Received; April 17, 2004, Accepted; July 1, 2004
To whom correspondence should be addressed: Hideyuki YAMADA, Ph. D., Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel. +81-92-642-6585, Fax. +81-92-642-6588, E-mail: yamada@senou.phar.kyushu-u.ac.jp


Regular Article

Enhancement of Acetaminophen Cytotoxicity in Selenium-binding Protein-overexpressed COS-1 Cells

Takumi ISHIDA¹, Masamiki ABE¹, Kazuta OGURI² and Hideyuki YAMADA¹
¹Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan
²Faculty of Pharmaceutical Sciences, Kyushu University of Health and Welfare, Miyazaki, Japan

Summary: The role of selenium-binding protein (SeBP), which has a high ability to associate with acetaminophen (AAP), on the cytotoxicity of AAP was studied. To clarify this issue, we examined the cytotoxic effect of AAP using COS cells stably expressing SeBP. Expression of SeBP enhanced the susceptibility of the cells to AAP-induced cytotoxicity. Several clones of SeBP-expressed COS cells were obtained, and they exhibited different degrees of susceptibility toward AAP. It was found that there is an inverse correlation between the expression level and the cell viability ($r = -0.872$). On the other hand, no increase in toxicity was observed in the SeBP-expressed cells treated with N-acetyl-p-quinone imine (NAPQI), which is an active metabolite of AAP. These results show that SeBP is an important factor in AAP hepatotoxicity. Moreover, our data suggest that the toxic mechanism of AAP differs from that of NAPQI.

Key words: acetaminophen-binding protein; selenium-binding protein; stable expression; acetaminophen; N-acetyl-p-quinone imine; cytotoxicity

Introduction

Acetaminophen (AAP) is widely used as a safe analgesic and antipyretic at therapeutic doses. However, many reports have shown that AAP overdosing can cause hepatotoxicity and even death in humans.¹,² A dose of AAP two to three times greater than the maximal amount recommended can cause hepatotoxicity, and higher doses result in potentially fatal centrilobular necrosis.¹,³ Previous studies have suggested that AAP-induced liver injury may be related to protein arylation,⁴ and change in oxidative stress,⁵ calcium regulation,⁶ mitochondrial homeostasis,⁷ transcription pathways,⁸ proinflammatory signaling⁹,¹⁰ and cell death pathways.¹¹ However, the molecular mechanism(s) of AAP-induced hepatotoxicity is not yet fully understood, although it is known that the AAP metabolite, N-acetyl-p-quinone imine (NAPQI), plays a role.

Metabolic activation of AAP to NAPQI is catalyzed by many isoforms of cytochrome P450 including CYP1A2, 2E1 and 3A4.¹¹,¹² Although NAPQI is inactivated by glutathione S-transferase (GST) via conjugation with reduced glutathione, NAPQI accumulates when the glutathione level is low. After consumption of glutathione, NAPQI can covalently bind to a number of intracellular target proteins, resulting in a variety of forms of cellular dysfunction.¹²,¹³ Of the cellular proteins capable of associating with AAP, AAP-binding protein (AAPBP) is of particular interest as far as AAP cytotoxicity is concerned. Bartolone et al.²⁵ reported previously that the covalent binding of AAP to hepatic proteins in mice was highly selective to 44 and 58 kDa proteins. In addition, a good correlation between AAP binding to the 58 kDa AAPBP and cellular damage by AAP has been reported in the mouse²⁶ and human.²⁷ AAPBP is known to be present in the liver²⁸ and highly homologous with selenium-binding protein (SeBP).²⁷ The deduced amino acid sequence of AAPBP differs from SeBP by only 14 residues. Although the physiological role of SeBP remains unknown, it is suggested that this protein is involved in the inhibition of cell proliferation and tumor suppression.²⁹ Based on these reports, SeBP is expected to have a similar function as AAPBP and to be one of the important factors in AAP-induced...
hepatotoxicity.

