行政院國家科學委員會專題研究計畫 成果報告

氮氧化物促進癌化及惡化轉移之作用及機轉(二)
研究成果報告(精簡版)

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行政院國家科學委員會專題研究計畫期末成果報告

氮氧化物促進癌化及惡化轉移之作用及機轉(二)

Study of tumor promoting and malignant progression effect of nitrogen oxides in vitro and in vivo

計畫編號：NSC 95-2320-B-040-021

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一、摘要

空氣中的氮氧化物(NOx)在以前一直被認為僅從燃燒等廢氣產生，是一種體外污染物，最近有許多研究顯示，NOx與生物體中蛋白之相互作用扮演著重要的生理角色(1,2), NO在生體內可與superoxide產生peroxynitrite (3)，在cigarette smoke fracction中也發現peroxynitrite的產生(4)，peroxynitrite已被証實顯示具有神經毒性(5,6)，上皮細胞傷害(7)及脂質過氧化作用(5)等，peroxynitrite也被證實為一種oxidative stress-inducing compound，能造成人類細胞DNA傷害(4,8),但此是否煙霧或空氣污染物中NOx造成肺癌之原因，仍未被證實，過去我們的研究中除了發現NOx會攻擊DNA產生 8-NO_2-G(9)外，我們也發現NOx能促進MRC-5 細胞生長及S phase分佈增加(10)，本研究過去的工作發現氣體性NOx對人類肺纖維細胞MRC-5 之增生作用，其作用係經由加強Cyclin/CDK活化及Rb磷酸化(11)；從以上結果及許多相關研究，顯示NOx可能有tumor promotion的作用，因此我們將進一步探討NOx在腫瘤促進作用的角色。由之前的結果顯示NOx有抑制B(a)P (benzo[a]pyrene)致細胞死亡之作用(12)，此作用經由抑制JNK1 及增加Bcl-2 及Mcl-1 之途徑。因此本研究的工作將探討NOx是否有促進肺癌細胞侵入轉移及惡化作用，我們用NOx促進下探討A549 肺癌細胞在migration及invasion作用及其在鼠體中之轉移作用及其機轉；緊接之工作在於釐清NOx所調控肺癌惡化增生之分子機轉，以確立NOx促進肺癌增生、惡化、轉移、侵入之重要性。

由以上的研究，我們將首先提出NOx致癌作用之角色可能在於它的腫瘤促進作用及惡化轉移。

Keywords: malignant progression; lung cancer; migration; invasion; iNOS; metalloproteinase; melanoma; metastasis.

二、材料與方法

【一】NOx gas-saturated medium 製備 (9-11)

PBS 以N2 gas 去氧30 分鐘後, antheutic NO gas (含N2) 通入PBS 中 30 分後,其飽和液以Gries reagent 分析NOx (NO + NO2) 濃度,處理細胞之濃度依此濃度稀釋加入細胞培養基中, 分析cell free system 及在cell system 中NOx 之差異為細胞經外生性NOx 刺激產生之NO。

【二】NOx對於肺癌細胞侵入轉移之影響

(1) Gelatiin Zymography

將0.1% Gelatin-8% SDS-PAGE電永膠片置於含有電泳緩衝液的電泳槽中，將細胞培養後不含胎牛血的培養液與5×染劑均勻混和後，注入膠片中，分別

以 100V 與 140V 的電壓進行電泳分離。結束電泳分離後，以 washing buffer 在室溫下沖洗 30 分鐘 2 次，然後加入 reaction buffer 在 37℃恆溫箱中反應 12 小時，最後反應完的膠片以染色液染色 30 分鐘，再以退色液退染，觀看結果。

(2) Cell-Matrix adhesion 分析

首先以 typeⅣ collagen coating 24well 培養皿整夜，並以含有 2% FBS 的 PBS block 住非專一性的結合 2 小時，再將處理過 NOx 的 5 種細胞以 trypsin 打下，subcluture 至 24well 的培養皿中，25 分鐘後以 PBS 沖洗掉未貼附的細胞，待細胞正常生長 16 小時後，以 MTT assay 測量吸附細胞的比例。

