GLUTATHIONE
Synthesis during development and metabolism in experimental hypertension

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Academic Dissertation

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their roman numerals:

I Levonen A-L, Lapatto R, Saksela M, Raivio KO. The expression of gamma-glutamylcysteine synthetase during development. *Pediatr. Res.* 47:266-270, 2000.

II Levonen A-L, Lapatto R, Saksela M, Raivio KO. Human cystathionine gamma-lyase: developmental and in vitro expression of two isoforms. *Biochem. J.* 347:291-295, 2000.

III Levonen A-L, Laakso J, Vaskonen T, Mervaala E, Karppanen H, Lapatto R. Down-regulation of renal glutathione synthesis by systemic nitric oxide synthesis inhibition in spontaneously hypertensive rats. *Biochem. Pharmacol.* 59:441-443, 2000.

IV Levonen A-L, Laakso J, Mervaala E, Karppanen H, Lapatto, R. Glutathione metabolism in spontaneously hypertensive rats. (submitted)

In addition, some previously unpublished results are presented.

LIST OF NONSTANDARD ABBREVIATIONS

ARDS	acute respiratory distress syndrome
BSO	buthionine sulfoximine
CGL	cystathionine γ-lyase
CGL-L	cystathionine γ-lyase, long form
CGL-S	cystathionine γ-lyase, short form
cGPx	cytosolic glutathione peroxidase
CLD	chronic lung disease
CuZnSOD	copper-zinc superoxide dismutase
EC-SOD	extracellular superoxide dismutase
eNOS	endothelial nitric oxide synthase
G6PDH	glucose-6-phosphate dehydrogenase
GCS	γ-glutamylcysteine synthetase
γ-GT	γ-glutamyl transpeptidase
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSNO	S-nitrosoglutathione
GSSG	oxidized glutathione
H_2O_2	hydrogen peroxide
iNOS	inducible nitric oxide synthase
MnSOD	manganese superoxide dismutase
MTF-1	metal-regulatory transcription factor 1
NAC	N-acetylcysteine
$NO^{\bullet}$	nitric oxide
NOS	nitric oxide synthase
$O_2^{\bullet -}$	superoxide
$OH^{\bullet}$	hydroxyl radical
$ONOO^{-}$	peroxynitrite
OTC	L-2-oxothiatzolidine-4-carboxylate
RDS	respiratory distress syndrome
ROS	reactive oxygen species
RNS	reactive nitrogen species
SOD	superoxide dismutase
SHR	spontaneously hypertensive rat
WKY	Wistar-Kyoto rat

SUMMARY

Reactive oxygen species (ROS) have been implicated in many pathological situations, from complications of oxygen therapy in preterm infants to diseases in adult medicine, including hypertension. Glutathione (GSH) is one of the most important endogenous antioxidants in the cell. Its synthesis is dependent on the activity of the rate-limiting enzyme, γ-glutamylcysteine synthetase (GCS), and the availability of the substrate, cysteine, which is either derived from the diet or protein catabolism, or synthesized from methionine in the liver by the transsulfuration pathway.

The present study was designed to investigate the ability of the premature neonate to synthesize glutathione, and to generate cysteine through the transsulfuration pathway. To this end, the expression of GCS in human tissues relevant to neonatal oxidative injury as well as the hepatic expression of two forms of cystathionine γ-lyase (CGL), the last enzyme of the transsulfuration pathway, were studied during development. Moreover, the ability of the two mRNA isoforms to code for active enzyme was assessed. The role of glutathione in hypertension was studied in a genetic animal model of hypertension, the spontaneously hypertensive rat (SHR).

The mRNA expression and enzyme activity of GCS were studied in lung, liver and kidney tissue, and CGL in liver tissue derived from abortions, neonatal autopsies or transplantations. The ability of the two CGL mRNA forms to code for active enzyme was investigated by transfecting the corresponding cDNAs into eukaryotic cells. The activity of GCS and three other enzymes involved in GSH metabolism, glutathione peroxidase (GPx), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH) were measured in the kidney, myocardium and liver of SHR and its normotensive control strain, Wistar-Kyoto (WKY).

GCS was expressed and active in all tissues studied from early 2nd trimester. Strong mRNA expression of both CGL isoforms was detected from the 19th gestational week onwards, while enzyme activity was detected only in adult liver. Only the longer CGL isoform was enzymatically active in transfected cells. In the kidney of SHR, nitric oxide synthase (NOS) inhibition caused a decrease in GCS activity, which was further augmented by a high sodium intake.

In conclusion, GCS appears to be fully functional from early stages of development in tissues studied implying that GCS does not limit GSH synthesis in preterm neonates. However, as CGL activity is absent from fetal and neonatal liver, insufficient synthesis of cysteine may account for low GSH levels in premature infants. In an experimental animal model of hypertension, NOS inhibition down-regulates GSH synthesis, which may aggravate hypertensive kidney injury.

INTRODUCTION

Reactive oxygen and nitrogen species (ROS and RNS, respectively) are implicated in many pathological situations. These range from clinical complications of premature infants related to oxygen therapy, such as chronic lung disease, to problems in adult medicine, such as cardiovascular diseases. While ROS and RNS are produced even in normal cellular metabolism, their production in pathophysiological situations exceeds the capacity of the cell to provide defense against their damaging effects. Thus the balance between ROS production and the antioxidant defense is critical in determining the extent of the damage caused by these highly reactive molecules.

Glutathione (γ-glutamylcysteinylglycine, GSH) is one of the most important antioxidants present in the cell. It has a number of other functions, such as detoxification of xenobiotics, regulation of the redox-state of sulfhydryl groups of proteins, as well as participation in DNA, protein, and leukotriene synthesis. Cells synthesize GSH by two ATP-dependent enzymes, γ-glutamylcysteine synthetase (GCS), and glutathione synthetase. GCS is the rate-limiting enzyme and it is feed-back inhibited by GSH. GCS is also regulated by ROS and RNS. The rate of GSH synthesis may also be limited by the availability of one of its three precursor amino acids, cysteine, which is either provided in the diet or synthesized in the liver from methionine in a metabolic pathway called transsulfuration.

This series of studies were undertaken to elucidate the role of the enzymes involved in glutathione synthesis during human development and in experimental hypertension.

REVIEW OF THE LITERATURE

Reactive oxygen and nitrogen species

Reactive oxygen and nitrogen species (ROS and RNS, respectively) include a number of highly reactive molecules (Table 1). Some of these species are defined as free radicals, any atom or molecule having an unpaired electron in its outer orbit (Halliwell and Gutteridge 1989). Others, however, are not radicals but nevertheless are active metabolites of oxygen and nitrogen. Examples of such molecules are hydrogen peroxide (H_2O_2), which reacts with divalent cations in the Fenton reaction, thereby forming the very reactive hydroxyl radical ($OH^{\cdot}$) (Halliwell and Gutteridge 1989), and peroxynitrite ($ONOO^-$), formed by the reaction of superoxide ($O_2^{\cdot -}$) and nitric oxide ($NO^{\cdot}$).

Production

ROS are generated in the normal aerobic metabolism of cells in a variety of reactions (Table 2). The main source of ROS is mitochondria, where oxygen is reduced to water in the electron transport chain (Halliwell and Gutteridge 1989). About 1% of the oxygen reduced by mitochondria is converted to $O_2^{\cdot -}$ (Turrens 1997), at the level of NADH dehydrogenase (Turrens and Boveris 1980) or coenzyme Q (Cadenas et al. 1977). Mitochondrial $O_2^{\cdot -}$ can be further dismutated to H_2O_2 (Fridovich and Freeman 1986). With regard to hyperoxic lung injury in neonates, it is of interest to note that mitochondrial production of ROS is increased in hyperoxia (Freeman and Crapo 1981).

Table 1. *Reactive oxygen and nitrogen species*

Reactive oxygen species (ROS)		Reactive nitrogen species (RNS)	
$O_2^{\cdot -}$	superoxide	$NO^{\cdot}$	nitric oxide
$OH^{\cdot}$	hydroxyl	NO_2	nitrogen dioxide
$LOO^{\cdot}$	lipid peroxyl	N_2O_3	dinitrogen trioxide
$LO^{\cdot}$	lipid alkoxyl	$ONOO^-$	peroxynitrite
H_2O_2	hydrogen peroxide	ONOOH	peroxynitrous acid
HOCl	hypochlorous acid		

Table 2. *Cellular sources of ROS*

Source	Localization
Electron transport system	Mitochondria
NADPH oxidase Cyclo-oxygenases Lipoxygenases	Plasma membrane
Xanthine oxidase Catecholamines Riboflavin Transition metals ($Fe^{2+/3+}$, $Cu^{1+/2+}$)	Cytosol
Oxidases	Peroxisome
Nuclear membrane electron transport (cytochromes P-450, and b_5)	Endoplasmic reticulum

Besides mitochondria, important cellular sites of ROS generation relevant to the present study are membrane-bound NAD(P)H oxidases. NAD(P)H oxidases generate large amounts of $O_2^{\cdot -}$ in activated inflammatory cells (Rossi 1986), but in vascular cells the enzyme responsible for NADPH oxidase-like activity appears to be different and produces $O_2^{\cdot -}$ in much smaller quantities (Mohazzab et al. 1994). While the role of different ROS-generating systems in the vasculature is far from clear, evidence suggests that NADPH oxidase-like activity is a contributing source of ROS in endothelial cells (Mohazzab et al. 1994), vascular smooth muscle cells (Griendling et al. 1994), and intact aortas (Rajagopalan et al. 1996) in response to a variety of stimuli such as angiotensin II and cytokines. This may have implications in hypertension, as angiotensin II is a potent vasoconstrictor and some forms of hypertension are associated with elevated levels of this peptide.

Nitric oxide is synthesized from L-arginine in a reaction catalyzed by the enzyme nitric oxide synthase (NOS), which utilizes molecular oxygen and NADPH as co-substrates (Griffith and Stuehr 1995). There are three isoforms of this enzyme, type I (neuronal nitric oxide synthase, nNOS), type II (inducible nitric oxide synthase, iNOS), and type III (endothelial nitric oxide synthase, eNOS). Type I and III are referred to as the constitutive form (cNOS). These isoforms are continuously present in the cell and they can be activated by Ca^{2+}. NOS I and NOS III generate low fluxes of $NO^{\bullet}$, whereas the Ca^{2+} independent iNOS, originally discovered in cytokine-induced macrophages, is a high-output enzyme induced by cytokines, LPS and other bacterial products. Although termed

as constitutive, NOS I and III are also subject to regulation. Expression is enhanced by, for example, estrogens (NOS I and III), shear stress, TGF-β1, and high glucose (NOS III) (Forstermann et al. 1998). Interestingly, under certain circumstances NOS I (Pou et al. 1992) and NOS III (Vasquez-Vivar et al. 1998) can generate $O_2^{\cdot -}$, which may have important implications in vascular diseases characterized by endothelial dysfunction, such as hypertension.

Cellular effects of ROS and RNS

ROS and RNS may cause peroxidation of lipids, denaturation of proteins and damage to nuclear acids (Figure 1). Thus almost all cellular components can be injured by them (Freeman and Crapo 1982). In cellular membranes, ROS cause peroxidation of polyunsaturated fatty acids. This reaction is initiated by abstraction of a hydrogen atom from the lipid molecule and is followed by autocatalytic propagation, in which lipid hydroperoxides and lipid radicals are formed. This cascade can be terminated by the reaction of two peroxyl radicals or the reaction of a peroxyl radical with an antioxidant molecule. $NO^{\cdot}$ can also terminate a lipid peroxidation process by reacting with a peroxyl radical (Wink and Mitchell 1998). However, in the presence of superoxide, nitric oxide forms peroxynitrite, a powerful oxidant capable of initiating lipid peroxidation (Radi et al. 1991, Hogg and Kalyanaraman 1999).

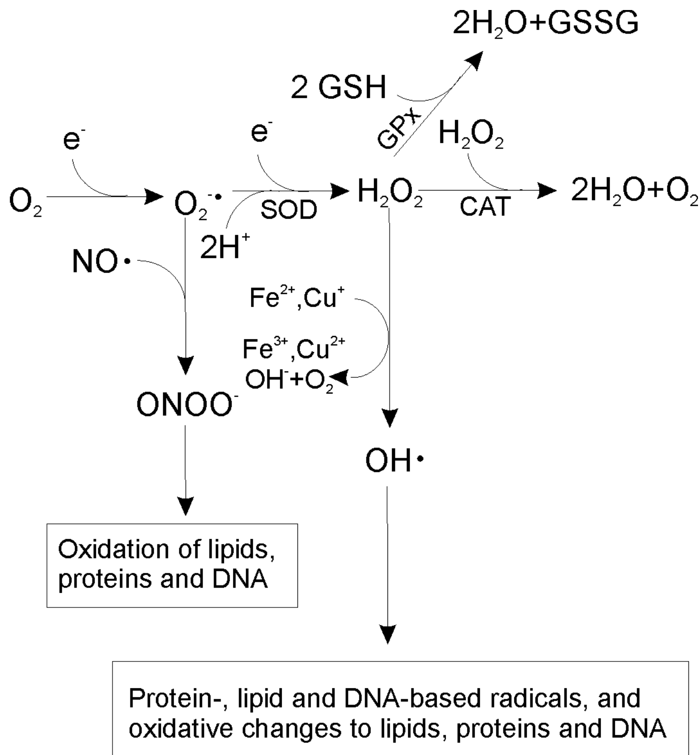

Figure 1. *Sources of reactive oxygen species and antioxidant pathways*

Proteins can undergo numerous covalent changes upon exposure to oxidants (Dean et al. 1997). For example, radical-mediated oxidation induces the formation of amino acyl carbonyl groups (Stadtman 1990). The sulfhydryl moiety of cysteine is highly prone to oxidative attack leading to formation of disulfide bonds. Peroxynitrate causes oxidative modification of a number of amino acid residues (Ischiropoulos and al-Mehdi 1995). These reactions lead to protein inactivation and, ultimately, to proteolytic degradation. However, some modifications, such as thiol-disulfide exchange or tyrosine nitration, are fairly specific and these are postulated to be regulatory (Gilbert 1984, Ischiropoulos 1998).

