden als indicator bij een actuele blootstelling. De Cd waarden gemeten in urine waren niet gecorreleerd met de Cd waarden in de nier.

In ***hoofdstuk 3*** wordt het niveau van oxidatieve stress in de nieren van muizen blootgesteld aan maximaal 100 mg $CdCl_2$/l beschreven. Genexpressie studies van pro- en antioxidanten en kwantificatie van GSH, AsA en MDA werden uitgevoerd. Onze studie toonde aan dat een blootstelling aan lage Cd concentraties veranderingen in de anti- en pro-oxidatieve systemen in de cel veroorzaakt, wat uiteindelijk kan leiden tot adaptatie en overleving. Een

bifasische stress respons werd gemeten. Een belangrijke rol blijkt ook weggelegd voor *Prdx2* en *Nox4*, die totnogtoe niet bestudeerd werden in een chronisch blootstellingsexperiment aan relatief lage Cd concentraties.

In ***hoofdstuk 4*** wordt de morfologie van de nieren en meer specifiek van de proximale tubulaire cellen beschreven van muizen die chronisch blootgesteld werden. Aan de hand van preliminaire studies van de nieren van muizen blootgesteld aan maximaal 100 mg $CdCl_2$/l konden we afleiden dat enkel kleine veranderingen zich voordeden; daarom werd dit morfologisch gedeelte uitgebreid met een blootstellingsexperiment gedurende 16 weken aan zeer hoge (250 en 500 mg $CdCl_2$/l) Cd concentraties. De nieren van muizen blootgesteld aan deze hoge concentraties, vertoonden schade aan mitochondriën en basolaterale invaginaties.

In ***hoofdstuk 5*** worden de immunohistochemische observaties besproken voor wat betreft MT en de fibrotische merkers fibronectine, collageen I en α-SMA, en het effect van Cd-blootstelling op hun expressie. De expressie van MT, een zwaar metaal- en radicaalvanger, was dosis- en tijdsafhankelijk. Cd-geïntoxiceerde nieren van muizen die blootgesteld werden aan 100 mg $CdCl_2$/l of meer vertoonden stijgende immunoreactiviteit voor wat betreft α-SMA en de extracellulaire matrix merker fibronectine.

Algemene discussie

In onze geïndustrialiseerde samenleving worden zeer snel nieuwe technologieën ontwikkeld. De toenemende productie van goederen, de productieprocessen en de stijgende consumptie zijn een bedreiging voor onze omgeving en onze samenleving. Vervuilende stoffen kunnen onze gezondheid schaden. Verder kunnen ze interfereren met belangrijke lichaamsprocessen, het functioneren van organen beïnvloeden en ernstige ziektes of zelfs de dood tot gevolg hebben.

Cadmium, dat gecommercialiseerd werd in de 20^{e} eeuw door het gebruik in de industrie, vormt een substantiële bedreiging voor onze gezondheid, samen met andere toxische elementen zoals lood, kwik en arseen (Järup, 2003; WHO, 2000). Bovendien hebben verscheidene populatiestudies in België, Zweden en Japan aangetoond dat naast de occupationeel blootgestelde arbeiders ook individuen die via hun omgeving blootgesteld worden, matige tot ernstige gezondheidsproblemen kunnen ondervinden door Cd. De gevolgen van een blootstelling aan Cd kunnen ernstig zijn: tubulaire nierschade, osteoporose (Äkesson et al., 2006; Järup, 2002; Nordberg, 2004), leverdysfunctie (El-Demerdash et al., 2004; WHO, 2000) en longkanker (Nawrot et al., 2006; Verougstraete et al., 2003). Het meeste geviseerde orgaan in een chronische Cd blootstelling is de nier. Daarom is het belangrijk om de mechanismen te begrijpen die leiden tot Cd nefrotoxiciteit. Met behulp van een dierenmodel, dat chronisch aan Cd blootgesteld wordt, kan dit gebeuren. Een overzicht van de resultaten is gegeven in tabel 6.2.

Tabel 6.2 *Schematisch overzicht van de belangrijkste resultaten in deze thesis*

		Exposure time (weeks)				
Cd conc (mg $CdCl_2$/l)	Experiment	1	4	8	16	23
0						
10	Gen expr.	↓ *Hmox1* ↙ *Bcl2*		↑ *Mt1* ↗ *Mt2*		↑ *Mt1-Mt2-Gpx4-Nox4* ↓ *Prdx2*
	IHC				↑ MT	↑ MT
	Morf.				lys-vac	lys-vac
100	Gen expr.	↑ *Mt1-Mt2* ↙ *Bcl2* ↗ *Prdx2*		↑ *Mt1-Mt2* ↓ *Sod1-Bcl2* ↙ *Prdx2*		↑ *Mt1-Mt2-Gpx4-Hmox1-Nox4-Cat* ↙ *Prdx2*
	IHC		↑ MT		↑ MT-fibr- α-SMA	↑ MT-fibr- α-SMA
	Morf.				lys-vac ↓ Fe partikels proteinurie	lys-vac proteinurie
250	IHC				↑ MT-fibr- α-SMA ↗ Coll I	
	Morf.				lys-vac- mitoch. + basol. schade-edema	
500	IHC				↑ MT-fibr- α-SMA ↗ Coll I	
	Morf.				lys-vac- mitoch. + basol. schade-edema ↓ Fe partikels	