(3) 細胞移動性分析

利用 48 well Boyden chamber的分析方法，lower chamber為含有 0.1% gelatin 的 conditioned medium，將細胞處理 NOx後，subculture並計算細胞數，然後注入固定量的細胞(10^4-1.5×10^4)於 upper chamber，待細胞移動 5 小時以後，取下薄膜，以甲醇固定細胞 10 分鐘，風乾 5 分鐘之後，以Giemsa(1：20)染色 1 小時，最後固定住薄膜，擦拭掉薄膜之上層細胞，在顯微鏡底下隨機選取視野，作移動細胞數之統計。

(4) Wound healing 分析

將細胞種於 6 well plate 中(1×10^6cell/well)以含 1%FBS之DMEM 培養基培養，24 小時後以yellow tip在每個well中劃出 2 條傷口，以PBS洗 2 次，在每個well加入不同濃度之NOx，觀察 0、6、12、24 小時傷口癒合情形。

(5) Electrophoretic Mobility Shift Assay

先將細胞全部的nuclear extract protein 準備好，NF-kB gel shift oligonucleotide 用T4 polynucleotide kinase和(γ-^{32}P) ATP (3,000Ci/mmol at 10mCi/ml)標記起來，使用 1μg Nuclear extracts protein 和含有 20% glycerol、 5mM $MgCl_2$、2.5mM EDTA、2.5mM DTT、250mM NaCl、50mM Tris-HCl (PH 7.5)、0.25mg/ml poly(dl-dC)‧poly(dl-dC)的溶液以及NF-*k*B antibiotic(anti-p65, anti-p50)在室溫下一起作用 10 分鐘之後加入^{32}P-labeled consensus oligo在室溫下作用 20 分鐘，將Sample 放到 4% acrylamide gel使用 0.5 X TBE buffer用 25mA跑 1 小時 30 分，之後在 80℃的環境下乾膠 2 小時，曝光約 24-48 小時。

【三】癌症轉移之動物實驗-NOx促進轉移作用

(1) 首先利用黑色素瘤細胞(B16-F10)，在有無 NOx 處理一週之後，將細胞打入六週大 C57/BL6 小黑鼠的鼠蹊部(腹腔注射)，3 週後觀察比較有無處理 NOx 下，是否黑色素瘤細胞有轉移到肺臟的能力。

(2) 再利用 A549 (human lung adenocarcinoma cell)細胞，在不同劑量NOx處理下(1, 5, 10μM)，再將A549細胞處理不同劑量NOx(1, 5, 10μM)一週之後，將NOx-induced A549 細胞打入六週大 C57/BL6 小黑鼠的鼠蹊部(腹腔注射)及尾靜脈(每次靜脈注射5隻)， 3週後犧牲, 取肺臟、腦及肝臟切片染色(利用Cytokeratin 8免疫染色觀察adenocarcinoma)，觀察比較不同劑量NOx處裡下，是否NOx-induced A549細胞有轉移到肺臟及其他器官的能力。

三、結果

(一) 氣體性NOx影響肺癌細胞之生長情形

利用MTT assay測試氣體性氮氧化物(NOx)影響A549、H1299、H1355肺癌細胞之生長情形：三種細胞暴露在不同劑量(0、1、2.5、5μM)之氣體性氮氧化物(NOx)下24小時，利用MTT分析肺癌細胞生長情形，結果發現，細胞暴露在濃度1、2.5、5μM

之氣體性氮氧化物(NOx)24小時後，對於肺癌細胞並無毒性作用。(Fig. 1)

(二) 氣體性氮氧化物(NOx)影響人類肺癌細胞之移動

由初步傷口癒合實驗測試不同肺癌細胞在受到氣體性氮氧化物(NOx)刺激下，細胞migration的能力觀察。測試3種不同的肺癌細胞(Fig.2)：A549細胞在無血清(serum free)情形下，受到5μM氣體性氮氧化物(NOx)刺激，24小時後結果發現，NOx能刺激 A549 細胞的migration(Fug.2)；此外H1299與H1355兩株肺癌細胞則發現，在無血清下處裡5μM濃度的飽和氣體氮氧化物溶液，24小時觀察細胞移動的情形，初步發現H1299與H1355細胞migration情形不顯著。

