

ROLE OF CYTOSOLIC GLUTATHIONE S- TRANSFERASES IN PROTECTION AGAINST ACETAMINOPHEN-INDUCED LIPID PEROXIDATION IN WEANLING RATS

A. ALLAMEH,* M. NIKSERESHT, AND F. KHEYRDOOSH

*From the Department of Biochemistry, Faculty of Medical Sciences,
Tarbiat Modarres University, P.O. Box 14155-4838, Tehran, I.R. Iran.*

ABSTRACT

Resistance of the weanling rat to acetaminophen (APAP)-induced hepatotoxicity is manifested with regard to a surge in APAP-glutathione (GSH) conjugate formation in the liver [Allameh et al. *Mech Aging Dev* 95(1997)71]. The present study was conducted to assess the role of this detoxification pathway in APAP-induced lipid peroxidation in the liver. Lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS) in rat liver homogenate was observed to be increased due to a decrease in hepatic cellular GSH concentration. Cellular GSH content was relatively lower in growing liver and further decreased in rats treated with either GSH-depleting agents or APAP, whereas adult animals under APAP treatments suffered significantly less depletion of GSH. APAP injection to weanling rats pre-treated with diethylmaleate (DEM) aggravated lipid peroxidation. Administration of a single large dose of APAP (500 mg/kg b.w.) to weanling rats, 3 h before sacrifice, which caused 46% GSH depletion, resulted in a 25% increase in lipid peroxidation. Pre-treatment of growing rats with DEM, 30 min before APAP, caused about 70% depletion in GSH content as a result of which there was a further increase (approx. 1.6 fold) in lipid peroxide formation (control: 37.40; experimental: 60.76 nmol malondialdehyde formation/g tissue). GSH S-transferase activity is not necessarily a determinant of APAP toxicity in adult animals. Unlike adults, in growing tissues the enzyme activity is induced single overdose of APAP. When these data are discussed in relation to our earlier study, it could be concluded that APAP-dependent induction of GSH S-transferases is responsible for increased APAP-GSH conjugate formation which facilitates inactivation of NAPQI as well as other toxic metabolites of lipid peroxidation.

MJIRI, Vol. 13, No. 3, 213-217, 1999

Keywords: Acetaminophen; hepatotoxicity; lipid peroxidation; weanling rat; glutathione; glutathione S-transferase.

* Corresponding author.

INTRODUCTION

Paracetamol or acetaminophen (APAP) is a commonly used mild analgesic known to cause liver necrosis in animals and humans at high single overdoses.¹⁻³

APAP is primarily metabolized by conjugation with glucuronic acid and sulfate in the liver of many animal species. A relatively small proportion of the drug (<4%) is bioactivated through the cytochrome P-450 route to a reactive intermediate known as N-acetyl-p-benzoquinoneimine (NAPQI).⁴⁻⁶ NAPQI in small amounts is detoxified as a glutathione conjugate. When the NAPQI is formed in large amounts, it causes the depletion of cellular glutathione stores and is thereby available to bind to cellular macromolecules leading to cytotoxic effects.^{7,8}

NAPQI can react in more than one way, and multiple mechanisms of hepatotoxicity have been proposed for APAP.^{9,11} Studies on the mechanism of APAP-induced hepatotoxicity show a good correlation between the extent of covalent binding of the APAP reactive metabolite to centrilobular hepatocytes and the extent of lethal cell injury.^{12,13}

NAPQI also possesses oxidative properties and may induce oxidative stress as an additional mechanism of cellular injury.¹⁰ Concerning APAP, oxidative stress is the cause of the following changes; lipid peroxidation, depletion of protein and non-protein-SH groups, and calcium homostasis.¹⁴⁻¹⁶ Lipid peroxidation is a process beginning with free radicals and leads to oxidation of the unsaturated fatty acids which are important components of the tissue phospholipids, particularly in the plasma membrane.¹⁷ Lipid peroxidation has been observed after APAP administration *in vivo* in mice and rats and *in vitro* in mouse and rat hepatocytes exposed to APAP.¹⁸⁻²²

It is well established that the pharmacokinetics of APAP in young children are different from that of adults. Earlier, clinical studies showed that in children under the age of 10 years, the predominant pathway for APAP detoxification is sulfation, and glucuronidation is less developed.²³ In growing rats, despite having an overall deficiency in drug metabolizing factors, such as cytochrome P-450, reduced GSH and cytosolic GSH S-transferase possess the capacity to efficiently detoxify hepatotoxic chemicals. Our recent work in developing rats has thrown some light on the mechanisms involved in detoxification of APAP in rat liver at early ages.

