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Antioxidative effect of a chymotrypsin inhibitor from *Momordica cochinchinensis* (Cucurbitaceae) seeds in a primary rat hepatocyte culture

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Abstract: The antioxidative activity of a chymotrypsin-specific potato type I inhibitor from *Momordica cochinchinensis* (MCoCI) (Cucurbitaceae) has been investigated using the primary rat hepatocyte system. *tert*-Butyl hydroperoxide (*t*-BHP) was used to induce oxidative stress. Pretreatment of hepatocytes with MCoCI for 24 h significantly reversed *t*-BHP-induced cell damage, and the associated glutathione depletion and lipid peroxidation. The activities of glutathione-S-transferase and superoxide dismutase were also increased. These results suggested that MCoCI possessed antioxidative activity which may account for some of the pharmacological effects of *Momordica cochinchinensis* seeds, the traditional Chinese medicine known as Mubiezhi, from which MCoCI was isolated.

Keywords: chymotrypsin inhibitor; hepatocytes; *Momordica cochinchinensis*; oxidative stress; *tert*-butyl hydroperoxide; Mubiezhi

INTRODUCTION

Protease inhibitors have been shown to be capable of preventing carcinogenesis in various *in vivo* and *in vitro* model systems [1,2]. They suppress carcinogenesis by a number of mechanisms, among them the prevention of the formation of reactive oxygen species [3]. Protease inhibitors also have a hepatoprotective effect against ischaemia/reperfusion injury which is believed to be closely related to the generation of oxygen free radicals [4]. Several naturally occurring chymotrypsin inhibitors [5] and synthetic protease inhibitors [6] are capable of decreasing H_2O_2 formation by activated human polymorphonuclear leukocytes (PMNs), and also superoxide anion radical production by differentiated HL-60 cells [7]. In PMNs, free radical-induced lipid peroxidation can also be prevented by urinastatin through inhibition of the proteases, especially elastase [8].

A comparison between the different types of protease inhibitor shows that the most potent of the anticarcinogenic protease inhibitors on a molar basis are those with the ability to inhibit chymotrypsin or chymotrypsin-like proteases [1]. The study by Frenkel *et al.* [5] also shows that the chymotrypsin-specific inhibitors are most effective in inhibiting the formation of H_2O_2 in stimulated PMNs. In our study of the bioactive compounds from the seeds of *Momordica cochinchinensis* (Cucurbitaceae), a novel potato type I chymotrypsin inhibitor MCoCI was isolated [9]. This study reports the antioxidative effect of MCoCI. The isolated rat hepatocyte culture was employed, with *tert*-butyl hydroperoxide (*t*-BHP) to provide the oxidative stress [10]. The cell viability, amount of lipid peroxidation, levels of reduced glutathione (GSH) and oxidized glutathione (GSSG), as well as the activities of various antioxidant enzymes, including catalase, superoxide dismutase (SOD), glutathione-S-transferase (GST) and glutathione reductase (GR) were evaluated after treatment with MCoCI.

MATERIALS AND METHODS

Materials

William's medium E, Hank's balanced salt solution (HBSS), collagen, collagenase, insulin, dexamethasone, *t*-BHP, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), sodium dodecyl sulfate, thiobarbituric acid and all the chemicals for the enzyme assays were purchased from Sigma. Cell culture materials, including fetal bovine serum, RPMI 1640 medium, penicillin and streptomycin sulfate were obtained from Invitrogen.

Purification of MCoCI from the Seeds of *Momordica cochinchinensis*

The chymotrypsin-specific protease inhibitor, MCoCI, was isolated from the seeds of *Momordica cochinchinensis* by chromatography on chymotrypsin-Sepharose 4B and subsequently by C18 reversed-phase HPLC, as described by Tsoi *et al.* [9].

Isolation and Culture of Primary Rat Hepatocytes

Rat hepatocytes were isolated by two-stage collagenase perfusion as described by Seglen [11]. Only preparations with cell viability greater than 90% were used for subsequent experiments. Cells were seeded onto culture plates precoated with collagen at a density of 2×10^5 cells/well for 24-well plates (for cytotoxicity assay) and 1×10^6 cells/well for 6-well plates (for biochemical assays). For experiments to measure the antioxidant enzyme activities, hepatocytes were seeded onto 100 mm

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dishes at a density of 1×10^8 cells/dish. The cells were cultured in William's medium E supplemented with 0.3 μM insulin, 0.1 μM dexamethasone, 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C, 90% humidity and 95% O_2. All experiments were performed 24 h after cell attachment to allow the formation of a monolayer of cells.