In DNA, ROS and RNS can cause structural alterations in DNA such as base pair mutations, rearrangements, deletions, insertions and sequence amplification (Wiseman and Halliwell 1996). $OH^{\bullet}$ is especially damaging, modifying purine and pyrimidine bases of DNA as well as the sugar backbones of DNA.

ROS and RNS are not only deleterious, but they can serve as signaling molecules during normal cellular metabolism (Finkel 1998). ROS have been shown to affect multiple cell signaling pathways such as protein kinase C, signal transducer and activator of transcription (STAT) factors and mitogen activated protein (MAP) kinases, which link extracellular stimuli to cellular responses such as cell proliferation, differentiation, and cell death (Suzuki et al. 1997, Griendling and Harrison 1999). Several transcription factors, including AP-1, AP-2 and NF-κB, are redox-regulated (Dalton et al. 1999). Moreover, the activity of metabolic enzymes may be regulated by the oxidation state of critical thiol groups (Gilbert 1984). $NO^{\bullet}$ exhibits a number of regulatory properties ranging from the regulation of the mitochondrial respiration through inhibition of cytochrome c oxidase (Torres et al. 1995), to the cGMP-dependent vasorelaxation, the first $NO^{\bullet}$-dependent signaling event discovered (Ignarro et al. 1987). Thus ROS and RNS work as signaling molecules in multiple cellular sites and metabolic events, and the physiological role of oxidants in cell regulation and their mechanisms of action are only emerging.

Defense against ROS and RNS

Antioxidant enzymes

Superoxide dismutases (SODs), first described by McCord and Fridovich (1969), are metalloproteins that dispose effectively and specifically of superoxide anions (Figure 1). These enzymes facilitate the dismutation of two otherwise repulsive $O_2^{\cdot -}$ radicals, which join to form hydrogen peroxide (Fridovich 1978). In eukaryotes, three different forms of SODs have been characterized: a copper and zinc containing form (CuZn-SOD) that is localized in the cytosol, a manganese containing form (MnSOD) in the mitochondria (Slot et al. 1986) and a copper- and zinc-containing extracellular form (ECSOD) in the extracellular matrix (Marklund 1984).

Catalase, localized mainly in peroxisomes in the cell, enhance the reaction by which two hydrogen peroxide molecules decompose to water and oxygen (Figure 1) (Fridovich 1978). The enzyme does not metabolize alkyl hydroperoxides (Chance et al. 1979). Its high K_m value for H_2O_2 and compartmentalization to peroxisomes implies that its role is mainly to protect the cell against H_2O_2 produced in these organelles. The role in H_2O_2 catabolism at low rates of H_2O_2 production is probably of minor importance (Jones et al. 1981).

The glutathione redox cycle is a central mechanism in scavenging alkyl hydroperoxides, but it is also active in metabolizing H_2O_2 (Chance et al. 1979). The enzymes in the cycle include glutathione peroxidase (GPx) and glutathione reductase (GR). Because GPx has a lower K_m value for H_2O_2 than catalase, it is considered more important when low levels of H_2O_2 are produced.

Recently, a peroxiredoxin (Prx) family of proteins has been shown to possess peroxidase-like activity (Kang et al. 1998). These enzymes do not use glutathione as a cofactor but rely on thioredoxin as a source of reducing equivalents for the reduction of H_2O_2. The biological importance of Prx in H_2O_2 disposal remains to be elucidated.

Scavengers and antioxidant molecules

There are a number of molecules capable of inhibiting oxidation reactions due to ROS either by preventing their formation or by scavenging them, thus inhibiting the chain-reaction. Metal chelating agents, such as desferrioxamine, or proteins, such as transferrin, ferritin, metallothionein and ceruloplasmin, sequester metal ions and thus prevent the formation of noxious oxidants by the Fenton reaction (Halliwell and Gutteridge 1989). Many antioxidants are low molecular weight compounds that are either synthesized by the cells or derived from the diet. An important lipid-soluble antioxidant is α-tocopherol (vitamin E), which acts in cellular membranes as a chain-breaking antioxidant of lipid peroxidation by donating hydrogen to peroxyl radicals. α-tocopherol works in concert with water-soluble ascorbate (vitamin C), which re-reduces the tocopheroxyl radical

(Buettner 1993). Ascorbate also scavenges effectively superoxide, singlet oxygen and hydroxyl radicals. In addition, it can spare intracellular GSH (Meister 1994). Other endogenous small molecular weight antioxidants include uric acid, which is synthesized by xanthine dehydrogenase and bilirubin, produced by heme oxygenase (Halliwell and Gutteridge 1989).

Glutathione

Glutathione (L-γ-glutamyl-L-cysteinylglycine) (Figure 2) is a tripeptide present in virtually all animal cells (Meister and Anderson 1983). It is usually the most abundant intracellular small molecular weight thiol, present in the millimolar range in mammalian cells. The peptidic γ-linkage between glutamic acid and cysteine is thought to protect the tripitide from degradation by aminopeptidases. Glutathione is also less prone to oxidation than cysteine, making it an ideal compound for maintaining intracellular redox potential. Glutathione exists either in reduced (thiol, GSH) or oxidized (disulfide, GSSG) form (Figure 2). GSH is the predominant form, and GSSG content is usually less than 1% of GSH. In the cell, almost 90% of glutathione is in the cytosol, 10% in the mitochondria and a small percentage in the endoplasmic reticulum and in the nucleus (Meister 1991).

Mitochondria appear to have a distinct pool of GSH that is resistant to GSH depletion (Meister 1991). Mitochondria do not synthesize GSH themselves but import it through an as yet unidentified ATP-dependent mechanism (Fernandez-Checa et al. 1998). As mitochondria do not contain catalase, GSH-dependent reactions are thought to be the main mechanism by which mitochondria dispose of hydrogen peroxide (Meister 1991), though the recent characterization of the thioredoxin-redox system implies that these could be involved as well (Pedrajas et al. 1999). Hydrogen peroxide can also diffuse to the cytosol and be metabolized there.

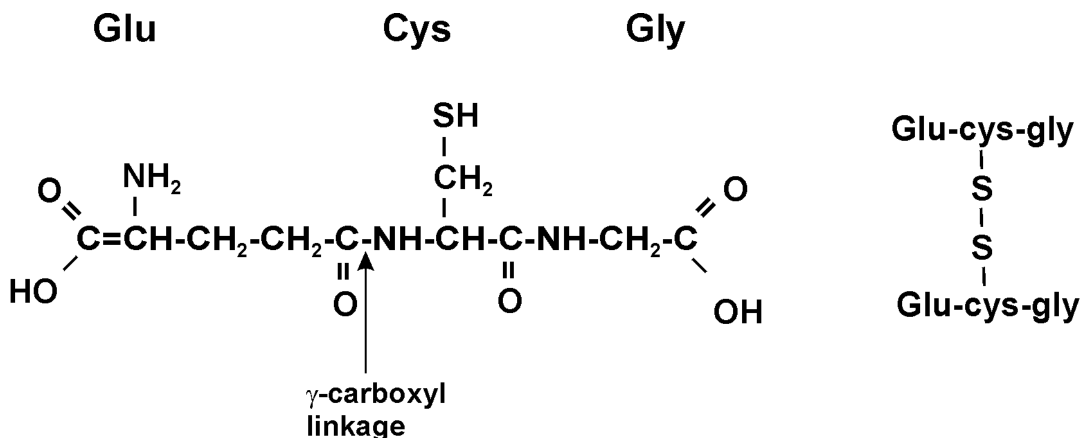

Figure 2. *Glutathione and glutathione disulfide*

Antioxidant function of GSH

Disposal of hydrogen peroxide and lipid peroxides is catalyzed by isoforms of GSH peroxidase (GPx). As a consequence, GSH is oxidized to GSSG, which is then reduced back to GSH by GSSG reductase at the expense of NADPH, thereby forming a redox cycle (Figure 3). The main pathway for NADPH regeneration is the pentose phosphate shunt. In addition to GPx, GSH-S-transferases may dispose of lipid peroxides in the cell (Awasthi et al. 1980). In addition to enzymatic disposal of peroxides, GSH can also react non-enzymatically with $OH^{\bullet}$, N_2O_3 and $ONOO^-$ (Wink and Mitchell 1998).

The family of glutathione peroxidases is comprised of four distinct selenoproteins. The classical or cytosolic glutathione peroxidase (cGPx) was the first mammalian selenoprotein to be characterized (Flohe et al. 1973) (Rotruck et al. 1973). Later on, phospholipid hydrolipid glutathione peroxidase (PHGPx) (Ursini et al. 1982, Brigelius-Flohe et al. 1994), plasma GPx (pGPx) (Takahashi et al. 1987), a gastrointestinal form of GPx (GI-GPx) (Chu et al. 1993), and non-selenium dependent GPx (Shichi and Demar 1990, Fisher et al. 1999) were identified. All glutathione peroxidases reduce hydrogen peroxide and alkyl hydroperoxides, but their specificities for the hydroperoxide differ. They use GSH as thiol substrate, but other substrates, such as thioredoxin (Bjornstedt et al. 1994) may also be used. The abundance of different GPx isoforms may reflect the different roles of hydroperoxides in signaling in various tissues, cells and cellular compartments (Brigelius-Flohe 1999).

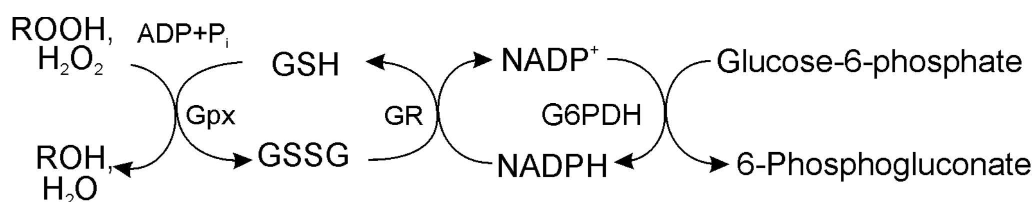

Figure 3. *Glutathione redox cycle.*

Two strains of cGPx knockout mice have been created independently (Ho et al. 1997, de Haan et al. 1998). The mice grew and developed normally and did not show any histopathologies, indicating a limited role of cGPx during normal development and under physiologic conditions. Furthermore, these mice did not show increased susceptibility to hyperbaric oxygen (Ho et al. 1997). However, when stressed with paraquat, cGPx (-/-) mice died faster than controls (de Haan et al. 1998). The mice were also susceptible to ischaemia-reperfusion injury (Yoshida et al. 1997). These studies imply that cGPx is important in the protection against oxidative stress.

Recently, GSH has been shown to play a major role in the protection against cytotoxic effects of $ONOO^-$ (Ma et al. 1997). The protective mechanism is yet to be elucidated. The direct interaction of $ONOO^-$ with the thiol group of GSH is rapid enough, in view of the high intracellular concentration, to make it significant (Koppenol et al. 1992). It has also been proposed that GPx (Sies et al. 1997) and other selenoperoxidases (Arteel et al. 1998) can act as peroxynitrite reductases, thereby preventing cellular damage caused by peroxynitrite.

Detoxifying functions of GSH

Detoxification of xenobiotics is one of the major functions of GSH. Toxic electrophiles conjugate with GSH either spontaneously or enzymatically in reactions catalyzed by GSH S-transferases (Whalen and Boyer 1998). GSH S-transferases are a family of enzymes expressed in all human tissues, though the expression of different isoenzymes is variable (Awasthi et al. 1994, Mainwaring et al. 1996). In the liver, GSH-S transferases account for as much as 5-10% of the total soluble protein (Whalen and Boyer 1998). Thus, the major pathway for GSH utilization in the liver is through transferase reactions. The conjugation of electrophiles with GSH is irreversible, and the resulting conjugates are excreted from the cell. GSH conjugates can then be used for resynthesis of GSH through the mercapturic pathway. In addition to exogenous substances, many endogenously formed compounds, such as prostaglandins and leukotrienes, conjugate with GSH by a similar mechanism (Wang and Ballatori 1998).

