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This paper is dedicated to Professor Włodzimierz Ostrowski

2-Methyl-thiazolidine-2,4-dicarboxylic acid protects against paracetamol induced toxicity in human liver derived HepG2 cells*

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The effects of 2-methyl-thiazolidine-2,4-dicarboxylic acid (CP) on paracetamol-induced toxicity were investigated and evaluated in a human liver derived HepG2 cell line. Incubation of the cells with CP (2 mM and 10 mM) drastically attenuated the GSH and cysteine depletion caused by toxic concentrations of paracetamol (1 mM and 5 mM). When CP (10 mM) was introduced alone into the medium, the level of malondialdehyde and the reactive oxygen species were maintained at the control levels with a simultaneous increase of non-protein sulfhydryl in the cells. Thus, the results of our work prove that CP is a non-toxic precursor of cysteine and GSH, and successfully prevents paracetamol toxicity in HepG2 cells.

Paracetamol (APAP) is a commonly used analgesic and antipyretic drug which produces hepatic centrilobular necrosis when administered in high doses. The toxicity of APAP is believed to be due to the reactive metabolite *N*-acetyl-*p*-benzoquinone-imine (NAPQI) formed by the cytochrome P450 mixed function oxidase system [1] (Scheme 1). The participation of reactive oxygen species (ROS) in the pathogenesis of APAP toxicity was also demonstrated [2]. Glutathione, the major cellular non-protein thiol compound, plays a very important role in the detoxication of the reactive metabolites both, NAPQI and ROS [3, 4]. In therapeutic, low doses, APAP is detoxicated in hepatocytes in the reaction with active sulfate (PAPS), while NAPQI reacts with GSH and is subsequently

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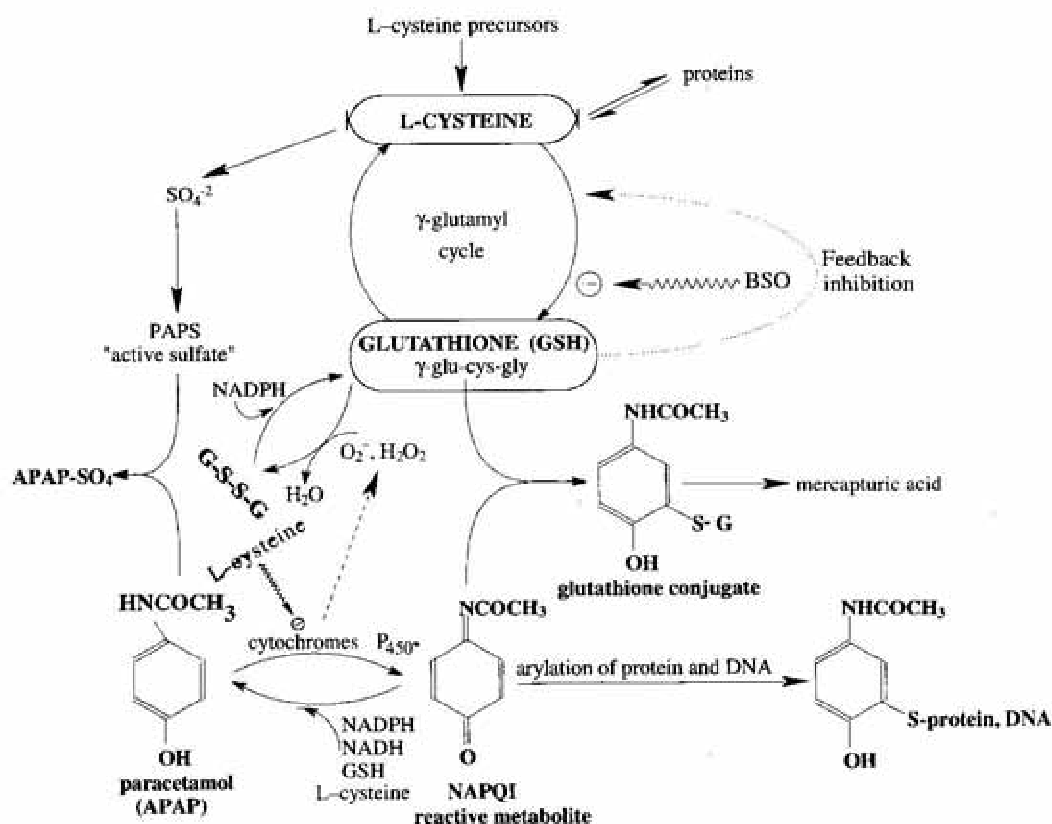
Abbreviations: APAP, paracetamol (acetaminophen); BSO, buthionine sulfoximine; CP, 2-methyl-thiazolidine-2,4-dicarboxylic acid; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); GSH, reduced glutathione; GS, glutamine synthetase; LDH, lactate dehydrogenase; MDA, malondialdehyde; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NPSH, non-protein sulfhydryl; PAPS, adenosine-3′-phosphate 5′-phosphosulfate; ROS, reactive oxygen species.