Oxidative Stress and Cell Viability

After incubation with various concentrations of MCoCI in William's medium E for 24 h, the hepatocytes were washed with HBSS to remove unabsorbed MCoCI. Afterwards, oxidative stress was induced by culturing the cells in William's medium E containing 500 μM *t*-BHP for 3 h. Cell viability was measured by MTT assay. After the cells were washed twice with phosphate buffered saline (PBS), 400 μl of 0.5 mg/ml MTT in RPMI 1640 medium was added to each well. The formazan crystals formed in active metabolic cells were extracted with 400 μl 10% sodium dodecyl sulfate in 10 mM HCl after 2 h of incubation at 37 °C. The absorbance was determined at 540 nm.

Biochemical Assays

For malondialdehyde (MDA) determination, the cells were treated with *t*-BHP in HBSS instead of William's medium E, as the colour of William's medium E would influence the colorimetric determination of MDA. MDA released into the medium was measured spectrophotometrically at 532 nm after reaction with thiobarbituric acid [12]. Cellular GSH content was determined by the DTNB spectrophotometric method [13]. Hepatocytes were trypsinized, sonicated and deproteinized by trichloroacetic acid. After centrifugation, the supernatant was treated with DTNB for 15 min before the absorbance at 412 nm was measured. The medium GSSG content was determined after incubation with DTNB in the presence of GR and NADPH at 37 °C for 30 min [13]. The absorbance of the mixture was determined at 412 nm.

Measurement of Antioxidant Enzyme Activities

Hepatocytes were washed twice with PBS, and then trypsinized and lysed by sonicating on ice. After centrifugation, the supernatant was used for enzyme determination. Catalase activity was determined at 240 nm with 10 mM H_2O_2 in 50 mM sodium phosphate, pH 7.0. SOD activity was determined by following the increase in absorbance at 550 nm in a reaction mixture containing 10 μM cytochrome C, 50 μM xanthine, 0.1 mM EDTA, 50 mM potassium phosphate, pH 7.8 and 6 mU xanthine oxidase in the presence of cell lysate. GST activity was determined in a reaction mixture containing 5 mM GSH, 1 mM 1-chloro 2,4-dinitrobenzene (CDNB) and cell lysate (50 μg protein/ml) in 0.1 M potassium phosphate, pH 8.5. The rate of decrease in absorbance at 340 nm was recorded. The GR activity of the cell lysate (50 μg protein/ml) was determined in 1 mM EDTA, 50 μM NADPH, 1 mM GSSG, 0.1 M potassium phosphate, pH 7.0. The oxidation of NADPH was followed by the decrease in absorbance at 340 nm.

Statistical Analysis

The results obtained were expressed as the mean ± standard error of three experiments, each performed in triplicate. Statistical analysis was performed by Student's *t*-test with one-way analysis of variance. Significant difference was taken as $p < 0.05$.

RESULTS

Effect of MCoCI Pretreatment on *t*-BHP-induced Hepatocyte Damage

The oxidative stress induced by 500 μM *t*-BHP caused over 80% of hepatocytes to die after 3 h of incubation (Figure 1). Such damage could be partially prevented by pretreating the cells with MCoCI for 24 h in a dose-dependent pattern (Figure 1). At a concentration of 100 μg/ml, MCoCI reinstated cell viability from 18% to 91%. A time-course study showed that significant protection could be observed after 6 h of incubation with 100 μg/ml MCoCI: 50% cell viability could be observed (data not shown).

Effect of MCoCI on *t*-BHP-induced Lipid Peroxidation, GSH Depletion and GSSG Increase

The addition of *t*-BHP (500 μg/ml) for 3 h stimulated lipid peroxidation as indicated by a 2.8-fold increase in the amount of MDA released into the medium (Figure 2). Pretreating the cells with MCoCI at doses ranging from 12.5 to 100 μg/ml inhibited MDA formation. For example, at 100 μg/ml, MCoCI caused a 52% reduction in MDA formation compared with that caused by *t*-BHP alone (Figure 2). Treatment with *t*-BHP also led to a reduction in intracellular GSH content by 85%. Such a decrease could be prevented by MCoCI pretreatment in a dose-dependent pattern (Figure 3) such that at 100 μg/ml, MCoCI could restore the GSH content to the control level. Hepatocytes treated with *t*-BHP also showed a 2.8-fold increase in the level of GSSG, which could also be prevented by pretreatment of MCoCI dose-dependently (Figure 4).

