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Allicin Modulates the Antioxidation and Detoxification Capabilities of Primary Rat Hepatocytes

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Abstract

The effect of allicin, an active ingredient of garlic, on lactate dehydrogenase (LDH) leakage, lipid peroxidation, glutathione (GSH) content, and GSH-related enzyme activity was investigated in primary hepatocytes. In this study, allicin was synthesized in our laboratory as an experimental material, and primary hepatocytes isolated from Sprague-Dawley rats were used as an experimental model. According to the results, hepatocytes treated with 10 μM allicin did not differ from the control on LDH leakage during various incubation times. When the hepatocytes were treated with 10 μM allicin, their levels of thiobarbituric acid reactive-substances (TBARS) did not differ significantly from that of the control within the 8-h incubation. However, the TBARS values of hepatocytes treated with 30 and 50 μM allicin were higher compared to the control after incubation for 4 h and 8 h, respectively. The hepatocyte intracellular GSH content was significantly higher than that of the control after 30 μM allicin treatment, but treatment with 50 μM allicin caused a significant GSH depletion after incubation for 4 h or longer. In addition, when hepatocytes were treated for 24 h with 10 or 30 μM allicin, glutathione peroxidase (GPx) activity was significantly increased compared to that of the control, whereas 50 μM allicin treatment for 24 h or longer significantly decreased the GPx activity. Glutathione reductase (GRd) activity was significantly increased when the hepatocytes were treated with 10 μM allicin for 24 h, but GRd activity significantly decreased when the hepatocytes were treated with 50 μM allicin. However, hepatocytes treated for 24 h with 10 or 30 μM allicin showed significantly increased glutathione S-transferase (GST) activity compared to the control. These results suggest that 10 μM allicin potentially enhances the antioxidation and detoxification capabilities of primary rat hepatocytes.

Keywords: allicin, antioxidation, detoxification, primary rat hepatocytes

Introduction

Garlic (大蒜 dà suàn; the bulb of *Allium sativum* [Liliaceae]) is used in the diet as a vegetable or spice, but also has a long history of medicinal use. Garlic has benefits as an antibiotic, anti-atherosclerotic, and has anti-proliferative properties for treating cancer (Cutler and Wilson, 2004; Ogita et al., 2007; Chu et al., 2012). The principle strong odor and active ingredient of fresh garlic is believed to be allicin (diallyl thiosulfinate) and its degradation products (Block, 1985). Intact allicin does not exist in the garlic bulb, but its precursor allicin (*S*-allyl-*L*-cysteine sulfoxide) is likely stored in the cytoplasm. When garlic is cut and/or homogenized, the

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active enzyme system alliinase, most of which is stored in the vacuole, converts alliin to allicin (Lancasater and Collin, 1981). The biological activity of allicin is attributed to antioxidant activity or thiol disulfide exchange (Chung, L. Y., 2006). In addition, a previous study indicated that allicin rapidly reacts with free thiol groups and enters biological membranes (Miron, T., et al. 2010).

One of the more abundant non-protein thiols is glutathione (GSH) in mammalian systems. It is the major intracellular thiol protein, and its levels are organ-dependent, varying between 2–10 mM (Tanabe et al., 2004). In the GSH redox system, GSH is a substrate for glutathione peroxidase (GPx). GPx plays an important role in protecting cell proteins and cell membranes from oxidative stress. In addition, glutathione reductase (GRd) is a flavoprotein that regenerates GSH and provides reducing power for various thio-coupled transferases and peroxidases (Lehninger, A.L., 2005). It has been shown that GSH and its related enzyme system play an important role in hepatic antioxidation and drug metabolism. Previous studies have shown that allicin can decrease free radical scavenging to lower lipid peroxidation (Prasad et al., 1995). Allicin can reverse ethanol-induced hepatocytoxicity by enhancing hepatic glutathione (GSH) and GSH-related enzyme system expression (Wang et al., 1998). In a short-term study, allicin selectively expresses the glutathione S-transferase (GST) gene in the murine gastrointestinal tract and liver (Andorfer et al., 2004).

