行政院國科會專題研究計畫成果報告
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計畫名稱：荷爾蒙與含硫氨基酸調控肝細胞麩甘氨酸合成之探討
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一、中文摘要

已知麩甘氨酸（glutathione, GSH）在胞內扮演有多樣且重要的角色，本研究即針對肝細胞內麩甘氨酸爲對象探討荷爾蒙與含硫氨基酸對麩甘氨酸合成之影響，肝細胞分別在含0.1 mM或1.0 mM含硫氨基酸的培養液中爲期六天，同時各培養液再分別依有無加入胰導素或甲基脫氫皮質醇（dexamethasone）分爲四小組，分析比較胞內麩甘氨酸濃度、麩甘氨酸相關酵素活性及蛋白表現。結果：在1.0 mM甲硫氨酸及半胱氨酸下，胞內麩甘氨酸隨培養時間增加，第四天達到最高，至於在0.1mM甲硫氨酸及半胱氨酸培養下，麩甘氨酸濃度則遞減，顯示培養液甲硫氨酸及半胱氨酸的充分供應有助於維持肝細胞內麩甘氨酸的合成；至於荷爾蒙方面，結果則指出胰導素或甲基脫氫皮質醇的效應正好相反，無論含硫氨基酸濃度胰導素均可增加胞內麩甘氨酸水準，至於甲基脫氫皮質醇則是抑制，在麩甘氨酸合成決定酵素－γ-glutamylcysteine synthetase 活性分析上，則顯示出胰導素增加胞內麩甘氨酸與其增進γ-glutamylcysteine synthetase活性有關，至於甲基脫氫皮質醇則是因爲抑制了γ-glutamylcysteine synthetase活性；在麩甘氨酸過氧化酶（GSH peroxidase）、麩甘氨酸還原酶（GSH reductase）及麩甘氨酸硫轉移酶（GSH S-transferase）活性方面，則不受胰導素、甲基脫氫皮質醇與含硫氨基酸影響。

關鍵詞：胰島素、甲基脫氫皮質醇、含硫氨基酸、麩甘氨酸、γ-glutamylcysteine synthetase

Abstract

Glutathione (GSH), a tripeptide, plays diverse roles in cells. It is not only important in drug detoxification but also in protecting cells against oxidative damage. Elevating the amount of cellular GSH is good for cells. Normal cells have a regulatory mechanism on GSH synthesis and the GSH concentration in hepatocytes is normally ranged from 2 to 4 mM. In this study, we intend to study whether sulfur amino acid level and hormones modulate hepatic GSH synthesis. Hepatocytes were cultured in media contained 1.0 mM or 0.1 mM each of methionine and cysteine in the presence or absence of 5 mg/L insulin and/or 1 μM dexamethasone, for up to 6 d. For all treatments, samples were taken on d 1, 3, 4 and 6. Cells cultured with 1.0 mM methionine and cysteine had increased intracellular GSH and reached maximum at d4. However, intracellular GSH decreased gradualiy in cells treated with 0.1 mM methionine and cysteine. The presence or absence of insulin or dexamethasone in medium affected hepatic GSH level over the entire experimental period. Insulin significantly increased intracellular GSH level after d1. In contrast, dexamethasone decreased hepatic GSH level. As examined the γ-glutamylcysteine synthetase activity, cells treated with insulin had higher enzyme activity than cells treated with dexamethasone. However, enzyme activity was similar in cells incubated with 1.0 mM methionine and cysteine or in cells incubated with 0.1 mM methionine and cysteine. No difference in GSH peroxidase, GSH reductase and GSH S-transferase activities between treatments was noted in this study.