Scheme 1. A schematic representation of the relationship between paracetamol biodegradation and glutathione and L-cysteine metabolism.

excreted as mercapturic acid [5] (Scheme 1). On the other hand, in the case of an APAP overdose, the two cosubstrates become rapidly depleted, and the excess of NAPQI forms covalent bonds with DNA and proteins, which leads to cytotoxicity [6]. This means that in the course of the metabolism of toxic doses of APAP there occurs excessive "trapping" of the sulfur coming from the two cosubstrates: GSH and active sulfate. A consequence of the above events is the depletion of the pool of cysteine, which is an amino acid both limiting GSH biosynthesis in the cells [7] and providing sulfates. Thus, in order to prevent APAP toxicity one should introduce cysteine into the cells as an antidote. However, it is not possible to administer free cysteine due to its neurotoxicity [8]. Also, GSH alone cannot be employed as a drug since it is not transported across cellular membranes [9]. For this reason investigations have been undertaken on hepatoprotective properties of 2-methyl-thiazolidine-2,4-dicarboxylic acid (CP), a product of cysteine and pyruvate condensation (Scheme 2), which can supply the cells with free cysteine in a reverse reaction.

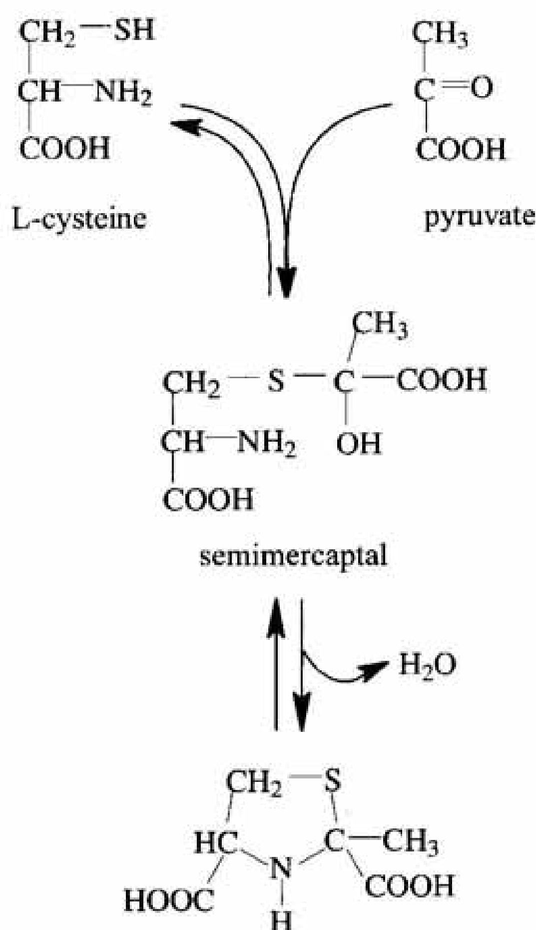
Our previous *(in vivo)* studies on this compound have determined its protective activity in ethanol [10] and paracetamol intoxication in mice [11].