Maintenance of intracellular thiol status by GSH

Accumulating evidence suggests that the GSH-GSSG redox couple is important in regulating cellular proteins through reversible disulfide bond formation (Dalton et al. 1999). The formation of inter- and intramolecular disulfides as well as mixed disulfides between protein SH-groups and GSH, that is, protein glutathiolation, has been implicated in regulation of enzyme activity and transcription. Protein cysteinyl thiols react with GSSG. As the GSH/GSSG ratio usually exceeds 100, small increases in GSSG concentration will promote oxidation of protein cysteinyl thiols, shifting the equilibrium of thiol-disulfide exchange significantly in the direction of mixed disulfide formation and the formation of intra- and intermolecular disulfide bonds. Reduction of mixed disulfides is enzyme-mediated by catalysts such as thioredoxin, glutaredoxin and protein-disulfide isomerase (Cotgreave and Gerdes 1998). Many signal transduction pathways and transcription factors fundamental for cell growth, differentiation and apoptosis, appear to be redox-regulated. It has also been postulated that the thiol-disulfide equilibrium within the cell may regulate certain metabolic pathways by activating and inactivating enzymes (Gilbert 1982).

Protein thiol groups may also be modulated by S-nitrosothiols such as S-nitrosoglutathione (GSNO) (Stamler and Hausladen 1998). S-nitrosothiols are formed by the reaction of $NO^{\bullet}$-derived species with thiols, and it has been suggested that these play a role in the storage and transport of $NO^{\bullet}$ (Girard and Potier 1993). GSNO is able to modify protein thiol groups by both protein S-nitrosation and S-thiolation, and these modifications in critical thiols have been postulated to be mechanisms by which $NO^{\bullet}$ controls cellular processes (Ji et al. 1999).

GSH in cysteine storage

One of the important functions of GSH is to store cysteine because it is less prone to oxidation than cysteine (Meister 1991). The γ-glutamyl cycle (Figure 4) allows GSH to serve as a source of cysteine (Meister and Anderson 1983). GSH is exported by a carrier-mediated transporter and transmembrane protein γ-glutamyl transpeptidase (γ-GT) transfers the γ-glutamyl moiety of GSH to an amino acid, preferably cystine thereby forming γ-glutamyl amino acid and cysteinylglycine. γ-glutamyl amino acid can then be transported back into the cell. Inside the cell, the γ-glutamyl amino acid can be further metabolized by γ-glutamylcyclotransferase to release amino acid and 5-oxoproline, which then can be converted by 5-oxoprolinase to glutamate and used for resynthesis of GSH. Extracellular cysteinylglycine may be either split extracellularly by dipeptidase to form cysteine and glycine, or it can be transported into the cell as such, hydrolyzed and used for resynthesis of GSH.

The liver has been identified as the central organ in the interorgan homeostasis of GSH. The liver is not only able to synthesize GSH from its constituent amino acids glutamine, cysteine and glycine, but it also has an unique capacity to synthesize cysteine from methionine through the transsulfuration pathway (Reed and Orrenius 1977, Beatty and Reed 1980). Furthermore, liver epithelial cells have a high capacity for GSH efflux (Ookhtens and Kaplowitz 1998), with sinusoidal GSH export as the major determinant of plasma levels of low molecular weight thiols. GSH is excreted from a sinusoidal cell by carrier-mediated transporters that are yet to be characterized in detail (Ookhtens and Kaplowitz 1998). Certain stress hormones, such as adrenaline and phenylephrine, have been shown to enhance sinusoidal efflux of GSH, making more hepatic GSH available to systemic circulation (Sies and Graf 1985). In human, the exported GSH is mainly degraded to its constituent amino acids by γ-GT and dipeptidase in the liver (Hinchman and Ballatori 1990), and the main small molecular weight thiol in plasma is cysteine (or its oxidized form cystine) (Speisky et al. 1990). Plasma cysteine can then be used for GSH resynthesis in extrahepatic tissues. Alternatively, GSH can be used through the γ-glutamyl cycle in extra-hepatic tissues rich in γ-GT, such as kidney (Griffith and Meister 1979), and lung (Martensson et al. 1989).

The lung epithelium has been shown to have high levels of γ-GT activity and it utilizes extracellular GSH from alveolar lining fluid (Berggren et al. 1984). γ-GT expression is increased in rat lung epithelial cells by oxidants such as menadione and t-butylhydroquinone (Kugelman et al. 1994), suggesting that γ-GT may play a role in the protection against oxidative stress in the lung. However, oxidative stress has no effect on γ-GT activity in human type II alveolar epithelial cells (A549 cells) (Rahman et al. 1998). Furthermore, only rare columnar epithelial cells in bronchi and terminal bronchioles are γ-GT immunopositive in mouse lung (Lieberman et al. 1995). Therefore, the involvement of γ-GT in the regulation of lung GSH levels during oxidative stress *in vivo* remains unproven.

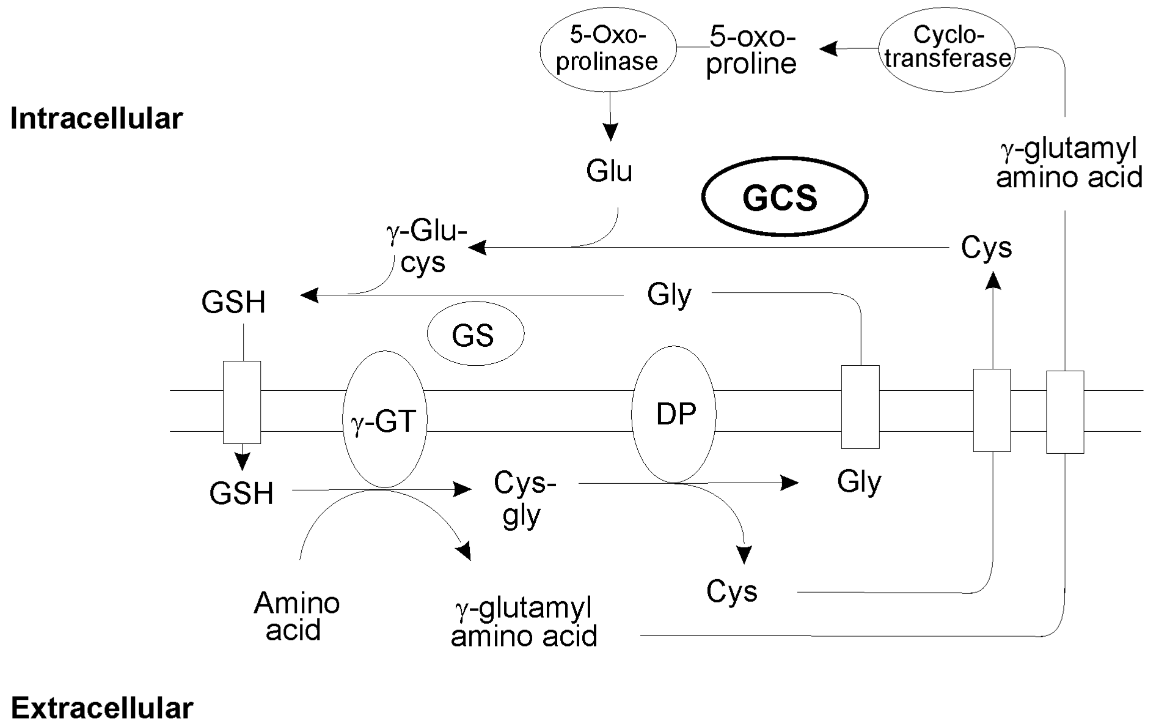

Figure 4. ***g**-glutamyl cycle. Abbreviations: DP, dipeptidase; Cys, cysteine; GCS, **g**-glutamylcysteine synthetase; GS, glutathione synthetase; **g**-Glu-cys, **g**-glutamylcysteine; **g**-GT, **g**-glutamyl transpeptidase; Glu, L-glutamic acid; Gly, glycine, GSH, glutathione.*

Glutathione biosynthesis

GSH is synthesized from cysteine, glutamate and glycine by the consecutive actions of two ATP-dependent enzymes, γ-glutamylcysteine synthetase (GCS, EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3) (Meister 1995) (Figure 4). Both enzymes are exclusively cytosolic. The first step in GSH biosynthesis catalyzed by GCS is rate-limiting, while GSH synthetase apparently has no regulatory role (Lu 1999). Physiologically, GCS is regulated either by competitive, non-allosteric inhibition by GSH (Richman and Meister 1975) or by the availability of GSH precursor, cysteine (Deneke and Fanburg 1989). While the intracellular glutamate concentration is several-fold higher than the K_m value of GCS for glutamate, the intracellular cysteine concentration approximates the K_m value for cysteine (0.1-0.3 mM) (Lu 1999).

g-glutamylcysteine synthetase

The mammalian γ-glutamylcysteine synthetase (GCS) is a heterodimer consisting of a heavy (GCS_h, 73 kDa) and a light (GCS_l, 28 kDa) subunit, and these can be dissociated under non-denaturing conditions by dithiothreitol (Seelig et al. 1984). All the catalytic activity of the enzyme, as well as the site for feed-back inhibition by GSH, resides in GCS_h, while GCS_l serves a regulatory function (Huang et al. 1993a, Huang et al. 1993b). When the light subunit is present, the K_m for L-glutamate of rat kidney holoenzyme is reduced from 18 mM to 1.4 mM and the K_i for GSH is increased from 1.8 mM to 8.2 mM. The kinetics of the recombinant human enzyme are similar (Misra and Griffith 1998). Since intracellular levels of L-glutamate and GSH are 1-3 mM and 1-10 mM, respectively, it is likely that the presence of the light subunit is needed for full activity of GCS *in vivo* (Huang et al. 1993a, Huang et al. 1993b).

GSH synthesis is enhanced through upregulation of GCS in a variety of cells and organs after exposure to agents that cause oxidative stress (Table 3, see (Lu 1999) and (Rahman and MacNee 1999)). These agents increase GCS_h mRNA levels mostly through increased transcription, but in some cases through mRNA stabilization as well. Oxidative agents increase also the transcription of GCS_l. Thus, upon oxidative stress there appears to be a concomitant induction of both subunits, enhancing GSH synthesis and thereby increasing cellular tolerance against oxidative stress.

Table 3. *Agents that induce γ-glutamylcysteine synthetase and elevate glutathione (modified from Lu, (Lu 1999), and Rahman and MacNee (Rahman and MacNee 1999)).*

Agent	Cell/organ
Oxidants	
H_2O_2	Human alveolar type II cells (A549)
Dimethylnaphthoquinone	Rat lung cells (L2)
t-Butylhydroquinone	Human hepatocyte cells (HepG2)
Hyperoxia	A549 cells, rat lung
Pyrrolidine dithiocarbamate	HepG2 and bovine aortic endothelial cells
β-naphthoflavone	HepG2 cells
Cytokines	
TNF-α	HepG2, A549 cells
IL-1β	endothelial cells
Heavy metals	
Cadmium	A549 cells
Mercury	Rat kidney
NO donors	
S-nitroso-N-penicillamine	Bovine aortic endothelial and rat vascular smooth muscle cells
DETA NONOate	Bovine aortic endothelial and rat vascular smooth muscle cells

Nitric oxide increases intracellular GSH by inducing GCS in rat vascular smooth muscle cells (Moellering et al. 1998) and bovine endothelial cells (Moellering et al. 1999a). NO donors also increase GSH concentration in rat lung fibroblasts, possibly through a similar mechanism (White et al. 1995). In vascular smooth muscle cells, $NO^{\bullet}$ fluxes at or above 8 nM/s increase the mRNA expression of both subunits (Moellering et al. 1998). In endothelial cells, lower rates of production, 1-3 nM/s, corresponding to the rate required for NOS III to elicit vasodilation (Kanai et al. 1995), are sufficient to induce GCS (Moellering et al. 1999a). The induction of GCS by $NO^{\bullet}$ appears to be a cGMP independent event (Moellering et al. 1998, Moellering et al. 1999a).

The 5'-flanking regions of both GCS subunits have been cloned and sequenced (Figure 5) (Mulcahy et al. 1997, Moinova and Mulcahy 1998). Putative activator protein-1 (AP-1), nuclear factor kappa B (NF-κB), activator protein-2 (AP-2), and several antioxidant responsive elements (ARE; also referred to as electrophil responsible element or EpRE), have been identified in the promoter region of the GCS_h gene (Mulcahy and Gipp 1995, Mulcahy et al. 1997). The basal transcription of the gene is driven by sequences between -202 and +22, containing a consensus TATA box (Mulcahy et al. 1997). AP-1 or AP-1-like

responsive elements residing in the region of -817 to +82 of the promoter sequence have been shown to be important for transcriptional induction by various agents that increase ROS production, such as TNF-α (Morales et al. 1997, Rahman et al. 1999), menadione (Rahman et al. 1996) and H_2O_2 (Rahman et al. 1996), while NF-κB appears to have no effect on induction by menadione or H_2O_2 (Rahman 1998), or by TNF-α (Morales et al. 1997, Rahman et al. 1999). Distal ARE/EpRE (ARE 4, Figure 5) sequences have been shown to be important in the induction of GCS_h by electrophilic compounds β-naphthoflavone (Mulcahy et al. 1997) and pyrrolidine dithiocarbamate (PDTC) (Wild and Mulcahy 1999) through post-translational activation of transcription factor Nrf2 (Wild et al. 1999, Itoh et al. 1999). In addition, the metal responsive element (MRE), which is also involved in the regulation of general cellular stress response, appears to be crucial for GCS_h gene expression in response to heavy metals as well as for the constitutive expression of the gene in the liver (Gunes et al. 1998).

