活性氧化物種對游離輻射和抗藥性之影響和其調節機制
Modulation of cellular responses to ionizing radiation and drug resistance by reactive oxygen species (ROS)

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執行單位：國立成功大學醫學院醫學系放射線學科

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活性氧化物種對游離輻射和抗藥性之影響及其調節機制

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一、中文摘要

目的：生物體受到外在的刺激如游離輻射或具有細胞毒性的化學藥物時，會產生活性氧化物種，造成氧化傷害。麸胱甘肽(Glutathione, GSH)是生物體內非常重要對抗氧化損傷的抗衡機制系統，來自身體內生合成的麸胱甘肽主要受到麸胱甘肽合成酵素(γ-glutamylcysteine synthetase heavy subunit, γ-GCS)的調控，由於有些腫瘤本身γ-GCS就已高度表達，本一年期的計畫欲研究調控生物體內γ-GCS來影響活性氧化物種的產生，研究其對鉑金類化學藥物抗藥性的影響及作用機轉。

材料與方法：小細胞肺癌的細胞株SR3A，外送入麸胱甘肽合成酵素催化鏈(γ-GCSh)的基因後，經過篩選，得到三株高度穩定表達γ-GCSh的細胞，分別命名為SR3A-13, SR3A-14, SR3A-15，測量這些細胞株發現如預期所假設的，其細胞內的活性氧化物種均比母細胞株較為減少。本研究利用這些細胞株，測量其對化學藥物鉑金(Cisplatin)的抗藥性，並測量細胞內此化學藥物和金屬銅的含量，佐以西方墨點試驗法和核醣核酸內切酵素保護定序法定量及分析、小干擾核糖核酸干擾mRNA的轉譯作用等方法，研究調節生物體氧化還原作用影響抗藥性的分子作用機轉。

結果：三株高度穩定表達γ-GCS的細胞，對化學藥物鉑金(Cisplatin)的抗藥性反而減少，其作用機制經過西方墨點試驗法和核醣核酸內切酵素保護定序法定量及分析後，發現是透過增加細胞膜上的銅離子輸送蛋白(human copper transporter 1, hCtr1)之故。利用藥物或小干擾核糖核酸等方法將細胞內的麸胱甘肽減少，則細胞對於化學藥物鉑金(Cisplatin)的抗藥性會增加。雖然這些細胞內金屬銅的攝入量在測量時是增加的，但是細胞卻無銅離子過多中毒的徵兆，反而呈現出生物體缺乏銅離子的各式典型生化反應，同時生物體內負責輸出銅離子的蛋白量並無改變，顯示藉由麸胱甘肽影響細胞內活性氧化物種的生成，會影響細胞內銅離子的量減少，此平衡狀態的改變導致銅離子輸送蛋白在細胞膜的表現增加。

結論：藉由麸胱甘肽影響細胞內活性氧化物種的生合成，會影響細胞對化學藥物鉑金的抗藥性，我們推測麸胱甘肽可能會與細胞內銅離子形成螯合複合體，增加細胞內的麸胱甘肽可以耗損細胞內的銅離子，繼而改變生物體內微妙的銅離子平衡狀態，導致負責化學藥物鉑金輸入細胞內的主要蛋白—銅離子輸送蛋白在細胞膜的表現增加，繼而影響抗藥性。