(三) 氣體性NOx影響A549細胞之移動及轉移能力(Dose dependent)

利用傷口癒合實驗測試氣體性氮氧化物(NOx)影響A549細胞之移動能力情形：A549細胞暴露在不同劑量(0、1、2.5、5μM)之氣體性氮氧化物(NOx)下24小時，利用傷口癒合實驗分析A549細胞移動情形，結果發現，細胞暴露在濃度1、2.5、5μM之氣體性氮氧化物(NOx)24小時後，與control組對照可發現，A549細胞暴露在5μM NOx下可發現細胞移動情形最為顯著。(Fig. 3A、3B)

利用Boyden chamber實驗測試氣體性氮氧化物(NOx)影響A549細胞之移動能力情形：A549細胞暴露在不同劑量(0、1、2.5、5μM)之氣體性氮氧化物(NOx)下24小時，利用Boyden chamber實驗分析A549細胞移動情形，結果發現，細胞暴露在濃度1、2.5、5μM之氣體性氮氧化物(NOx)24小時後，與control組對照可發現，A549細胞暴露在5μM NOx 下可發現細胞穿透Boyden chamber情形最為顯著。(Fig. 3C、3D)

(四) 氣體性氮氧化物(NOx)對於誘導人類肺癌細胞iNOS表現及iNOS對於肺癌細胞移動轉移的影響

將人類肺癌細胞A549暴露於氣體性氮氧化物下所誘發細胞移動轉移之影響，可能來自於氣體性氮氧化物所誘導iNOS的增加所導致，因此針對iNOS加入其抑制劑L-NAME，並分析iNOS蛋白及RNA之表現(Fig.4A、4B)，結果發現氣體性氮氧化物能誘導iNOS的增加，且iNOS蛋白及RNA之表現受到其抑制劑L-NAME所抑制。緊接再利用傷口預合實驗測試及Boyden chamber實驗測試氣體性氮氧化物(NOx)所誘導之iNOS影響A549細胞之移動轉移能力(Fig.4C、4D)，結果發現A549細胞之移動轉移能力，會受到其iNOS抑制劑L-NAME處理後而被抑制，證實iNOS之重要性。

(五) 氣體性NOx影響A549細胞MMP-2及TIMP-2之表現

經由初步Zymography分析法，觀察A549細胞在受到濃度5μM氣體性氮氧化物(NOx)刺激下，細胞表現MMP-2與MMP-9的能力。在無血清(serum free)下處裡5μM濃度的飽和氣體氮氧化物溶液，0, 3, 6, 9, 12, 24小時收及細胞培養基，利用Zymography分析法，觀察MMP-2與MMP-9的情形，初步發現與無處理NOx下相比較，發現氣體性氮氧化物(NOx)能刺激正常A549肺癌細胞MMP-2的表現量增加，隨後在無血清(serum free)下處裡5μM濃度的飽和氣體氮氧化物溶液，0, 3, 6, 9, 12, 24小時收細胞lysate，利用Western blot分析，觀察TIMP-2之表現，結果發現氣體性氮氧化物(NOx)隨著暴露時間增加，能抑制A549細胞TIMP-2之表現。(Fig.5A)，Fig.5B為量化之結果。

