Intracellular glutathione regulates Andrographolide-induced cytotoxicity on hepatoma Hep3B cells

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Andrographolide (ANDRO), a diterpenoid lactone isolated from the traditional herbal plant Andrographis paniculata, was reported to induce apoptosis in hepatoma Hep3B cells in our previous study (Ji LL, Liu TY, Liu J, Chen Y, Wang ZT. Andrographolide inhibits human hepatoma-derived Hep3B cells growth through the activation of c-Jun N-terminal kinase. Planta Med 2007; 73: 1397–1401). The present investigation was carried out to observe whether cellular reduced glutathione (GSH) plays important roles in ANDRO-induced apoptosis. ANDRO initially increased intracellular GSH levels which then decreased later, while inhibition of cellular GSH synthesis by L-Buthionine-(S,R)-sulfoximine (BSO) augmented ANDRO-induced cytotoxicity and apoptosis in Hep3B cells. On the other hand, the thiol antioxidant dithiothreitol (DTT) rescued ANDRO-depleted cellular GSH, and abrogated ANDRO-induced cytotoxicity and apoptosis. Furthermore, BSO pretreatment augmented ANDRO-decreased expression of antioxidant protein thioredoxin 1 (Trx1), while DTT reversed this decrease. Further results showed that ANDRO increased the activity of the GSH-related antioxidant enzyme glutathione peroxidase (GPx) and the production of intracellular reactive oxygen species (ROS). Taken together, this study demonstrates that the intracellular redox system plays important roles in regulating the cytotoxicity of ANDRO on hepatoma Hep3B cells.

Keywords: andrographolide, hepatoma Hep3B cell, apoptosis, glutathione

Introduction

Oxidative stress occurs as a result of an imbalance between the production of reactive oxygen species (ROS) and their neutralization by antioxidants. Intracellular glutathione (GSH), a ubiquitous reducing sulphydryl (–SH) tripeptide, is one of the most abundant antioxidants in cells and tissues. GSH is involved in many important cellular functions including chemical detoxification and antioxidant defense by the formation of less-toxic GSH-xenobiotic conjugates via GSH S-transferase or de-activation of ROS either via direct GSH–ROS interaction or via the activity of glutathione peroxidase (GPx).1,2 Owing to its critical importance, GSH is generally maintained at high (mM) concentrations in mammalian cells.3
are reports that cellular GSH depletion augments the toxic effects of various chemotherapeutic agents on cancer cells, which demonstrate the important roles of GSH in cancer therapy.4–6

Andrographolide (ANDRO; Fig. 1) is a major bioactive ingredient isolated from Andrographis paniculata, which is a traditional herbal medicine used for the treatment of various diseases including respiratory infection, fever, bacterial dysentery and diarrhea for centuries in many Asian countries, such as China and India.7 Recently, there has been increasing research interest in the anti-cancer effects of ANDRO.8–10 Hepatocellular carcinoma (HCC) is one of the commonest malignancies world-wide. As the therapeutic outcome of HCC is very poor in most countries and the annual incidence is nearly equal to annual mortality, HCC is considered a lethal disease.11,12 Our previous report showed that ANDRO caused hepatoma Hep3B cell death via inducing apoptosis,13 which indicates the potential application of ANDRO in treating HCC.

The present study was designed to investigate whether intracellular GSH content and its modulation are involved in regulating the cytotoxicity of ANDRO on Hep3B cells. We found that the sensitivity to ANDRO-induced cytotoxicity was related to the intracellular GSH content and the regulation of cellular GSH modulated ANDRO-induced cytotoxicity.

Materials and methods

Cells and reagents

The hepatoma-derived Hep3B cell line was obtained from the American Type Culture Collection (ATCC, Manassaa, USA). Cells were cultured in MEM (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (ExCell Biology, Cranford, NJ, USA), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere (5% CO2).

Andrographolide was isolated from Andrographis paniculata by Dr Xu Chong and identified by IR, NMR, MS. The purity of the compound was more than 98% as determined by high pressure liquid chromatography (HPLC) analysis.13 Rabbit polyclonal antibody against Trx1 was from Cell Signaling Technology (Danvers, MA, USA) and rabbit polyclonal antibody against actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase-conjugated goat anti-rabbit IgG(H+L) was purchased from Jackson ImmunoResearch (West Grove, PA, USA). GSH, oxidized glutathione (GSSG) and NADPH were purchased from Roche Diagnostics GmbH (Mannheim, German). Other reagents unless indicated were from Sigma Chemical Co. (St Louis, MO, USA).