Gen expr. = gen expressie; IHC = immunohistochemie; Morf. = morfologische veranderingen; fibr. = fibronectine; coll I = collageen I; lys = verhoging van het aantal lysosomen; vac = stijgende vacuolisatiegraad; mitoch. + basol. schade = mitochondriale en basolaterale schade; ↑ stijging (in expressieniveau); ↓ daling; ↗ kleine stijging (randgeval van significantie); ↙ kleine daling (randgeval van significantie). Sod2 expressieniveaus bleven stabiel, MDA, GSA, GSSG en DHA niveaus verschilden niet en AsA waarden daalden concentratie-onafhankelijk. U-Cd waarden kunnen als 'realistisch' beschouwd worden.

In deze thesis hebben we het experimenteel opzet van een Cd blootstelling in muizen beschreven. Het gebruik van muizen als proefdiermodel voor Cd-intoxicatie is betrekkelijk nieuw, daar voorgaande studies zich meestal toespitsten op ratten. Het grote voordeel van een muizenmodel, met name de mogelijkheid om met knock-outs te werken, was het doorslaggevende argument om voor dit model te kiezen. Verschillende aspecten van Cd intoxicatie in de nieren werden in dit project belicht. De Cd waarden die gemeten werden in urine, zijn realistisch (in vergelijking met de humane situatie); Cd waarden in het bloed die lager zijn dan 10 µg Cd/l, zijn dat ook. Verder bevestigde ons model de stelling dat enkel de Cd concentratie in de nieren een betrouwbare merker is voor een chronische blootstelling, terwijl Cd-waarden in het bloed een indicatie geven over een actuele blootstelling. Hoewel vele studies de Cd-waarden in urine gebruiken als een indicator van de totale lichaamslast, kon dit niet bevestigd worden in onze studie. De relatief korte blootstellingsperiode kan een verklaring hiervoor zijn. Wanneer men aan lage Cd concentraties blootgesteld wordt, duurt het een tijdje vooraleer de urinaire Cd waarden (lineair) stijgen. Deze bevindingen tonen aan dat ons model een goed model is om de mechanismen met betrekking tot een chronische blootstelling aan (relatief) lage Cd concentraties te bestuderen.

Het verschijnen van eiwitten in de urine van muizen die blootgesteld waren aan 100 mg $CdCl_2$/l na 16 weken was een indicatie van renale schade op functioneel niveau. De Cd-concentratie in de niercortex die op dat moment gemeten werd was 45.5 ± 2.8 µg/g. Deze proteïnurie kan van tubulaire of glomerulaire/tubulaire origine zijn; aangezien op morfologisch vlak geen tekenen van glomerulaire schade gedetecteerd werden, is tubulaire proteïnurie het meest waarschijnlijk. Een verhoging van andere biomerkers (NAG, ALP, glucose) voor Cd toxiciteit werden evenwel niet in de urine gedetecteerd.

Aangezien men veronderstelt dat oxidatieve stress een sleutelrol heeft met betrekking tot vroege proximale tubulaire nierschade, onderzochten we deze mogelijkheid ook in ons model. Ondanks zulke bevindingen in voorgaande studies, waren er geen indicaties van oxidatieve stress als aanzet voor renale schade (proteïnurie). Hogere MDA niveaus, een indicator van lipidenperoxidatie,

werden niet gedetecteerd in de nieren van muizen blootgesteld tot 100 mg $CdCl_2$/l gedurende 23 weken. GSH-depletie werd ook niet gemeten, alhoewel dit zich zou manifesteren wanneer de defensieve respons onvoldoende is om de Cd-stress op te vangen. Onze studie toonde wél aan dat de nieren in staat zijn om met de Cd-geïnduceerde stress om te gaan en dat zij zich aanpassen aan deze stress situatie. Een chronische blootstelling aan Cd concentraties tot 100 mg $CdCl_2$/l veroorzaakte een bifasische defensieve respons in de nier. Na 1 week blootstelling was een eerste stress respons meetbaar; de tweede na 23 weken, wat doet vermoeden dat de nieren in staat zijn zich aan te passen aan Cd-stress. Verder speelt MT zonder twijfel een cruciale rol in de detoxificatie van Cd en het blijft belangrijk gedurende de hele blootstellingsperiode. Dit was op het niveau van gen- en eiwitexpressie merkbaar. Verder bleek *Prdx2* betrokken bij de vroege Cd respons, terwijl *Nox4* opgereguleerd werd na 23 weken. Deze veranderingen in pro- en antioxidatieve systemen in de cel kunnen leiden tot adaptatie en overleving.

