行政院國家科學委員會專題研究計畫成果報告

# 環境中氮氧化合物與亞硝基化合物之毒理學研究：一氧化氮基因毒性分子機制之探討(3/3)

# Studies on the molecular mechanisms of genotoxicity of nitric oxide (NO)

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中文摘要

NO 在以前一直被認為僅從燃燒的廢氣產生，是一種體外的污染物，並不受研究者的重視。最近生物醫學的進步，藥理與生化學家研究出 NO 是一種重要的內生性傳遞物質，它可在自體細胞合成，具有很強的生物功能，諸如血管的擴張鬆弛，血壓的調控，神經訊息的傳遞，基因的表現等等。其重要性很受注目，1992 年 Science 雜誌就把 NO 選為年度風雲分子(The Molecular of the Year)。自此這個不起眼的簡單分子，就搖身一變為大名鼎鼎，如雷貫耳的奇異分子。GSNO(S-nitrosoglutathione) 為細胞內抗氧化物 GSH 攜帶一氧化氮之化合物，當它釋出一氧化氮時，可對細胞造成 DNA 傷害。已有許多報告指出一氧化氮自由基可造成細胞凋亡，但 GSNO 引起細胞凋亡之詳細機制尚未被研究清楚。在本實驗中，我們使用人類腸癌細胞株，來探討 GSNO 對細胞之毒性及細胞凋亡之機制。我們發現，細胞給予 GSNO 處理之同時，若一起加入銅離子($Cu^{++}$)，則細胞死亡率較單獨處理 GSNO 明顯減少。據此我們亦證實了 $Cu^{++}$ 在細胞外可促使 GSNO 釋出 NO・，由於 NO・不穩定，在極短時間內轉變為硝酸鹽及亞硝酸鹽，因而降低進入細胞內 NO・之量，而減少細胞的傷害。NO・調控細胞凋亡過程中之基因表現，是我們想要嘗試了解的，以西方墨點分析來偵測基因表現的變化，結果發現 Bad、Bax 及 c-Jun 等蛋白的表現皆有增加之現象，而 p27 及 Bcl-2 的表現卻有被抑制的現象。為了解 NO・在參與細胞內訊息傳遞所扮演之角色，我們也對 PKA 及 PKC 之表現進行探討，結果發現 PKA 與 PKCζ之表現會被 NO・抑制，此結果告訴我們在 GSNO 引起之細胞凋亡中，存在一種特殊的調節機制，而 PKA 及 PKCζ可能扮演一重要角色。

關鍵詞：一氧化氮，細胞凋亡，空氣污染物

**Abstract**

In this study, the amount of S-nitrosoglutathione (GSNO) was measured spectrophotometrically at 334 nm. Spontaneous decrease of absorbency at 334 nm was detected when GSNO was exposed to 37 °C and a high pH (pH 8.0). We investigated the catalytic roles of various metal ions on the decomposition of GSNO. The degradation of GSNO (0.5 mM) was enhanced by the presence of $Cu^{2+}$ and $Ni^{2+}$ ions. The amount of NO release from GSNO degradation was estimated by the Griess reaction based on nitrite accumulation. The results indicate that nitrite production was elevated by at least 2-fold in the presence of $Cu^{2+}$. Our study further indicates that $Cu^{2+}$ enhance GSNO-induced apoptosis in human colon adenocarcinoma (HT 29) cells. We also found that copper ions

modulate the expression of bad, *bax*, and *bcl-2* in GSNO-treated HT 29 cells. The levels of *bax* and bad proteins were significantly elevated by about 4- to 6-fold when compared with mock-treated cells at 24 h after combined treatment of GSNO plus $Cu^{2+}$ or $Ni^{2+}$. On the other hand, significant inhibition of *bcl-2* occurred in HT 29 cells with simultaneous treatment of GSNO with $Cu^{2+}$ (or $Ni^{2+}$). It seemed that $Cu^{2+}$ ($Ni^{2+}$) could enhance the decomposition of GSNO that liberated NO to activate the pathways. Our results demonstrated that the apoptotic effects induced by GSNO was promoted by $Ni^{2+}$ and $Cu^{2+}$ through two different mechanisms: by depletion of intracellular GSH level and by triggered of NO release from GSNO which then promoted the NO-induced apoptotic cell death in human cells.