The basal expression of the GCS_l gene is directed by a consensus AP-1 site at -340:-334 of the promoter sequence (Moinova and Mulcahy 1998). It is also involved in β-naphthoflavone-mediated induction together with the EpRE site at -301:-291 (Moinova and Mulcahy 1998). As in GCS_h, Nrf2 appears to mediate the induction through EpRE, possibly through complex formation with Maf proteins and JunD (Wild et al. 1999).

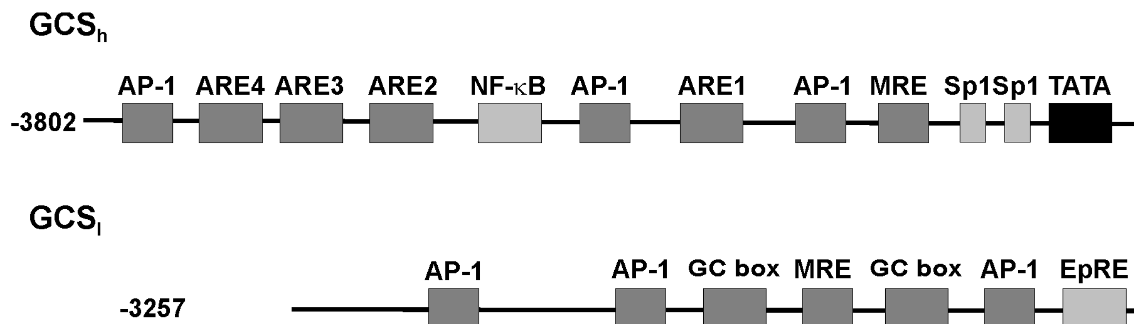

Figure 5. *Promoter regions of GCS_h and GCS_l genes.*

The availability of cysteine as a determinant of GSH synthesis

The hepatic GSH level appears to be largely dependent on dietary cysteine and methionine, derived from dietary protein. In rat liver, starvation for 48 hours reduces liver GSH to between two thirds and one half of the normal levels. The GSH levels are quickly replenished upon refeeding (Tateishi et al. 1974). Liver GCS activity and mRNA expression are down-regulated in a dose-dependent manner by high sulfur amino acid or protein content in the diet (Bella et al. 1996, Bella et al. 1999a, Bella et al. 1999b). Thus, substrate availability rather than GCS activity appear to regulate GSH synthesis in the liver.

Cysteine is transported into the cell by sodium-dependent A or ASC systems (Bannai 1984). Intracellular cysteine concentrations can be increased also by cystine, which is reduced intracellularly to yield cysteine. The transport of cystine is distinct from that of cysteine, and it utilizes the Xc^- transport system (Bannai 1986). This system is sodium-independent and mediates one-to-one exchange to glutamate. Xc^- transport system has been shown to be induced by various oxidants and hyperoxia (Deneke et al. 1989, Miura et al. 1992), as well as by $NO^{\bullet}$ (Li et al. 1999) leading to increased GSH synthesis and intracellular GSH levels.

Transsulfuration pathway

The liver is the main site for cysteine biosynthesis, which occurs through the transsulfuration pathway. In transsulfuration, methionine is sequentially converted into cysteine via several enzymatic steps (Figure 6.). The first step is the ATP-dependent activation of methionine to S-adenosylmethionine (SAM), and it is catalyzed by methionine adenosyltransferase. Subsequent demethylation and removal of the adenosyl moiety yields homocysteine. Homocysteine condenses with serine to form cystathionine in a reaction catalyzed by cystathionine synthase. Cleavage of cystathionine, catalyzed by cystathionine γ-lyase, releases free cysteine. In this pathway, methionine and homocysteine are readily interconvertible, but the subsequent step, the formation of cystathionine, is irreversible.

Availability of methionine appears to be the principal determinant regulating the activity of transsulfuration and remethylation pathways. The main regulatory control appears to be exerted at the level of homocysteine: when methionine is needed, homocysteine is remethylated by methionine synthase or betaine-homocysteine methyltransferase; when methionine is in excess, metabolism of homocysteine via the cystathionine synthase reaction is accelerated (Selhub 1999). Interestingly, cystathionine β-synthase is a heme protein that under reducing conditions exhibits a 1.7-fold lower activity than under oxidizing conditions (Taoka et al. 1998). It has been postulated that under oxidative stress, homocysteine transsulfuration is favored over remethylation, thereby increasing the supply of cysteine for GSH synthesis. However, liver methionine

adenosyltransferase is known to be inactivated upon oxidative and nitrosative stress (Corrales et al. 1991, Avila et al. 1997, Sanchez-Gongora et al. 1997), which leads to a decreased production of S-adenosylmethionine and subsequent decrease in liver GSH levels (Lu 1998).

The last enzyme of the transsulfuration pathway is cystathionine γ-lyase (CGL, γ-cystathionase, EC 4.4.1.1). It catalyzes the conversion of L-cystathionine into L-cysteine, α-ketobutyrate and ammonia. Mammalian CGL is a pyridoxal 5' phosphate (PLP) dependent enzyme consisting of four subunits each binding one molecule of PLP (Matsuo Y 1958, Steegborn et al. 1999). Although the three-dimensional structure of human CGL has not been elucidated, sequence similarity suggests that the overall fold of the enzyme would resemble that of the other members of the γ-family of PLP-dependent enzymes cystathionine β-lyase and cystathionine γ-synthase (Alexander et al. 1994, Clausen et al. 1996, Clausen et al. 1998, Steegborn et al. 1999). Two monomers are in close contact sharing active site residues forming an active dimer. Two of these dimers then interact to form a CGL tetramer.

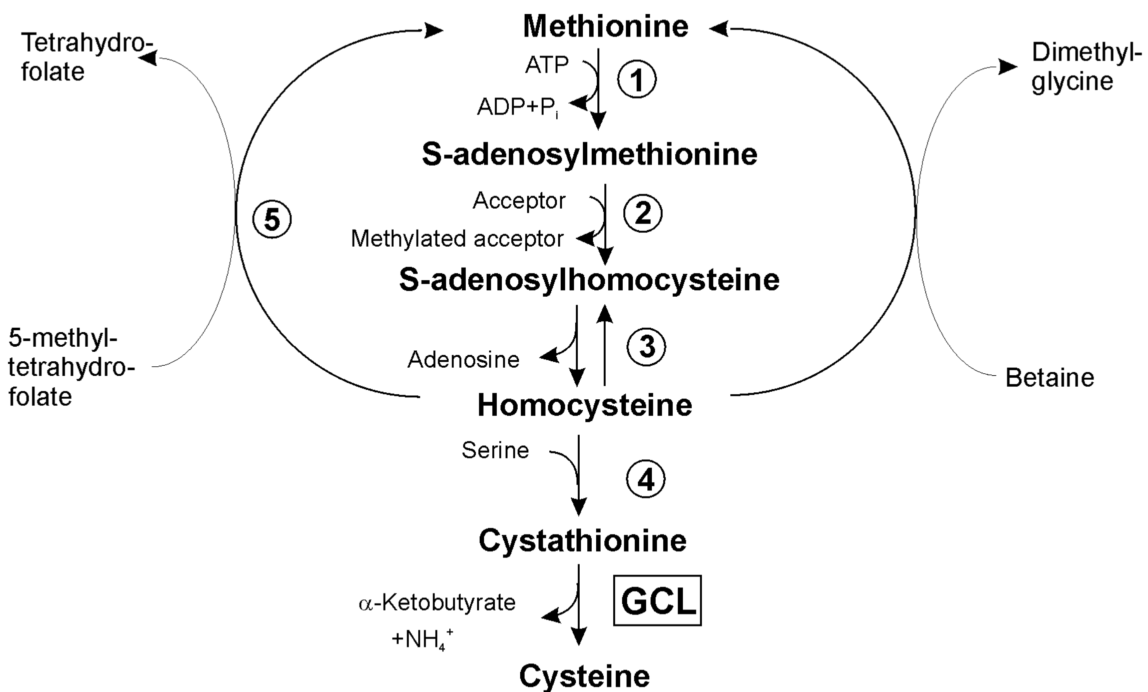

Figure 6. *Transsulfuration pathway. Numbers and abbreviations: 1) Methionine adenosyltransferase, 2) Various methyltransferases, 3) S-adenosylhomocysteine hydrolase, 4) cystathionine* **b**-*synthase, 5) methionine synthase, 6) betaine-homocysteine methyltransferase, CGL, Cystathionine* **g**-*lyase.*

Both human (Lu et al. 1992) and rat (Erickson et al. 1990) cDNA for CGL have been cloned. Two forms of human mRNA for CGL have been characterized, of which the shorter form has an internal deletion of 132 base pairs. As the human CGL gene has not been localized, it is not known whether these two forms are products of different genes or splice variants. Furthermore, it is not known whether both mRNAs are translated. As it has been shown that CGL activity is absent from human fetal liver (Sturman et al. 1970), it was proposed that the subunit composition of the tetrameric CGL regulates the activity, and that the postnatal increase in CGL activity is caused by a change in relative expression of the two isoforms (Lu et al. 1992). The absence of CGL activity in the liver of preterm neonates has been shown to be associated with lowered cysteine levels and, subsequently, impaired GSH synthesis (Vina et al. 1995).

ROS and antioxidants in pathophysiology

Neonatal problems

Preterm infants may be deficient in pulmonary surfactant and are at risk for the respiratory distress syndrome (RDS) during the first days of life. Such infants often require ventilator therapy with high inspired oxygen concentrations to provide adequate oxygenation. Prolonged ventilator treatment is associated with the development of chronic lung disease (CLD). The typical morphologic changes include airway epithelial damage, bronchial smooth muscle cell hyperplasia and obstruction, as well as alveolar destruction and interstitial fibrosis. Predisposing factors include immaturity, high ventilator pressures, and high inspired oxygen concentrations. The histologic findings in CLD are markedly similar to pulmonary oxygen toxicity in experimental animals (Frank 1992, Abman and Groothius 1994). Preterm neonates at risk for development of CLD have shown an enhanced inflammatory reaction in the lungs with an associated increase in pulmonary microvascular permeability (Groneck et al. 1994). Inflammatory cells contribute to the lung injury by releasing proteolytic enzymes and ROS, which damage pulmonary cells and alter their function (Pierce and Bancalari 1995).

There is ample indirect evidence that ROS are associated with CLD. The amount of lipid peroxidation products, pentane and ethane, in expired air of very low birth weight infants is increased, and the amounts are significantly higher in patients with a poor outcome (Pitkänen et al. 1990). The amount of protein carbonyls, a marker for protein oxidation, in tracheal aspirates of those infants developing CLD is markedly higher than those that do not (Varsila et al. 1995). Infants that subsequently develop CLD have increased plasma levels of allantoin,

a non-enzymatic oxidative product of uric acid (Ogihara et al. 1996), as well as increased concentrations of lipid peroxidation products (Ogihara et al. 1999). Also RNS may play a role in the pathogenesis of CLD, as plasma nitrotyrosine levels are increased (Banks et al. 1998).

Apart from CLD, ROS have been ascribed a role in other common problems of prematurity, such as retinopathy of prematurity (ROP), and necrotizing enterocolitis (NEC) (Warner and Wispe 1992). The association of ROP, which is a developmental vascular disorder of the retina, with prolonged oxygen therapy is well established, leading to the hypothesis that increased free radical formation in hyperoxia would cause aberrant vessel formation (Kretzer and Hittner 1988). However, arterial oxygen levels in premature infants requiring oxygen therapy are lower than in healthy neonates breathing room air, and experimental data suggest that the fluctuation in arterial oxygen levels and degree of hypoxia may have more influence on proliferative retinal disease than extended hyperoxia (Penn et al. 1995). The pathogenesis of NEC is multifactorial, but ischemia and subsequent reperfusion with increased ROS production are likely to contribute to the injury (Warner and Wispe 1992).

Taken together, the association of ROS with neonatal lung injury is fairly well established. In other neonatal clinical complications, more clinical and experimental data are needed to confirm the involvement of ROS in pathophysiological processes.

Antioxidant defense of the newborn

It has been demonstrated in a number of animal species that a several-fold increase in the activities of fetal lung antioxidant enzymes, MnSOD and CuZnSOD, catalase and GPx, normally occurs during the final 15-20% of gestation (Frank 1991). This has been considered a defense mechanism against air breathing and relative hyperoxia after birth. Insufficient activity of antioxidant enzymes in the lung of the preterm neonate is thought to aggravate the damage resulting from ventilatory care. However, coordinated induction of antioxidant enzymes does not appear to take place in the human lung (Fryer et al. 1986, Strange et al. 1988, Strange et al. 1990, McElroy et al. 1992, Asikainen et al. 1998). The expression of MnSOD, CuZnSOD, catalase and GPx in human infants born prematurely is similar to that in adults, which indicates that preterm neonates are better adapted for life in an oxygen-containing environment than previously suspected.

