Glutathione Reductase Is Inhibited by Acetaminophen-glutathione Conjugate *in vitro*: Possibly an Important Mechanism in Acetaminophen Liver Injury

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Short title: Inhibition of glutathione reductase by APAP-SG in vitro

Summary

Acetaminophen is one of commonly used analgesics and antipyretics. It is a safe drug at therapeutic doses but in the overdose, it causes liver injury which may lead to acute liver failure. The aim of the present work was to investigate a new mechanism contributing to the toxic acting of acetaminophen especially to explore the possible inhibition of glutathione reductase through an acetaminophen-glutathione conjugate. The acetaminophen-glutathione conjugate was synthesized by the reaction between glutathione and N-acetyl-p-benzoquinone imine and purified by column chromatography. The inhibition effect of the conjugate on two types of glutathione reductase (from yeasts and rat hepatocytes) was tested spectrophotometrically. We synthesized the conjugate and found that the enzyme activity was reduced significantly after treatment with different concentrations of acetaminophen-glutathione conjugate in both yeast and hepatocyte glutathione reductases (0.014 U/ml); the enzyme activity (from hepatocytes) was decreased to 79±7 %, 67±2 % and 39±7 %, in 0.37, 1.48 and 3.7 mM concentration of the conjugate, respectively. We found that glutathione reductase was dose-dependently inhibited by the conjugate of acetaminophen and glutathione. Our finding results in new consequences in description of acetaminophen toxicity and, in addition, explains previously published findings which have not been fully explainable till now.

Keywords: acetaminophen toxicity; glutathione reductase; glutathione; hepatotoxicity.

Introduction

Acetaminophen (APAP) is one of the presently mostly used analgesics and antipyretics. It is considered to be a safe drug when used at therapeutic doses. On the other hand, the acetaminophen overdosing is the most frequent cause of acute liver failure in men (Lee 2004). Hence, the mechanisms of acetaminophen toxicity have been studied very intensively recently.

At therapeutic doses, acetaminophen is detoxified by three major pathways in the liver. The most of APAP dose is conjugated with glucuronate and sulfate (about 80% and 10%, respectively). Remaining part of APAP is oxidized by cytochrome P450 to a toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI). This compound is detoxified by either spontaneous or enzyme-catalyzed reaction with glutathione (GSH) resulting in a conjugate, 3-(glutathion-S-yl)acetaminophen (APAP-SG). In APAP overdose, the glucuronidation and sulfation pathways are saturated, acetaminophen is being oxidized to NAPQI in much higher extent and GSH stores become depleted. Consequently, NAPQI binds to various proteins, the APAP-protein adducts are produced, and due to GSH depletion, there is an increase of oxidative stress in the cell (Jollow *et al.* 1973). These actions result in hepatocellular death seen as centrilobular necrosis in the liver (Jaeschke and Bajt 2006; Mitchell *et al.* 1973).

So far, numerous mechanisms contributing to the hepatocyte injury have been found. Except of GSH depletion and reactive oxygen species (ROS) production, the lipoperoxidation, mitochondrial permeability transition pore opening (Kon *et al.* 2004) and impairment of mitochondrial respiration have also been mentioned. Despite the processes cited above, the crucial causation of the toxicity remains unknown (Jaeschke and Bajt 2006; Kaplowitz 2004). Till now, two possible theories have been postulated – the oxidative and the metabolic one. The oxidative theory proposes the explanation of the damage by an increase of oxidative stress, the latter one by binding of NAPQI to SH-groups of proteins supposing their function

to be impaired (James *et al.* 2003). Unfortunately, neither the oxidative nor the latter theory explain the entire toxicity found in acetaminophen treated liver cells at all points.

As we have recently described (Rousar *et al.* 2009), acetaminophen toxicity is linked to reduced activity of glutathione reductase (GR) *in vitro*. It is a crucial enzyme in glutathione metabolism because it reduces glutathione disulphide (GSSG) back to the reduced form, GSH. Thus, this enzyme is essentially important during oxidative stress, where the level of GSSG increases and the inhibition of glutathione reductase could be a principal mechanism in acetaminophen toxicity. Since the cause of the enzyme inhibition remains unknown, the aim of our work is focused on an attempt to find and describe the reason of decreased activity of glutathione reductase (using two different types of glutathione reductases, i.e. from yeast and from rat hepatocytes); and especially, to prove the hypothesis that APAP-SG conjugate may play an important role in the mechanism of APAP toxicity. The experiments were carried out *in vitro.* The outcomes were aimed to serve as the preliminary data for following testing in cells and *in vivo*.