In addition, allicin shows remarkable anti-viral, anti-fungal, and anti-bacterial activity (Zhou et al., 1997; Kim, Y. S., et al., 2012; Cutler and Wilson, 2004). Allicin significantly inhibits human platelet aggregation (Liao, F. and Jiao, L., 2000) and lowers atherosclerosis risk from hyperlipidemia and hypertension in rats fed with a high cholesterol diet (Ali et al., 2000). Recently, anti-cancer research showed that allicin decreases GSH levels to inhibit proliferation of the human breast cancer cell line MCF-7 (Hirsch et al., 2000). Zhang et al. (Zhang et al., 2010) showed that allicin induces gastric cancer cell apoptosis by decreasing the mitochondrial membrane potential ($\Delta\Psi m$) and activating caspase-3, -8, and -9. Allicin also induces apoptosis by regulating caspase-independent or -dependent apoptotic signaling pathway in various cancer cells (Zhang et al., 2006; Park et al., 2005; Oommen et al., 2004; Bat-Chen et al., 2010) and autophagic cell death in human liver cancer cell line (Chu et al., 2012). However, data regarding the optimum concentration of allicin and its effects on antioxidation and detoxification capabilities in normal hepatocytes are limited.

Therefore, the present study used a liver perfusion method and primary rat hepatocyte culture to investigate the effect of various allicin concentrations and incubation times on detoxification capability, and the antioxidation system.

Materials and Methods

Chemicals

Diallyl disulfide (DADS) was purchased from Tokyo Kasei (GC purity: 93%). L-15 media, insulin, transferrin, fetal bovine serum (FBS), and pencillin/streptomycin solution were obtained from Gibco Laboratories (Grand Island, NY, USA). Trypsin inhibitor, bovine serum albumin (BSA), N-2-hydroxy-ethylpiperazine-N2-ethane sulfuric acid (HEPES), potassium chloride (KCl), sodium chloride (NaCl), glucose, phenol red, galactose, potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), pyruvate, Triton X-100, collagen, trypan blue, β-nicotinamide adenine dinucleotide (reduced form, β-NADPH), β-nicotinamide adenine dinucleotide phosphate (reduced form, β-NADPH), sodium dodecyl sulfate (SDS), 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), reduced glutathione (GSH), oxidized glutathione (GSSG), 1-chloro-2,4-dinitrobenzene (CDNB), sodium azide (NaN_3) and GSH reductase, and iodoacetic acid (IAA) were purchased from Sigma Chemical Co. (St, Louis, MO, USA). Collagenase (type I) and percoll solution were purchased from The Worthington Biochemical Co. and Pharmacia LKB (Piscataway, NJ, USA), respectively.

Synthesis, purification, and identification of allicin

According to the method of Iberl et al. (Iberl et al., 1990), diallyl disulfide is an initiator of allicin synthesis. The diallyl disulfide was selected from oxidation by hydrogen peroxide to prepare cured allicin. Allicin purification was performed using a Sephadex LH-20 column (25-100 μm) equilibrated with elution methanol (60%) in water containing 0.1% formic acid. The eluted substances were evaluated by performing HPLC and ^{1}H-NMR (Sheen and Wu, 1997). Allicin purity was greater than 80%.

Hepatocyte isolation and culture

Male Sprague-Dawley rats (aged 8 weeks) were used for hepatocyte isolation. The animals were housed in stainless-steel grid cages with an artificial 12 h

light/dark cycle. Rats had free access to food (PMI Feeds, St Louis, MO, USA) and water. Rats were treated in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996).

Rat hepatocytes were prepared by collagenase perfusion as described previously (Berry and Friend, 1973; Bonney et al., 1974; Sheen et al., 1996). Cells (2.5×10^6) were plated on 60-mm collagen-coated plastic tissue culture dishes (Falcon Labware) and incubated in a 37°C humidified incubator (NUAIRE Co., MN, USA) in an air atmosphere. The media was changed 4 h after plating to the same culture media that contained 2.0 g/L BSA instead of FBS. Media was changed once each day, 20 h after plating.

Treatment

Twenty hours after plating, the cells were treated with 0, 10, 30, or 50 μM allicin (final concentration) for 4, 8, or 24 h. After 24 h treatment with allicin, fresh media without allicin was added for an additional 48 h of culture. At various experimental time intervals, the reaction was stopped by removing the media and washing with cold phosphate buffered saline (PBS). Cells were removed using a cell scraper for further analyses.

LDH leakage assays and microscopic examination

The effect of various allicin concentrations on the viability of primary rat hepatocytes was evaluated by LDH leakage and microscopic examination. Intra- and extracellular LDH activity was assayed according to the methods of Moldus et al. (1978). Phase contrast photomicrographs of the monolayer cultures were obtained for morphological examination using a Nikon Diaphot 300 inverted stage microscope equipped with phase contrast and camera attachments.