Little is known about the ontogeny of the enzymes important for glutathione synthesis. The activities of GCS are significantly lower in the kidneys of neonatal mice, but the liver and lung have levels similar to those of adults (Harman et al. 1990). A similar trend has also been reported in rats (Tsui and Yeung 1979). In human fetal erythrocytes (Lestas and Rodeck 1984), leukocytes (Lavoie and Chessex 1998), and liver (Rollins et al. 1981), GCS activities are in the same range as in adults.

Cystathionine γ-lyase activity (Sturman et al. 1970), as well as immunoreactive protein (Gaull et al. 1972), is absent from human fetal liver, although other enzymes of the transsulfuration pathway are present. In rats, activity in the liver is low during fetal development and increases rapidly during the last 3 days of gestation (Heinonen 1973). The rate of GSH synthesis from methionine is 6 times lower in fetal than in adult rat hepatocytes, presumably due to the low CGL activity (Pallardo et al. 1991). In preterm infants, plasma cysteine levels are much lower than in full term newborns (White et al. 1994, Vina et al. 1995), reflecting low cystathionase activity. Studies on very low birth weight infants during the first week of life, when they are mostly on parenteral nutrition, have shown that plasma cysteine levels decrease more, relative to term reference values, than those of any other amino acid (Van Goudoever et al. 1995). In addition, concentration of GSH in plasma and bronchoalveolar lavage fluid is inversely proportional to gestational age, suggesting that GSH deficiency is present in the lungs of preterm infants and that the deficiency increases with the degree of prematurity (Jain et al. 1995).

The role of ROS and RNS in the development of hypertension

It is clearly established that $NO^{\bullet}$ plays a critical role in several renal processes, including regulation of renal plasma flow, glomerular filtration rate, renin and angiotensin generation, and sodium excretion (Kone and Baylis 1997). All three types of NOS are expressed in the kidney in a cell type-specific manner and are subject to complex and distinct control mechanisms. The role of each NOS isoform in renal processes is somewhat unclear, since most studies have been done using non-specific NOS inhibitors. However, NOS I and NOS III appear to be involved in the regulation of renal plasma flow and glomerular filtration rate, and NOS II in tubular sodium transport (Kone and Baylis 1997, Gabbai and Blantz 1999). Thus $NO^{\bullet}$ appears to be critical in normal sodium excretion and renal hemodynamics, and impediment of its actions leads to disturbance in kidney homeostasis that may promote hypertension.

Both human essential hypertension as well as many experimental animal models of hypertension, such as the spontaneously hypertensive rat (SHR), are associated with increased production of ROS and/or low levels of antioxidants. Increased production of ROS, notably $O_2^{\bullet -}$, leads to inactivation of $NO^{\bullet}$, thus

preventing its vasodilatory action (Gryglewski et al. 1986). The evidence for involvement of ROS in human hypertension is indirect: essential hypertension has been shown to be associated with increased plasma levels of lipid peroxidation products (Russo et al. 1998), and clinical studies show that ascorbic acid improves endothelium-dependent vasodilation and reduces blood pressure in hypertensive patients (Taddei et al. 1998, Duffy et al. 1999). In SHR, increased production of $O_2^{\cdot -}$ has been shown in mesenteric arterioles (Suzuki et al. 1995), and a synthetic membrane-permeable SOD mimetic normalizes the blood pressure (Schnackenberg et al. 1998). Also xanthine oxidase inhibitors attenuate the rise in blood pressure in SHR, suggesting that xanthine oxidase may be a source of ROS generation that is associated with an increasing arteriolar tone in SHR (Nakazono et al. 1991) (Suzuki et al. 1998). It should be noted, however, that xanthine oxidase inhibitors do not inhibit exclusively xanthine oxidase. Another possible source of ROS in SHR is dysfunctional NOS III (Cosentino et al. 1998).

While the kidney has been shown to be crucial in the development and maintenance of hypertension, the possible involvement of renal ROS production in hypertension has drawn relatively little attention. Angiotensin II increases $O_2^{\cdot -}$ production in vascular smooth muscle cells and adventitial fibroblasts (Griendling et al. 1994, Pagano et al. 1998), as well as in kidney mesangial cells (Jaimes et al. 1998). Thus at least in angiotensin II-dependent hypertension, there appears to be an increase in renal $O_2^{\cdot -}$ production, which is likely to attenuate $NO^{\cdot}$-mediated effects. In SHR, increased salt intake leads to an increase in blood pressure and in renal xanthine oxidoreductase activity, but the increase is mainly in the dehydrogenase form, which does not produce ROS (Laakso et al. 1998). Studies on ROS in the kidney face technical problems similar to those in studies on $NO^{\cdot}$, because the specific site and the source of ROS is important to know in order to understand their role in renal processes.

Increased oxidative stress in SHR may be caused not only by enhanced production but also by lowered antioxidant capacity. In this respect, it is of interest that increased $O_2^{\cdot -}$generation in the myocardium of SHR has been shown to be associated with low activities of MnSOD and CuZnSOD, implying decreased antioxidant protection (Ito et al. 1995). Little is known about GSH synthesis or the enzymes of the GSH redox cycle in SHR in comparison to WKY, and the reports are somewhat controversial. Myocardial GCS has been reported to be higher in SHR that in WKY (Carlos et al. 1998). GPx activity has been reported to be either higher (Cabell et al. 1997), similar (Yuan et al. 1996) or variable depending on age (Batist et al. 1989), and hepatic GPx activitity lower (Kitts et al. 1998) in SHR in comparison to WKY. However, no systematic studies have been made to elucidate the activities of all the enzymes in GSH redox cycle or GSH synthesis in these animals.

The role of ROS and RNS in the secondary effects of hypertension

Hypertension injures blood vessels, thereby causing end-organ damage. While many steps in the process are not completely understood, recent advances in the understanding of the chain of events have made it possible to construct a tentative model of hypertensive kidney damage (Luft et al. 1999). According to this model, the primary signaling event is increased blood flow through small arteries, which has been shown to increase connective tissue production and promote medial hypertrophy, probably through proliferation of both endothelial and vascular smooth muscle cells (Tulis et al. 1998). The endothelial layer acts as a signal transduction interface for hemodynamic forces: both shear stress (the tangential force due to blood flow) and cyclic strain (circumferential stress due to transmural pressure) initiate numerous growth-promoting pathways, e.g. release of growth factors (Chien et al. 1998). Furthermore, cyclic strain increases the formation of ROS, presumably by activation of NAD(P)H oxidase (Howard et al. 1997). This leads to an upregulation of leukocyte chemoattractants, such as monocyte chemotactic protein-1 (MCP-1) (Wung et al. 1997), as well as adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) (Cheng et al. 1998). Infiltration of the permeabilized endothelium by leukocytes initiates an inflammatory cascade leading to vascular and mesangial cell proliferation and hypertrophy, increased coagulation and matrix production, and ultimately end-stage renal failure.

While mechanical forces are crucial in initiating the events leading to vascular remodeling and subsequent end-organ damage, other mediators are involved as well. Rats transgenic for both human angiotensinogen and renin genes (dTGR), develop severe cardiac and renal damage and die by 7 weeks of age, despite an only modest increase in blood pressure (Luft et al. 1999). These effects are reversed by angiotensin converting enzyme (ACE) inhibitors and angiotensin II type 1 (AT-1) receptor inhibitors and, interestingly, by PDTC (Muller et al. 2000). The latter is an inhibitor of NF-κB (Schreck et al. 1992, Liu et al. 1999), and has also been shown to be a non-specific scavenger of ROS as well as to increase cellular GSH levels through induction of GCS (Wild and Mulcahy 1999, Moellering et al. 1999b). These results indicate that angiotensin II contributes to hypertensive vasculopathy. Indeed, angiotensin II affects growth-promoting processes directly as well as indirectly through synthesis of growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor β (TGFβ) (Raij 1999), and these synthetic pathways appear to be redox-regulated (Sundaresan et al. 1995, Du et al. 1999).

Nitric oxide appears to antagonize the effects of angiotensin II in the vasculature in many ways. It is a powerful inhibitor of vascular smooth muscle and mesangial cell growth as well as extracellular matrix production (Du et al. 1999, Raij 1999, Garg and Hassid 1989). These effects have been elegantly shown *in vivo* in NOS III knockout mice subjected to hemodynamic injury (Rudic et al. 1998). Nitric oxide inhibits activation of the redox sensitive transcription

factor NF-κB in response to pro-inflammatory cytokines, which in turn prevents transcription of chemokines and adhesion molecules and subsequent leukocyte infiltration (Zeiher et al. 1995, De Caterina et al. 1995, Peng et al. 1995). Nitric oxide has also been shown to downregulate ACE synthesis (Higashi et al. 1995), AT-1 receptors in vascular tissue (Ichiki et al. 1998), and TGF-β1 expression (Craven et al. 1997). Thus the antagonistic interaction of $NO^{\bullet}$ and angiotensin II, and the balance between these two, appears to be important in the development of end-organ damage.

In conclusion, it is reasonable to hypothesize that in hypertensive end-organ damage the effects of angiotensin II override the effects of $NO^{\bullet}$. Increased production of ROS upon stimulation by angiotensin II may initiate a pro-trophic and pro-inflammatory cascade, which ultimately leads to end-organ damage. While the role of glutathione in this process is yet to be established, many of these pathways are potentially affected by intracellular thiol status.

OBJECTIVES OF THE STUDY

The hypothesis underlying this series of studies is that glutathione plays an important role in antioxidant defense, exemplified by two clinical situations, preterm birth and hypertension. Therefore, studies were performed to assess the ability of the premature neonate to synthesize glutathione, and to generate its rate-limiting substrate, cysteine, through the transsulfuration pathway. Furthermore, the role of glutathione in hypertension was studied in a genetic animal model of hypertension, the spontaneously hypertensive rat.

The specific aims were:

1. To study the mRNA expression and activity of the rate-limiting enzyme of GSH synthesis, γ-glutamylcysteine synthetase, in human fetal, neonatal and adult lung, liver, and kidney.
2. To study the mRNA expression and activity of cystathionine γ-lyase in the human fetal, neonatal, and adult liver, to assess the ability of its two mRNA isoforms to code for active enzyme, and to elucidate whether alternative splicing accounts for the two isoforms.
3. To study GSH metabolism in the spontaneously hypertensive rat and to determine the role of $NO^{\bullet}$ in the regulation of GSH synthesis in this experimental model.

MATERIALS AND METHODS

Human samples

Fetal lung, liver, and kidney samples (15 to 19 gestational wk) (N=6) were obtained from legal abortions. The samples between 26-42 gestational weeks were obtained from neonatal autopsies performed within 12 h of death. The causes of death were respiratory distress syndrome (RDS) (N=2), congenital heart disease (N=1), respiratory failure and hydronephrosis (N=1), and meconium aspiration (N=1). Adult lung tissue samples (N=3) were obtained from macroscopically normal tissues of lung cancer patients undergoing lung surgery and from donor lungs of single lung transplantations. Adult liver tissue (N=4) was obtained from partial liver transplantations. Kidney tissue (N=5) was obtained from renal biopsies or from cadaver donors.

The study protocol was approved by the Ethical Committees of The Hospital for Children and Adolescents, and Department of Thoracic and Cardiovascular Surgery, University of Helsinki, Helsinki, Finland.

Experimental animals

In the first set of experiments, 47 inbred nine-week-old male SHR rats (Harlan Sprague Dawley Inc, IN, USA) weighing 240 - 250 g were divided into six groups. The animals were kept three weeks on diets containing 0.2, 1.1 or 6.0 % of NaCl (w/w of the diet) with or without N^{ω}-nitro-L-arginine methyl ester (L-NAME, 0.025 % in the diet, providing approximately 20 mg/kg body weight/day). In the second experiment, 20 nine-week-old SHR, weighing 190 - 250 g, were divided into two groups, receiving 6.6 % NaCl in diet with or without isosorbide-5-mononitrate (IS-5-MN, 0.1 % w/w of the diet, providing 60-70 mg/kg body weight/day) for eight weeks. In the third experiment, fourteen eight-week-old SHR rats (Harlan Sprague Dawley Inc, IN, USA) weighing 240 - 250 gm were divided into two groups. They were kept for 6 weeks on diets containing either 0.3 % (low-salt) or 2.6% (high-salt) NaCl (w/w of the diet). The control Wistar-Kyoto rats (n=8) were kept on a diet containing 0.3% NaCl.

The procedures and protocols for the animal studies were in accordance with the institutional guidelines and were approved by the Animal Experimentation Committee of the Intitute of Biomedicine, University of Helsinki, Finland.

The systolic blood pressure and heart rate of the rats were measured with the use of a tail-cuff analyzer (Apollo-2AB Blood Pressure Analyzer, Model 179-2AB, IITC Life Science) as described (Vaskonen et al. 1997). The digital values for systolic blood pressure and heart rate were evaluated automatically from the analog data by a microprocessor. Before the measurements the rats were warmed for 10 to 20 min at 28°C to make the pulsations of the tail artery detectable. The average of three readings was used. To minimize the stress-induced fluctuations in blood pressure, all measurements were taken by the same person in the same quiet environment between 2 and 6 p.m.