It is speculated that at early ages APAP-GSH conjugate formation increases in response to APAP and acts as a protective mechanism against APAP-induced hepatotoxicity.²⁴ This is induced in compensation of other systems which are generally believed to be underdeveloped at the early stages of life.^{25,26}

To our knowledge, the role of hepatic cytosolic GSH S-transferase activity and age-related differences in GSH depletion (due to APAP treatment) in the prevention of lipid

peroxidation (formation of malondialdehyde: MDA) has not been investigated.

MATERIALS AND METHODS

Chemicals

Acetaminophen, reduced glutathione (GSH), 1,4-dichloro-nitrobenzene (CDNB), thiobarbituric acid (TBA), diethyl maleate (DEM) and di-thiobis-nitrobenzoic acid (DTNB) were the products of Sigma Chemical Co. St. Louis, U.S.A. All other chemicals and reagents used in this study were from E. Merck, Germany.

Animals and treatments

Albino rats of Wistar strain were used in all experiments. Breeding and housing of the rats was carried out at the animal house of Tarbiat Modares University. Animals were maintained under controlled lighting and temperature and were fed a commercial pellet available locally.

All young adult and weanling rats used for the experiments were determined to be male. At the time of APAP treatment the age of adults was 10-12 weeks and weanling rats aged 22 ± 3 days. APAP was dissolved in ethanol/propylene glycol (1:4 by volume) and injected as a single i.p. dose to young adult and weanling rats. The volume of ethanol injected to each animal was the same at all dose levels.

Lipid peroxidation

Concentrations of TBARS were compared in liver homogenates prepared from weanling and adult rats treated with different doses of APAP viz. 250, 500 or 700 mg/kg b.w. Each rat was treated with a single i.p. dose of APAP. Matched age-groups were injected an equal amount of vehicle alone and considered as control groups. All the animals were sacrificed 3 hours after APAP treatment, and livers were removed immediately and processed for measuring liver malondialdehyde (MDA) concentration. Whenever indicated, rats were injected with 0.5 mL of DEM/kg b.w. 30 min before administration of APAP. The time-course effects of APAP on lipid peroxide formation in weanling rat livers was studied in animals treated with a single dose of 500 mg/kg and sacrificed at time intervals of 3, 24 and 48 hours after injection. TBARS in the homogenate were measured using TBA reagent.²⁷ The MDA formed was measured colorimetrically using an extinction coefficient of $1.56 \times 10^5 \text{ cm}^2$.

Hepatic GSH and GSH S-transferase

Liver samples obtained from control and APAP-treated rats were homogenized and processed for measuring non-protein-SH groups. Liver GSH was measured using Ellman's reagent according to the method described by Sedlak and Lindsay.²⁸

A small portion of the tissues were processed for

Table I. Effect of a single dose of acetaminophen on liver GSH, GSH S-transferase and MDA formation.

| Treatment | GSH ($\mu\text{mol/g}$ liver) | | GSH S-transferase (U/mg protein) | | MDA (nmol/g tissue) | |
|------------|-----------------------------------|------------------|-------------------------------------|------------------|------------------------|------------------|
| | Adult | Weanling | Adult | Weanling | Adult | Weanling |
| Control | 5.90 \pm 0.38 | 4.19 \pm 0.20 | 0.77 \pm 0.06 | 0.54 \pm 0.04 | 26.46 \pm 1.7 | 37.40 \pm 2.3 |
| APAP | 5.65 \pm 0.19 | 3.02 \pm 0.14* | 0.79 \pm 0.04 | 0.76 \pm 0.04* | 28.15 \pm 1.6 | 47.01 \pm 1.9* |
| APAP + DEM | 3.36 \pm 0.4* | 1.24 \pm 0.09* | 0.80 \pm 0.05 | 0.69 \pm 0.02* | 39.54 \pm 3.9* | 60.76 \pm 2.0* |

All rats received a single i.p. dose of APAP (500 mg/kg b.w.) prepared in ethanol: propylene glycol (1:4). DEM-treated animals were given 0.5 mL/kg b.w. 30 min prior to APAP injection. Control rats were given an equal volume of vehicle alone.

All parameters were measured 3 h after treatment. For details see *Materials and Methods*.

Data are expressed as mean \pm SEM, n=4. * p <0.05, significantly different from control group.

preparation of cytosolic fraction as described earlier.²⁹ The activity of liver cytosolic GSH S-transferase was measured according to the procedure of Habig et al. using CDNB as the substrate.³⁰ The protein concentration in cytosolic fractions was measured according to the procedure of Bradford,³¹ using bovine serum albumin as standard.