Materials and Methods

Chemicals

Glutathione reductase (from *Sacch. cerevisiae*), glutathione reduced, glutathione disulphide, sodium phosphate buffer, potassium phosphate buffer, hydrochloric acid, acetaminophen and NADPH were purchased from Sigma-Aldrich (USA).

Preparation of acetaminophen-glutathione conjugate

The APAP-SG conjugate was synthesized according to the method of Thatcher and Murray (Thatcher and Murray 2001), the separation of the conjugate was performed as a modification of the method described by Allameh and Alikhani (Allameh and Alikhani 2002). Sodium

hydroxide solution (8 g in 250 ml of distilled water) was added to the solution of silver nitrate (4.224 g in 65 ml of distilled water) during 1 minute at room temperature. Mixture was stirred for 10 minutes and the precipitated silver oxide was filtered off using glass sinter and washed with several portions of water (300 ml), acetone (300 ml) and diethyl ether (300 ml). The yield was almost quantitative (2.85 g).

NAPQI: Acetaminophen (0.428 g) was suspended in 100 ml of dry chloroform and just prepared silver oxide (2.2 g) was added. The suspension was stirred for 2 h at room temperature under argon atmosphere. Then, the reaction mixture was filtered using glass sinter and the yellow filtrate of NAPQI was used promptly in the next reaction step.

APAP-SG conjugate: Glutathione (0.857 g) was dissolved in 250 ml of 0.1 M sodium phosphate buffer pH 7.4 (3.549 g Na_2HPO4) and freshly prepared NAPQI solution in chloroform was added dropwise with vigorous stirring during 15 minutes. The mixture was stirred for 1 h at room temperature. Reaction mixture was separated and aqueous layer was washed with chloroform (50 ml) and twice with ethyl acetate (50 ml); water was evaporated using vacuum evaporator at 40 °C. Residue of the mixture was stirred in methanol (250 ml) for 3 h and filtered using glass sinter to remove undissolved sodium phosphate buffer. Methanol was evaporated using vacuum evaporator (40 °C).

APAP-SG conjugate was separated using column chromatography on Silicagel 60 (Merck, Germany) where the separation of the reaction residue containing APAP, APAP-SG and GSSG was performed; mobile phase consisted of methanol/water (9:1). The fractions (10 ml) were collected after separation and analyzed by TLC chromatography according to Allameh and Alikhani (Allameh and Alikhani 2002). The detection was carried out using TLC on Silicagel 60 F254 (Merck, Germany) with methanol/water (9:1) as a mobile phase; bands were visualized by $\lambda = 254$ nm (APAP, APAP-SG) or after reaction with 0.2 % ninhydrin (APAP-SG, GSSG). The Rf values for APAP, APAP-SG and GSSG were approximatelly

0.9, 0.7 and 0.3, respectively. The Rf values of GSSG and APAP were determined after comparison with standard values; the band of GSH was not proved what confirms the conversion of GSH to GSSG during described procedure. APAP-SG conjugate was obtained as a solid by desiccation of the fractions with proved APAP-SG only.

Preparation of hepatocyte lysates

Hepatocytes were isolated from male albino Wistar rats (250-280 g; Biotest, Czech Republic) by collagenase perfusion (Berry *et al.* 1991). The viability of freshly isolated hepatocytes was more than 90 % as confirmed by trypan blue exclusion. Isolated hepatocytes were suspended in Williams' E medium and diluted to final density of 10^6 cells per ml. The cells were sonicated (Bandelin Sonopuls sonicator, Germany) and the lysates were centrifuged (4°C, 10 min, 10000 g). The inhibition of glutathione reductase was tested in supernatant which was diluted in distilled water to gain the final GR activity similar to samples containing yeast glutathione reductase. All animals received care according to the guidelines set by the Institutional Animal Use and Care Committee of the Charles University, Prague, Czech Republic.