Introduction

Due to the diverse and important role of GSH, the regulatory mechanism of GSH synthesis attracts extensive attention in the recent years. It is known that GSH synthesis can be regulated via different pathways, by feedback mechanism [1] or by modulation of GSH efflux [2] or the γ-glutamylcysteine synthetase (GCS) activity [3-5]. GCS, the rate-limiting enzyme in de novo GSH synthesis, is believed to play a crucial role in regulating cellular GSH content. Many factors, such as GSH itself, hormones, GSH depleting agents, GSH conjugating xenobiotics and oxidative stress have been demonstrated to be the GCS regulators [5, 6-10]. The rat GCS is

composed of one heavy (73 kDa) and one light (30 kDa) subunit. Evidence indicated the heavy chain exhibits all of the catalytic activity and the feedback inhibition by GSH [11], whereas the light subunit is enzyme inactive but with important regulatory function [12]. Regulatory mechanism on GCS heavy subunit has been demonstrated to act on either gene transcription [13] or post translation [3,]. Mulcahy et al. [14] reported that GCS heavy subunit gene expression in HepG2 cells in the presence of ß-naphthoflavone is mediated by a distal antioxidant response sequence containing an embedded tetradecanoylphorbol-13-acetate-responsive element. GCS activity can also be regulated via phosphorylation of heavy subunit by protein kinase A, protein kinase C and Ca^{2+}/calmodulin-dependent kinase II [15].

Increased intracellular GSH content and GCS activity are correlated well with increased cell survival against oxidative stress [16,17]. However, GCS could be negatively controlled by the GSH ($K_i \cong 2.5$ mM) [1]. Recently, we noticed GSH level of rat hepatocytes cultured in L15 increases from 2.0 mM of freshly isolated cells to 6.0 mM on d 6 after plating [18]. Increase in GSH biosynthesis disappeared when the sulfur amino acid concentration in medium decreased to 0.1 mM. But we did not know whether the decrease in GSH content was simply due to the limited supply of amino acid for GSH synthesis or was related to GCS protein. However, both insulin and dexamethasone which are commonly supplemented in the cell culture medium are also the possible factors contributing to the hepatic GSH content during culture [4, 19]. The elevation of cellular GSH content during culture can be resulted from the action of both hormones. In this study, we intend to examine the effect of sulfur amino acids and hormones on hepatic GSH content as well as the role of GCS played in GSH biosynthesis. At the same time, we also investigated the effect of insulin and dexamethasone on GSH biosynthesis and on several GSH-related enzyme activity, including GSH peroxidase, GSH reductase and GSH S-transferase.

Results and Discussion

Cellular GSH content. In cells supplemented with 1.0 mM each of methionine and cysteine, reduced GSH content gradually increased up to d 3, then decreased (**Fig. 1**). When the amino acids concentrations were 0.1 mM each of methionine and cysteine, GSH decreased at the first 24 h followed by increasing to the initial GSH level at the next 48 h, then gradually decreased and only 20% of the initial GSH level was left at d 6. Regardless of the absence or presence of dexamethasone, insulin supplementation increased hepatic GSH content compared to cells lack of hormones or supplementation with dexamethasone alone. Cells incubated with dexamethasone alone had the lowest cellular GSH level among the treatment.

GCS activity. Although 0.1 mM each of methionine and cysteine supplementation could not maintain cellular GSH content, GCS activity was similar to cells incubated with 1.0 mM of amino acids (**Fig. 2**). Results indicate that the decrease in GSH content in cells cultured with low sulfur amino acids during the culture period is unrelated to GCS protein but is probably due to the limited supply of cysteine for GSH biosynthesis. To compare the effect of hormones, we found that GCS activity change pattern over the entire culture period was parallel to that of cellular GSH content. Insulin supplementation increased GCS activity and dexamethasone suppressed enzyme activity.

Other GSH-related enzyme activities. In this study, GSH peroxidase, GSH reductase and GSH S-transferase activities were also determined. Over this 6-d period, the change pattern was different among these three enzymes. GSH peroxidase activity was gradually decreased after plating, GSH S-transferase kept constant, however, GSH reductase activity was gradually increased (data not shown).