關鍵字：活性氧化物種、麸胱甘肽、麸胱甘肽合成酵素(γ-GCS)、銅離子輸送蛋白、抗藥性。
二、英文摘要

**Abstract:**
It has been appreciated that exposure of eukaryotic cells to chemotherapeutic agents or ionizing radiation induces steady-state increases of reactive oxygen species (ROS). ROS which consist of o xyl radicals including superoxide anion, hydroxyl radical and hydrogen peroxide, play important roles in regulating many normal physiological functions. Evidence has accumulated showing that redox signals are activated in response to drug treatments of human malignancies. Glutathione (GSH) system is an important intracellular antioxidant that regulates physiological redox conditions by counteracting oxidative stress. Studies have established that γ-glutamylcysteine synthesis heavy subunit (γ-GCS), which is the rate-limiting enzyme for the biosynthesis of glutathione, is an important regulator of intracellular redox conditions. Previous studies have demonstrated that treating cultured cells with cisplatin upregulated the expression of glutathione and its de novo rate-limiting enzyme, γ-glutamylcysteine synthetase (γ-GCS). It has also been shown that many CDDP-resistant cell lines exhibit high levels of γ-GCS/γ-GCSI and GSH. Since GSH system is the major intracellular regulator of redox conditions, these results have been taken into considerations that elevated levels of γ-GCS/GSH are responsible for the CDDP resistance. In contrast to this context, we found that overexpression of GSH by transfection with expression recombinant plasmid containing the γ-GCS plasmid conferred sensitization to CDDP through upregulation of human copper transporter 1 (hCtr1), which is also known as transporter for CDDP. Depleting GSH levels in these transfected cells reversed CDDP sensitivity with concomitant reduction of hCtr1 expression. While expression of Cu transport was also upregulated in the transfected cells, no enhanced sensitivity to Cu toxicity were observed in these cells. We also observed that these GSH-overproducing cells exhibited biochemical signature of Cu deficiency. Our results redefine a new role of GSH in the regulation of CDDP sensitivity. Overproduction of GSH depleted bioavailable Cu pool, leading to upregulation of hCtr1 and sensitization of CDDP transport and cell killing. These findings provide a previously undiscovered new role of GSH in the regulation of CDDP sensitivity.

**Key Words:** Reactive oxygen species (ROS), glutathione, cisplatin, γ-glutamylcysteine synthetase heavy subunit (γ-GCS), human copper transporter 1 (hCtr1).

三、研究成果報告

**Introduction:**

Cellular metabolism is critical for the generation of energy in biological systems; however, as a result of electron transfer reactions, reactive oxygen species (ROS) are generated in aerobic cells. Although low amounts of ROS are easily tolerated by the cell, abnormally high levels of ROS induce oxidative stress (1). It has been appreciated that exposure of eukaryotic cells to chemotherapeutic agents or ionizing radiation induces steady-state increases of reactive oxygen species (ROS). ROS which consist of o xyl radicals including superoxide anion, hydroxyl radical and hydrogen peroxide, play important roles in regulating many normal physiological functions including intracellular oxygen metabolism, immune-mediated attack of pathogens, signal transduction/gene expression pathways, as well as being present and worsening a number of human pathological conditions. Glutathione (GSH) system is an important intracellular antioxidant that regulates physiological redox conditions by counteracting oxidative stress. Studies have established that γ-glutamylcysteine synthesis heavy subunit (γ-GCS), which is the rate-limiting enzyme for the biosynthesis of glutathione, is an
important regulator of intracellular redox conditions (2).

Cisplatin (CDDP) has been recognized as an important antitumor agent because of its activity against human malignancies, including testicular, ovarian, cervical, bladder, head and neck, and small cell lung cancers (SCLC) (3). However, many patients eventually relapse and develop resistance to the treatment. It is well known that CDDP acts on multiple cellular targets representing diverse signal transduction pathways. It is therefore conceivable that multiple mechanisms have been proposed for CDDP resistance, including reduction of drug transport and increased DNA adduct tolerance and repair.

Another CDDP-resistance mechanism that has been widely described in the literature is the detoxification through glutathione (GSH) system. GSH is the most abundant thiol-containing antioxidant. Previous studies demonstrated that exposure of cultured cells to CDDP led to the development of CDDP resistance that were closely correlated with increased cellular GSH levels (4). Moreover, GSH depletion by buthionine-sulfoximine (BSO) is associated with increased sensitivities to CDDP (5). In many case when γ-GCS mRNA contents were measured, elevated levels of γ-GCS mRNA were also correlated with CDDP resistance. These studies have been widely taken to suggest that intracellular GSH levels play an important role in regulating CDDP resistance. However, these studies were frequently used drug–treated cells and the observations were mostly correlation in nature. To investigate the cause-effect relationship between elevated GSH and CDDP sensitivity, we studied the CDDP sensitivity of those γ-GCS-transfected cells and explored the mechanisms.

Methods and Materials:

Chemicals and Reagents. CDDP, arsenite, copper, doxorubicin, vinblastine, melphalan, chlorambucil, and BSO were purchased from Sigma (St Louis, MO). Construction and preparation of recombinant adenovirus, AdE1.tTA;γGCSh, were described previously (6).