Glutathione reductase activity assay

The principle of the method is the reduction of oxidized glutathione by glutathione reductase in the presence of NADPH (Carlberg and Mannervik 1975). Activity of both yeast and hepatocyte GR was determined at 25 °C in 0.2 M potassium phosphate buffer (pH 7.5) by monitoring of NADPH absorbance decline (λ = 340 nm) using well-plate spectrophotometer INFINITE M200 (Tecan, Austria). The volumes of solutions were 50 µl GR (0.014 U/ml), 25 µl GSSG (3.7 mM) and the assay was started by addition of 50 µl NADPH (0.7 mM); the values in the brackets mean the final concentrations of a compound in a well. The decline of

absorbance was monitored during 20 minutes and the results were presented as a dependence of absorbance ~ time. (1 Unit was defined as an amount of the enzyme which will reduce 1 μmole of oxidized glutathione per minute at pH 7.6 at 25 °C.)

Estimation of GR inhibition by APAP-SG conjugate

The inhibition of both yeast and hepatocyte glutathione reductase activities by APAP-SG conjugate were assayed in well plates. The stock solution of APAP-SG (100 mM) was prepared. Then, the solutions with various concentrations of APAP-SG (5 mM, 10 mM, 20 mM, 40 mM, 50 mM) were prepared by dilution in distilled water. 10 μl of each solution were added to the mixture of GR (50 μl) and GSSG (25 μl) to assess the inhibition effect. The measurement was started by addition of NADPH (50 μl) and monitored by $\lambda = 340$ nm spectrophotometrically for 20 minutes by 25 °C. Control samples were prepared by identical protocol, the distilled water (10 μl) was added instead of APAP-SG conjugate.

Statistical analysis

All experiments were repeated at least two times with negligible differences among results. The results were processed by one-way ANOVA test, followed by Bonferroni post-test. The results are expressed as the mean ± SD (GraphPad Prism 4.03 for Windows, GraphPad Software, USA). $p < 0.05$ was considered as significant.

Results

Purified APAP-SG conjugate was used to estimation of possible inhibition effect in two types of glutathione reductase (0.014 U/ml) – yeast and from rat hepatocytes. The results show (Fig. 1) that the activity of both types of GR was decreased similarly in comparison to control. The enzyme activity was inhibited to 52.7 ± 1.5 % in yeast GR and to 48.1 ± 8.8 %

in rat hepatocyte GR. Regarding it, the other experiments were carried out only with GR from rat hepatocytes.

We analyzed also blank samples in all experiments. The blank samples did not contain GSSG and the obtained absorbance signals were subtracted in both control and APAP-SG-treated samples. The results show that the blank signal accounts for only 2 % and 4 % of absorbance decrease in control and APAP-SG-treated samples, respectively (Fig. 2). Although we did not purified the hepatocyte GR, the values of blank signals showed, that our conditions of GR assay were rather specific.

The inhibition effect on hepatocyte glutathione reductase was tested in a number of APAP-SG concentrations. We found, that the enzyme activity was decreased proportionally related to increasing APAP-SG concentration (Fig. 3). The GR activity was inhibited to 79.8 ± 7.0 %, 72.1 ± 0.5 %, 66.9 ± 2.2 %, 48.0 ± 7.1 % and 39.5 ± 7.4 % in 0.37 mM, 0.74 mM, 1.48 mM, 2.96 mM and 3.7 mM APAP-SG, respectively.

Discussion

Acetaminophen toxicity is a complex process where many mechanisms contribute to the hepatocyte impairment. Since the acetaminophen overdosing is one of the mostly found causations of acute liver failure (Lee 2004), the estimation of APAP toxicity has been studied very intensively.

Recently in this field, a number of review and original papers have been published in which new mechanisms contributing to the liver damage have been described. However, none of the mechanisms explains the cause of the hepatocyte damage completely (Jaeschke and Bajt 2006; James *et al.* 2003).

