行政院國家科學委員會專題研究計畫成果報告

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一、 中文摘要

空氣污染中存在許多致癌物質，NOx(NO、NO_2、NO_3...)是其中一種。過去有研究指出，NO 及 NO_2 會造成哺乳類動物肺部的損傷；此外也有研究指出 NO 及 NO_2 與細胞的增生分化也有相關。而這兩者都癌化有關。本篇實驗發現，暴露氣體性 NOx(1 μM)之下會引起人類肺纖維細胞(MRC-5)的增生，利用流式細胞儀與細胞毒性測試方法發現，細胞週期中的 S 期及細胞數目都有增加。

許多研究證實 NO 在細胞訊息傳遞中是一重要因子。在本篇研究中我們想了解氣體性 NOx 促使 MRC-5 細胞增生的訊息傳遞路徑，利用西方墨點法結果發現：(1)iNOS 被活化；(2)活化 MAPK cascade(MEKK1、MKK4、JNK、p38MAPK)；(3)一些轉錄因子被活化(NF-κB、phospho-ATF2、c-Jun/c-Fos)；(4)細胞週期蛋白表現增加(Cyclins、Cdks)；(5)細胞週期的抑制蛋白表現下降(p27、p21、p16)。此外，本實驗中發現氣體性 NOx 與 MRC-5 細胞的抗凋謝死亡也有相關，利用西方點墨法結果發現：(1)抗凋謝死亡蛋白表現增加(IAP、phospho-Bad)；(2)促使細胞凋謝死亡蛋白表現下降(Cytochrome c、Caspase 3)。因此我們推論氣體性氮氧化物可能經由促使人類肺纖維細胞增生與抑制人類肺纖維細胞凋謝死亡來導致肺癌的產生。

Abstract

There are many carcinogen in air pollution, including NOx (NO, NO_2, NO_3). Several data have suggested that NO and NO_2 could induce injury in mammalian lung cells. Formation of NO and NO_2 have been shown to stimulate cells proliferation and differentiation. These are contributing to multistage carcinogenesis. We demonstrated that exposure of human lung fibroblast cells (MRC-5) to gaseous NOx saturation solution (1 μM) would induce cell poliferation. We used flow cytometry and MTT assay to clarify the gaseous NOx induced cell poliferation. In this study, we found that gaseous NOx can increase cell cycle at S phase and cell number.

Many studies have identified NOx as having a critical role in cellular signaling. In this study, we focused on elucidating the mechanism of gaseous NOx signaling in MRC-5 cells. We found that NOx-induced activation of (1) inducible nitric oxide synthase (iNOS), (2) MAPK cascade (MEKK1, MKK4, JNK, p38MAPK), (3) transcription factors (NF-κB, phospho-ATF2, c-Jun/c-Fos), (4) cell cycle proteins (Cyclins, Cdks),and decreasing of cell cycle inhibitors (p27, p21, p16). We alos found that gaseous NOx could activate anti-apoptotic factors (IAP, phospho-Bad) and decrease expression of apoptotic proteins (Cytochrome c, Caspase 3). In conclusion, we suggest that gaseous NOx could play an important role in carcingogenesis by inducing human lung fibroblast cells proliferation and anti-apoptosis.