Cell viability assay

After various treatments, cells were incubated with 500 µg/ml 3-(4,5-dimethyl thiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) for 4 h. The functional mitochondrial dehydrogenases in survival cells can convert MTT to blue formazan, which was dissolved in 10% SDS–5% iso-butanol–0.01 M HCL.14 The optical density was measured at 570 nm with 630 nm as a reference and cell viability was normalized as a percentage of the control.

Measurement of cellular GSH

The content of cellular GSH was determined with the 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) assay according to the reported method with a minor modification.15 Briefly, after treatment, cells were harvested in metaphosphoric acid (5%) buffer. The reaction mixture contained EDTA (1 mM), NADPH (0.24 mM), glutathione reductase (0.06 U), DTNB (86 µM) and samples. Yellow 5-thio-2-nitrobenzoic acid (TNB) formation was monitored at 412 nm. GSSG was determined after elimination of GSH with 2-vinylpyridine. The levels of GSH were calculated from the difference between concentrations of total glutathione (GSH+GSSG) and GSSG. The intracellular levels of GSH were calculated based on cellular protein concentration.

Thiol depletion assay

Thiol depletion assay was performed according to the reported method with minor revision.16 GSH (100 µM)
was incubated with ANDRO (100 μM) at 37°C in phosphate buffer (pH 7.0) for various indicated times, and then GSH was determined. The reaction mixture contained metaphosphoric acid (1.67%) buffer, DTNB (0.4 mg/ml) and samples. Yellow 5-thio-2-nitrobenzoic acid (TNB) formation was monitored at 412 nm. GSH amounts were normalized as the percentage of control.

**DNA fragmentation assay**
DNA fragmentation assay was performed according to a previously described method with minor modifications. Briefly, cells were lysed with buffer containing 10 mM Tris-HCl (pH8.0), 10 mM EDTA, 150 mM NaCl, 0.4% SDS and 100 μg/ml proteinase K. The fragmented DNA in the lysate was extracted with phenol/chloroform/isopropyl alcohol (25:24:1, v/v), and then precipitated for 5–10 min in liquid nitrogen with chilled 100% ethanol and 3 M sodium acetate. The DNA pellet was saved by centrifuging at 17,000 g for 15 min at 4°C and then washed with 70% ethanol and resuspended in Tris-HCl (pH 8.0) with 100 μg/ml RNaseA at 37°C for 1 h. The DNA fragments were separated by 2% agarose gel electrophoresis, stained with ethidium bromide and photographed under UV light.

**Western blot analysis**
Cells were lysed in lysis buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 20 mM NaF, 0.5% NP-40, 10% glycerol, 1 mM phenylmethyl sulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml peptatin A. Proteins were separated by SDS-PAGE and blots were probed with appropriate combination of primary and horseradish peroxidase (HRP)-conjugated secondary antibodies. Proteins were visualized by enhanced chemiluminescence (ECL, Amersham). For repeated immunoblotting, membranes were stripped in 62.5 mM Tris (pH 6.7), 20% SDS and 0.1 M 2-mercaptoethanol for 30 min at 50°C.

**Glutathione peroxidase assay**
After treatment, cells were harvested in cold phosphate buffer (pH 7.0), sonicated (2 × 5 s) in ice, and centrifuged at 4000 g for 10 min. The supernatant was used for the enzymatic assays. GPx activity was assayed according to a published method using H2O2 as substrate. One unit of enzyme activity was defined as the utilization of one micromolar GSH per minute, and the activity of GPx was calculated based on cellular protein concentration.

**Cellular ROS detection**
Intracellular ROS were measured according to a reported method with a minor modification. Cells were incubated with 20 μM H2DCFDA and 50 μM ANDRO for the indicated times. After treatment, cells were washed with PBS buffer and immediately lysed in lysis buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 20 mM NaF, 0.5% NP-40, and 10% glycerol. The whole-cell lysates were centrifuged (10,000 g, 5 min, 4°C) and 100 μl of lysate was transferred to a Blackwall clear bottomed 96-well plate. Fluorescence was immediately read at an excitation of 485 ± 20 nm, emission 525 ± 20 nm using a Biotech FL600 spectrophotometer (Biotech Instruments, Winooski, VT, USA). The protein concentrations in the supernatant were assayed, and all the results were calculated as units of fluorescence per microgram of protein. The data were reported as DCF fluorescence per microgram of protein (% of control) corresponding to the increase of fluorescence per microgram of protein associated with ANDRO-treated cells compared to untreated cells.

**Statistical analysis**
All values were expressed as mean ± SEM. Differences between groups were assessed by one-way analysis of variance using the SPSS v.16.0 software package for windows. P < 0.05 was considered a statistically significant difference.