The former theory explaining a cause of APAP toxicity is a metabolic theory. It is linked to increased NAPQI production during metabolic phase in the APAP overdose. After GSH

stores are depleted, NAPQI binds to various proteins and the APAP-adducts are produced (Jollow *et al.* 1973; Qiu *et al.* 1998). This was believed to impair the protein function. However, none or only a modest change in their activity was found (Pumford *et al.* 1997). Therefore, the metabolic theory as a principal mechanism of APAP toxicity was ruled out.

The proposal of the latter, oxidative theory, is that the GSH depletion is followed by enhanced oxidative stress in a short period of time which leads to the hepatocyte injury. Indeed, it was supported by discovery of increased lipoperoxidation, ROS production or increased synthesis of nitric oxide (NO) (Hinson *et al.* 1998; Jaeschke *et al.* 2003; Knight *et al.* 2001). However, it is questioned if the oxidative stress is a cause or just a response to decreased glutathione levels. Bajt *et al.* proved that the increased ROS production follows the GSH depletion (Bajt *et al.* 2004). Another issue was to determine the localization of ROS production. The role of Kupffer cells, increased NO production or mitochondria as a source of ROS were investigated. Nevertheless, the answer to the role of ROS as the main cause of cell death has not been confirmed definitely yet (Jaeschke and Bajt 2006; James *et al.* 2003). The present work is subjected on the estimation of a newly proposed principle contributing to the acetaminophen toxicity. We observed recently that the glutathione reductase activity was reduced in rat hepatocytes treated with acetaminophen (Rousar *et al.* 2009). This inhibition was proved to be dose-dependent. We ascribed the reason of GR inhibition to impairment of the enzyme by ROS or toxic aldehydes, as described in several published papers (Ochi 1990; Vessey and Lee 1993). Despite this, we have proposed another theory of the cause of GR inhibition. We have hypothesized that the inhibition could be caused by direct effect of APAP-SG, the conjugate of glutathione and acetaminophen (or NAPQI more precisely).
To prove our hypothesis, we decided to prepare APAP-SG conjugate by organic synthesis. The solution with APAP-SG was added to the mixture containing glutathione reductase and

the decline of NADPH absorbance was measured. Results were compared to control samples in which volume and content of added solution were absolutely identical except of APAP-SG presence.

We tested two types of glutathione reductase – commercial from yeast *Sacch. cerevisiae* and just prepared from rat hepatocytes. In accordance to our hypothesis, we repeatedly observed after analysis of the experimental data, that both types of GR were inhibited by the APAP-SG largely; 2.96 mM APAP-SG was able to inhibit glutathione reductase activity (0.014 U/ml in both types of GR) by about 50%. Since both glutathione reductases are genetically very far, we can conclude, that the described inhibition by APAP-SG is a universal effect.

The assessed concentrations of APAP-SG (0.37 – 3.7 mM) may be comparable to the APAP-SG levels occurring in hepatocytes. In APAP overdose, the levels of APAP in the cells are in milimolar range (Mitchell *et al.* 1973); the GSH levels in hepatocytes are found in milimolar range as well (Pastore *et al.* 2003). After the glucuronidation and sulfation pathways are satured, the most of the dose is oxidized to NAPQI. Thus, here described effect of APAP-SG on the GR activity is likely to occur in the cells. The possibly substantial role of GR in the acetaminophen toxicity is supported by the work of Armesto *et al.* (Armesto *et al.* 1993) who demonstrated that a drug, lobenzarit, enhances GR activity in mice. Since lobenzarit was proved to have hepatoprotective properties in APAP overdose, this effect may be caused just by decreasing of the GR inhibition present in APAP-treated hepatocytes.

We propose, the cause of in present work observed GR inhibition by APAP-SG might be due to similar principle as in case of inhibition of glutathione reductase by S-nitrosoglutathione (Becker *et al.* 1995). The authors presented that S-nitrosoglutathione was capable to inhibit the glutathione reductase activity by reversible and/or irreversible mechanism. This was likely associated to a binding of NO to active site of the enzyme after broken bond between GSH and NO. It follows, that GR is able to react with a compound of chemical structure

related at least to a half of glutathione disulphide molecule. Since APAP-SG conjugate disposes of structure similar to a part of GSSG in fact, it is possible that the mechanism of the inhibition could be analogous as well.