Histology

Two groups of weanling and adult rats were given a single i.p. dose of APAP (500 mg/kg b.w.) and killed 48 h later by decapitation. Small liver sections from the right lobes were placed into 10% freshly prepared formalin. The tissues were allowed to fix for 48 h before being dehydrated, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin for histopathological examination.

RESULTS

Table I shows that a single i.p. dose of 500 mg APAP/kg b.w. injected to adult rats has little effect on hepatic cellular GSH concentration at 3 hours, whereas APAP administered to weanling rats under similar conditions caused about 27% depletion in liver GSH concentration. APAP injection to DEM-pretreated rats caused about 43 and 70% depletion in cellular GSH content in weanling and adult livers respectively (Table I).

In growing liver, APAP-dependent changes in GSH (approximately 28% depletion) resulted in a 25.7% increase in lipid peroxidation (thiobarbituric acid reacting substances; TBARS). Lipid peroxidation was further increased (up to 62%) in weanling rats treated with DEM prior to APAP.

Time-course studies showed that the concentration of TBARS in liver is returning to normal values in experimental groups sacrificed either 24 or 48 hours after APAP injection.

The level of TBARS measured in liver homogenates obtained from control animals was greater in weanling rats as compared to that of adults (weanling: 37.40, adult: 26.46

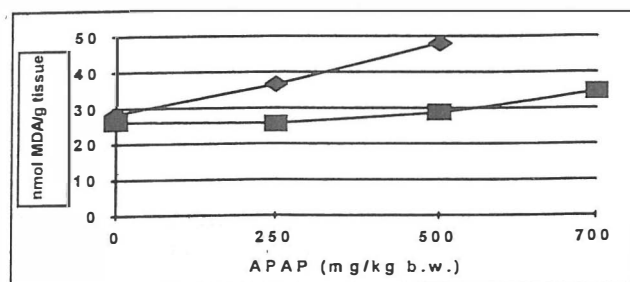


Fig.1. Dose-dependent effect of acetaminophen on lipid peroxidation in adult and weanling rat liver. Each point is the mean value of 4 analyses obtained from 4 separate livers either from a weanling { -○-○- } or young adult { -□-□- } rat. Animals were killed 3 h after administration of a single i.p. dose of APAP (mg/kg b.w.). Preparation of liver homogenate and measurement of MDA is as described in *Materials and Methods*.

nmol MDA/g tissue). Figure 1 shows that the difference in MDA formation in weanling rats treated with a single dose of either 250 or 500 mg APAP/kg was statistically different (p <0.05). Administration of a single dose of APAP, i.e. 700 mg/kg, to adults resulted in approximately 24% increase in lipid peroxidation in liver homogenate. Growing rats treated with an equal dose of the drug die within 2 h, the reason for which is not well understood. Histopathological examinations carried out on livers of these animals showed no gross change in these tissues.

APAP treatment alone of adult rats neither lowered GSH levels nor increased lipid peroxidation as compared to their control groups. In the case of adults, the concentration of TBARS was significantly increased when GSH concentrations were depleted with DEM treatment prior to APAP (Table I).

The role of hepatic cytosolic GSH S-transferase in modulation of lipid peroxidation induced by APAP in the two age-groups was investigated by measuring the enzyme activity in different experimental groups. A single overdose

of APAP resulted in a differential induction in hepatic cytosolic GSH S-transferase activity in the two age-groups. The enzyme activity in growing livers was increased significantly (approx. 40%) as compared to control animals, whereas in adult tissues the enzyme activity remained within normal range (Table I).

Histological observations

No evidence of hepatic necrosis was observed in light microscopy in weanling and adult rats treated with 500 mg APAP/kg.

DISCUSSION

APAP is a commonly available over-the-counter analgesic and a frequent cause of intentional and unintentional poisoning. After ingestion of a single APAP dose exceeding 140 mg/kg, children develop toxicity much less frequently than adults.³²

It has been reported that APAP is less toxic in newborn mice and rats than in older animals. The covalent binding of APAP to liver proteins has been shown to correlate with liver necrosis. Measurement of hepatic necrosis, APAP-protein binding and liver GSH depletion indicated that APAP was less toxic to neonatal mice than adult animals.³³ The data presented here confirm those reported by others^{33,34} and support the concept that the ability of newborn animals to detoxify reactive metabolites of APAP by GSH conjugation develops earlier than the development of the enzyme system responsible for the production of the toxic metabolite.