Cell Cultures. SR3A and the development of its γ-GCS stably transfected cell lines, SR3A-13, SR3A-14, and SR3A-15, have been described previously (2). The cells were grown in DMEM containing 10 % fetal calf serum at 37 °C in 5% CO₂ atmosphere except otherwise described and 400 μg/ml of G418 for the transfected lines.

Determination of Drug Sensitivity. Cells grown in 96 well plates (10⁴ cells/ml medium) were continuously exposed to various concentrations of drugs for 72 hr incubation, 200 μl of MTT (0.5 mg/ml, Sigma) was added to each well, and the plate was incubated for 4 hr. The medium was removed and the formazan products were solubilized with 120 μl DMSO. The cell contents were measured by the absorbance at 570 nm. The IC₅₀ value (μM) was calculated by the Hill plot method with linear regression.

Measurements of Cu and CDDP Uptake. 10⁶ cells were plated in a 12 well plate. After 24 hr, cells were treated with 20 μM CDDP or 2 μM ⁶⁴CuSO₄ and the plates were incubated at 37 °C on 5 % CO₂ up to 4 h. After incubation for various time intervals, plates were placed on ice and rinsed three times with 3 ml ice cold PBS. Cell lysis buffer (0.1 % Triton-X100 and 1 % SDS in PBS) in a volume of 800 μl was added to the wells, and the radioactivity of cell lysates was determined by scintillation counter. For measurement of CDDP contents, cell lysates were acidified with 200 μl 0.3N HCl and determined in an atomic absorption spectrometer (SpectrAA300, Varian, Palo Alto, CA).
RNase Protection Assay and Immunoblots. The procedures for preparation of RNA and RNase protection assay have been described previously (2;7). The western blots were carried out according to the procedures previously described (7) using the following antibodies: rabbit polyclonal antibodies against hCtr1 (8), ATP7A (Orbigen, San Diego, CA, 1:500), ATP7B (Orbigen, 1:500), Cu/Zn SOD (Calbiochem, Darmstadt, Germany, 1:5,000) and mouse monoclonal β-actin (Pierce, Rockford, IL) antibody.

Measurements of ROIs, CCO and SOD1 Activities. Relative reactive oxygen species levels were measured by flow cytometry using dihydrorhodamine 123 (Invitrogen) according to the procedure described previously (2). CCO activity was determined using an assay kit obtained from Sigma according to the procedure provided by the vendor. SOD1 activity was measured by the procedure described by Chen et al (9).

Results:
Elevated Expression of GSH in γ-GCSH-Transfected Cells Sensitizes Cells to CDDP But Not Copper. We previously performed transfection of γ-GCSH recombinant cDNA into a human SCLC cell line, SR3A. Several γ-GCSH overproducing cell lines were obtained. Among them, SR3A-13, SR3A-14, and SR3A-15, exhibited 5.1-, 9.6- and 2.2- fold, respectively, increased γ-GCSH mRNA levels, corresponding to 3.0-, 3.9- and 2.7- fold increases of GSH levels as compared with those in SR3A cell line. As consistence with previous study, all the transfected cells exhibited resistance to the alkylating agents, melphalan, and chlorambucil. Interestingly, these cells exhibited elevated sensitivities to CDDP, but not to Cu except SR3A-15, toxicity. However, not all the γ-GCSH-transfected cells exhibited resistance to doxorubicin. These results demonstrated that elevated expression of GSH by transfection enhanced the sensitivities to CDDP.

γ-GCSH-Transfected Cells Showed Increased Transport Activities of CDDP and Cu. To investigate whether enhanced CDDP sensitivity was due to increased drug accumulation, we determined the rates of drug uptake. SR3A and its three transfected lines were treated with 20 μM CDDP, and 2 μM Cu64. After different time intervals, the cellular CDDP and Cu contents were measured by atomic absorption spectroscopy and scintillation counting, respectively. The rates of CDDP and Cu64 transport were both greater in the transfected cells than that in the untransfected SR3A cells (Fig. 1).