**Keywords**: Apoptosis, Nitric Oxide, Air pollutant.

## Introduction

Nitric oxide (NO) is produced from macrophages or macrophage-like cells by inflammatory stimulation, and has various effects on the immune system. Previous studies have demonstrated that the induction of nitric oxide synthase by LPS plus interferon-γ (INF-γ) or interleukin-1 (IL-1) causes apoptosis in various types of cells [1-4]. Other studies including ours have indicated that high concentrations of NO induce wild-type *p53* protein accumulation and apoptosis [5-9]. Recent studies have demonstrated that *p53* performs an essential role in response to NO generated either from an NO donor or from over expression of NOS. Over expression of wild-type *p53* in human tumor cell lines results in down-regulation of NOS gene expression, as well as enzymatic activity [10]. Such an observation implies that an increased level of NOS expression in human tumor samples may be a loss of *p53*-mediated NOS gene regulation due to functional inactivation of wild-type *p53*. Little is known about the pathways leading to formation of RSNO in biological tissues. NO does not react with sulfhydryl groups directly, but a potent nitrosating species ($NO_x$) is formed upon reaction of NO with oxygen. There is general agreement that the biologic activity of nitrosothiols, e.g., smooth muscle relaxation or inhibition of platelet aggregation, is due to release of free NO, but the mechanisms involved in the decomposition of these compounds are unclear. Remarkable variations have been reported in the stability of different nitrosothiols in aqueous solution at physiological pH levels, with their half lives ranging from a few seconds (S-nitroso-L-cysteine) to hours (GSNO). However, more recent studies indicate that nitrosothiols are stable in aqueous solutions and only decompose and release NO in the presence of trace metals. Thus, $Cu^{2+}$ ions have been shown to trigger decomposition of S-nitroso-N-acetyl penicillamine (SNAP) and GSNO, and

the effect of copper ions was found to be potentiated by reductants such as ascorbate. We further assessed the effects of $Cu^{2+}$ and $Ni^{2+}$ ions on the modulation of *bax*, *bcl-2*, and bad gene expression in response to GSNO treatment. The described reactions may have important implications for the biochemistry and physiology of NO–mediated biological processes.

**Result and discussion**

As shown in figure 1A, GSNO was stable at room temperature (25 °C) for at least 96 h. In contrast, the absorbance of 334 nm apparently decreased at 48 h when GSNO was exposed to 37 °C. Such results imply that NO may be released from GSNO at 37 °C in a cell culture system. GSNO (0.5 mM) solution was then prepared with potassium phosphate buffer (50 mM) to different pH values and then exposed to 37 °C or 25 °C at the indicated times (Figure 1B). This experiment demonstrated that GSNO degradation was more rapid at a higher pH (pH 8.0) when exposed to 37 °C. Our results indicate that decomposition of GSNO (0.5 mM) was more rapid in the presence of $Cu^{2+}$ and $Ni^{2+}$ (Figure 2) when compared to other metal ions (data not shown).

The cytotoxicity of $Cu^{2+}$ after 24-h treatment was evaluated in HT 29 cells. We found that a concentration of $Cu^{2+}$ of less than 10 μM was non-toxic to HT 29 cells (Figure 3A). As described above, $Cu^{2+}$ (10 μM) strongly enhances GSNO decomposition; the level of NO production in HT 29 cells was further estimated with the Griess reaction by determination of nitrite accumulation (Figure 3B). Our results indicate that nitrite production was elevated by at least 2-fold in the presence of $Cu^{2+}$ (Figure 3B). In order to investigate whether $Cu^{2+}$ could modulate NO-induced apoptosis, the percentage of apoptotic cells was calculated by TUNEL assay, which further demonstrated that $Cu^{2+}$ enhances GSNO-induced apoptosis (Figure 3C). In order to further demonstrate the critical role of $Cu^{2+}$ in intracellular GSNO decomposition, HT 29 (Figure 4) cells were pretreated with GSNO (1 mM) for 6 h, washed twice with PBS, and then treated with $Cu^{2+}$ (2.5 μM) at the indicated times. Other groups were treated with either GSNO (pretreated for 6 h then washed with PBS), or $Cu^{2+}$ (treated consistently) as a comparison group. We found that exogenous added $Cu^{2+}$ significantly augments the apoptotic effect induced by GSNO (Figure 4).