Enzyme activity measurements

Frozen human samples were homogenized in 100 mM Tris-HCl buffer (pH 8.2) containing 50 mM KCl, 20 mM $MgCl_2$ and 2 mM EDTA. The homogenates were centrifuged at 14,000 x g for 30 min at 4°C. The liver and lung homogenates were filtered through P-10 gel filtration columns (Pharmacia) and kidney homogenates through Micro Bio-Spin 6 Column (Bio-Rad, Hercules, CA). GCS activity was measured as described (Nardi et al. 1990), with modifications. Briefly, the assay mixture (final volume 100 µl) containing 10 mM ATP, 6 mM DTT, 3 mM L-cysteine and 15 mM L-glutamic acid in 100 mM Tris-HCl buffer was preincubated at 37°C for 15 min to ensure complete reduction of thiols. The reaction was initiated by addition of the sample. The samples were incubated for 15-30 min at 37°C. After the incubation 50 µl of the mixture were added to 50 µl of 30 mM monobromobimane (Thiolyte®, Calbiochem, La Jolla, CA) in 50 mM N-ethylmorpholine (pH 8.4) and left to react at room temperature for 5 min. The reaction was stopped by addition of 10 µl 100% TCA and the precipitated protein was spun down at 14,000 g for 5 min. 5 µl of supernatant were injected into a Waters Novapak C-18 HPLC column (4 µm, 3.9 x 150 mm) running an isocratic mobile phase consisting of 4% acetonitrile, 0.25% acetic acid and 0.25% perchloric acid, pH 3.7. The fluorescent product was detected using Shimadzu RF-10AxL spectrofluorometer (Shimadzu Corporation, Kyoto, Japan) with excitation and emission wavelengths of 394 nm and 480 nm, respectively. The quantity of γ-glutamylcysteine was measured by comparison with γ-glutamylcysteine (Bachem, Bubendorf, Switzerland) standards derivatized and analyzed as above. Enzyme activity was normalized on the basis of protein content, which was determined using Bio-Rad DC protein assay kit.

For cystathionine γ-lyase activity measurements, the cultured cells were harvested and suspended in ice-cold 10 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA. After 3 freeze-thaw cycles the non-soluble debris was spun down and the supernatant used for activity measurement. Frozen liver samples were homogenized in 30 mM sodium phosphate buffer (pH 7.0), centrifuged at 14,000 x g for 30 min at 4°C, and filtered through P-10 gel filtration columns (Amersham Pharmacia Biotech) to remove endogenous amino acids. Enzyme activity was measured as described (Heinonen 1973). The specificity of the assay was confirmed using propargylglycine, an inhibitor of CGL.

Spectrophotometric assays were used to measure GPx, GR and G6PDH activities. GPx was measured by following the oxidation of NADPH in the presence of t-BOOH, GSH and GR (Beutler 1975b), and GR and by following NADPH oxidation in the presence of GSSG (Beutler 1975a) at 340 nm. G6PDH was measured by following the reduction of NADP in the presence of glucose-6-phosphate (Löhr and Waller 1974).

mRNA detection

Total RNA was extracted from 293T cells using RNeasy mini kit (Qiagen). For the extraction of total RNA from tissues, the guanidinium thiocyanate/cesium chloride method was used (Chirgwin et al. 1979).

For the detection of CGL by Northern blotting, RNA was fractionated on agarose-formaldehyde gels at 10 μg/lane. Following capillary transfer onto nylon filters (Hybond-N, Amersham Pharmacia Biotech), the blots were hybridized using standard methods (Sambrook et al. 1989) with [^{32}P] labeled (DuPont) complementary RNA probe corresponding to nucleotides 874-1106 of the published sequence (Lu et al. 1992). Following hybridization and washes, the filters were exposed to Kodak BioMax MR autoradiography films (Eastman Kodak Co.) The filters were stripped and reprobed with [^{32}P] labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA probe transcribed from p-TRI-GAPDH-plasmid (Ambion).

For quantification of RNA from fetal, neonatal and adult tissues, the ribonuclease protection assay (RPA II, Ambion Inc) was used. For the detection of GCS subunits, ^{32}P-radiolabeled antisense RNA probes were transcribed from the PstI-NcoI fragment of GCS_h (nucleotides 1375-1628) (Gipp et al. 1992), and the HindII fragment of GCS_l cDNA (nucleotides 583-888) (Mulcahy and Gipp 1995), and hybridized with 10 μg (liver and kidney) or 20 μg (lung) total RNA at 42°C overnight. In order to detect GCL-L and CGL-S, templates for radiolabeled RNA probes were generated by amplifying 301-bp and 169-bp fragments of CGL-L and CGL-S using the forward primer 5'-GCAAGTGGCATC-TGAATTTG-3' and reverse primer 5'-CCCATTACAACATCACTGTGG-3' flanking the deletion site. The resulting fragments were cloned into the pCR 2.1 cloning vector (Invitrogen) and digested with SpeI. Using T7 RNA polymerase

and $[^{32}P]$UTP, these vectors yield 397- and 265-bp radiolabeled run-off transcripts and 299- and 167-bp protected fragments for CGL-L and CGL-S, respectively. To normalize for RNA content, the samples were hybridized with RNA probes transcribed from human β-actin cDNA (pTRI-Actin-Human, Ambion Inc.). Following RNase A+T_1 digestion, the protected fragments were separated on 5% polyacrylamide/8 M urea gels and exposed to Kodak BioMax MR autoradiography film (Eastman Kodak Co, Rochester, NY).
For RT-PCR, liver RNA samples were reverse transcribed using random hexamer primers (Promega) and amplified using the same primers that were used for creating probes for the ribonuclease protection assay.

In vitro expression of CGL

Cell culture and transfections

Human embryonic adenovirus-transformed kidney cells (293T cells) were maintained in Dulbecco’s modified Eagle’s medium with 862 mg/l Glutamax I™ (Gibco BRL) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were seeded at a density of 4 x 10^6/10-cm plate 20 h before transfection. Cultures were grown at 37°C in a humidified atmosphere with 5% CO_2. Transient transfections were performed using FuGENE™ 6 Transfection Reagent (Roche Molecular Biochemicals) according to the manufacturer’s protocol. Cells were transfected with 6 µg of pcDNA-CGL-S, pcDNA-CGL-L or pcDNA3. Transfection efficiency was monitored by cotransfection of 1.5 µg of pCMVβ (Clontech) β-galactosidase expression vector and measuring β-galactosidase activity as described (Rosenthal 1987). Cells were harvested 48h after transfection.

Plasmids carrying cystathionine g-lyase isoforms

Human cystathionine g-lyase cDNA (HCL-1, GenBank acc. #52028) was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA, cat. no. 79761). This cDNA clone represents the shorter CGL isoform. However, the original clone obtained from ATCC lacked the 3’ end (nucleotides 1106-1194) of the reported sequence of the short form of CGL (Lu et al. 1992). The missing nucleotides of the coding sequence, a Kozak consensus translation initiation sequence and restriction sites were added to the sequence by PCR. The sequences of the primers used were as follows: forward 5’-CTCTGGTACCGCGACC*ATGCAGGAAAAAGACGCCTCCTC* and reverse 5’-CGTCGGTACC*CTAGCTGTGAATTCTTCCACTTGGAGGGTGTGC* (sequences corresponding to the human CGL are italicized and KpnI restriction sites are underlined). pcDNA-CGL-S was created by cloning the resulting DNA fragment into the KpnI site of the mammalian expression vector pcDNA3 (Invitrogen).

In order to clone the longer isoform CGL-L, 1 µg of total human liver RNA was reverse transcribed using random hexamer primers (Promega) and PCR amplified as above. The CGL-S and CGL-L clones were sequenced using ABI prism automated sequencer (Perkin-Elmer).

PCR of genomic DNA

Our assumption was that the two CGL forms are splice variants and the 132 bp fragment missing from CGL-S is flanked by two introns. DynaZyme EXT DNA Polymerase (Finnzymes) was used to obtain the PCR fragments. For the amplification of the 5' intron, 100 ng of human genomic DNA were amplified using forward primer 5'-GCAAGTGGCATCTGAATTTG-3' and reverse primer 5'-ACAATATGTGCACAGCCTTC-3'. The resulting ca. 2.3 kb fragment was cloned into pCR-XL-TOPO vector (Invitrogen) and sequenced from both ends of the insert. The 3' intron was amplified using forward primer 5'-AACCCCACCCAGAAGGTGATTG-3' and reverse primer 5'-GCAGAATAC-ATAGAAATATCAGCTCC-3'. PCR amplification resulted in ca. 5 kb DNA fragment, which was then cloned and sequenced as above.

Statistical Analysis

For comparing the expression of GCS in fetal, neonatal and adult tissues, the Mann-Whitney U-test was used with a level of $P \leq 0.05$ chosen to indicate significant differences. The Spearman rank correlation test was used to assess the correlation between enzyme activity and mRNA levels.

The CGL activity in cells transfected with CGL-S, CGL-L or empty plasmid were compared with Student's t-test. Data were expressed as mean ± S.E.M. Linear regression was used to assess the correlation between the mRNA expression of CGL-S and CGL-L during development.

In studies on experimental animals, comparisons between treated and non-treated animals were carried out using Student's t-test. The activities were expressed as mean ± S.D. The relationship between GCS activity and blood pressure was assessed using Pearson's linear correlation coefficient.

All statistical analyses were performed with the SPSS 8.0 program (SPSS Inc.).

RESULTS

The expression of GCS

In the liver, both GCS mRNA species were detectable using Northern blotting, whereas in other tissues only the ribonuclease protection assay was sensitive enough to detect GCS_l and GCS_h. Neither mRNA expression of GCS_l and GCS_h nor the GCS activity in the fetal and neonatal liver differed significantly from adult samples. The mean activity of GCS was three to four times higher in the liver than in other tissues studied (4.4 nmol/min/mg protein).

In the lung, the mRNA expression of GCS_h was at the same level in fetal, neonatal and adult groups, whereas GCS_l expression was higher in the neonates than in the fetuses and the adults ($p=0.03$). The mRNA expression of GCS_l was highest in two neonatal patients (gestational age 26 weeks) who died of RDS. Also the highest specific activity (1.9 nmol/min/mg protein) in the lung was measured from one of the RDS patients. There was no statistically significant difference in the GCS activity in fetal, neonatal and adult groups.

The mRNA expression of GCS_h in the kidney was at the same level in all groups. The GCS_l expression was significantly lower in the fetuses than in the neonates and the adults ($p=0.02$), but GCS_l mRNA expression did not correlate with enzyme activity ($r=-0.03$). The GCS activity in the adults was statistically significantly higher than in the fetuses and the neonates ($p=0.03$), but this may be accounted for by the variation in the small number of samples.

Developmental and *in vitro* expression of CGL

The expression of CGL during development

CGL activity was only detected in adult liver tissue (68±9 nmol cysteine/h/mg protein), whereas activities in fetal, preterm and term neonatal liver samples were undetectable. In contrast, mRNA expression was detected from the 19th gestational week onwards, and the mRNA levels were comparable to those of adult liver samples. The expression of the two mRNA forms varied in parallel ($r=0.94$, $p<0.001$). Since in the ribonuclease protection assay the length and the specific activity of the probe affect the intensity of the signal, exact quantification of mRNA is not possible by this method. However, the results of the RT-PCR suggest that the longer form is more abundant in the liver throughout development.

Splice variation on cystathionine g-lyase

Amplification of the 5’ intron from genomic DNA resulted in ca. 2.3 kb PCR fragment, in which the first 93 nt from the 5’ end as well as 71 nt from the 3’ end were similar to the reported cDNA sequence (Lu et al. 1992). A BLAST sequence similarity search of the GenBank database did not show similarity to any other known nucleotide sequences. At the putative exon-intron boundaries, consensus U2-type GT-AG 5’ donor and acceptor sequences (Sharp and Burge 1997) were found (Figure 7).