Recent studies have demonstrated that many copper transporters, including import transporter (hCtr1) and export transporters (ATP7A and ATP7B), are also involved in CDDP transports (10). To investigate the roles of these CDDP transporters in CDDP accumulation in the transfected cells, we performed RNase
protection assay to determine the steady-state mRNA levels of these transporters. Fig. 2A shows that levels of hCtr1 mRNA were elevated in the transfected cells, whereas the levels of ATP7A and ATP7B mRNA show no significant difference as compared with those in SR3A cells. Elevated expression of hCtr1, but not ATP7A and ATP7B, was also evidenced by immunoblottings (Fig. 2B). Immunofluorescent microscopy shows that the overexpressed hCtr1 in the transfected cells were mainly associated with cytoplasmic membrane (Fig. 2D). These results demonstrated that the sensitization of γ-GCSh-transfected cells to CDDP was associated with increased hCtr1 expression.

To substantiate these results, we turned to γ-GCSh-inducible expression system using recombinant adenoviral vector AdE1.tTA.γGCSh (6) which contains two expression cassettes: one constitutively expresses tetracycline (tet)-regulatable transactivator (rTA) and the other contains a γ-GCSh expression cassette whose expression is under the control of rTA. In the absence of tet, the constitutively expressed rTA binds to the promoter of γ-GCSh and activates the transcription of γ-GCSh; whereas in its presence, expression of γ-GCSh was inhibited. SR3A cells were transduced with AdE1.tTA.γGCSh in the presence (+) or absence (-) of tet for 24 or 48 hrs. As expected, high levels of γ-GCSh were expressed in cells grown in the absence of tet, as compared with those treated with tet (6). hCtr1 expression was elevated in the γ-GCSh-overexpressing cells (Fig. 2C). These results confirmed that elevated γ-GCSh levels induce the expression of hCtr1. Low levels of hCtr1 upregulation were also observed in cells treated with tet which suppress the induced expression of γ-GCSh. The mechanism of this induction is not known, perhaps due to oxidative stress associated with adenoviral transduction (6).

Reduced Levels of Reactive Oxygen Species Is Not Responsible for the Induction of hCtr1 in the γ-GCSh-Transfected Cells. Two possible mechanisms can account for the upregulation of hCtr1 expression by the overexpression of GSH. The first possibility is that, as GSH is the major antioxidant that regulates intracellular redox conditions, induction of hCtr1 expression in the GSH-overproducing cells may be a redox responsive mechanism. To test this possibility, we treated SR3A cells with different concentrations of catalase, another antioxidant that scavenges H$_2$O$_2$. H$_2$O$_2$ is relatively stable in comparison with other reactive oxygen species (O$_2$• and •OH) and can diffuse across cellular membrane. Addition of catalase to the medium can reduce intracellular ROS levels in cultured cells. However, an excess of catalase is toxic because it tips redox balance toward oxidized conditions.

SR3A cells were treated with different doses of catalase for 16 hrs. Cells
were harvested and divided into two aliquots: one aliquot was used for the analysis of redox conditions using ROS sensitizing fluorescence probe; and the other was used for measurements of hCtr1, ATP7A, and ATP7B levels by western blots. Fig. 3A shows that catalase at concentrations of 1,000 and 1,250 U/ml reduced intracellular ROS levels by 13.7% and 15.4%, respectively. No elevated expression of hCtr1, ATP7A, and ATP7B was observed under these conditions (Fig. 3B), indicating that reduced redox conditions did not enhance the expression of these transporters. However, levels of hCtr1, ATP7A and ATP7B were increased about 2-fold in cells treated with the high concentrations (2,000 and 2,250 U/ml) of catalase (Fig. 3B), which showed appreciably elevated intracellular ROS levels, i.e., 1.6% and 2.5% (Fig. 3A). These results were consistent with the possibility that hCtr1 expression is sensitive to elevated, but not reduced, ROS conditions.