利用分析 RNA 與 protein 表現，觀察 iNOS 抑制劑處理細胞後 MMP-2 與

TIMP-2 之表現，發現 iNOS 抑制劑隨著處理時間增加，能抑制 MMP-2 之表現並回復 A549 細胞 TIMP-2 之表現(Fig. 5C、5D)。

(六) 參與調控氣體性NOx影響A549細胞移動之轉錄因子

由上述結果可發現，5μM 濃度的氣體性氮氧化物(NOx)可刺激 A549 細胞之移動，而造成細胞移動可能是經由活化 MMP-2 與抑制 TIMP-2 所照成的結果，而調控 MMP-2 表現之轉錄因子有 NF-κB、c-Fos 及 c-Jun，因此利用分析細胞核內蛋白表現(Fig.6A)，並利用 iNOS 抑制劑加入來確認 iNOS 的角色(Fig.6B)。最後再利用 Electrophoretic Mobility Shift Assay(EMSA)分析在細胞核中，NF-κB 及 AP-1 之表現情形，結果發現，A549 細胞受到 NOx 刺激下，NF-κB 先在 6 小時有被活化的表現，在 9 與 12 小時有下降趨勢，但在 24 小時又被活化(Fig.7A)；而 AP-1 之表現則在 24 小時表現較為明顯(Fig.7B)。因此證實 NOx 刺激 A549 細胞產生 MMP-2 可能是先經由活化 NF-κB，之後經由一連串訊息傳遞路徑刺激下再度活化 NF-κB 與 AP-1。

四、討論

經由本研究結果可發現，氣體性氮氧化物(NOx)會影響A549細胞之移動能力(Fig.1~Fig.4)；而且暴露在5μM濃度的NOx下，隨著暴露時間的增加，A549細胞分泌之MMP-2也有增加的趨勢(Fig.5)，相對的，抑制MMP-2活性的tissue inhibitor of metalloproteinases (TIMPs)(13)，經由Western blot分析後也發現，隨著暴露NOx時間增加，TIMP-2之表現也受到抑制(Fig.5)，並經由iNOS抑制劑加入來證實iNOS在氣體性氮氧化物誘導人類肺癌細胞移動轉移上扮演重要角色。此外過去研究指出，在Macrophage中，抑制NF-κB的peptide可以降低MMP-2、MMP-9的表現(14)；在一些黑色素瘤細胞中，NF-κB可藉由活化MT-MMP的表現而去活化proMMP-2，增加癌細胞侵入的能力(15)；在單核細胞中發現，AP-1會調控MMP-2與MMP-9基因的轉活化，進而影響到MMP-2與MMP-9的表現(16)。因此經由Fig.7 EMSA分析後發現，調控NOx影響A549細胞移動之轉錄因子，可能是經由先經由活化NF-κB，之後經由一連串訊息傳遞路徑刺激下再度活化NF-κB與AP-1，而這二階段式的活化致使MMP-2的最終表現增加，達到影響癌細胞之移動。本部份研究之結果也以總圖說明之(Fig.8)。

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六、結果

圖一、氣體性氮氧化物(NOx)對於人類肺癌細胞生長的相關性

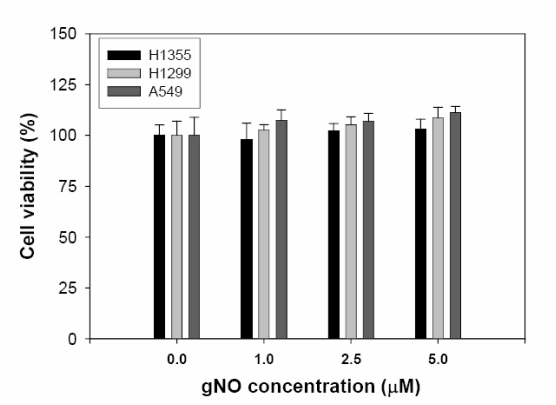


Fig. 1. Effects of gNO on cell viability of three NSCLC cells (H1355, H1299 and A549). Cells (10^5 cells/well) were treated with various concentrations (0, 1.0, 2.5 and 5.0 μM) of gNO for 24 h. Cell viability was analyzed by MTT assay. The result represents the average of three independent experiments ± SD. *p< 0.05, **p< 0.01 compared with the control.