We have previously clarified a full length cDNA of inducible rat SeBP. In that experiment, only a cDNA was cloned from rat liver in spite of using primers common to mouse SeBP and AAPBP.\(^{30}\) Although the physiological function of this inducible SeBP is also unclear, it is expected to resemble that of mouse AAPBP because of the high homology (93\%) of both proteins. Therefore, we constructed cells stably expressing SeBP, and estimated the role of SeBP on AAP cytotoxicity using these cells.

**Methods**

**Materials:** COS-1 cells were purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). AAP, NAPQI and G418 solution were purchased from Sigma (St. Louis, MO, USA), Nacalai Tesque INC. (Kyoto, Japan) and PAA Laboratories (GmbH, Linz, Austria), respectively. The cDNA of rat liver SeBP (Kyoto, Japan) and PAA Laboratories (GmbH, Linz, Austria) was purchased from NUNC Int., New York, USA) for propagation. Each of them was transferred to a 3.5 cm plate (Nalge NUNC Int., New York, USA) and TransIT-\textregistered LT1 (Mirus Corp., Madison, WI, USA) were purchased from the sources indicated. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Dulbecco’s phosphate-buffered saline (PBS), trypsin-EDTA solution and NaHCO\(_3\) were obtained from Invitrogen Corp. (Carlsbad, CA, USA). The cell-count kit WST-1 was purchased from Wako Pure Chemical Industries, Co. Ltd. (Osaka, Japan). Rabbit anti-serum against purified rat SeBP was prepared by the methods described elsewhere.\(^{34}\)

**Construction of SeBP expression vector and its stable expression in COS-1 cells:** SeBP cDNA was subcloned into the p\textsc{TargetTM} mammalian expression vector (Promega Corp., Madison, WI, USA) and TransIT-\textregistered LT1 (Mirus Corp., Madison, WI, USA) were purchased from the sources indicated. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Dulbecco’s phosphate-buffered saline (PBS), trypsin-EDTA solution and NaHCO\(_3\) were obtained from Invitrogen Corp. (Carlsbad, CA, USA). The cell-count kit WST-1 was purchased from Wako Pure Chemical Industries, Co. Ltd. (Osaka, Japan). Rabbit anti-serum against purified rat SeBP was prepared by the methods described elsewhere.\(^{34}\)

**Stable expression of SeBP in COS-1 cells:** To produce cell lines stably expressing SeBP, p\textsc{TargetTM} mammalian expression vector containing SeBP cDNA was transfected to COS-1 cells, and the transformants were selected using G418. As the result, 12 surviving clones (COS-SeBP) were obtained. Immunoblot analysis of the cytosolic protein from 6 randomly selected COS-SeBP cells and COS-Mock cells is shown in Fig. 1A. SeBP with a molecular mass of 54 kDa was

**Measurement of cytotoxicity:** Mock-transfected (COS-Mock) or SeBP-expressed (COS-SeBP) COS-1 cells were seeded onto 96 well plates at 8 × 10\(^4\) cells/well. After 24 hr incubation in an atmosphere of 5% CO\(_2\) at 37°C, a switch was made to AAP- or NAPQI-containing medium. For this purpose, AAP or NAPQI was directly added to Dulbecco’s modified Eagle’s medium containing 0.45% glucose, 0.2% NaHCO\(_3\) and 10% FBS. Cell viability was assessed using the WST-1 cell-count kit. Briefly, each sample of cells grown on 96 well plates was washed with 150 μL PBS and then 100 μL medium supplemented with 10% FBS was added. A working solution (10 μL, supplement attached to the kit) containing 0.33% (w/v) 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) and 0.007% (w/v) 1-methoxy-3-methylphenazinium methosulfate was then added to each well following incubation for 2.5 hr. The absorbance due to living cells was measured at 405 nm with a reference wavelength of 620 nm using a plate reader NJ-2300 (Nalge NUNC Int., New York, USA).