The exploration of glutathione reductase inhibition by APAP-SG lead to many consequences, which have been believed to play crucial roles in entire acetaminophen toxicity, or even change the point of view on them completely. Indeed, the conjugate of acetaminophen (or more precisely NAPQI) and glutathione occurs always once NAPQI has been formed by cytochrome P450. Moreover, NAPQI production is even catalyzed by an enzyme, glutathione-S-transferase (GST) (Coles *et al.* 1988). The conjugation of APAP and GSH has been considered till now as a protective mechanism which defends the hepatocyte against the binding of NAPQI to various proteins. The conjugate is consequently transported out of the cell to the bile by MRP-2 (Multidrug resistance-associated protein-2) localized in the canalicular membrane of hepatocyte (Chen *et al.* 2003).

Despite generally accepted mechanisms described above, several original works involved results that were not anticipated before. In 2000, the work of Henderson *et al.* described the estimation of APAP toxicity in GST-pi knockout mice (Henderson *et al.* 2000). GST-pi is an isoenzyme of glutathione-S-transferase which is proved to catalyze the formation of APAP-SG conjugate in the liver (Coles *et al.* 1988; Henderson and Wolf 2005). Remarkably, the results showed that GST-pi knockout mice were much more resistant to APAP toxicity than wild-type mice. The explanation of that phenomenon was attributed to GST-catalyzed redox cycling and enhancement of oxidative stress, or to the role of GST-pi as an inhibitor of the stress inducible Jun N-terminal kinase (Henderson *et al.* 2000).

Another work was concerned in testing of susceptibility of transport-deficient hyperbilirubinemic rats to acetaminophen (Silva *et al.* 2005). This strain of rats is deficient in MRP-2, thus, the hepatocytes possess higher levels of intracellular glutathione that should

have been transported to the bile usually. The treatment of MRP-2 deficient rats with acetaminophen resulted in a discovery that mutant rats were more resistant than wild-type of rats. The cause of this finding was recognized as a consequence of increased intracellular GSH level. Therefore, the inhibitor of glutathione synthesis was used to decrease cellular glutathione levels and results showed that the resistance to acetaminophen decreased and was much lower than in wild type mice. The published explanation was that increased expression of certain cytochrome P-450 isoenzymes produced even more NAPQI which caused even larger impairment.

Both of these works have brought rather unexpected outcomes. The expectations of the authors in the article of Henderson *et al.* were that the absence of GST-pi enzyme had to lead to much larger liver damage (Henderson *et al.* 2000); the finding of increased susceptibility to acetaminophen in transport-deficient rats published in the latter work was a little surprising too (Silva *et al.* 2005). However, the results could be explained convincingly regarding the finding published in this paper.

Firstly, the hepatocytes from mice lacking GST-pi produce much lower amount of APAP-SG conjugate certainly (Coles *et al.* 1988). Hence, lower concentration of APAP-SG can lead to slighter inhibition of GR; indeed, this is supported by different recoveries of glutathione after comparison of wild-type and GST-pi knockout mice treated with acetaminophen (Henderson *et al.* 2000). Hepatic glutathione levels were measured for 5 hours after APAP treatment. The results showed that GSH concentrations were decreased in both of strains although GSH levels remained higher at all time points in knockout mice compared to wild-type. In addition, the GSH levels recovered to near pretreatment levels within 5 h in GST-pi null mice, however, GSH remained depleted in wild-type mice. The authors tested the expression of glutathione biosynthetic enzymes, γ-glutamylcysteine synthetase and glutathione synthetase, which were essentially unchanged. Hence, the GSH recovery cannot be explained

in this way and it is likely that the diverse GSH recovery between mice strains could be influenced by different GR activity.

Secondly, the MRP-2 deficiency in rats likely evoked an accumulation of APAP-SG in the hepatocytes leading to the subsequent deepening of the GR inhibition (Silva *et al.* 2005). Obviously, an attribute of enhanced NAPQI formation is important too, but the increased toxicity may be due to increased production of APAP-SG again.