Elevated GSH Levels Reduces Bioavailable Pool of Cu That Upregulates hCtr1 Expression. Glutathione can form a GSH-Cu(I) complex by directly interacting with its internal cysteine-SH residue. The formation of this complex is almost a spontaneous reaction and requires no enzymatic involvement, resulting in reduced intracellular bioavailability of Cu. It has been well-established that hCtr1 expression is regulated by intracellular available Cu levels (10). Expression of hCtr1 is upregulated in Cu-depleted cultured cells but downregulated under Cu-replete condition (10). To test the possibility whether upregulation of hCtr1 in the γ-GCSH-transfected cells is due to the reduction of available Cu, we analyzed three biochemical markers associated with Cu deficiency. (i) We examined the enzymatic activity of the well-characterized Cu-dependent enzyme, Cu,Zn superoxide dismutase (SOD1), which is reduced in Cu-deficient animals (11;12). As shown in Fig. 4A, the activity of SOD1 was significantly reduced in the SR3A-13, SR3A-14, and SR3A-15 cells as compared with that in SR3A cells. (ii) The mitochondrial cytochrome C oxidase (CCO) requires Cu for its activity. It has been demonstrated that reduced CCO activities is associated with Cu depletion (13). We found that CCO activity was significantly reduced in the γ-GCSH-transfected cells as compared with that in the non-transfected cells (Fig. 4B). (iii) Ceruloplasmin (Cpm) is a copper-containing ferroxidase that plays an important role in mammalian iron homeostasis. This protein utilizes the bound Cu ions to couple iron oxidation with four-electron reduction of dioxygen. Cultured cells grown in Cu-depleted medium severely comprise the incorporation of seven Cu atoms into the Cpm as it transverses the secretary pathway, resulting in increased apo-Cpm at the expense of holo-Cpm. This phenomenon is also
observed in the serum from animals of intestinal epithelial cell-specific ctr1 knockout mice (14). We performed immunoblot analysis of Cpm from cultured media of γ-GCSh-transfected cells following SDS-PAGE under non-reducing conditions which resolve the apo- and holo-enzymes into molecular mass corresponding to 135 kDa and 85 kDa, respectively. As shown in Fig. 4C, almost all Cpm secreted into medium from cultured SR3A cells was in holo-enzyme form. However, the ratios of holo-Cpm to apo-Cpm were significantly reduced in the γ-GCSh-transfected cells. Thus, the analyses of three Cu-containing enzymes strongly suggested that elevated GSH levels reduced intracellular available Cu and led to the induction of hCtr1 expression, thus increasing the uptake of CDDP and the sensitization of cells to CDDP toxicity. Reduction of GSH Levels in the γ-GCSh-Transfected Cells Reverses CDDP Sensitivity and hCtr1 Expression.

Fig. 4. Measurements of biochemical signature for Cu deficiency in the γ-GCSh transfectected cells. (A) SOD1 activities and protein levels; (B) CCO activities; and (C) ceruloplasmin.

To validate the above results, we used BSO to deplete intracellular GSH levels and analyzed the sensitivities of the treated cells to Cu and CDDP. Fig. 5B shows that BSO greatly sensitized SR3A cells to CDDP (90% decrease in IC50 value as compared with those in the untreated cells). Increased CDDP sensitivity is correlated with increased expression of hCtr1 (Fig. 5C). In contrast, the IC50 values of CDDP in the BSO-treated SR3A-13, SR3A-14, and SR3A-15 cells increased to the level comparable to that of untreated SR3A (Fig. 5B). Strikingly, expression levels of hCtr1 were reduced in the BSO-treated transfected cells (Fig. 5C). These results demonstrated that treating the γ-GCSh-transfected cells with GSH-depleting agent reversed the acquired CDDP sensitivity, further supporting the role of GSH in mediating CDDP toxicity. In comparison, BSO treatment increased sensitivity to Cu toxicity, regardless of γ-GCSh-transfected or non-transfected cells (Fig. 5A).

In summary, our results showing that it is feasible to modulate cellular sensitivities to CDDP treatment by using the Cu chelator, GSH, has important implications in cancer chemotherapy using platinum-based antitumor agents. Development of small molecules targeting...
the pool of ntracellular available Cu may be a novel approach toward improving the efficacy of these important antitumor agents.

四、參考文獻:

References:


五、計畫成果自評：

本計畫原先的構想是以三年的時間探討調控活性氧化物種對游離輻射和抗藥性之影響和其調節機制之研究，因為僅通過一年之經費，故先研究調控活性氧化物種對抗藥性之影響。

這是第一篇藉由外送合成麩胱甘肽基因來影響細胞內活性氧化物種的生合成，會影響細胞對化學藥物鉑金抗藥性的報告。我們推測細胞內的麩胱甘肽可能與細胞內銅離子形成螯合複合體後，改變生物體內微妙的銅離子平衡狀態，導致銅離子輸送蛋白在細胞膜的表現增加，因為銅離子輸送蛋白是細胞內負責輸送化學藥物鉑金的主要蛋白，此項研究有助於發展其他創新抗癌藥物時的重要參考。