GSH S-transferase isozyme and GSH synthetase expression. Rat GSH S-transferase is composed of two subunits in homodimer or heterodimer characteristic [20]. Although GSH S-transferase activity maintained constant over the entire culture period, it may not mean that all the GSH S-transferase isozyme expression is unchanged. Immunoblotting analysis revealed that GSH-S-transferase Ya and Yc isozymes levels constantly decreased post plating and was not different among the hormone supplemented groups (**Fig. 3**). Yb

expression, however, was dependent on the hormone supplementation. The level of Yb in cells incubated with dexamethasone maintained constant for up to d 6, instead, in the presence of insulin, Yb quickly decreased after d 4. A dramatic change was noted on the inducible form of GSH S-transferase, Yp, expression (**Fig. 4**). Only small amount of the Yp expression was detected in freshly isolated hepatocytes, but subsequently increased with time. No matter whether the cells were incubated with 1.0 or 0.1 mM each of methionine and cysteine, Yp was first detected at d3, then quickly increased up to d 6. Interestingly, Yp level in the presence of low sulfur amino acid concentration was much higher than that in cells incubated with high amino acid concentration. Results suggested the expression of this inducible GSH S-transferase isozyme can be up-regulated in the presence of low methionine and cysteine supplementation. Certainly, the modulation may be not related directly to the sulfur amino acids but is due to the low cellular GSH level or the potential stress during culture. It deserves further study. The expression of Yp was also noted to be suppressed in the presence of dexamethasone. Insulin, however, had no influence on the expression of this GSH S-transferase isozyme. Regardless of sulfur amino acids and hormones treatment, GSH synthetase maintained similar level over this 6-d experiment (data not shown).

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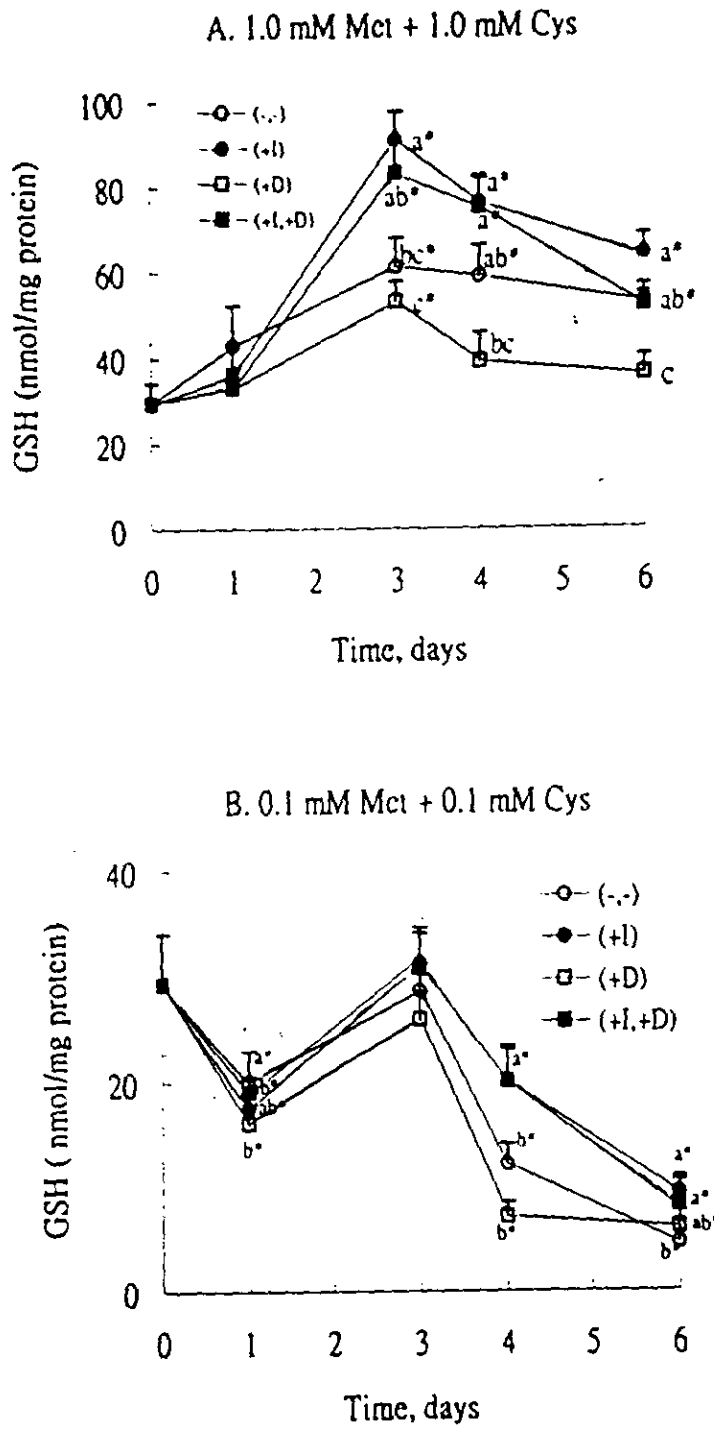