Naast de gemeten proteïnurie, die waarschijnlijk niet veroorzaakt is door oxidatieve stress, werden op morfologisch vlak enkele kleine veranderingen (vacuolisatie, meer lysosomen) geobserveerd in nieren van muizen blootgesteld aan maximaal 100 mg $CdCl_2$/l gedurende 23 weken. Het was overduidelijk dat de veranderingen zich beperkten tot de proximale tubuli. Verder konden de veranderingen sterk variëren van cel tot cel, van tubulus tot tubulus en van dier tot dier. Aan de andere kant toonde onze studie aan dat de nieren van muizen blootgesteld aan 100 mg $CdCl_2$/l verhoogde immunoreactiviteit vertonen voor wat betreft de fibrose-merkers fibronectine en α-SMA. Tubulaire cel en nucleaire hypertrofie, welke ook karakteristiek zijn voor tubulointerstitiële fibrose, werden evenwel niet gedetecteerd. Verder waren er ook geen tekenen van epitheliaal-naar-mesenchymale transitie: de epitheelcellen kleurden niet positief voor α-SMA. De muizen die lagere Cd concentraties toegediend kregen, vertoonden geen verhoogde immunoreactiviteit voor deze merkers in hun nieren.

Tekenen van gevorderde proximaal tubulaire celschade kon worden geobserveerd wanneer muizen werden blootgesteld aan zeer hoge (250 en 500 mg $CdCl_2$/l) Cd concentraties. Mitochondriën vertoonden zwelling en degeneratie

en basolaterale invaginaties gingen verloren. In sommige tubuli waren veel meer mitochondriën te zien (= fissie), wat een vroege indicatie kan zijn van celdood (apoptose). Zwelling in het cytosol was ook geregeld merkbaar. De immunoreactiviteit voor de fibrose- merkers was meer uitgesproken in vergelijking met de reactiviteit in de nieren van muizen blootgesteld aan 100 mg $CdCl_2$/l. Dit toont aan dat een blootstelling van muizen aan 250 en 500 mg $CdCl_2$/l substantiële nierschade veroorzaakt, die (waarschijnlijk) nooit zal gevonden worden in de humane situatie.

Toekomstperspectieven

Er zijn nog andere aspecten van Cd toxiciteit die interessant kunnen zijn om in ons model te onderzoeken. We hebben reeds preliminaire experimenten uitgevoerd met betrekking tot magnetische resonantie-beeldvorming (MRI) van Cd-geïntoxiceerde (gefixeerde) nieren (0, 100 en 500 mg $CDCl_2$/l gedurende 16 weken; n=2). Een donkere band in de corticomedullaire regio van nieren van muizen blootgesteld aan 500 mg $CdCl_2$/l en oedeem in de nieren van muizen blootgesteld aan 100 (en soms 500) mg $CdCl_2$/l werden gedetecteerd met behulp van MRI. Een uitgebreide studie met gefixeerde nieren van muizen die blootgesteld werden aan lage en hoge Cd concentraties gedurende verschillende maanden, en *in vivo* beeldvorming van Cd-geïntoxiceerde muizen zouden kunnen leiden tot de ontwikkeling van een niet-invasieve studiemethode met betrekking tot Cd nefrotoxiciteit.

Sommige knockout muismodellen zijn ook erg interessant om de onderliggende mechanismen van Cd toxiciteit te onderzoeken. In de eerste plaats denk ik dan aan de megaline knockouts, aangezien megaline de belangrijkste CdMT transporter is in de PTC. Een knockout model met betrekking tot de endo/lysosomale acidificatie of transport lijkt ook erg interessant aangezien een defect ter hoogte van de endo/lysosoom trafficking of verzuring aan de basis kan liggen van de nefrotoxiciteit.

Een andere interessante benadering is de proteoomanalyse van urine en nieren van met Cd geïntoxiceerde dieren. Proteomica is dé techniek om biomerkers te ontdekken, en is steeds belangrijker geworden in toxicologische en epidemiologische studies (Nordberg, 2004). Dit kan resulteren in de detectie van (nieuwe) biomerkers of het kan indicaties geven om mogelijke mechanismen van Cd toxiciteit in meer detail te onderzoeken.