A commonly used antidote to paracetamol intoxication is *N*-acetylcysteine [12]. Its toxicity although, it is regarded to be low, should not be neglected [13].

In the present study the protective action of CP against the toxicity of APAP has been studied *in vitro* on the human hepatoblastoma HepG2 cells. These cells retain drug metabolizing capabilities, including the cytochrome P450-dependent mixed function oxidases, and the activity of conjugating glucuronic acid and sulfate [14].

MATERIALS AND METHODS

Chemicals. 2-Methyl-thiazolidine-2,4-dicarboxylic acid (CP) was synthesized according to Schubert [15] in the Laboratory of Organic Synthesis of the Jagiellonian University (Cracow, Poland). L-Cysteine, reduced glutathione, dithiothreitol, NADPH, glutathione reductase, 5,5-dithiobis(2-ni-



Scheme 2. The non-enzymatic formation of thiazolidine-2,4-dicarboxylic acid from L-cysteine and pyruvate.

trobenzoic acid) (DTNB), 2,7-dichlorofluorofluorescein, hydroxylamine, Na-ADP, imidazole HCl, L-glutamine acid, γ-glutamyl-hydroxylamate, paracetamol, sodium arsenate and the lactate dehydrogenaese kit 340LD were obtained from Sigma (St. Louis, MO, U.S.A.). Ninhydrin was from Merck (Darmstadt, Germany), and 2,7-dichlorofluorescein diacetate was purchased from Molecular Probes (Eugene, OR, U.S.A.).

Cultures and incubation of HepG2 cells for toxicity studies. Hep-G2 cells were routinely grown in Dulbecco's modified Eagle's medium (Biochrom KG, Berlin) supplemented with 10% (v/v) fetal calf serum (Boehringer, Mannheim), 3 µg/ml insulin (Boehringer, Mannheim), 10 U/ml penicillin and 100 µg/ml streptomycin (Sigma). They were grown in a humidified atmosphere of 5% CO_2 and split 1:4 every 7 days (seeding density 5×10^6 cells/175 sq cm flask). The medium was renewed routinely 3–4 days after passage. The cells were collected from confluent cultures 7–10 days after being subculture for enzyme activity measurements as indicated in the Results.

For studies on paracetamol toxicity and the protective properties of CP, HepG2 cells were treated for 10 days with 1 mM APAP alone and with 1 mM APAP and 2 mM CP in the first experiment, and with 5 mM APAP alone and 5 mM APAP with 10 mM CP in the second experiment. The control cells were treated with 0.9% NaCl for the same period of time. In order to determine the effect of CP alone, in another experiment HepG2 cells were treated with 10 mM CP for 2 h. The viability of the cells in suspensions was assessed by Trypan B exclusion (typically > 90%).

The exponentially growing cells were collected by trypsinization, washed with 0.1 M phosphate buffer, pH 7.4, and the cells were homogenized in the above buffer (10^8 cells/1 ml end volume of homogenate). After homogenization a portion of the homogenate was immediately treated with the same volume of cold 5% trichloroacetic acid because HepG2 cells express a very high cell surface γ-glutamyltranspeptidase activity [16] which can break down the GSH released from the cells. In the CCl_3COOH supernatant, GSH levels were measured using the GSH-reductase recycling assay of Tietze [17], cysteine and cystine were determined according to Gaitonde [18] and NPSH was measured by the reaction with DTNB [19]. The homogenate served for determinations of lipid peroxidation measured as thiobarbituric acid positive reactants (malondialdehyde) according to Ohkawa *et al.* [20], as well as of reactive oxygen species according to Bondy & Guo [21], using 2,7-dichlorofluoroscein diacetate. The activity of glutamine synthetase was measured as described by Bondy & Guo [21] and the activity of lactate dehydrogenase (LDH) using the UV test from Sigma, No. 340LD.