Figure 1. The effect of hormones and various level of methionine and cysteine on GSH content in rat hepatocytes. Hepatocytes were isolated from 8-wk-old male Sprague-Dawley rats. After isolation, cells were maintained in L 15 cell culture medium for 4 h then changed to medium contained 1.0 or 0.1 mM each of methionine and cysteine in the presence or absence of insulin and/or dexamethasone for up to 6 d. Values are means ± SD, n=5. *Treatment means in a medium significantly different form time 0 ($P<0.05$). ^{abc}Treatment means not sharing a letter among hormonal supplementation differ significantly ($P<0.05$).

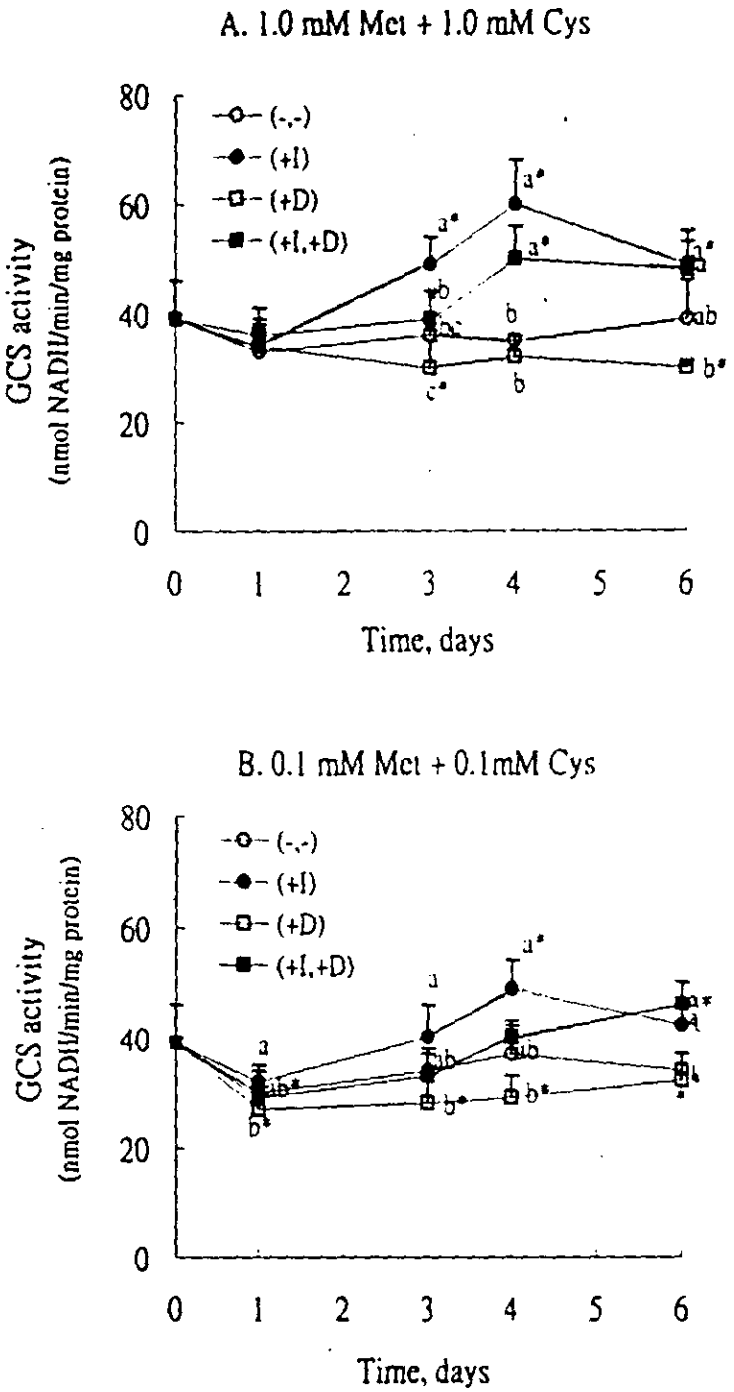

Figure 2 GCS activity in rat hepatocytes cultured for up to 6 d. Values are means ± SD, n=4. *Treatment means in a medium significantly different form time 0 ($P<0.05$). ^{abc}Treatment means not sharing a letter among hormonal supplementation differ significantly ($P<0.05$).