Lipid peroxidation

Lipid peroxidation of hepatocytes was elevated by thiobarbituic acid-reactive substrate product (TBARS) formation. Hepatocyte TBARS levels were assayed using the method of Fraga et al. (1988). Cytosolic protein concentrations were determined using the method of Lowry et al. (Lowry et al., 1951).

GSH and related enzyme assays

Samples for GSH determination were prepared by adding 1 ml 10% perchloric acid, which contained 2 mM 1,10-phenanthroline, to each dish. The plates were scraped, and the contents centrifuged for 10 min at 10,000 × *g*. Acid-soluble GSH in the hepatocellular supernatant was measured using HPLC (Reed et al., 1980). GSH peroxidation (GPx) activity was determined spectrophotometrically using hydrogen peroxide (H_2O_2) as a substrate (Lawrence and Burk, 1976). GSH reductase (GRd) activity was measured as described by Bellomo et al. (1987). GSH S-transferase (GST) activity in the hepatic cytosol was assayed according to the method of Habig et al. (1974) using CDNB as substrate. Protein concentration of the cells was determined as previously described (Lowry et al., 1951).

Statistical analysis

Statistical analysis were performed using SAS software (SAS Institute). ANOVA and Duncan's multiple comparison were used to determine significant differences among means. $P < 0.05$ was considered statistically significant.

Results

LDH leakage assays and microscopic examination of cell viability

The results of the LDH leakage assays are shown in Figure 1, and 10 or 30 μM allicin (LDH leakage was 13.3±5.2 and 14.8±4.3%, respectively) did not differ significantly from control during the 24 h incubation (control LDH leakage was 12.7 ± 3.2%). However, when treated with 50 μM allicin for 4 h, LDH leakage was 19.8 ± 3.5% and was significantly greater than that in the control (control LDH leakage was 7.0 ± 3.1%)

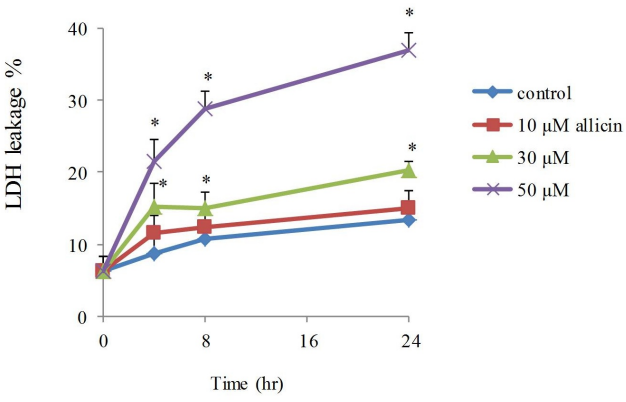

Figure 1. The LDH leakage of various concentrations of allicin were added primary rat hepatocytes. Hepatocytes isolated from each animal were divided equally and cultured in media containing 0 (control), 10, 30, or 50 μM allicin. Values are expressed as the mean ± SD from 4 rats. *$P < 0.05$ between control and treatment at the same time interval (ANOVA and Duncan's multiple comparison tests).