One major finding of our study is the observation that GSH conjugation in weanling rats is induced in response to APAP treatment. Age-related differences in the rate of APAP-GSH formation in the liver depends neither on cellular GSH concentration nor on the basal activity of liver GSH-transferase. Hence, a possible reason could be APAP-dependent induction in hepatic cytosolic GSH S-transferase which is confined to weanling rats (Table I). The GSH conjugation pathway catalyzed by GSH S-transferases is responsible for modulation of lipid peroxide formation which increased a few hours after administration of a single dose of APAP (Table I and Fig. 1).

The rate of lipid peroxide formation in adult and growing tissues differed according to the extent of GSH depletion. But the loss of GSH alone can not explain the toxicity of APAP, since classic GSH-depleting agents deplete GSH and increase the sensitivity of the cell to toxins, but do not cause toxicity by themselves.¹⁰

Following APAP treatment, adult rats suffered relatively less lipid peroxide formation. In contrast, TBARS formation in weanling rat liver has been increasing depending on the dose of the drug (Fig. 1). APAP treatment of growing rats, on one hand caused lipid peroxide formation in the liver, while on the other hand increased the activity of cytosolic GSH S-

transferases (Table I). When the concentration of TBARS has reached about 60 nmol/g tissue, the activity of GSH S-transferase was also induced to about 40%. Administration of APAP alone to adult rats failed to change any of the three parameters investigated, but there was a significant increase in the concentration of TBARS when GSH was at its minimum, i.e. 1.24 nmol/g tissue. A drop in the concentration of TBARS in liver homogenates prepared from immature rats killed 24 or 48 hours after APAP injection could be an explanation for histopathological results showing no damages in livers. Therefore it would seem that inducibility of liver GSH S-transferases in growing animals is a developmental process which could be considered as an alternative mechanism for protection of cellular macromolecules against APAP-induced liver damage.

A 40% induction in enzyme activity is probably limited to a particular class or rather isoforms of GSH S-transferases which are specifically induced at early ages.³⁵ The part played by hepatic microsomal GSH S-transferase in detoxification of APAP in immature liver is not well known, although it has been proved that in adult rats, this enzyme plays a major role in protection against liver damage by covalently binding to NAPQI.³⁶

ACKNOWLEDGEMENT

We thank Professor L.F. Prescott for his valuable suggestions.

REFERENCES

1. Boyer TD, Rouff LJ: Acetaminophen-induced hepatic necrosis and renal failure. *J Am Med Assoc* 218: 440, 1971.
2. Vermeulen NPE, Bassems RM, VanDer Straat R: Molecular aspects of paracetamol-induced hepatotoxicity and mechanism-based prevention. *Drug Met Rev* 24: 367, 1992.
3. Nelson SD: Mechanisms of the formation and disposition of reactive metabolites that can cause acute liver injury. *Drug Met Rev* 27: 147, 1995.
4. Miner DJ, Kissinger PT: Evidence for the involvement of N-acetyl-p-benzoquinone imine in acetaminophen metabolism. *Biochem Pharmacol* 28: 3285, 1975.
5. Dahlin DC, Miwa GT, Lu AYH, Nelson SD: N-acetyl-P-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. *Proc Natl Acad Sci USA* 81: 1327, 1981.
6. Harvison PJ, Guengerich GP, Rashed MS, Nelson DS: Cytochrome P-450 isozyme selectivity in the oxidation of acetaminophen. *Chem Res Toxicol* 1: 47, 1988.
7. Lauterburg BH, Mitchell YR: Toxic doses of acetaminophen suppress hepatic glutathione synthesis in rats. *Hepatology* 2: 8, 1982.
8. Prescott LF: Paracetamol overdose: pharmacological considerations and clinical management. *Drugs* 25: 290, 1983.
9. Mathews AS, Hinson JA, Roberts DW, Tomford NR:

- Comparison of covalent binding of acetaminophen and the regioisomer 3'-hydroxyacetanilide to mouse liver protein. *Toxicol Letters* 90: 77, 1997.
10. Hinson JA, Pumford NR, Nelson SD : The role of metabolic activation in drug toxicity. *Drug Met Rev* 26: 395, 1994.
 11. Holtzman JL: The role of covalent binding to microsomal proteins in the hepatotoxicity of acetaminophen. *Drug Met Rev* 27: 277-297, 1995.
 12. Black M: Acetaminophen hepatotoxicity. *Ann Rev Med* 35: 577-593, 1984.
 13. Jollow DJ, Mitchell JR, Potter WZ, Davis DC, Gillette JR, Brodie BB: Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. *J Pharmacol Expt Ther* 187: 185, 1973.
 14. Potter WZ, Davis DC, Mitchell JR, Jollow DJ, Gillette JR, Brodie BB: Acetaminophen-induced hepatic necrosis. III. Cytochrome P-450-mediated covalent binding *in vivo*. *J Pharmacol Expt Ther* 187: 203, 1973.
 15. Albano E, Rundgren M, Harvison PJ : Mechanism of N-acetyl-p-benzoquinone imine cytotoxicity. *Mol Pharmacol* 28: 301, 1985.
 16. Moore M, Thor H, Moore G, Nelson S: The toxicity of acetaminophen and N-acetyl-p-benzoquinoneimine in isolated hepatocytes is associated with thiol depletion and increased Ca²⁺. *J Biol Chem* 260: 1335 - 1340, 1985.
 17. Wendel A, Feuerstein S: Drug-induced lipid peroxidation in mice. I. Modulation by mono-oxygenase activity, glutathione and selenium status. *Biochem Pharmacol* 30: 2513, 1981.
 18. Younes M, Sause C, Singers CP, Lemoine R: Effect of desferrioxamine and diethyl dithiocarbamate on paracetamol-induced hepato- and nephrotoxicity. The role of lipid peroxidation. *J Appl Toxicol* 8: 261-265, 1988.
 19. Fairhurst S, Barber DJ, Clark B, Horton AA: Studies on paracetamol induced lipid peroxidation. *Toxicology* 23: 249-259, 1982.
 20. Albano E, Poli G, Charpotto E, Biasi F, Dianzani MV: Paracetamol-stimulated lipid peroxidation in isolated rat and mouse hepatocytes. *Chem Biol Interactions* 47: 249, 1983.
 21. Kyle ME, Miccadei S, Nakae D, Farber JL: Superoxide dismutase and catalase protect cultured hepatocytes from the cytotoxicity of acetaminophen. *Biochem Biophys Res Commun* 149: 889, 1987.
 22. Ozdemirler G, Aykac G, Uysal M, Oz H: Liver lipid peroxidation and glutathione-related defence system in mice treated with paracetamol. *J Appl Toxicol* 14: 297-299, 1994.
 23. Miller RP, Roberts RJ, Fischer LJ: Kinetics of acetaminophen elimination in newborn children and adults. *Clin Pharmacol Ther* 19: 284, 1976.
 24. Allameh A, Vansoun EY, Zarghi A: Role of glutathione conjugation in protection of weanling rat liver against acetaminophen-induced hepatotoxicity. *Mechanism of Aging and Dev* 95: 71, 1997.
 25. Dutton GJ: Biochemical Development of the Fetus and Neonate, In: Jones CT, (ed.), *Drug Metabolism and Development*. Amsterdam: Elsevier, p. 69, 1982.
 26. Behroozikha M, Saeedi M, Allameh A: Comparison of aflatoxin B1-DNA binding and glutathione conjugate formation by liver preparations from rats of different ages. *Cancer Lett* 66: 69, 1992.
 27. Ohkawa H, Oshishi N, Yagi K: Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 95: 351, 1979.
 28. Sedlak J, Lindsay RH: Estimation of total, protein-bound and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* 25: 205, 1968.
 29. Allameh A, Saxena M, Raj HG: Differential effects of butylated hydroxyanisole on metabolism of aflatoxin B1 *in vitro* by liver and lung microsomes. *Cancer Lett* 40: 49, 1998.
 30. Habig WH, Pabst MJ, Jakoby WB: Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. *J Biol Chem* 25: 7130, 1974.
 31. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248, 1976.
 32. Webster PA, Roberts DW, Benson RW: Acetaminophen toxicity in children, diagnostic confirmation using a specific antigen biomarker. *J Clin Pharmacol* 36: 397-402, 1996.
 33. Hart JG, Timbrell JA: The effect of age on paracetamol hepatotoxicity. *Biochem Pharmacol* 28: 3015, 1979.
 34. Green MD, Fischer LJ: Hepatotoxicity of acetaminophen in neonatal and young rats. *Toxicol Appl Pharmacol* 74: 125, 1984.
 35. Allameh A, Amini A, Farzami B: Differences in the activities and subunits of glutathione S-transferase in newborn and adult liver. *Proceedings of the 16th International Congress of Biochemistry and Molecular Biology, New Delhi, 1994*.
 36. Yonamine M, Aniyō Y, Yokomakura T, Koyama T, Nagmine T, Nakanishi H: Acetaminophen-driven activation of liver microsomal glutathione S-transferase of rats. *Jpn J Pharmacol* 72: 175, 1996.