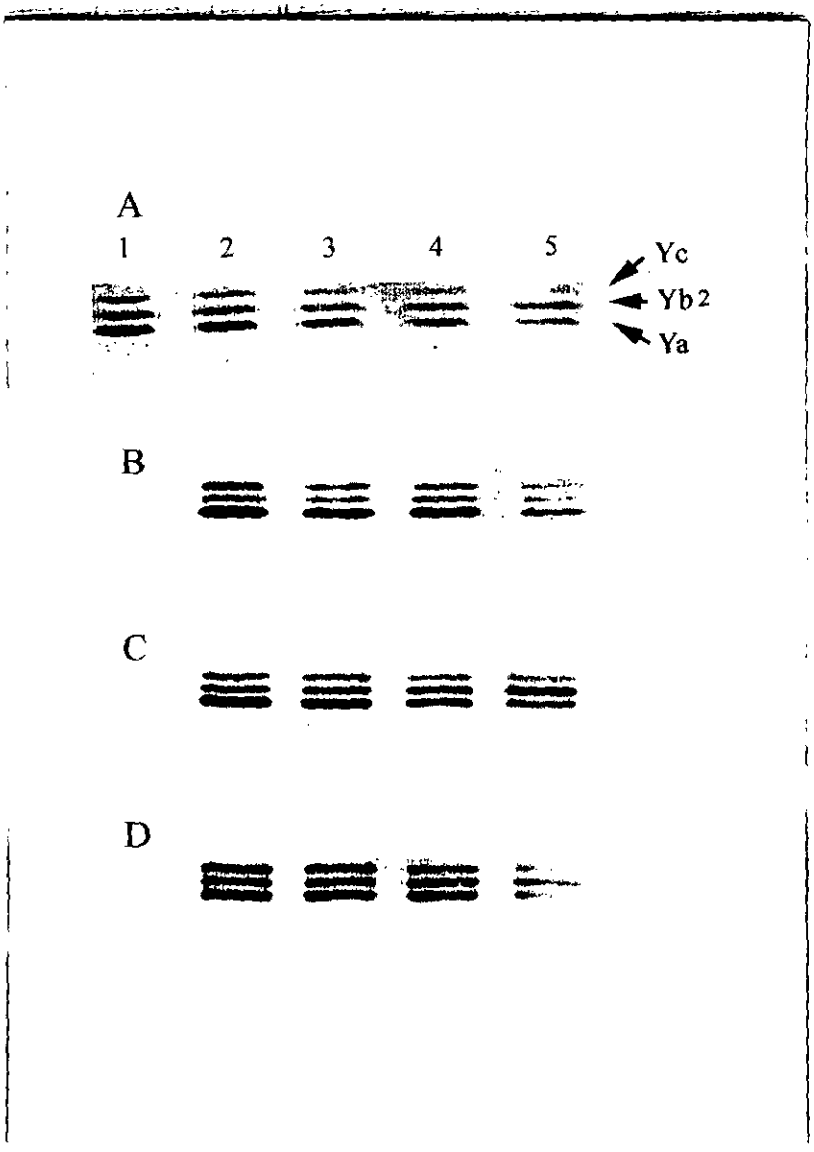

Figure 3. The effect of insulin and dexamethasone on GSH S-transferase Ya, Yb and Yc expression in rat hepatocytes cultured in the presence of 1.0 mM each of methionine and cysteine. For each lane 1 μg of cytosolic protein was applied to SDS-polyacrylamide gels. After transferred the proteins to PVDF membranes, GSH S-transferase isozymes were immunostained by antibody-linked peroxidase activity. Lane 1, cytosol from freshly isolated hepatocytes; lanes 2-5, samples from hepatocytes cultured for 1, 3, 4 and 6 d, respectively. Panel A-D, cytosol from cells maintained in medium contained both of insulin and dexamethasone, insulin alone, dexamethasone alone or in the absence of insulin and dexamethasone, respectively.