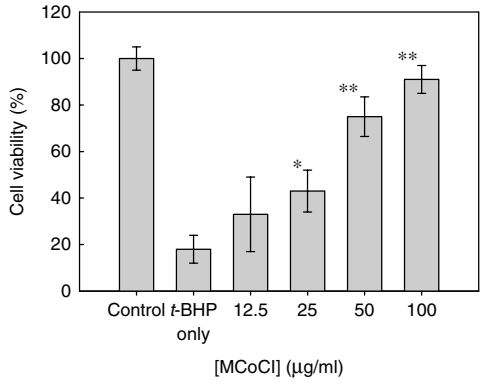

Figure 1 Effect of MCoCI pretreatment on *t*-BHP-induced cell damage. Hepatocytes were pretreated with different concentrations of MCoCI for 24 h prior to challenge with 500 μM *t*-BHP for 3 h. Cell viability was determined by the MTT assay. The results are expressed as mean ± standard error from three experiments, each performed in triplicate. $^*p < 0.05$, $^{**}p < 0.01$ when compared with cells treated with *t*-BHP only.

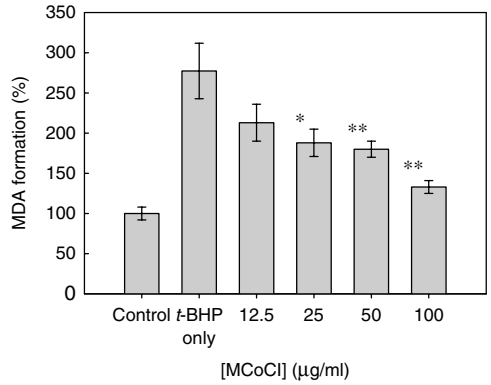

Figure 2 Effect of MCoCI pretreatment on *t*-BHP-induced lipid peroxidation. Hepatocytes were pretreated with MCoCI for 24 h prior to challenge with 500 μM *t*-BHP for 3 h. MDA leakage into the culture medium was determined by the thiobarbituric acid reagent. The results are expressed as mean ± standard error from three experiments, each performed in triplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared with cells treated with *t*-BHP only.

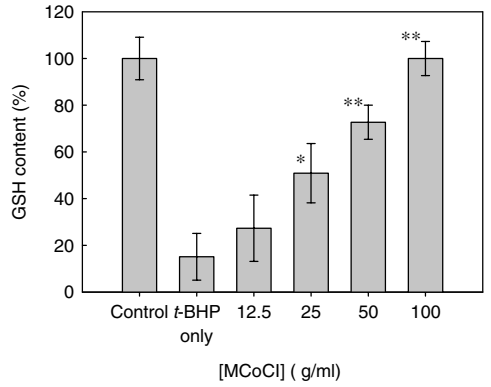

Figure 3 Effect of MCoCI pretreatment on *t*-BHP-induced GSH depletion. Hepatocytes were pretreated with MCoCI for 24 h prior to prior to challenge with 500 μM *t*-BHP for 3 h. Afterwards, the cells were trypsinized and lysed. The GSH content was determined spectrophotometrically after reacting with DTNB. The results are expressed as mean ± standard error from three experiments, each performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$ when compared with cells treated with *t*-BHP only.

Effect of MCoCI on Antioxidant Enzyme Activities

The effect of MCoCI on the four antioxidant enzymes is shown in Table 1. The enzymatic activities of GST and SOD increased by 60% and 20%, respectively, after incubation of the hepatocytes with MCoCI (100 μg/ml) for 24 h at 37 °C. On the other hand, no significant change was observed for the activities of GR and catalase.

DISCUSSION

Mubiezhi, made from the seeds of *Momordica cochinchinensis,* is a traditional Chinese medicine

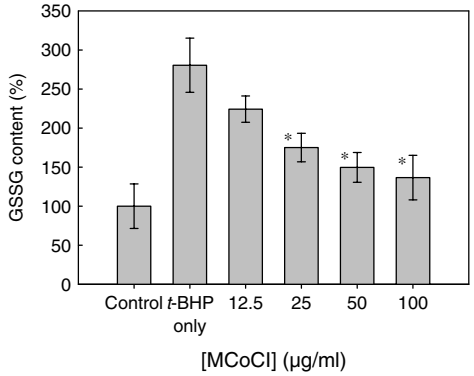

Figure 4 Effect of MCoCI pretreatment on *t*-BHP-induced GSSG formation. Hepatocytes were pretreated with MCoCI for 24 h prior to challenge with 500 μM *t*-BHP for 3 h. GSSG released into the culture medium was converted to GSH by GR, before being measured with DTNB. The results are expressed as mean ± standard error from three experiments, each performed in triplicate. $^{*}p < 0.01$ when compared with cells treated with *t*-BHP only.