圖二、氣體性氮氧化物(NOx)影響人類肺癌細胞之移動

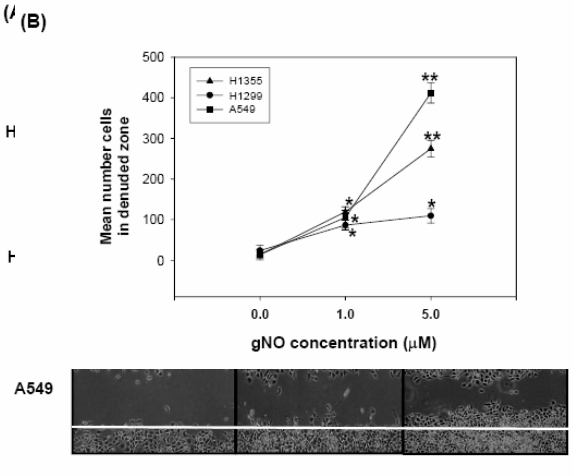
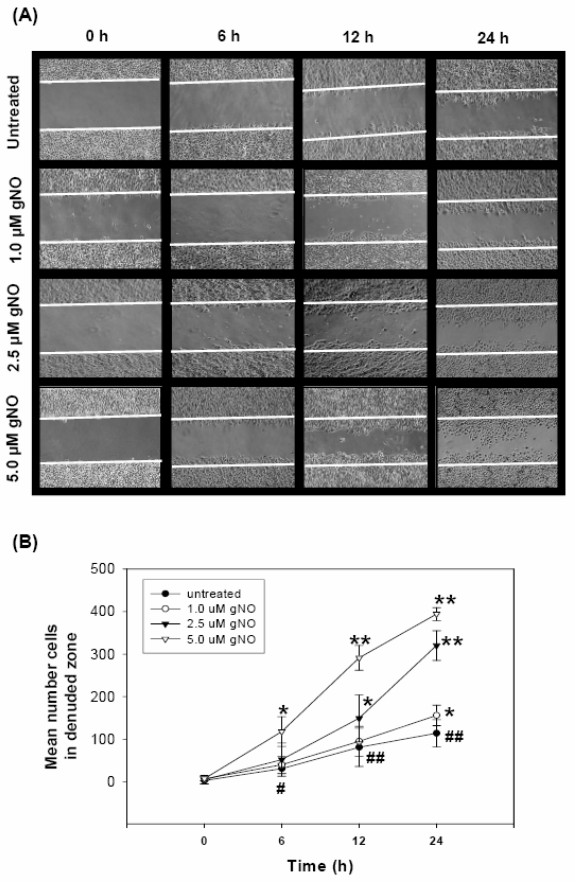


Fig. 2. Effects of gNO on wound healing ability of three NSCLC cells (H1355, H1299 and A549). Monolayers of culture cells treated with gNO (at 1.0 and 5.0 μM) or without (untreated) were scraped and the number of cells in the denuded zone (i.e., wound) was quantitated after 24 h under a light microscopy. (A) Representative photomicrographs of the denuded zone 24 h after scraping in the untreated and gNO-treated groups. White lines indicate the wound edge. (B) Quantitative assessment of the mean number of the three cell types in the denuded zone and represents the average of three independent experiments ± SD. *p< 0.05, **p< 0.01 compared with the untreated group.