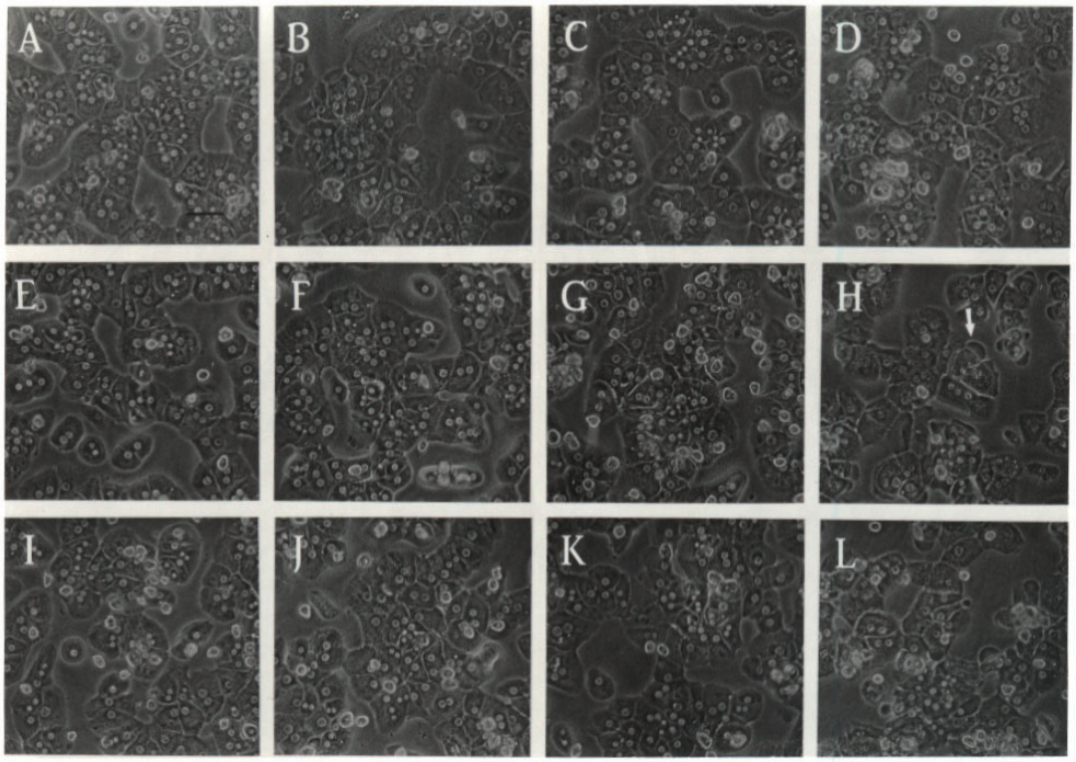

Figure 2. Morphology changes after various concentrations of allicin were added to primary rat hepatocytes. Hepatocytes isolated from each animal were divided equally and cultured in media containing 0 (control), 10, 30, or 50 μM allicin. (400× magnification under inverted stage microscope equipped with phase contrast). A-D, Hepatocytes were cultured for 4 h after treatment with 0 (control), 10, 30, or 50 μM allicin, respectively. E-H, Hepatocytes were cultured for 24 h after treatment with 0 (control), 10, 30, or 50 μM allicin, respectively. I-L, Hepatocytes were cultured for 48 h after treatment with 0 (control), 10, 30, or 50 μM allicin, respectively. Arrow in H shows bleb formation. Scale bar = 50 μm.

($P < 0.05$). However, upon microscopic examination, we found no significant changes in the morphology of hepatocytes treated with 10 or 30 μM allicin compared to control (Figure 2). However, when treated with 50 μM allicin, there were significant changes in hepatocyte morphology, such as membrane blebbing after 8 and 24 h (Figure 2H & 2L).

Lipid peroxidation

Lipid peroxidation of primary rat hepatocytes was evaluated by thiobarbituric acid-reactive substances (TBARS) production. According to the TBARS levels shown in Figure 3, 10 or 30 μM allicin had TBARS levels of 4.0 ± 0.1 and 4.2 ± 0.6 nmol/mg cytosolic protein, respectively, and did not differ significantly from the control after 24 h incubation (control TBARS level is 3.8±0.3 nmol/mg cytosolic protein). However, when treated with 50 μM allicin, the TBARS level after 4 h was significantly higher compared to control ($P < 0.05$). At 24 h, the TBARS level was 5.9 ± 0.4 nmol/mg cytosolic protein with 50 μM of allicin.

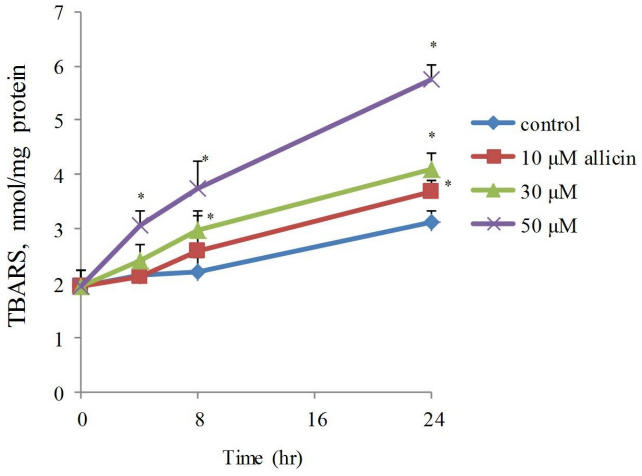

Figure 3. Effect of various concentrations of allicin on thiobarbituric acid reactive substances (TBARS) production in primary rat hepatocytes

Hepatocytes isolated from each animal were divided equally and cultured in media containing 0 (control), 10, 30, or 50 μM allicin. Values are expressed as the mean ± SD from 4 rats. *$P < 0.05$ between control and treatment at the same time interval (ANOVA and Duncan's multiple comparison tests).