Statistical significance of difference between the paracetamol and 0.9% NaCl, as well as between paracetamol and paracetamol + CP treated cells was calculated by the Student's *t*-test.

RESULTS

Preliminary studies carried out with human hepatoblastoma cells (HepG2) showed that CP introduced into the medium at a concentration of 10 mM (Fig. 1) did not affect

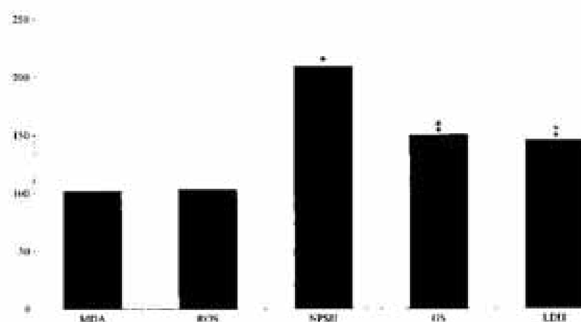


Figure 1. Effect of 2-methyl-thiazolidine-2,4-dicarboxylic acid (CP) at 10 mM concentration in medium on the levels of lipid peroxidation (MDA), reactive oxygen species (ROS), nonprotein sulfhydryl (NPSH), as well as the activities of lactate dehydrogenase (LDH) and glutamine synthetase (GS) in cultured HepG2 cells.

For details see Materials and Methods. The viability of HepG2 cells treated for 2 h with 10 mM CP was 100%. Control values (0.9% NaCl treated cells): MDA, 1.35 ± 0.036 nmol/mg protein; ROS, 45.66 ± 1.52 pmole/min per mg protein; GS, 0.42 ± 0.016 µmol/mg protein; LDH, 1.73 U/mg protein; NPSH, 18.00 nmol/mg protein. $*P < 0.05$; $**P < 0.001$

the process of lipid peroxidation; the MDA and ROS values were maintained at the control levels as compared to 0.9% NaCl treated cells. Simultaneously, the cell level of NPSH was increased, similarly as the activity of LDH and GS. The activity of the latter enzyme is regarded as particularly susceptible to peroxidative damage and arylation [21, 22]. Thus, these results mean that CP, as a precursor of cysteine, when introduced to the medium at a high, 10 mM concentration, seems to have a beneficial effect upon the cells and to prevent peroxidative damage.

Further studies on APAP toxicity and protective properties of CP (Fig. 2) demonstrated that 1 mM concentration of APAP in the medium resulted in an increase of the ROS level in the HepG2 cells and an increase of cystine content, whereas the levels of GSH and MDA were unchanged as compared to 0.9% NaCl treated cells. This means that the most sensitive, the increased ROS and cystine levels are the primary index of APAP toxicity. When administered simultaneously, 1 mM APAP and 2 mM CP resulted in a drop of cellular ROS, and a significant increase of GSH and cysteine concentrations as compared to APAP treated cells. At higher APAP concentration (5 mM) in the medium (Fig. 3) both MDA and ROS increased as compared to 0.9% NaCl treated cells, pointing to an intensified process of lipid peroxidation. At the same time the level of GSH was significantly decreased, free cysteine in the cells became undetectable, and cystine, its oxidized form, was increased. When the medium was supplemented simultaneously with 5 mM APAP and 10 mM CP, peroxidative damage was inhibited, the levels of MDA and ROS were lowered. A parallel increase of GSH and cysteine concentration values, well above the control levels, was also observed. At 1 mM APAP concentration (Fig. 2) no effect was observed on the activity of GS, and the slight drop in LDH activity could be corrected in the presence of 2 mM CP in the medium. A higher, 5 mM APAP concentration, resulted in a drop of LDH and GS activity which could not be counterbalanced by the presence of CP in the medium (Fig. 3).