Table 1 Effect of MCoCI Pretreatment on the Activity of Different Antioxidant Enzymes

[MCoCI] (μg/ml)	Activity (%)			
	GST	SOD	GR	Catalase
0	100 ± 10	100 ± 11	100 ± 12	100 ± 13
6.25	120 ± 12	115 ± 13	110 ± 10	106 ± 13
25	145 ± 10[b]	122 ± 8[a]	110 ± 9	110 ± 8
100	160 ± 9[b]	120 ± 5[a]	105 ± 7	108 ± 10

Hepatocytes were treated with MCoCI for 24 h. The cell lysate was prepared for the determination of enzyme activities. The results are expressed as the mean ± standard error from three experiments, each performed in triplicate.
[a] $p < 0.05$.
[b] $p < 0.01$ when compared with control.

which has been used for treating boils, pyodermas, mastitis, tuberculous cervical lymphadenitis, ringworm infections, freckles, sebaceous cysts, haemorrhoids and haemangiomas [14]. Besides some low molecular weight bioactive components, the seeds of *Momordica cochinchinensis* are also rich in protease inhibitors. Several trypsin inhibitors [15,16] and a chymotrypsin inhibitor (MCoCI) [9], with molecular weights ranging from 3000 to 7500, have been isolated. However, it remains to be determined whether these protease inhibitors are responsible for the pharmacological effects of Mubiezhi.

In the present investigation, the antioxidative effect of the chymotrypsin-specific inhibitor MCoCI was investigated. A prerequisite for pharmacological applications of protease inhibitors is the lack of cytotoxicity. Using the primary hepatocyte culture system, it was found that MCoCI did not

have any toxic effect at doses found to be effective in inhibiting the oxidative damage due to *t*-BHP.

t-BHP is commonly used to induce oxidative stress in toxicological and pharmacological studies. Incubation of rat hepatocytes with 500 μM *t*-BHP for 3 h led to significant oxidative damage, as shown by the loss of cell viability, GSH depletion and MDA formation. Such results agreed with previous studies using the same system [10,17]. *t*-BHP is metabolized intracellularly by microsomal cytochrome P450 reductase to form free radicals inside the cells. A pretreatment scheme was used in the present study in which unabsorbed MCoCI was removed before *t*-BHP challenge. Thus, any antioxidative activity observed should occur intracellularly, and not as a consequence of reacting with *t*-BHP directly in the culture medium. The effect may be explained by activation of a downstream signalling pathway resulting either from the absorption of MCoCI into the cells or the binding of MCoCI onto a specific receptor on the hepatocytes. Gabexate mesilate, a synthetic protease inhibitor of 417 daltons, can enter the hepatocytes and is useful in preventing ischaemia/reperfusion injury of the human liver [4]. Being a much larger molecule with a molecular mass of 7514 daltons [9], MCoCI may not diffuse easily through the membrane. Although the mechanism for the internalization of MCoCI remains unknown, it is obvious that MCoCI could protect cells against acute oxidative stress induced by the exogenous agent *t*-BHP.

The antioxidative activity of MCoCI can be demonstrated by inhibiting *t*-BHP induced lipid peroxidation. The proposed antioxidant could be located near the site of free radical generation and scavenges the free radicals immediately after they are generated; or it might act through enhancement of antioxidant enzymes. Direct scavenging of the oxidative species by MCoCI could not be demonstrated by using the simple *in vitro* free radical generation systems described in Yau *et al.* [10] (data not shown). In this regard, it is similar to the action of Bowman-Birk inhibitor in suppressing the production of superoxide anion radicals in differentiated HL-60 cells [7], but in contrast to the sweet potato trypsin inhibitor which has scavenging activities against 1,1-diphenyl-2-picrylhydrazyl and hydroxyl radicals [18]. On the other hand, the antioxidant enzymes, GST and SOD could be activated by 60% and 20%, respectively, after the cells were treated with MCoCI. Such activation may account for the antioxidative properties of MCoCI.

In conclusion, MCoCI possessed antioxidative activities in the rat hepatocyte culture system challenged with *t*-BHP-induced oxidative stress. Such an antioxidative effect of MCoCI may be responsible for some of the pharmacological actions of the traditional Chinese medicine Mubiezhi.

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