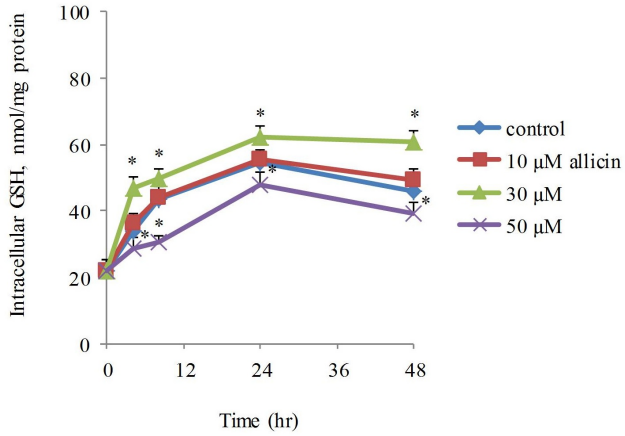

Figure 4. Reduced intracellular glutathione levels of various concentrations of allicin treated-primary rat hepatocytes. Hepatocytes isolated from each animal were divided equally and cultured in media containing 0 (control), 10, 30, or 50 μM allicin. Values are expressed as the mean ± SD from 4 rats. *P < 0.05 between control and treatment at the same time interval (ANOVA and Duncan's multiple comparison tests).

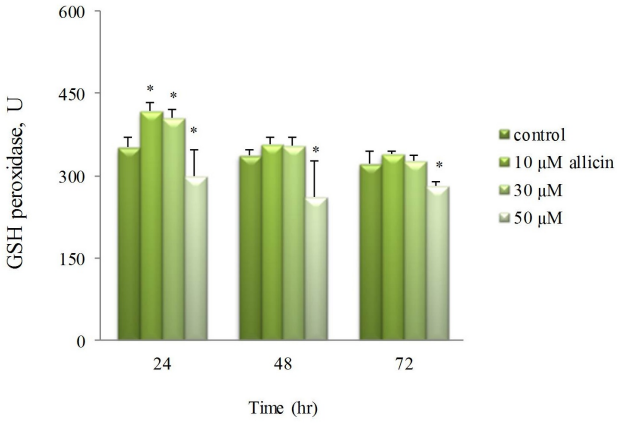

Figure 5. Glutathione peroxidase activity of various concentrations of allicin treated-primary rat hepatocytes. Hepatocytes isolated from each animal were divided equally and cultured in media containing 0 (control), 10, 30, or 50 μM allicin. GSH peroxidase activity is expressed as nmole NADPH oxidized/min/mg cytosolic protein. Values are expressed as the mean ± SD from 4 rats. *P < 0.05 between control and treatment at the same time interval (ANOVA and Duncan's multiple comparison tests).

GSH concentration

Figure 4 shows changes in intracellular GSH levels in primary rat hepatocytes at different incubation times with various allicin concentrations. When treated with 10 μM allicin, the intracellular GSH content of hepatocytes did not differ significantly from control after 48 h incubation. However, intracellular GSH content was significantly greater than control (P < 0.05) when the hepatocytes were treated with 30 μM allicin for 4 h or longer (intracellular GSH content was approximately 47–61 nmol/mg cytosolic protein). In addition, when treated with 50 μM allicin, the intracellular GSH level was lower compared to the control at 4 h or longer, but was not significantly lower than the control. Similarly, intracellular GSSG levels were significantly increased (P < 0.05) upon treatment with 30 μM allicin at 24 and 48 h, and deceased by treatment with 50 μM allicin.

GPx, GRd, and GST activity

The change in GPx activity towards H_2O_2 in primary rat hepatocytes treated with various concentrations of allicin is shown in Figure 5. When treated with 10 μM allicin, GPx activity at 48 and 72 h (356 ± 15 and 332 ± 14 U, respectively) were significantly higher than the control (298 ± 8 and 271 ± 8 U) (P < 0.05). However, when treated with 30 μM allicin, no significant changes in GPx activity were observed compared to controls. GPx activity in 50 μM allicin-treated hepatocytes was significantly (P < 0.05) lower compared to that of the control after 48 and 72 h (216 ± 24 and 182 ± 43 U, respectively).