圖三、氣體性 NOx 影響 A549 細胞之移動及轉移能力



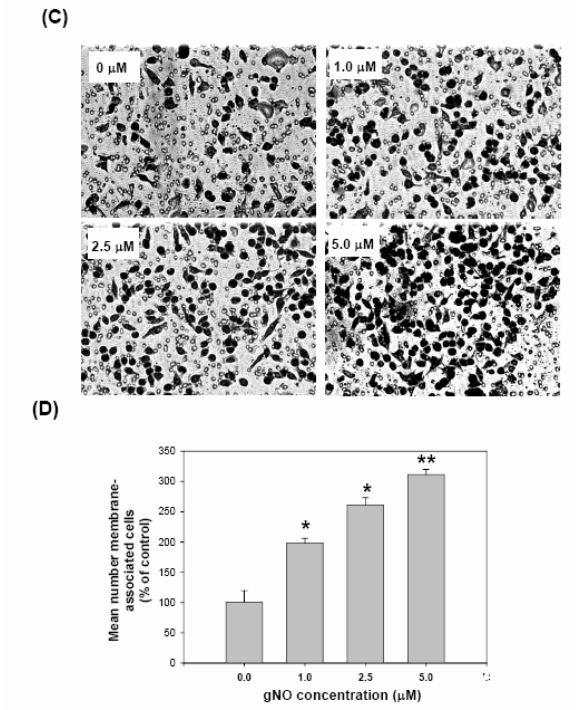


Fig. 3. Effects of gNO on A549 cell motility and invasion *in vitro*. Monolayers of A549 cells treated with gNO (at 0, 1.0, 2.5, and 5.0 μM) or without were scraped and the number of cells in the denuded zone (i.e., wound) was quantitated after indicated times (0, 6, 12 and 24 h) under a light microscopy. (A) Representative photomicrographs of the denuded zone 24 h after scraping in the untreated and gNO-treated groups. White lines indicate the wound edge. (B) Quantitative assessment of the mean number of cells in the denuded zone and represents the average of three independent experiments ± SD. #p< 0.05, ##p< 0.01 compared with the 0 h. *p< 0.05, **p< 0.01 compared with the respective time point of untreated group. (C) A549 cells treated with gNO (at 0, 1.0, 2.5 and 5.0 μM) for 24 h were plated in the upper chamber of a modified Boyden chamber coated with Matrigel containing a membrane, and the number of cells on the underside of the membrane was quantitated 6 h later under a light microscopy. Representative photomicrographs of the membrane-associated cells were assayed by Giemsa stain. The black part indicated the cells. (D) "% of control" represent denotes the mean number of cells in the membrane expressed as a proportion of that non-treated group and the average of three independent experiments ± SD. *p<0.05, **p<0.01 compared with the control.

圖四、氣體性氮氧化物(NOx)對於誘導人類肺癌細胞 iNOS 表現及 iNOS 對於肺癌細胞移動轉移的影響

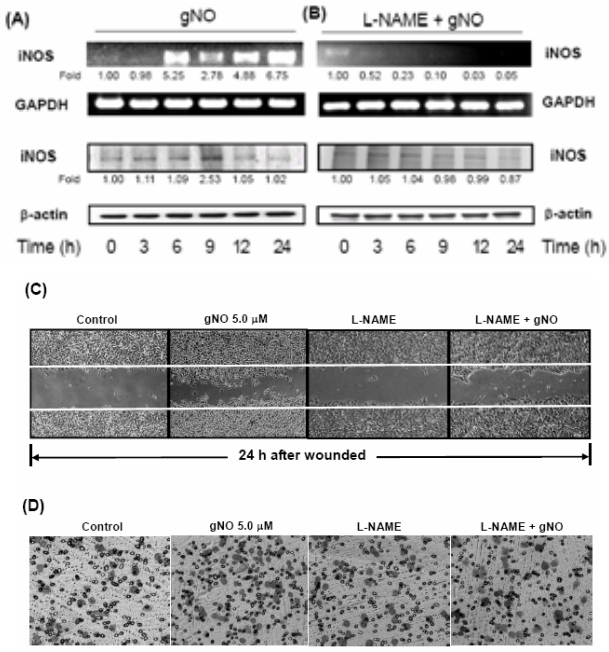


Fig. 4. I Effects of gNO on the mRNA and protein levels of iNOS. (A, B) A549 cells were treated 5.0 μM gNO in the (A) absence or (B) presence L-NAME (5.0 mM) for indicated times (0, 3, 6, 9, 12 and 24 h), and mRNA and protein levels of iNOS were determined by RT-PCR and Western blotting, receptively. GAPDH and β-actin were served as internal control. Determined expressions of the both levels were subsequently quantified by densitometric analysis with that of control being 1.00 fold as shown just below the gel data. (C) Effects of iNOS inhibitor on gNO-induced A549 cells migration and invasion. A549 cells were treated 5.0 μM gNO or without in the absence or presence L-NAME for 24 h. Cell migration was analyzed by wound-healing assay. White lines indicated the wound edge. (D) Cell invasion was analyzed by Boyden chamber assay. The black part indicated the membrane-associated cells. Results are representative of at least three independent experiments.