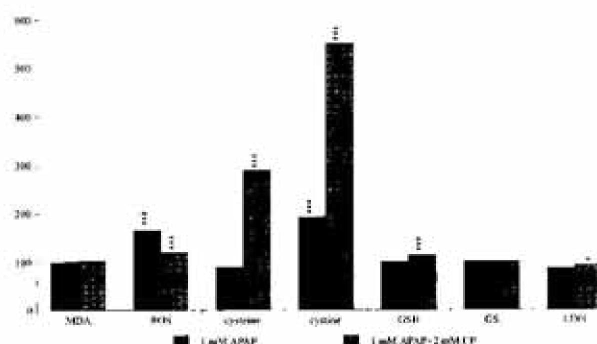


Figure 2. The protective effect of 2 mM concentration of 2-methyl-thiazolidine-2,4-dicarboxylic acid (CP) against the toxicity of 1 mM paracetamol (APAP) applied for 10-day to HepG2 cells culture.

For details see Materials and Methods. The viability of HepG2 cells treated for 10 days with 0.9% NaCl was 100% (control); 1 mM APAP the viability was 94% and of 1 mM APAP and 2 mM CP it was 97,5%. Control values (0.9% NaCl treated cells): MDA, 1.55 ± 0.049 nmoles/mg protein; ROS, 45.53 pmoles/min per mg protein; cysteine, 8.19 ± 0.82 nmoles/mg protein; cystine 0.29 ± 0.16 nmoles/mg protein; GSH, 20.00 ± 0.33 nmoles/mg protein; GS, 0.40 ± 0.012 µmoles/mg protein; LDH, 2.16 ± 0.094 U/mg protein. $*P < 0.05$; $**P < 0.02$; $***P < 0.001$

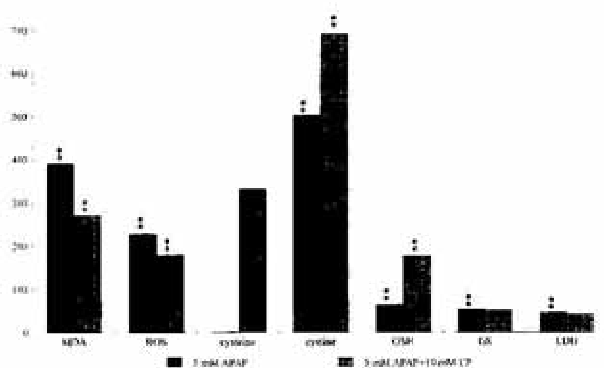


Figure 3. The protective properties of 10 mM concentration of 2-methyl-thiazolidine-2,4-dicarboxylic acid (CP) against the toxicity of 5 mM paracetamol (APAP) in the medium after a 10-day culture of HepG2 cells.

For details see Materials and Methods. Control values (0.9% NaCl treated cells): MDA, 2.70 ± 0.14 nmoles/mg protein; ROS, 90.19 ± 3.46 pmoles/min per mg protein; cysteine 3.76 ± 0.38 nmoles/mg protein; cystine, 0.36 ± 0.003 nmoles/mg protein; GSH, 19.30 ± 0.62 nmoles/mg protein; GS, 0.394 ± 0.013 µmoles/ mg protein; LDH, 2.05 ± 0.20 U/mg protein. $^{*}P < 0.05$; $^{**}P < 0.001$

DISCUSSION

Toxic doses of APAP depress the level of GSH in hepatocytes what is a prerequisite for the manifestation of toxicity of this drug [6]. The low level of GSH leads to excessive amounts of electrophilic NAPQI, which causes arylation of protein and oxidation of thiol groups within the cell [2]. In turn, this phenomenon may cause a loss of protein integrity and enzyme function, leading to cell death [1, 3].

It has been suggested that continuous GSH consumption for detoxication of NAPQI to thioeter glutathione-S-APAP and in the reaction of APAP with sulfates to APAP-SO_4 (Scheme 1), leads to a decrease endogenous cysteine level [23]. APAP toxicity is thus related to trapping of sulfur coming from these both cosubstrates and in consequence perturbing the cysteine pool. It has been demonstrated that APAP in toxic doses results in growth inhibition in rats as a consequence of the observed cysteine deficit [23].