**Statistics:** The statistical significance was calculated using either an unpaired Student’s \(t\)-test (comparison between two groups) or Fischer’s PLSD test (comparison between multiple sets).

**Results**

**Stable expression of SeBP in COS-1 cells:** To produce cell lines stably expressing SeBP, p\textsc{TargetTM} mammalian expression vector containing SeBP cDNA was transfected to COS-1 cells, and the transformants were selected using G418. As the result, 12 surviving clones (COS-SeBP) were obtained. Immunoblot analysis of the cytosolic protein from 6 randomly selected COS-SeBP cells and COS-Mock cells is shown in Fig. 1A. SeBP with a molecular mass of 54 kDa was
readily detected in all clones, whereas its expression in COS-Mock cells was not observed at all. Fig. 1B shows the expression level of SeBP estimated from the stained band. There were differences in the SeBP content of the 6 clones.

Effects of SeBP expression on AAP cytotoxicity:
The cell viability of COS-SeBP or COS-Mock cells following AAP treatment was measured by WST-1 assay. A typical time-course for the cell viability by 5 mM AAP treatment is shown in Fig. 2A. No cytotoxic effects of AAP were observed both in COS-Mock and COS-SeBP (clone 6) cells until 24 hr after AAP exposure. However, the cell viability of both types of cells was greatly reduced during the subsequent 24 hr, and the magnitude of this reduction was significantly greater in COS-SeBP cells than COS-Mock cells. Subsequently, a dose-response relationship for AAP cytotoxicity was measured using 3 clones and COS-Mock (Fig. 2B). The cytotoxicity was apparent from 5 mM AAP in COS-Mock and COS-SeBP cells. However, in COS-SeBP cells, the reduction in cell viability by 5 mM or more AAP was significantly greater than that in COS-Mock cells. When we compared the dose-response relationship in 6 different clones, an inverse correlation between the expression level of SeBP and the cell viability ($r = -0.872$) was observed (Fig. 3). These results show that SeBP plays a role in AAP-produced cytotoxicity.

Effects of SeBP expression on NAPQI cytotoxicity:
NAPQI, an active metabolite of AAP, is regarded as an important contributor to AAP hepatotoxicity. To obtain the information whether NAPQI requires SeBP to exert its toxic effects, the cytotoxicity of NAPQI in SeBP-expressed cells was examined. As shown in Fig. 4, a reduction in cell viability was found starting at 50 μM NAPQI in COS-Mock as well as COS-SeBP cells. No difference in susceptibility towards NAPQI was observed between COS-Mock and COS-SeBP cells. Cell viability in clone 6 was reduced somewhat by NAPQI in comparison with COS-Mock.

Discussion

In spite of numerous studies in terms of the toxic mechanism caused by an AAP overdose, it remains incompletely understood. However, it seems to be widely accepted that AAP cytotoxicity requires the formation of NAPQI, consumption of glutathione to be used with GST and covalent binding of NAPQI to a number of intracellular target proteins. This binding is believed to lead to a variety of forms of cellular dysfunction including mitochondrial damage and an increase in oxidative stress in this organelle, and ATP depletion.38,39) Furthermore, accumulation of calcium in nuclei and fragmentation of DNA has been observed.40,41) Recently, peroxynitrite formation and protein nitration have been postulated to be important factors in AAP toxicity.42–44) In the same way as the toxic mechanism, a manner of cellular damage caused by AAP overdose is also still obscure. Although it has long been demonstrated that AAP-induced cell damage involves oncosis or oncosis necrosis,45,46) many researchers have claimed that apoptosis plays a major role in AAP-induced hepatotoxicity or in AAP cytotoxicity in particular cell types.