Our hypothesis that APAP-SG conjugate is not only a product of acetaminophen detoxification but moreover it is a harmful agent, was supported by our results. In addition, we have documented, in cases described above, that increased level of APAP-SG due to GST-catalyzed reaction or due to blocked conjugate transport through MRP-2 resolved in deepening of hepatocyte impairment. For other support of our hypothesis, we sought to find another mechanism capable to enhance APAP-SG intracellular levels. We found a work where the acetaminophen toxicity was tested in mutant mice having elevated glutathione concentration (Rzucidlo *et al.* 2000). If our hypothesis was correct then the mice with elevated glutathione ought to be much more susceptible to acetaminophen toxicity.

In 2000, Dr. Rzucidlo *et al.* published a work focused on estimation of acute APAP toxicity in transgenic mice with elevated hepatic glutathione (Rzucidlo *et al.* 2000). They hypothesized that due to overexpression of glutathione synthetase and elevated GSH levels, the transgenic mice should be better protected against APAP toxicity. Surprisingly, the expectations were not fulfilled because transgenic mice showed significantly higher level of hepatotoxicity. More severe histopatological lesions in livers and higher levels of serum alanine aminotransferase activity were found in transgenic mice compared to control. The cause of this surprising finding was not fully explained again. However, regarding our discovery showed here, the mechanism of higher hepatotoxicity level in mice with elevated

glutathione is easily explainable by increased formation of APAP-SG conjugate and augmented inhibition of glutathione reductase.

Conclusions

The obtained results confirmed our hypothesis that glutathione reductase could be inhibited by APAP-SG conjugate. We supported it by published results of the other authors as well. Generally, depletion of GSH due to reaction with NAPQI in acetaminophen overdose is always related to acetaminophen toxicity. Since glutathione reductase is a crucial enzyme in maintenance intracellular GSH levels, the decrease of GR activity even raises hepatocyte impairment. In addition, it is generally accepted that the GSH exhaustion results in a number of consequent pathological processes (e.g. ROS production, peroxynitrite formation and/or lipoperoxidation) which lead to GSSG production and to the cell death. Regarding this, we conclude that we found an essentially important mechanism contributing to the acetaminophen toxicity in the liver.

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Abbreviations:

APAP, acetaminophen; APAP-SG, acetaminophen-glutathione conjugate; GR, glutathione reductase; GSSG, glutathione disulphide; GSH, glutathione (reduced form); GST, glutathione-S-transferase; MRP-2, multidrug resistance-associated protein-2; NAPQI, N-acetyl-p-benzoquinone imine; NO, nitric oxide; ROS, reactive oxygen species.

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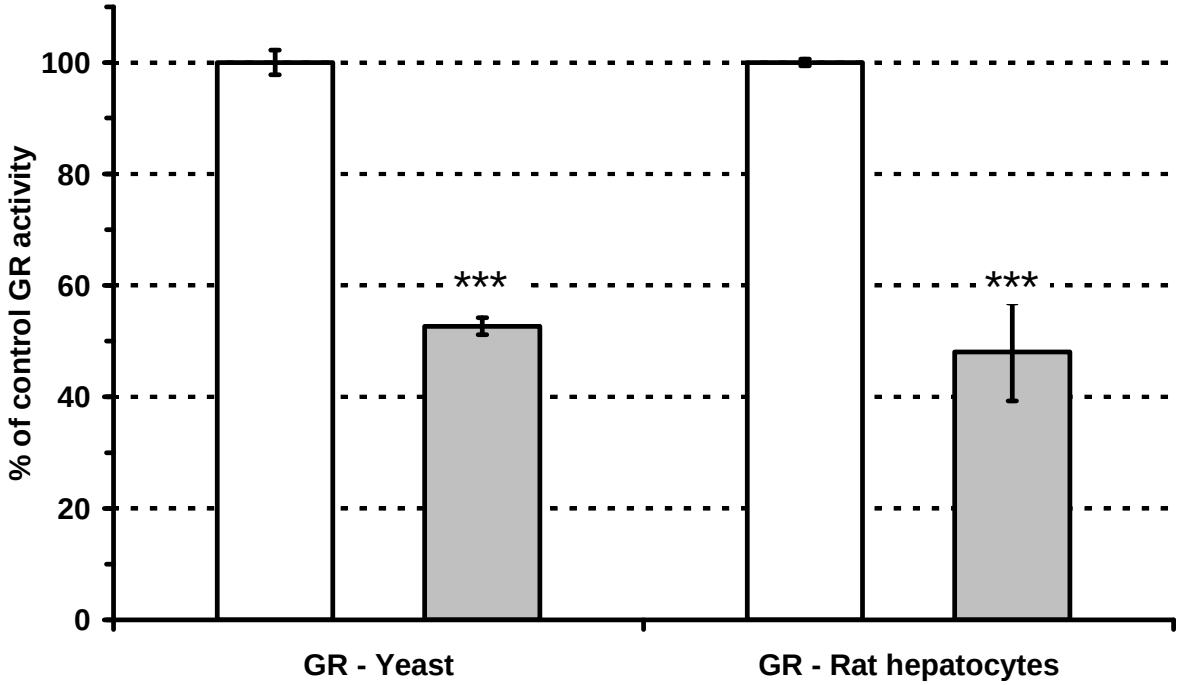
Fig. 1. Comparison of inhibition effect of acetaminophen-glutathione conjugate, APAP-SG (2.96 mM) on glutathione reductases from yeast and rat hepatocytes (0.014 U/ml). Glutathione reductases from yeast and rat hepatocytes were treated with a solution consisting of 2.96 mM APAP-SG conjugate (grey columns) and the decrease in absorbance (λ = 340 nm) was measured after addition of NADPH for 20 minutes. The results were evaluated and compared to control (white columns) which consisted of the same concentrations of all compounds, excluding APAP-SG conjugate. Results are expressed as mean ± SD. (n = 4; ***, $p < 0.001$, compared to control)