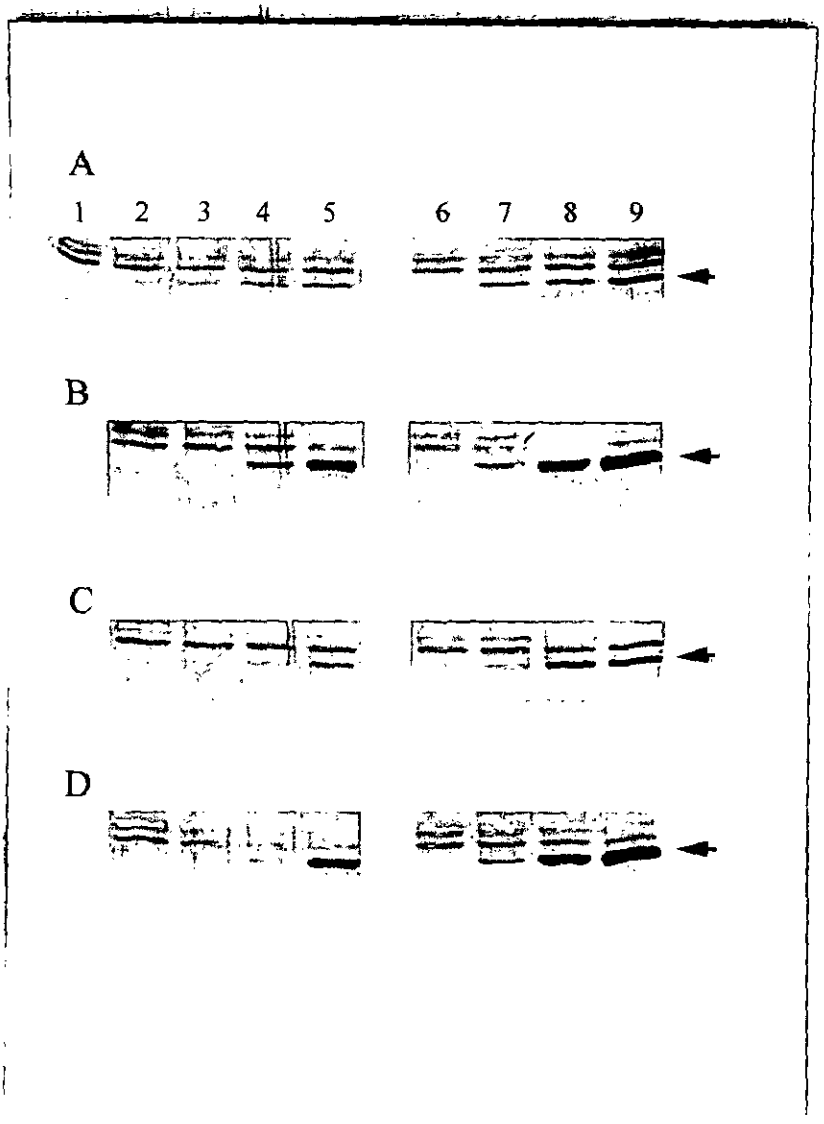

Figure 4. Immunodetection of Placental form of GSH S-transferase of hepatocytes with different levels of methionine and cysteine in the presence or insulin and dexamethasone (A), insulin alone (B), dexamethasone alone (C) or in the absence of insulin and dexamethasone (D). Data are shown the freshly isolated hepatocytes (lane 1), or cells cultured for 1 d (lanes 2,6), 3 d (lanes 3,7), 4 d (4,8) and 6 d (lanes 5,9) after the incubation with 1.0 mM (lanes 2-5) or 0.1 mM each of methionine and cysteine.