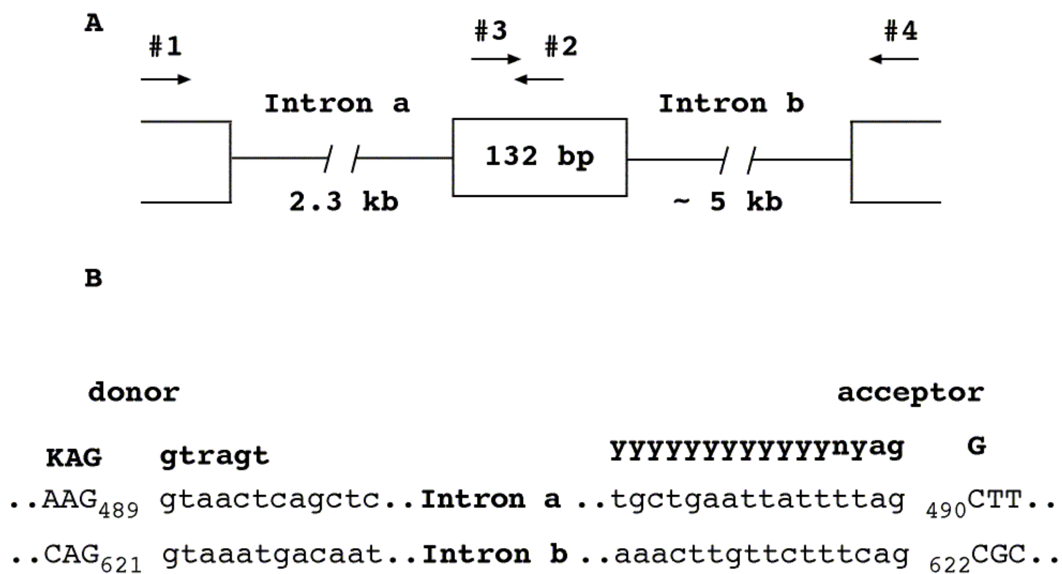

Figure 7. *(A) Exon-intron structure of the human cystathionine g-lyase gene at the site of splice variation. Exons are indicated as empty boxes and introns as thin lines. Primer positions are indicated by arrows numbered as follows: #1 and #2: The forward and reverse primers for amplification of intron a, #3 and #4: The forward and reverse primers for amplification of intron b. (B) Exon-intron junctions of the human cystathionine g-lyase gene. Sequences flanking exon-intron junctions are aligned with consensus splicing sequences. K indicates a or c, r indicates either purine (a or g), y indicates either pyrimidine (c or t), and n indicates any base. Exon sequences are in uppercase letters and intron sequences in lowercase letters. The intron positions in cDNA are shown as subscript numbers.*

Of the 5 kb DNA fragment resulting from the amplification of the 3' intron, 108 nucleotides at the 5' end and 41 nt from the 3' end were similar to the reported cDNA sequence and to no other sequence found in a BLAST search. The putative exon-intron junctions were consensus sequences (Figure 7) (Sharp and Burge 1997). The fragment missing in CGL-S appears to be coded by a single exon as the two genomic PCR fragments were overlapping.

Enzyme activity in 293T cells transfected with different cystathionine γ-lyase isoforms

There was a 1.5-fold increase in CGL activity 293T cells transfected with pcDNA-CGL-L compared to cells transfected with an empty pcDNA3 vector (Figure 8). Transfection with pcDNA-CGL-S did not cause any increase in activity. Although the change from basal activity was small, it was consistent and reproducible. Efficient transcription of both isoforms in transfected cells was verified by Northern blotting. The CGL mRNA expression of cells transfected with pcDNA-CGL-L and pcDNA-CGL-S was 7-fold and 11-fold, respectively, in comparison with basal CGL mRNA expression of mock-transfected cells. The β-galactosidase activities of the cells co-transfected with β-galactosidase expression vector were of the same order.

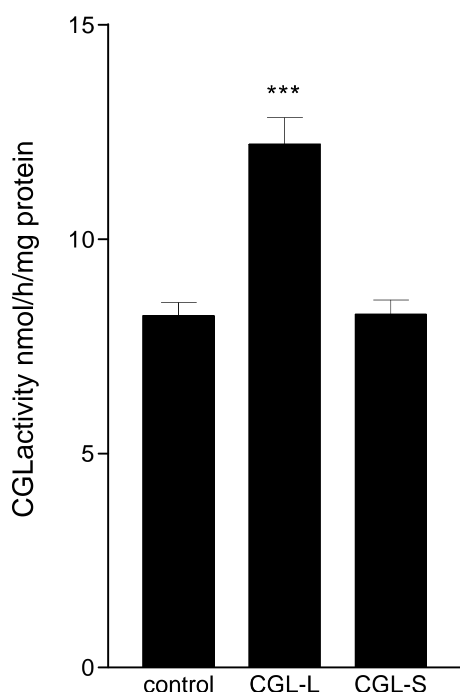

Figure 8. *Total cystathionine γ-lyase activity in 293T cells transfected with the two CGL isoforms.*
*Control cells were transfected with 6 μg of an empty pcDNA3 vector; CGL-L, cells transfected with 6 μg of pcDNA-GCL-L; CGL-S, cells transfected with 6 μg of pcDNA-GCL-S, N=11 in each group. Samples are from four independent experiments. Mean ± S.E.M. is given. Significance compared with control cells: *** P<0.001.*

Glutathione metabolism in the spontaneously hypertensive rat

The effect of NOS inhibition on enzymes involved in GSH metabolism in the kidney

L-NAME treatment led to a significant down-regulation of GCS activity (Figure 9). The reduction was largest in the high-sodium group. The GCS activity correlated negatively with the systolic blood pressure at the end of the follow-up period ($r = -0.60$, $p < 0.001$) and with the change in systolic blood pressure during the study ($r = -0.52$, $p < 0.001$).

L-NAME treatment had no effect on kidney GPx activity in any salt group (Table 4). Glutathione reductase activities were lower in rats treated with L-NAME, and this difference was statistically significant in the group receiving 1.1% NaCl in their diet.

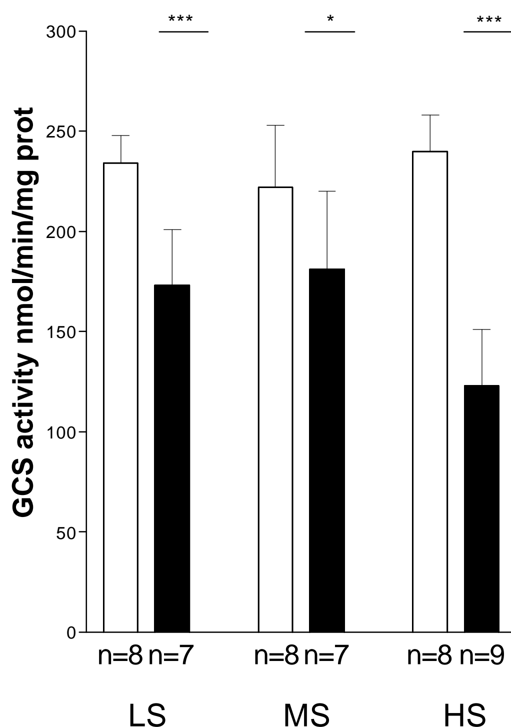

Figure 9. *g-glutamylcysteine synthetase activity in kidneys of spontaneously hypertensive rats treated with (solid bars) or without (open bars) N^{w}-nitro-L-arginine methyl ester and receiving a diet of variable NaCl content (LS=NaCl 0.2%, MS=NaCl 1.1%, HS=NaCl 6.0%), n indicates the number of animals in each group. Mean ± SD is given. * p < 0.05, *** p<0.001.*

The effect of NO$^{\bullet}$ donor on enzymes involved in GSH metabolism in the kidney

Isosorbide-5- mononitrate treatment did not have an effect on the renal GCS activity (190 ±35 and 191 ±36 nmol/min/mg protein with and without IS-5-MN, respectively) in the SHR. Also GPx and GR activities were unaffected by this treatment (Table 4).

Enzymes involved in GSH metabolism in SHR in comparison to WKY

In the kidney, the increases in GR and GCS activities, seen in both low salt and high salt SHR groups compared to WKY, did not reach statistical significance ($p=0.07$ and $p=0.11$, respectively). GPx and G6PDH activities were similar in all groups.

In the left ventricle of the heart, GPx, GR, and G6PDH activities did not differ between the groups (Table 5). GCS activity was below the detection limit of our assay. In the liver, the activities of GCS, GR and G6PDH were similar in all groups, whereas GPx was significantly lower in SHR.

Table 4. *Glutathione peroxidase (GPx), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH) activities in spontaneously hypertensive rats treated with L-NAME or isosorbide 5-mononitrate (IS-5-MN). *: $p<0.01$ with respect to control salt group.*

	GPx	GR	G6PDH
0.2% NaCl	46.5±6.0	88.9±13.6	15.8±0.8
0.2% NaCl + L-NAME	36.0±8.1	78.1±8.6	17.0±1.5
1.1% NaCl	45.7±6.4	89.6±6.1	15.3±1.0
1.1% NaCl + L-NAME	42.5±2.5	73.3±7.4*	17.5±1.4*
6.0% NaCl	43.8±5.0	86.8±8.0	15.3±1.0
6.0% NaCl + L-NAME	45.6±12.1	75.9±13.8	17.8±2.0*
6.6% NaCl	77.3±11.3	69.6±6.8	
6.6% NaCl + IS-5-MN	80.8±12.7	71.5±8.0	

Table 5. *Enzymatic activities of **g**-glutamylcysteine synthetase (GCS), glutathione peroxidase (GPx), glutathione reductase (GR), and glucose-6-phosphate dehydrogenase (G6PDH) in heart and liver homogenates. WKY: Wistar-Kyoto controls (n=8), SHR LS: spontaneously hypertensive rats on 0.3% NaCL diet (n=7), SHR HS: SHR on 2.6% NaCl diet (n=7). All data are mU/mg protein, and mean±S.D. are given. **: p<0.01.*

Group	Tissue	GCS	GPx	GR	G6PDH
WKY	Heart	n.d.	33.1±9.2	6.3±1.9	3.3±1.0
	Liver	9.1±1.7	119.3±17.9	47.3±12.3	9.9±2.5
SHR LS	Heart	n.d.	34.1±6.1	6.1±2.3	3.3±1.9
	Liver	8.7±1.1	89.1±36.1**		45.3±3.8
	8.5±1.5				
SHR HS	Heart	n.d.	33.7±2.3	6.5±1.5	2.5±1.1
	Liver	8.0±1.0	100.2±14.4**	45.4±3.0	8.9±1.7

Blood pressure

During the follow-up period of 6 weeks, the blood pressure in SHR increased 44±10 mmHg and 59±5 mmHg on low- and high-salt diets, respectively, whereas in control rats the blood pressure did not change. The increase in blood pressure in the high-salt group was significantly higher ($p<0.01$) than in the low-salt group.

DISCUSSION

The expression of enzymes involved in GSH synthesis during human development

The expression of γ-glutamylcysteine synthetase

Given the multitude of GSH-related cellular functions and especially the important role of GSH in cell proliferation (Cotgreave and Gerdes 1998), it is likely that the enzymes needed for GSH synthesis are expressed from early stages of embryonic development. Indeed, GCS has been shown to be expressed at the early head-fold stage (day 9.5) in rats (Trocino et al. 1995). Interestingly, high glucose concentration causes a decrease in its expression and a subsequent decrease in GSH concentration, which is associated with increased incidence of embryonic malformations and growth retardation. These effects are almost completely reversed by a GSH ester. Although the cDNAs for both subunits of mouse GCS have been cloned (Reid et al. 1997), GCS or GSH synthetase knock-outs have not been described. It is highly likely that such knock-outs are embryonically lethal. Mice lacking MTF-1, who have diminished hepatic expression of GCS, die *in utero* with hepatic failure (Gunes et al. 1998). In human liver (Rollins et al. 1981) and erythrocytes (Lestas and Rodeck 1984), GCS activities are at the same level in fetuses as adults, supporting the notion that GSH synthetic capacity is mature at early developmental stages. However, as GCS may be differentially regulated in various tissues, it is of importance to investigate the expression of GCS in those tissues that are critically exposed to ROS, such as the lung. The low concentration of GSH in the epithelial lining fluid of preterm newborn infants (Grigg et al. 1993) may reflect the immaturity of the lung to synthesize GSH efficiently.

Our results show that GCS mRNA expression and activity do not differ appreciably between fetal, neonatal and adult groups in the tissues studied. The highest GCS activity was measured in the liver, and the activities in the lung and in the kidney were lower but similar. As GCS is expressed and active in these tissues already in the second trimester, GCS appears to be fully operative also in preterm infants. It is of interest that GCS activity is two orders of magnitude higher in rat than in human kidney (Neuschwander-Tetri et al. 1997). This may explain why GCS activity is drastically increased postnatally in rats and mice (Tsui and Yeung 1979, Harman et al. 1990), but not in humans. Furthermore, γ-GT activity is also much higher in rat than in human kidney tissue (Hinchman and Ballatori 1990). These differences in tissue-specific expression of GCS and γ-GT between humans and rodents reflect differences in inter-organ GSH metabolism, as the kidney rather than the liver is the principal site of GSH salvage in rats.

The in vitro and developmental expression of cystathionine γ-lyase

Cystathionine γ-lyase activity (Sturman et al. 1970) has been shown to be absent from human fetal liver. As two mRNA forms of CGL have been found in human liver, it was postulated that changes in the relative expression of the two isoforms causes the postnatal increase in activity (Lu et al. 1992). It was proposed that the expression of the longer isoform would dominate during the fetal period and the protein coded by it would be inactive. Later on, the expression of the shorter mRNA form would increase causing the postnatal increase in activity. However, the shorter form of CGL lacks 44 amino acid residues, including the catalytically indispensable residues Asp187 and Asn188, which are conserved in all members of the γ-family of PLP dependent enzymes (Steegborn et al. 1999). This suggests that the shorter form would be inactive.

In our study, only those cells transfected with the cDNA corresponding to the longer form of CGL had increased enzymatic activity, although both mRNAs were efficiently transcribed. Transfection with the shorter form neither increased nor decreased the basal CGL activity. The deletion of the shorter form is at the catalytic area, while those residues interacting with the other subunits of the CGL tetramer remain intact. Therefore, it is likely that the shorter form would interact with the longer form, but does not inhibit its activity. However, the missing fragment in the shorter form of GCL would necessitate major movements of the secondary structure elements, which may result in a considerable alteration of the tertiary structure. Futhermore, as we have no means to detect CGL protein, it is possible that CGL-S protein is degraded rapidly, as has been shown for rat cystathionine β-synthase (Roper and Kraus 1992). Taken together, our findings do not support the postulated regulatory function of GCL-S.