The changes in GRd activity in primary rat hepatocytes treated with various allicin concentrations are shown in Figure 6. When treated for 24 h or longer with 10 or 30 μM allicin, GRd activities (GRd activities of 27–42 and 26–43 U, respectively) were significantly higher compared to that of the control (P < 0.05).

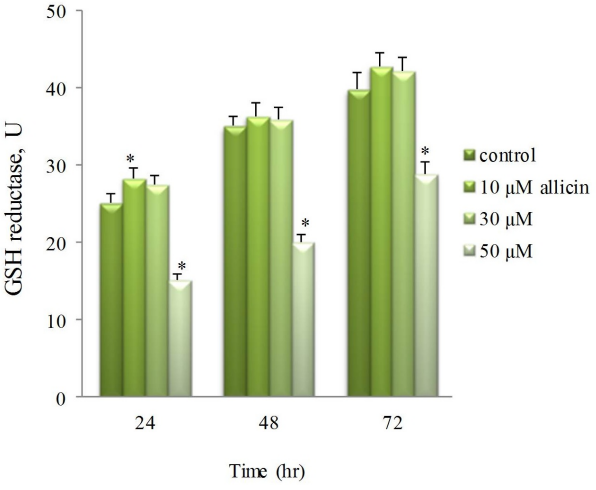

Figure 6. Effect of various allicin concentrations on glutathione reductase activity in primary rat hepatocytes. Hepatocytes isolated from each animal were divided equally and cultured in media containing 0 (control), 10, 30, or 50 μM allicin. GSH reductase activity is expressed as nmole NADPH oxidized/min/mg cytosolic protein. Values are expressed as the mean ± SD from 4 rats. *P < 0.05 between control and treatment at the same time interval (ANOVA and Duncan's multiple comparison tests).

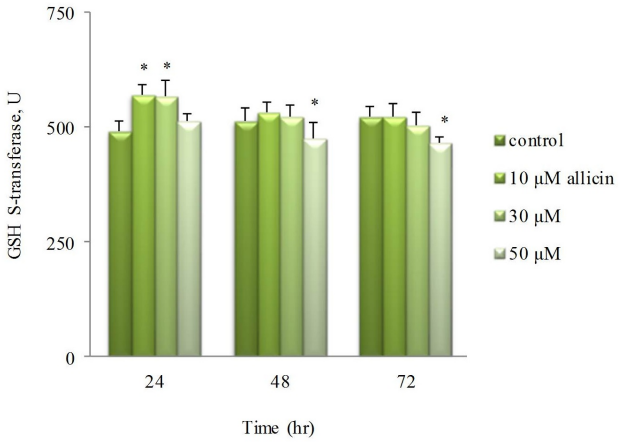

Figure 7. Glutathione S-transferase activity of various concentrations of allicin treated-primary rat hepatocytes. Hepatocytes were isolated from 8-week old male rats. Cells isolated from each animal were divided equally and cultured in media containing 0 (control), 10, 30, or 50 μM allicin. GSH S-transferase activity is expressed as nmole CDNB conjugate formed/min/mg cytosolic protein. Values are expressed as the mean ± SD from 4 rats. **P* < 0.05 between control and treatment at the same time interval (ANOVA and Duncan's multiple comparison tests).

However, GRd activities were significantly lower than that of the control ($P < 0.05$) in 50 μM allicin-treated hepatocytes after 24 hr.

The effect of various allicin concentrations and incubation times on GST activity towards CDNB in primary rat hepatocytes is shown in Figure 7. When treated for 72 h with 10 or 30 μM allicin, GST activity (619 ± 16 and 615 ± 21 U, respectively) was significantly higher compared to control (569 ± 18 U) ($P < 0.05$). In contrast, when treated with 50 μM allicin for 72 h, GST activity (520 ± 31 U) was significantly lower compared to control ($P < 0.05$).