Under normal physiological conditions the rate of GSH synthesis is governed by the availability of its precursor L-cysteine [7] (Scheme 1). The cysteine deficit related to APAP metabolism, exerts an inhibitory effect on GSH and sulfate biosynthesis [24, 25]. The concept that elevated levels of hepatic GSH can protect the liver against hepatotoxicity has encouraged the search for substances which — as cysteine precursors — would be able to increase intracellular levels of GSH and sulfate [24, 25].

In our previous investigations we found CP to have protective properties *in vivo* in ethanol and paracetamol intoxication in mice [10, 11].

The results of the present study show that HepG2 cells exposed to APAP and CP were able to take up CP, to use the cysteine supplied for GSH biosynthesis and in this way prevent peroxidative damage. Thus the protective mechanism of CP consists in supplementation of cysteine. For the HepG2 cells CP appears to be as a source of free cysteine, an excellent component of the medium. Cysteine supplementation makes possible GSH and active sulfates resynthesis, the two cosubstrates necessary for APAP detoxication [4, 7]. GSH has a protective effect not only in reactions of conjugation with NAPQI (Scheme 1) but also, as it has been demonstrated [25], in direct reduction of NAPQI by GSH. In addition, free cysteine is also able to directly reduce NAPQI back to APAP [26], thus diminishing the amount of the substrate for GSH conjugation. The increase of its oxidized form — cystine — triggered by APAP and observed in our studies provides a confirmation of this phenomenon (Figs. 2 and 3).

This is why constant supplementation of the cysteine deficit resulting from APAP metabolism seems to be a significant factor in prevention of APAP intoxication. Thus CP was found to be very effective as a cysteine precursor in HepG2 cells (Figs. 1, 2 and 3). The effect of CP as a source of cysteine for HepG2 cells exposed to APAP is also beneficial because due to an enzymatic defect, the cells are unable to use methionine as a source of cysteine [27, 28].

Summing up, the protective potential of CP against APAP toxicity resulting from cysteine supply makes it possible to detoxicate APAP and its electrophilic metabolite NAPQI. Yet it should be strongly emphasized that any intervention leading the cells to supplying the cells with cysteine is beneficial

only when the amino acid is provided in an optimal amount and at an optimal rate. It should be remembered that the level of GSH and cysteine, which like other compounds of a great biological importance, are strictly controlled within the cell through a feedback mechanism [29]. As demonstrated by the present study, the levels of cysteine and GSH are greater than the control values (in 0.9% NaCl treated cells) after administration of both CP alone (Fig. 1) and CP together with APAP (Figs. 2 and 3). This means that concentration of both GSH and cysteine in cultured HepG2 cells are markedly lower than optimal values resulting from the regulatory feedback mechanism. This can be also surmised on the basis of the fact that a high level of GSH and cysteine is a characteristic property of cells with neoplastic transformation (which also concerns HepG2 cells) [30]. Takamura *et al.* [31] demonstrated cysteine to markedly activate the growth of Ehrlich ascites tumor cells in cellular culture *in vitro*. The initially low level of GSH and cysteine in cultured HepG2 cells makes it possible both to study drug toxicity and to observe the protective activity of cysteine precursors, e.g. CP. It also makes possible to observe an increase in GSH and cysteine level and the simultaneous decreased cellular susceptibility to APAP toxicity. In the case of optimal concentration of cysteine and GSH in the cells, the introduction of additional cysteine which cannot be stored as GSH, will result in toxicity and a drop in GSH levels both in normal [32] and in neoplastic cells [33]. Thus the protective activity of cysteine precursors is possible only in cells with cysteine and GSH deficit [34]. Summing up, CP has proven to be a non-toxic source of cysteine in cultured HepG2 cells and to successfully protect them against APAP toxicity. This confirms the suggestion of its detoxicating and antioxidative properties.

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