In the present study, CGL activity was only detected in adult liver tissue, whereas activities in fetal, preterm and term liver tissues were undetectable. This is in accordance with an earlier report (Sturman et al. 1970). In contrast, strong mRNA expression was detected from the 19th gestational week onwards. That the mRNA expression of both subunits varies in parallel and the longer form is predominant throughout development further reinforces the notion that the postnatal increase in the CGL activity is not regulated by the change in the relative expression of the two isoforms. Although the discrepancy between CGL mRNA expression and enzyme activity could be accounted for by an inhibitor present in the crude extracts of fetal liver, or by CGL protein that is catalytically inactive, earlier studies do not support these views (Sturman et al. 1970, Gaull et al. 1972, Pascal et al. 1972). Rather, a likely explanation is post-transcriptional regulation of CGL gene expression, as has been shown for a number of enzymes during the development of the liver (Das et al. 1996, de Groot et al. 1987, de Groot et al. 1986, Hryb and Feigelson 1983).

Therapeutic possibilities of supplementation of GSH or its precursors

Theoretically, increasing intracellular GSH would be a rational approach to antioxidant therapy. As GSH itself is not efficiently transported into most cells, supplementation of GSH precursors, preferably the limiting substrate cysteine, would be more feasible. This is especially relevant to preterm neonates, who cannot synthesize cysteine themselves due to absent γ-cystathionine activity. However, cysteine is not very stable in intravenous solutions, and it has a narrow therapeutic window (Anderson and Meister 1987). Therefore, cysteine precursors could be used to augment cellular GSH.

N-acetylcysteine (NAC) has a thiol group that can directly scavenge certain oxidants. However, its major antioxidant effect is thought to be based on deacylation to cysteine, which is then used for GSH synthesis (Moldeus and Cotgreave 1994). NAC given intravenously is the established treatment for acetaminophen (paracetamol) poisoning (Vale and Proudfoot 1995). It is also used orally as a mucolytic agent (Grassi and Morandini 1976). There are a number of animal studies showing beneficial effects of NAC in various animal models of lung injury (Bernard et al. 1984, Leff et al. 1993, Sprong et al. 1998), but also opposite results have been reported (van Klaveren et al. 1997). In preterm guinea pigs, NAC ameliorates hyperoxic lung injury (Langley and Kelly 1993), but in mouse lung it does not protect against the detrimental effects of hyperoxia on pulmonary growth (Wilborn et al. 1996). In clinical studies, intravenous NAC have been shown to improve lung function in acute respiratory distress syndrome (ARDS) in some studies (Suter et al. 1994, Bernard et al. 1997, Jepsen et al. 1992), but others failed to see any effect (Domenighetti et al. 1997, Laurent et al. 1996). Given orally, NAC did not produce a sustained increase in GSH levels sufficient to increase the antioxidant capacity of the lungs (Bridgeman et al. 1994). In conclusion, both experimental studies with adult and preterm animals and clinical studies with adult patients have shown somewhat contradictory results, and no clear consensus exists, as to whether or not NAC is beneficial in the treatment of experimental or clinical lung injury.

With respect to preterm neonates, antioxidant therapy by NAC is an especially attractive option because of their relative cysteine deficiency. However, there are several potential problems. Nothing is known about the ability of preterm neonates to deacetylate NAC, and it is possible that their ability to deacetylate NAC and to utilize liberated cysteine for GSH synthesis is limited. Nevertheless, NAC may increase intracellular GSH through reduction of extracellular cystine to cysteine, thus augmenting cellular uptake of this amino acid. Moreover, thiols are strong reductants able to reduce transition metal ions, which participate in Fenton chemistry. They can also react to form reactive and potentially toxic thiyl radicals (Munday 1989). In addition, optimal function of a number of molecules such as cell membrane receptors and proteins involved in signal transduction may require a balance between oxidized and reduced thiols. Interference with the homeostasis

of these redox-sensitive pathways by excess amounts of reductants may alter the normal cell signaling that occurs upon oxidative stress (Cross et al. 1994).

Thiazolidines are potentially useful compounds for cysteine delivery. L-2-oxothiazolidine-4-carboxylate (OTC) is readily taken up by cells and metabolized by cellular 5-oxoprolinase to cysteine (Meister 1991). The efficacy of orally given OTC against pulmonary oxygen toxicity has been shown in protein-energy malnourished rats (Taylor et al. 1992). It has also been shown to improve the clinical outcome of ARDS patients (Bernard et al. 1997). OTC is not a thiol itself, so theoretically it does not have the extracellular adverse effects attributed to the thiol group that NAC might have. The clinical use of OTC is limited because it is not a registered drug. Futhermore, as the ontogeny of 5-oxoprolinase has not been studied, it is remains to be established whether or not preterm infants are able to metabolize it to cysteine.

Limitations of the study

All studies using human samples are fraught with technical and methodological difficulties. In our study, there was large variation in enzyme activities and mRNA levels between different individuals. This may in part be due to the fact that the period between the time of death and autopsy was variable, although we did not detect any correlation between the length of this time and enzymatic activity or mRNA expression. All infants were treated in intensive care units for up to five days before death, and all but one received oxygen therapy. Since both GCS mRNA expression and activity have been shown to be induced upon oxidative stress in lung epithelial cells (Shi et al. 1994, Tian et al. 1997), the upregulation of GCS_l in the lungs of 26 wk infants may well be caused by oxygen therapy. Thus, all the changes observed in mRNA levels do not necessarily reflect alterations of gene expression during development, but may be caused by disease processes or therapeutic interventions.

Glutathione metabolism in experimental hypertension

The interplay of $NO^{\bullet}$ and GSH in vascular function

There is ample evidence of a close interaction between $NO^{\bullet}$ and GSH metabolism. *In vitro*, $NO^{\bullet}$ increases intracellular GSH concentration by inducing GCS, as well as increasing cellular cystine uptake (Moellering et al. 1998, Moellering et al. 1999a, Li et al. 1999). Conversely, GSH is needed for proper function of NO synthases. In purified preparations of iNOS and nNOS, glutathione is needed for full enzyme activity (Stuehr et al. 1990, Hofmann and Schmidt 1995). Furthermore, $NO^{\bullet}$ synthesis is impaired in endothelial cells depleted of GSH (Ghigo et al. 1993), but increased in endothelial cells treated with NAC (Ramasamy et al. 1999). Thus $NO^{\bullet}$ and GSH synthesis appear to be regulated in a reciprocal manner.

In the vasculature, $NO^{\bullet}$ has a wide range of physiological functions, including inhibition of leukocyte adherence, chemotaxis, and vascular smooth muscle cell proliferation (Lane and Gross 1999). Intracellular thiol status appears to play a role in all of these effects. This is relevant to the end-organ damage due to hypertension, in which vascular smooth muscle cell proliferation and monocyte infiltration occur. Thiol antioxidants appear to have similar anti-inflammatory and antiproliferative effects to $NO^{\bullet}$ (Henkel et al. 1993, Wung et al. 1997, Du et al. 1999). In this respect, it is of interest that marked increases in monocyte infiltration into coronary vessels as well as in NF-κB activation and MCP-1 expression caused by chronic NOS inhibition in rats are effectively reversed by intraperitoneal administration of NAC (Usui et al. 2000).

In our study, chronic inhibition of NOS by L-NAME led to a down-regulation of GCS by 50% in the high salt group. Relatively little is known about the correlation of the level of GCS down-regulation and glutathione content *in vivo*. In a study by Drew *et al.* (Drew and Miners 1984), the effect of GCS inhibition by BSO on kidney GCS activity and GSH levels was studied in mice. From buthionine sulfoximine dose-response curves it could be estimated that down-regulation of GCS by 50% resulted in a reduction of GSH by 50%. Although the kidneys of adult mice appear to be unaffected by this degree of GSH deficiency for three weeks (Meister 1991), it is likely that in the case of secondary kidney damage due to hypertension, in which ROS appear to be a significant contributor, GSH depletion of this magnitude increases the damage.

It has to be emphasized that while GSH is the major intracellular thiol, many complementary molecules and systems exist in the cell which may compensate for the loss of GSH. Ascorbic acid, which is present in millimolar quantities, can replace GSH (Meister 1994). Catalase may substitute for the loss of GSH in the disposal of hydrogen peroxide. With respect to thiol redox-signaling, other redox-active molecules, such as thioredoxin, may compensate for GSH (Arrigo 1999). While the pharmacological effects of GSH augmenting drugs are not easily discerned, it would be interesting to see whether other drugs such as GSH ester or OTC could provide protection against the detrimental effects of chronic NOS-inhibition that is seen in the kidney of SHR, in the similar manner to NAC (Vaskonen et al. 1997, Usui et al. 2000).

There are a number obvious limitations in our study. Firstly, we did not measure kidney GSH levels. Thus, decrease in GCS regulation could possibly be due to down-regulation by high GSH levels. However, this seems unlikely, since inhibition of $NO^{\bullet}$ synthesis by L-NAME is associated with increased production of ROS both *in vitro* and *in vivo* (Niu et al. 1994, Usui et al. 1999, Usui et al. 2000). Secondly, nitrosoglutathione and other nitrosothiols were not measured, and thus their role in $NO^{\bullet}$ storage and transport is not known. Thirdly, the kidney has all three forms of NOS, and their specific functions and localizations are not fully elucidated. Therefore, inhibition by a non-specific NOS inhibitor and

measuring activity from total kidney homogenates does not provide information about the role of different NOS isoforms in the regulation of GSH synthesis, nor do we gain knowledge about the effect of NOS inhibition on GSH synthesis in the various cells in the kidney. Furthermore, given the complex roles of $NO^{\bullet}$ in the regulation of renal homeostasis such as sodium excretion, much of which is yet to be characterized in detail, it is not easy to envision what impact the inhibition of GSH synthesis would have on these mechanisms leading to renal hypertension.

The role of GSH in hypertension

There are relatively few reports on glutathione metabolism in human or experimental hypertension. When SHR rats have been compared to WKY, GPx activity has been reported to be either higher (Cabell et al. 1997), similar (Yuan et al. 1996) or variable depending on age (Batist et al. 1989), and hepatic GPx activity lower (Kitts et al. 1998) in SHR in comparison to WKY. The discrepancy may be at least partly be accounted for by differences between the ages of the rats in different studies, as it has been shown that GPx activity in myocardium decreases with advancing age (Batist et al. 1989).

Our results show that kidney GCS and GR activities tend to be higher in SHR than WKY, which implies an enhanced capacity to produce reduced GSH. It is of interest that even a small, 1.2-1.5 fold increase in GCS activity causes a 2-4 fold increase in intracellular GSH levels (Shi et al. 1994, Mulcahy et al. 1997). Considering the millimolar concentration range of intracellular GSH, GCS induction of this magnitude provides efficient protection against increased oxidative stress.

GR regulation under oxidative stress is not very well characterized. While it is known that GR is not increased upon exposure to hyperoxia in the lung (Pietarinen-Runtti et al. 1998), nor is it affected by cytokines known to cause increased oxygen radical formation (Kinnula et al. 1995), there may be tissue-specific differences in the regulation of GR. In the kidney, an increase in GR activity may provide an additional mechanism to maintain reduced GSH levels.

In the liver, GPx was lower in SHR than in WKY, which is in accordance with earlier studies (Cabell et al. 1997, Kitts et al. 1998). SHR liver has been shown to be more susceptible to GSH depletion by hydrogen peroxide (Kitts et al. 1998). However, decrease in liver GSH does not necessarily lead to increased oxidative damage (Kitts et al. 1998). Furthermore, as renal and cardiac damage are hallmarks of secondary damage caused by hypertension, and the liver is not affected, it is likely that the small decrease seen in our study in the liver GPx activity is of minor importance.

CONCLUSIONS

The main conclusions of this thesis are as follows:

1) In terms of activity and and mRNA levels, the expression of the rate-limiting enzyme of glutathione synthesis, γ-glutamylcysteine synthetase, is higher in the liver than in the lung and kidney, but does not vary appreciably between fetal, neonatal and adult samples. This indicates that glutathione synthetic capacity of the preterm neonate is not limited by the lack of this enzyme.
2) The mRNA expression of both the longer and shorter cystathionine γ-lyase isoform is abundant from the 19th gestational weeks onwards, whereas the enzyme activity is only present in adult liver. There is a close correlation between the mRNA expression of both isoforms during development. Only the longer isoform is enzymatically active when expressed in eukaryotic cells. These results suggest that cystathionine γ-lyase is post-transcriptionally regulated during development, and argue against the hypothesis that the subunit composition of cystathionine γ-lyase tetramer would regulate its activity during development.
3) Systemic inhibition of nitric oxide synthesis by L-NAME in spontaneously hypertensive rats down-regulates renal γ-glutamylcysteine synthetase activity, which is likely to aggravate secondary kidney damage in hypertension.
4) GSH recycling and synthesis appears not to be weaker in SHR in comparison to WKY. Rather, SHR tend to have higher renal GCS and GR activities implying better ability to increase reduced GSH. This effect may be an adaptive response to an increased oxidative stress.

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