圖五、氣體性 NOx 影響 A549 細胞 MMP-2 及 TIMP-2 之表現

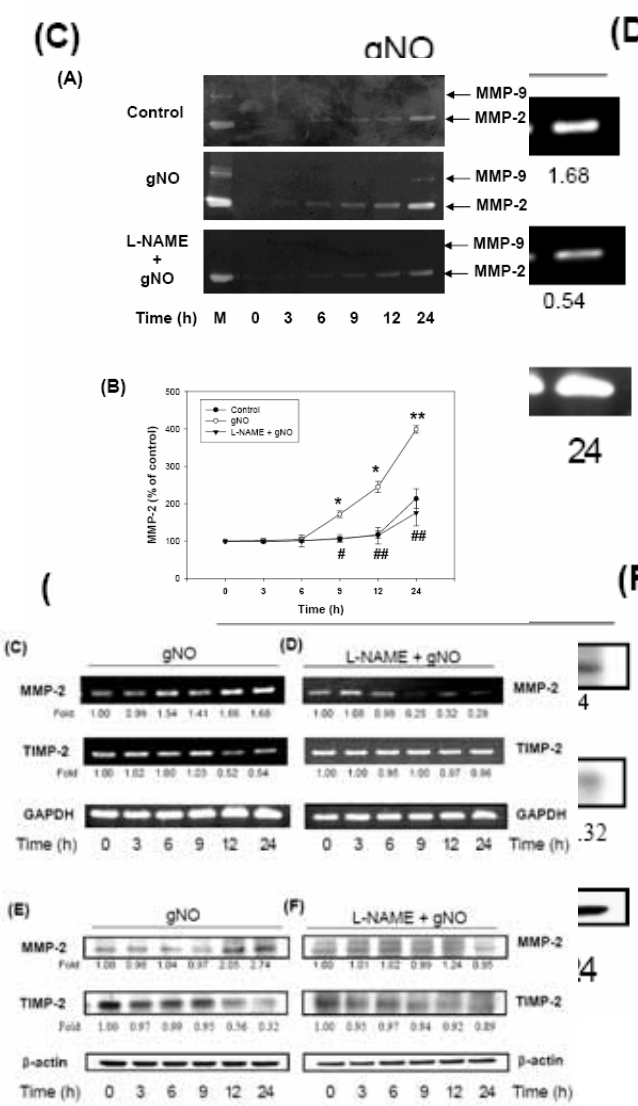


Fig. 5. Effects of gNO on MMPs activity and the mRNA and protein levels of MMP-2 and TIMP-2. (A) In time-dependent assays, various indicated times (0, 3, 6, 9, 12 and 24 h) of 5.0 μM gNO in the absence or presence L-NAME were treated on A549 cells in serum-free medium. The culture medium of A549 cells after being treated by gNO were subjected to gelatin-zymography to analyze the activity of MMPs as described in Materials and methods. (B) The determined activity of MMP-2 was quantitated by densitometric analysis using the untreated control as 100%. Data were represented as mean ± SD from three independent experiments. #p< 0.05, ##p< 0.01 compared with the 0 h. *p< 0.05, **p< 0.01 compared with the respective time point of gNO-treated group. (C, D) RT-PCR and (E, F) immunoblot analysis of the expression of MMP-2 and TIMP-2 in A549 cells treated with gNO. The cells were treated with 5.0 μM gNO in the (C, E) absence or (D, F) presence L-NAME and harvested at indicated times. GAPDH and β-actin were served as internal control.
Determined expressions of the mRNA and protein levels were subsequently quantified by densitometric analysis with that of control being 1.00 fold as shown just below the gel data.

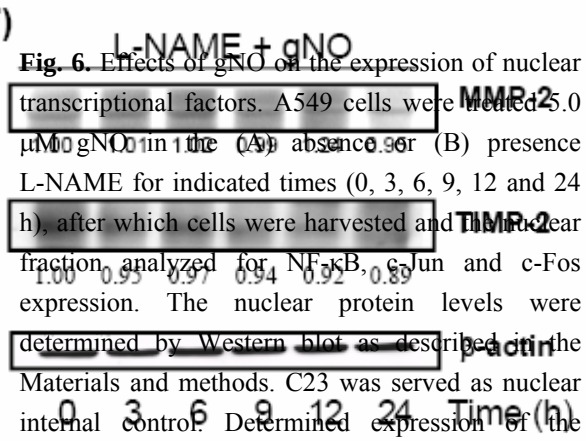


Fig. 6. Effects of gNO on the expression of nuclear transcriptional factors. A549 cells were treated 5.0 μM gNO in the (A) absence or (B) presence L-NAME for indicated times (0, 3, 6, 9, 12 and 24 h), after which cells were harvested and the nuclear fraction analyzed for NF-κB, c-Jun and c-Fos expression. The nuclear protein levels were determined by Western blot as described in the Materials and methods. C23 was served as nuclear internal control. Determined expression of the protein was subsequently quantified by densitometric analysis with that of control being 1.00 fold as shown just below the gel data.