**Results**

**Effects of ANDRO on GSH**
GSH is an important intracellular antioxidant, and Figure 2A shows that ANDRO (50 μM) enhanced cellular GSH levels during the early incubation period (3–6 h), which may be due to the cellular defensive response, and later (12–36 h) significantly decreased cellular GSH levels. The results also showed that ANDRO increased cellular GSSG levels during 6–12 h, which indicated possible ROS production in ANDRO-treated cells compared to untreated cells.

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cysteine ligase (GCL), formerly called γ-glutamyl-cysteine synthetase (γ-GCS), specifically and irreversibly, which is the rate-limiting enzyme of GSH synthesis.20 Figure 3A shows that with BSO (25 μM) pre-incubation for 12 h significantly depleted ANDRO-enhanced cellular GSH in Hep3B cells after 6 h treatment. Figure 3B shows that BSO (25 μM) pre-incubation for 6, 12 and 24 h significantly augmented ANDRO (50 μM)-decreased cell viability and changed the IC₅₀ of ANDRO after 24 h treatment. Our results further confirmed that cellular GSH was involved in the cellular defense against ANDRO.
DTT abrogated ANDRO-decreased cellular GSH and cell viability

In order to determine the importance of GSH in mediating ANDRO’s cytotoxicity, we investigated the protective effects of DTT, a well-known thiol antioxidant, against ANDRO on Hep3B cells. We found that DTT rescued the depletion of GSH in ANDRO-treated Hep3B cells. As shown in Figure 4A, ANDRO (50 µM) decreased cellular GSH to about 19.3% of control, while DTT rescued ANDRO-depleted cellular GSH to about 76.1% of control. Figure 4B shows that
DTT rescued the decrease of cell viability in ANDRO-treated Hep3B cells in a concentration-dependent manner, and DTT also obviously increased the cell viability in ANDRO-treated cells with BSO pretreatment. The results in Figure 4B also show that various concentrations of DTT alone had no significant toxicity on Hep3B cells.

**Effect of various antioxidants on ANDRO-decreased cell viability**

We also studied some other well-known antioxidants such as ascorbate (Vc), Trolox, mannitol and N-acetyl-L-cysteine (NAC) on the cytotoxicity of ANDRO on Hep3B cells. As shown in Figure 5A, cell viability was decreased to about 20.0% of control after incubation with 50 μM ANDRO for 24 h. Figure 5A also shows that 500 μM DTT reversed the cell viability to 72.5% of control, while Trolox (50 μM) also had some protective effects and reversed the cell viability to 28.5% of control, but Vc and mannitol both had no significant protective effects. NAC and DTT are both well-known thiol antioxidants, and further results in Figure 5B show that 5 mM NAC also inhibited ANDRO-induced cytotoxicity on Hep3B cells.

**Effects of GSH on ANDRO-induced apoptosis and Trx1 depletion**

To test whether GSH plays important roles in ANDRO-induced apoptosis, we analyzed the ANDRO-induced cytotoxicity on Hep3B cells.
apoptotic DNA ladder in Hep3B cells with either BSO or DTT co-incubation. Figure 6A shows that BSO (25 µM) pretreatment for 12 h significantly augmented ANDRO-induced apoptosis, while with DTT co-incubation, the ANDRO-induced apoptotic DNA ladder disappeared. Trx1 is an important cellular antioxidant protein, and Figure 6B shows that 25 µM BSO pretreatment augmented ANDRO-decreased expression of Trx1, while DTT co-incubation obviously reversed such decrease.

Effects of ANDRO on the activity of GPx enzyme and the production of ROS

GPx is an important GSH-related antioxidant enzyme, and we further observed the effects of ANDRO (50 µM) on the activity of GPx enzyme. As shown in Figure 7A, ANDRO enhanced GPx activity in a time-dependent manner, which indicated possible production of ROS induced by ANDRO. Cell-permeable H$_2$DCFDA can be de-acetylated to a non-fluorescent product, 2′,7′-dichlorodihydrofluorescein (H$_2$DCF) by cellular esterase, and then is oxidized by cellular ROS to a fluorescent product, 2′,7′-dichlorofluorescein (DCF). The results in Figure 7B show that ANDRO increased DCF fluorescence levels after cells were treated with ANDRO (50 µM) for 3 h, which further confirmed the production of ROS.