Fig. 2. Time course of the inhibition of glutathione reductase (from rat hepatocytes) by acetaminophen-glutathione conjugate, APAP-SG (2.96 mM). Glutathione reductase (0.014 U/ml) was treated with 2.96 mM APAP-SG conjugate (○, open circles) and the decrease in absorbance of NADPH (λ = 340 nm) was measured. After 20 minutes, the results were evaluated and compared to control (●, close circles) which consisted of the same concentrations of compounds, excluding APAP-SG conjugate. The blank samples without glutathione disulfide were also assessed - in the presence of the APAP-SG conjugate (□, open squares) and without APAP-SG conjugate (■, close squares).

Fig. 3. Inhibition of glutathione reductase (from rat hepatocytes) by acetaminophen-glutathione conjugate (APAP-SG). Ratio of resultant glutathione reductase activities (0.014 U/ml) to control signal in samples treated with various concentrations of APAP-SG conjugate (0.37 mM, 0.74 mM, 1.48 mM, 2.96 mM and 3.70 mM). The absorbance decrease of NADPH (λ = 340 nm) was monitored for 20 minutes. Results are expressed as mean ± SD. (n = 2-4; *, $p < 0.05$, ***, $p < 0.001$, compared to control)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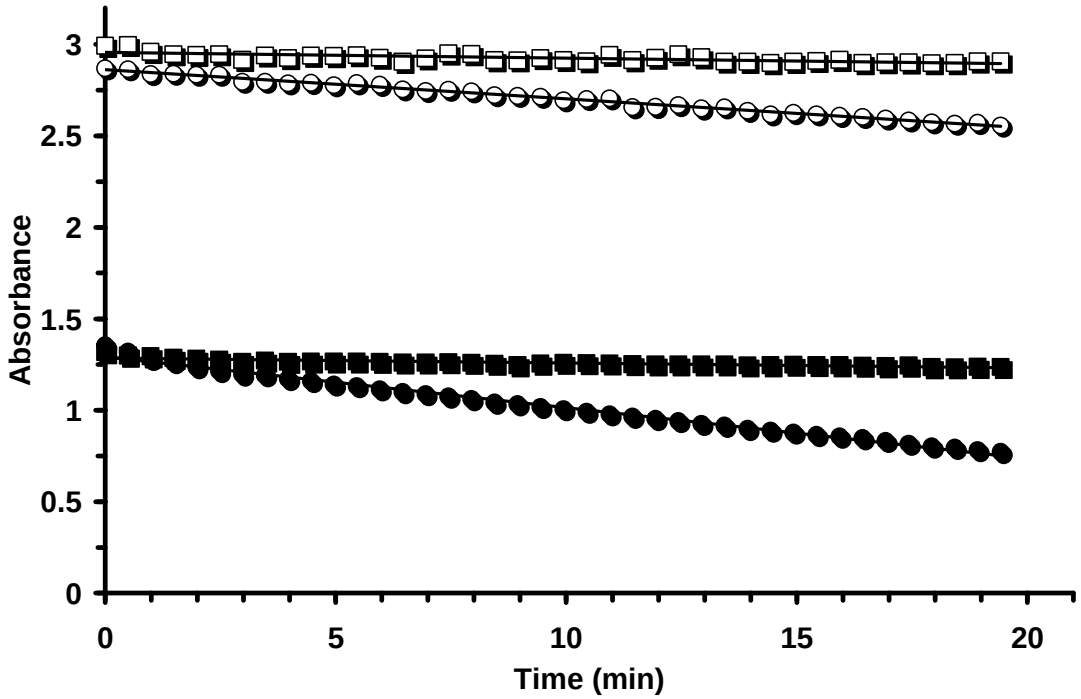
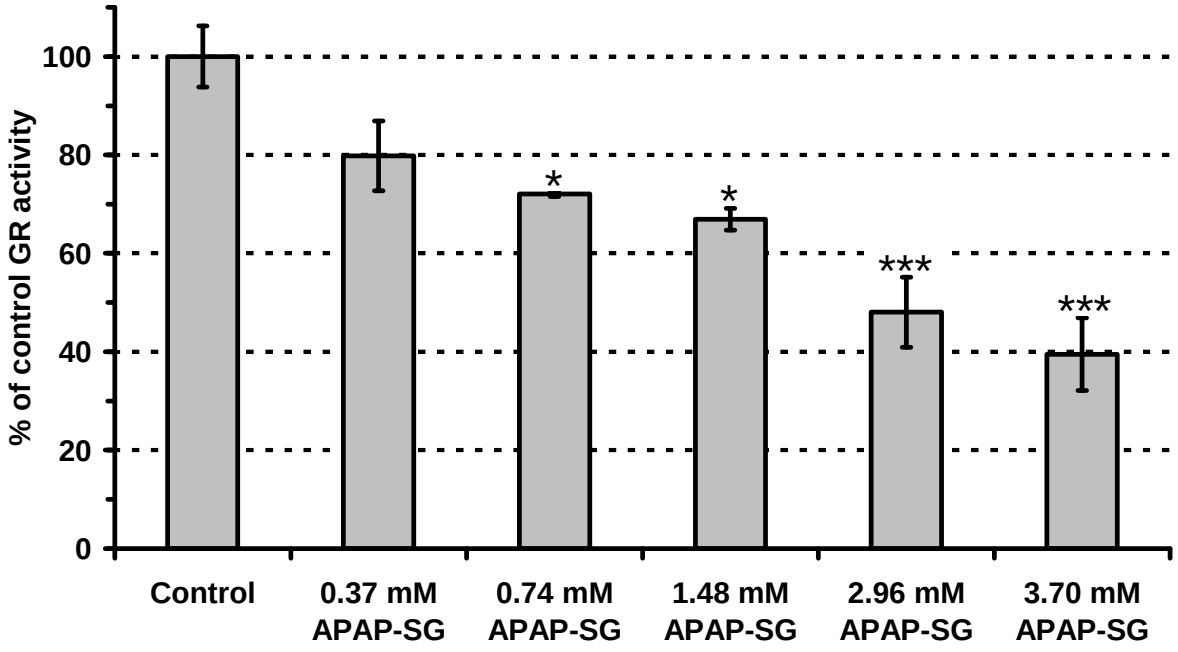
0
% of control GR activity
***
***
GR - Yeast
GR - Rat hepatocytes


3
2.5
2
1.5
1
0.5
0
Absorbance
0
5
10
15
20
Time (min)


% of control GR activity
100
80
60
40
20
0
*
*
***
***
Control
0.37 mM APAP-SG
0.74 mM APAP-SG
1.48 mM APAP-SG
2.96 mM APAP-SG
3.70 mM APAP-SG