In our recent studies, we demonstrated that *bcl-2* protein expression was down-regulated and *bax* protein expression was induced in NO-treated human cancer cells. Figure 5 shows that the expression of *bcl-2* was down-regulated in a time-dependent manner when cells were treated with GSNO (2mM). On the other hand, *bax* and bad proteins in HT 29 and COLO 205 cells were significantly elevated by GSNO treatment (Figure 5). By comparison, the alterations of *bax* and *bcl-2* protein expression were more

significant in the COLO 205 than HT 29 cell by GSNO treatment (Figure 5). We have gained further insight into the role of $Cu^{2+}$ and $Ni^{2+}$ involved in alteration of gene expression inGSNO-induced apoptosis. The levels of *bax* and *bad* proteins were significantly elevated by about 4- to 6-fold when compared with mock-treated cells at 24 h after treatment with GSNO plus $Cu^{2+}$ or $Ni^{2+}$ (Figure 6A and B). On the other hand, significant inhibition of *bcl-2* occurred in HT 29 cells when simultaneously treated with GSNO and $Cu^{2+}$ (or $Ni^{2+}$) (Figure 6C). In this figure, regulation of the *bax*, bad, and *bcl-2* levels in only GSNO-treated HT 29 cells was less significantly affected (Figure 6A, B, and C). It seems that $Cu^{2+}$ ($Ni^{2+}$) plays a critical role in decomposition of GSNO which clearly augments the effects on regulation of gene expression in apoptotic cells.

In our previous results, we demonstrated that antioxidants (such as L-N-acetyl-cysteine, LNAC) attenuate NO-induced apoptosis in human colon cancer cells. To test the protective mechanisms of antioxidants, we demonstrated that the intracellular level of glutathione (GSH) was elevated in cells after exposure to LNAC. Our results suggest that the protective effect of LNAC might be linked to its inducement of increases in cellular glutathione and *bcl-2* protein levels and to its suppression of cellular *bax* protein in treated cells. In this study, we considered the possibility that metals modulating apoptosis in NO-treated cells might arise from several mechanisms. In order to evaluate the roles of intracellular antioxidants (GSH) in cells exposed to NO and (or) combined treatment with metals, we therefore compared the levels of DNA fragmentation in cells after exposure to LNAC, GSNO, metals, and combined treatment. As shown in figure 7, DNA fragmentation was observed in GSNO (lane 4) and combined treatment of GSNO and metals (lanes 5 and 6). By contrast, LNAC protects apoptosis in cells exposed to GSNO (or combined treatment with GSNO plus metals) (lanes 7 to 9).

Our previous report shown that the intracellular level of GSH was elevated in cells after exposure to LNAC. We found that elevation of intracellular GSH level could attenuate NO-induced apoptosis in human cancer cells. Another study also demonstrated that apoptosis could be induced by GSH depleting agent (BSO) in human cholangiocytes. In this study, we further investigate whether promotion of NO-induced apoptosis by $Cu^{2+}$ and $Ni^{2+}$ were modulated through depletion of intracellular GSH levels. COLO 205 cells were treated either with BSO, GSNO, ions, or by different combination as indicated in the figure 8. The intracellular GSH levels were extremely depleted in cells treated with BSO (50 μM) (Figure 8A, lane 2) when compared to the control group (Figure 8A, lane 1). Interestingly, significant decreased of GSH levels were also observed in cells by combine treatment of GSNO either

with $Cu^{2+}$ or $Ni^{2+}$ (Figure 8A, lanes 5 and 7). We further demonstrated that apoptosis induction and intracellular GSH depletion were occurred simultaneously in cells by combine treatment of ions and GSNO (Figure 8A and B, lanes 5 and 7). However, less extent (15%) of cells undergoing apoptosis were observed in the BSO-treated group although the GSH levels were extremely depleted (Figure 8A and B, lane 2). Such results implied that $Cu^{2+}$ (or $Ni^{2+}$) mediated apoptotic effects induced by GSNO were partially through depletion of intracellular GSH level.

To further clarify such observations, the level of nitrite production was then measured in cells treated either by BSO plus GSNO (Figure 8C, lane 3) or ions plus GSNO (Figure 8C, lanes 5 and 7). We demonstrated that the level of nitrite production was consistently with the apoptotic effects induced by combine treatment of GSNO and ions (Figure 8B and C, lanes 5 and 7) whereas cells combine treated with GSNO and BSO do not (Figure 8B and C, lane 3). Collectively, our results suggest that the apoptotic effects induced by GSNO was promoted by $Ni^{2+}$ and $Cu^{2+}$ through two different mechanisms: by depletion of intracellular GSH level and by triggered of NO release from GSNO which then promoted the apoptotic cell death in human cells.

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成果自評

本計劃執行進度相當順利，此項計劃支援之相關研究成果計發表論文(SCI)共五篇如下：

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