Discussion

GSH is a ubiquitous reducing sulphydryl (-SH) tripeptide, which is the most abundant thiol antioxidant in hepatocytes. Cellular GSH conjugates toxic substances and predominantly participates in the detoxification of carcinogens, free radicals and peroxides, ultimately protecting cells and organs against toxicity. GSH also plays an important role in maintaining protein thiol groups, tocopherol and ascorbate in reduced states in cells. In the present study, we found that cellular GSH increased greatly when cells were incubated with ANDRO during the early time period (Fig. 2A), which may be due to a cellular defensive mechanism of counteracting the cytotoxicity of ANDRO and regulating the redox status in cells. However, after treatment with ANDRO for longer periods, cellular GSH was exhausted (Fig. 2A). There are reports that ANDRO may directly react with thiol of GSH in vitro, and the assumed hypothesis is that the α,β-unsaturated lactone moiety of ANDRO can react with GSH through the Michael addition reaction. The results illustrated in Figure 2B also confirmed the potential existence of the direct reaction of ANDRO with GSH in vitro.

BSO is a well-known inhibitor of GSH synthesis, and it has been reported that pretreatment with BSO augments the hepatotoxicity of many chemicals, which can be detoxified by GSH. In the present study, we found that 25 µM BSO significantly decreased ANDRO-induced early increase of cellular GSH. Further, the cytotoxicity of ANDRO unambiguously increased after cells were pretreated with BSO for various time (Fig. 3). Figure 6A shows that BSO pretreatment significantly augmented ANDRO-induced Hep3B cell apoptosis. All these results demonstrated the important roles of GSH in regulating the cytotoxicity of ANDRO on Hep3B cells. The present study showed that BSO augmented
ANDRO’s cytotoxicity, which indicates that ANDRO plus GSH depletion may be a better therapeutic strategy for HCC than ANDRO itself.

The glutathione antioxidant system is crucial for ROS defense in mammalian cells. We found that the exogenous antioxidant DTT reversed ANDRO-depleted cellular GSH. It also significantly prevented the cytotoxicity of ANDRO on Hep3B cells in a concentration-dependent manner with or without BSO pretreatment (Fig. 4). Figure 6A shows that the DNA apoptotic ladder nearly disappeared in Hep3B cells treated with ANDRO and DTT. DTT is a well-known dithiol agent and has been reported to prevent the cytotoxicity of some toxins. Possible explanations for the protective effects of DTT in our results are described below. One is that DTT can directly react with ANDRO in cells via thiol groups like GSH, leading to the detoxification of ANDRO. Another is that DTT can keep the cellular antioxidant defense system in a reduced status, such as glutathione or α-tocopherol (Ve), thus preventing the cytotoxicity of ANDRO. Further results showed that other antioxidants, such as Ve and mannitol, had no significant protective effects. However, there was weak protection against ANDRO-induced cytotoxicity by Trolox, which is an analogue of Ve (Fig. 5A). The results indicate the possible involvement of Ve in regulating the toxicity of ANDRO on Hep3B cells. NAC is a precursor of cellular GSH and can be converted into metabolites capable of stimulating GSH synthesis. Our results in Figure 5B show that 5 mM NAC significantly reversed ANDRO-induced cytotoxicity, which further confirmed the important role of GSH in regulating ANDRO-induced cytotoxicity on Hep3B cells.

Trx1 is a small, ubiquitous, thiol sulfhydryl (–SH) protein usually found in the cytoplasm that, along with the glutathione system, play important roles in maintaining the cytoplasm in a reducing environment. Trx serves as a co-factor in many thioredoxin reductase-catalyzed reductions, which reduces oxidized protein substrates in an NADPH-dependent process, and the Trx antioxidant system has been an attractive target for development of new cancer therapeutic agents.

We found that ANDRO decreased the expression of Trx1, and BSO augmented this decrease while DTT reversed such effects. All these results indicate that cellular GSH may play important roles in regulating the expression of Trx1, which may be involved in ANDRO-induced cytotoxicity on Hep3B cells.

Cellular GPx is a wide-spread enzyme found in many organisms throughout all kingdoms of life. It can change poisonous superoxide to non-toxic hydroxyl compounds via oxidizing GSH to GSSG. GPx was reported to play important roles in protecting cells against oxidative injury. Our results showed the significant increase of GPx activity in ANDRO-treated Hep3B cells (Fig. 7A), which may be due to the production of free radical species. H$_2$DCFDA is generally used to evaluate cellular production of ROS, and our results in Figure 7B further demonstrated that ANDRO induced the production of ROS in Hep3B cells.

It has been reported that ANDRO induced cell-cycle arrest and cell death via alteration of ROS in hepatoma HepG2 cells. The present study demonstrated that the cellular GSH redox system was involved in regulating the cytotoxicity of ANDRO on hepatoma Hep3B cells, and cells with GSH depletion by BSO were more sensitive to ANDRO treatment. Our results further defined the important roles of cellular GSH in regulating cancer therapy and also demonstrated a novel therapeutic proposal for the application of ANDRO plus BSO in HCC therapy.

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