Discussion

Several models have been devised for analyzing liver function *in vitro*. These include isolated liver perfusion, tissue slices, homogenates, and purified hepatocyte populations freshly isolated or in culture. Among these different models, cultures provide certain advantages. Specifically, cultures allow long-term studies on a liver cell population divested of non-parenchymal cells normally present in the organ. In addition, they allow for a controlled and simplified environment, as well as various experimental conditions and time courses. Hepatocyte cell culture will certainly continue to excite the interest of biologists and investigators. It is obvious that the availability of *in vitro* cultures with the functions of liver cells is valuable for understanding the regulation of certain metabolic activities and for elucidating the importance of factors that regulate hepatocytic specificity *in situ*. The most commonly studied functions are plasma protein production and enzymatic activities of liver-specific intermediary metabolism and xenobiotic metabolism. In previous studies, isolated and cultured primary rat hepatocytes provided a useful model for studying liver cell function in rat. LDH leakage assays and microscopic examination were used to determine the cell viability during prolonged incubation experiments. LDH leakage is an indicator of membrane damage and the assay is sensitive and easy to perform (Jurisic, V., 2003). Increased LDH leakage indicates more plasma membrane damage. Figure 1 shows that 10 or 30 μM allicin treatment for 24 h did not affect hepatocyte membranes. The results of microscopic examination (Figure 2) coincided with these results. An early visible indication of severe cell disturbance is the appearance of blebs on the outer surface of cell membrane (Lemasters et al., 1987; Simonin et al., 2007), which is not accompanied by immediate cell disruption (Scharfenberg et al., 1994). It was suggested that thiol oxidation of cytoskeleton proteins (Mirabelli et al., 1988) and plasma membrane rigidity (Florine-Casteel et al., 1991; Abe et al., 2009) might be intimately connected to, or precede, bleb formation. In our study, bleb formation and cell aggregation were observed 8 and 24 h after 50 μM allicin treatment. Therefore, allicin could also interact with plasma membrane functions by affecting these membrane proteins or enzymes. In addition, Zhang et al. (2010) have also shown that the inhibitory effect of allicin on tumor cell proliferation was associated with cell cycle arrest and apoptosis induction.

Plasma membranes consist of a phospholipid bi-layer. Lipid peroxidation of cellular membranes alters the polyunsaturated fatty acid components of membrane phospholipids (Yajima et al., 2009; Menendez et al., 2004). Poly fatty acid side chains are very susceptible to xenobiotics, such as free radicals, which can induce lipid peroxidation. Peroxidation causes damage to membrane lipids and proteins as well as depletion of antioxidants. Upon treatment with 50 μM allicin for 8 h, there were significantly increased blebs and TBARS levels ($P < 0.05$), as well as depletion of intracellular GSH after 24 and 48 h.

The GSH redox system plays a central role in several reactions. GSH depletion favors increases in the prooxidant/antioxidant balance in cells and increases the susceptibility to oxidative injuries. In nearly all tissues,

GSH is present in its reduced form, with less than 5% present as GSH-disulfide (GSSG). This is due to the thiol redox status, which is maintained by intracellular GRd and NADPH. When treated with 30 μM allicin for 4 h or longer, intracellular GSH levels were significantly higher compared to control (Figure 4; $P < 0.05$). This phenomenon is beneficial for the antioxidation and detoxification capacities of hepatocytes because GSH protects hepatocytes from the toxic effects of reactive oxygen species, such as free radical and peroxides (Scott, M. D., 2006). In contrast, when treated with 50 μM allicin for 24 or 48 hr, intracellular GSH levels were lower than controls. This indicates that damage had occurred in the hepatocytes, and results in GSH depletion.

GSH is used by GSH peroxidase to reduce H_2O_2 and organic peroxide from GSSG. Superoxide and H_2O_2, formed during metabolism, produce reactive oxygen species that lead to organic peroxide (Scott, 2006). GSH peroxidase and GSH reductase protect cell proteins and membranes against oxidatative stress (Meister, 1988). Compounds with an electrophilic center conjugate readily with GSH. GSH S-transferase catalyzes GSH interaction with many types of compounds. GST is a family of multifunctional enzymes that detoxify a wide variety of electrophilic xenobiotics (Asakura et al., 2007). The data in Figures 6, 7, and 8 show the activities of GST, GPx, and GRd were significantly affected by 50 μM allicin after 72 hr, except that GRd activity was significantly lower than control ($P < 0.05$). However, when cells treated with 10 and 30 μM allicin for 24, 48, and 72 h, GRd activity was higher than the control, suggesting that the enhanced GSH redox system increases GSH levels. Our results suggest that 10 μM allicin may potentially enhance the antioxidation and detoxification capability of primary rat hepatocytes.

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