Eindconclusie

Een blootstellingsstudie van zware metalen op cellulair en species niveau is van cruciaal belang als men de onderliggende mechanismen wil begrijpen die leiden tot schade. Zonder deze kennis is het niet mogelijk in te spelen op deze dreigingen.

Wanneer men met een milieuprobleem zoals Cd vervuiling te maken heeft, is het van belang om een betrouwbare en relevante studie te ontwikkelen. Voordien waren onderzoekers vooral geïnteresseerd om ernstige effecten van Cd-intoxicatie te onderzoeken. Hiervoor werden hoge Cd dosissen toegediend. In onze studie wilden we een balans vinden tussen het gebruik van tamelijk lage Cd concentraties waarbij vroege tekenen van deze intoxicatie in ons dierenmodel konden geobserveerd worden. We wilden een beter inzicht krijgen in de vroege effecten van Cd op de nier en in de cellulaire mechanismen met betrekking tot Cd nefrotoxiciteit.

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Curriculum Vitae

Curriculum Vitae

Sandy Thijssen was born on the 24th of September 1980 in Bilzen (Belgium). In 1998 she finished her secondary grade education (Latin-Sciences-Mathematics) at the Heilig Graf-instituut in Bilzen. Between 1998 and 2000 she studied at the Faculty of Biology at Hasselt University (the former Limburgs Universitair Centrum) in Diepenbeek. Between 2000 and 2002 she studied Biotechnology (Faculty of Sciences) in Gent. In July 2002 she graduated as Master (Licentiaat) in Biotechnology. In October 2002 she joined the group of Physiology of the Centre for Environmental Sciences at the University of Hasselt and transnationale Universiteit Limburg (tUL). During this period, the doctoral training program in Biomedical Sciences of the University of Hasselt was attended and successfully finished in 2006.

Publications in International Journals

Thijssen S., Maringwa J., Faes C., Lambrichts I., and Van Kerkhove E. (2007). *Chronic exposure of mice to environmentally relevant, low doses of cadmium leads to early renal damage, not predicted by blood or urine cadmium levels.* Toxicology 229: 145-56.

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Oral Presentations

Thijssen S., Maringwa J., Lambrichts I., Steels P., Van Kerkhove E. *Environmentally relevant doses of cadmium induce renal damage in mice.* Belgisch genootschap voor fundamentele en klinische fysiologie en farmacologie, 19th of November 2005, Antwerpen, Belgium

Thijssen S., Maringwa J., Van Kerkhove E. *The defence status of murine kidneys exposed to low concentrations of cadmium: from metal scavenging proteins to antioxidant detoxification.* Bullet Session, Belgische Vereniging voor Nefrologie (BNV/SBN), 16th of March 2006, Gent, Belgium.

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Poster Presentations

Thijssen S., Maringwa J., Lambrichts I., Steels P., Van Kerkhove E. *Chronic exposure of mice to environmentally relevant doses of cadmium induces early kidney damage.*
- ASN Renal Week, 15th of November 2005, Philadelphia, USA.
- Dag van het Onderzoek, 23rd of November 2006, Hasselt University, Belgium

Thijssen S., Maringwa J., Van Kerkhove E. *The defence status of murine kidneys exposed to low concentrations of cadmium: from metal scavenging proteins to antioxidant detoxification.*
- Belgische Vereniging voor Nefrologie (BNV/SBN), 16th of March 2006, Gent, Belgium.

Thijssen S., Maringwa J., Van Kerkhove E. *The defence status of murine kidneys exposed to low concentrations of cadmium: from metal scavenging proteins to antioxidant detoxification.*
- ERA/EDTA, XLIII Congres, July 15-18, 2006, Glasgow, Scotland.
- Dag van het Onderzoek, 23rd of November 2006, Hasselt University, Belgium

Awards

Award for the best poster presentation.
Thijssen S., Maringwa J., Van Kerkhove E. *The defence status of murine kidneys exposed to low concentrations of cadmium: from metal scavenging proteins to antioxidant detoxification.*
Belgische Vereniging voor Nefrologie (BNV/SBN), 16th of March 2006, Gent, Belgium.

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Science is organized knowledge.
Wisdom is organized life.
-**Immanuel Kant**-

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Beste Ivo, een dierenmodel gebruiken voor Cd-intoxicatie, is natuurlijk ook heel interessant op morfologisch gebied. Zodoende kwam ik dus al snel bij u terecht. Mijn kennis inzake histologie was zeer klein, en dus was het, zeker in het begin, niet evident om die histologische coupes -met een kritisch oog- te bekijken. Bedankt om mij op weg te helpen inzake elektronen- en lichtmicroscopie, en nog belangrijker, om mij een weg te banen in zoiets ingewikkeld als de nier.

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