圖七、參與調控氣體性 NOx 影響 A549 細胞移動之轉錄因子(二)

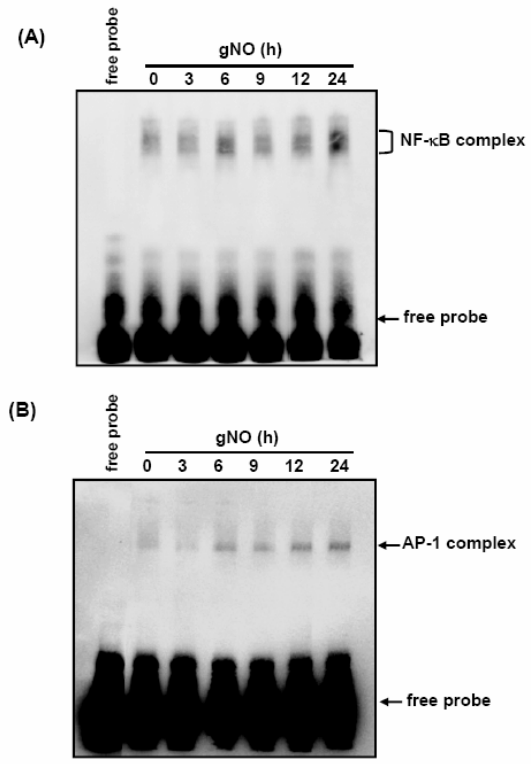


Fig. 7. Effects of gNO on NF-κB and AP-1 DNA binding. Cells were treated with 5.0 μM gNO for indicated times (0, 3, 6, 9, 12 and 24 h) and then nuclear extracts were analyzed for (A) NF-κB and (B) AP-1 DNA binding activity using biotin labeled NF-κB and AP-1 specific oligonucleotide by EMSA as described in Materials and methods. Lane 1 represented nuclear extracts incubated with unlabeled oligonucleotide (free probe) to confirm the specificity of binding. Results from three repeated and separated experiments were similar.

圖八、氣體性氮氧化物(NOx)誘導人類肺癌細胞移動轉移之機轉

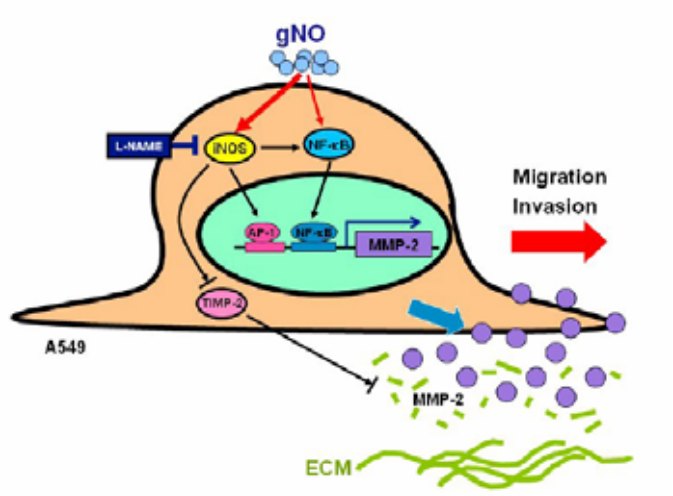


Fig. 8. Model showing pathways that mediate gNO-induced both the migration and invasion in A549 cells. gNO was shown to be capable of inducing A549 cells migration and invasion through the two mechanisms, one was the cell evens indirectly stimulated by the induction of iNOS (major pathway), followed by MMP-2/TIMP-2 imbalance involved the NF-κB or/and AP-1 (c-Jun) binding site and the induction of these transcriptional factors; and the other was directly the activation of NF-κB. See the text for discussion.