It is assumed that AAPBP is one of a major binding protein of NAPQI, and that the binding is trigger of cytotoxicity caused by AAP overdose. In fact, covalent binding of AAP or NAPQI in mice shows a highly selective to 58 kDa AAPBP. Additionally, a good correlation between AAP binding to the 58 kDa AAPBP and cellular damage by AAP has been reported in the mouse 38,47,48) and human.49) However, it is unclear that the binding of NAPQI to AAPBP is involved in either toxic or protective mechanism in the cytotoxicity by AAP overdose. This important problem is not still resolved because the physiological functions of SeBP and AAPBP are not fully elucidated. Morrison et al.31) suggested that SeBP may contribute to a reduction in DNA synthesis. They used five mouse mammary epithelial cell lines with different growth rates to examine the relationship between selenoprotein levels and inhibition of DNA synthesis. They found that the 58 kDa protein capable of being labeled with 75Se was one of the major selenoproteins, and its labeling intensity was most closely correlated with the degree of inhibition of DNA synthesis. It is, therefore, likely that SeBP enhances AAP cytotoxicity via its action on DNA synthesis. In this report, our results showed that the increased cytotoxicity by AAP overdose was produced by SeBP overexpression. As can be seen in Fig. 1A, the expression level of SeBP was not greatly different in rat liver cytosol and COS cells transfected with SeBP cDNA. Therefore, similar role of SeBP on AAP toxicity detected by this study would be expected in physiological conditions. The mechanisms involving SeBP with cell death by AAP have not been clarified. However, it was suggested that the expression of SeBP is one of the factors involved in the occurrence of the toxicity by AAP overdose.

Contrary to the result with treatment of AAP (Fig. 2), the overexpression of SeBP had no enhancing effect in the COS-1 cytotoxicity induced by NAPQI (Fig. 4). This discrepancy is not resolved in this study, however, it is raised a possibility that the cytotoxic mechanism(s) by AAP overdose is distinct from that by NAPQI. It has been reported that the antioxidant had only a slight effect on the toxicity of NAPQI in cultured rat hepatocytes, whereas it completely prevented that of AAP.50) This finding strongly suggests that AAP and NAPQI kill cultured hepatocytes by different mechanisms; thus, while AAP injures cells by oxidative stress, the same is not true for NAPQI in cultured rat hepatocytes, whereas it completely prevented that of AAP.51) This finding strongly suggests that AAP and NAPQI kill cultured hepatocytes by different mechanisms; thus, while AAP injures cells by oxidative stress, the same is not true for NAPQI in cultured rat hepatocytes, whereas it completely prevented that of AAP.51) The above authors showed that the incorporation of 75Se into 58 kDa
selenoprotein is increased following MEHP treatment, whereas 23 kDa and 15 kDa selenoproteins show a reduction in labeling. These data suggest that the physiological function of SeBP is stimulated by production of oxidative stress. Taking all above information together with our present data into consideration, the mechanism(s) by which AAP exhibits its cellular toxicity is likely to involve activating SeBP via AAP-induced oxidative stress or AAP binding to this protein. Since neither a SeBP requirement nor oxidative stress production is evident for NAPQI-induced toxicity, NAPQI seems to exhibit its effects by cellular protein modification which is independent of the toxic mechanism for AAP itself. Other possibility is also raised that exogenously added NAPQI would not be expected to produce the same effect as endogenously generated NAPQI and that a different covalent binding profile would be expected. Therefore, further studies are needed to clarify the difference of toxic manner in SeBP overexpressed COS-1 cells treated with AAP or NAPQI.

In conclusion, we have shown here that the overexpression of SeBP, which is highly homologous with AAPBP, enhances AAP cytotoxicity in COS-1 cells. Although the physiological role of SeBP and AAPBP remains unclear, it is possible that SeBP and AAPBP are one of the factors in the hepatotoxicity that occurs following AAP overdose.

Acknowledgements: The authors are very grateful to Miss A. Urayama for technical assistance. This work was supported in part by a Grant-in-Aid for Encouragement of Young Sciences from Japan Society for the Promotion Science (research No.: 13771422).

References