二、 緣由與目的

根據世界衛生組織的統計，最近二十年來全世界的癌症死亡率正快速上升，其中以肺癌的死亡率增加最快。而肺癌在台灣之發生率已居男性第二、女性第一(1)，但致肺癌之分子機轉尚不清楚；但對導致肺癌之危險因素，只知可能與抽煙、二手煙、家族史、暴露於環境中的致癌物質等多因素有關。空氣中含有污染性之 NOx(包括 NO_3、NO_2、NO...)中90%是來自汽機車燃燒燃料及工廠之廢氣(2,3)，所以 NOx 存在最早是由環境污染物分析，但對人類健康則少描述，只有從製造硝酸的工人或暴露於高濃度硝酸之農夫表現的一些症狀，如對鼻之刺激、肺部之不適等(4)。NOx 以前一直被認為僅從燃燒等廢氣產生，是一種體外污染物，最近發現生物體內內生性 NO 的產生，具有許多生物功能如調控血壓、神經訊息、基因表現等(5,6)。長期暴露 NO 或生物體感染發炎等因素造成體內 NO 濃度增加，使生物體產生基因毒性及許多副作用之研究也不斷被提出(7,8)，NO 會造成去氧核醣核酸上鹼基的 deamination 及 DNA strand 斷裂(9)。 最近有許多研究顯示，NO 與生物體中蛋白之相互作用扮演著重要生理角色(10, 11)，NO 在生體內可與 superoxide 產生 peroxynitrite(12)，peroxynitrite 已被證明顯示具有神經毒性(13, 14)、上皮傷害(15) 及脂質過氧化作用 (13) 等，peroxynitrite 也被證實為一種 oxidative stress-inducing compound，能造成人類細胞 DNA 傷害 (16, 17) ，生成 8-hydroxyquanine(8-OH-dG) 及 8-nitroquanine(8-NO_2-G) (18, 19)，但此是否為空氣污染中 NO 造成肺癌的原因，仍未被證實，本研究室已證實氣體性 NO 會攻擊人類肺纖維細胞(MRC-5 cell)的 DNA 而產生 8- NO_2-G(20)。最近有研究報告指出人類細胞暴露NO或過度表現 iNOS 都會造成 p53 大量累積(21)；且在 human cancer，NOS 的表現和活性都有增加的現象，顯示了 NO 在促癌化過程扮演重要的角色(22)。然而 NO 促進細胞增生方面的研究卻是很少(23-25)。早在 1984 年就有研究發現(26)，在長期且低劑量暴露在 NO_2 的環境下，會使人類 type Ⅰ 的肺泡細胞受傷，但同時也發現 type Ⅱ 的肺泡細胞卻有增生的現象。而在 1991 年的 Science 中也提到，NO 可能經由增加血管增生與使 DNA 突變的方式促使癌細胞生長(27)。在 1996 年有研究證實 NO 可以調控果蠅細胞的增生(28)，而 2000 年時有學者提出 NO 與 cGMP 可調控中樞神經系統軸突的成長(29)。此外，當外來給予 NO 時，也會促使皮膚角質化細胞的增生與分化(30)；NO 也能夠刺激內皮細胞的生長(31)。有研究指出，ROS(如 H_2O_2)能促使人類肺纖維細胞(MRC-5)的增生(32)；而在最近有研究提出ROS與NOx可以促使老鼠肝臟 stellate 細胞的增生(33)。最近幾年有研究指出 NO 是可以抑制細胞凋亡。在 1994 年 Mannick 等人首次提出內生性NOS與外來給予低劑量的NO donor 可以抑制人類 B lymphocytes 的 apoptosis(34)。而相類似的結果也在 splennocytes(35)、eosinophils(36)、ovarian follicles(37)、cardiac myocytes(38)及內皮細胞中發現；隨後在 1997 年 Kim 等人也發現，NO 抑制 apoptosis 是經由降低 caspase 3 的活性(39)；在 1998 年 Kim 等人又發現，NO 會抑制 Bcl-2 的切割與 cytochrome c 的釋放而達到 anti-apoptosis 的作用(40)；而在 1999 年 Jianrong 等人發現，NO 會抑制老鼠肝臟細胞經由 TNF-α 所誘導的 apotosis，是經由抑制 caspase 3、caspase 8 的活化及阻斷粒腺體的 dysfunction(41)。所以本研究進一步想證實 MRC-5 細胞暴露 NO 後，是否會經由活化 iNOS 所產生的 NO，進而活化 MEKK1 及 Raf 而誘導下游訊息傳遞路徑？此外是否經由活化 iNOS 所產生的 NO 也能夠活化 PI3K 訊息傳遞路徑？而這些路徑的活化是否能促使人類肺纖維細胞(MRC-5)的增生？以及是否氣體性 NOx 能使人類肺纖維細胞對抗細胞凋亡？也依此來探討是否氣體性 NOx 促使人類肺纖維細胞的增生與人類肺癌的產

生有關。

三、 結果與討論

將 MRC-5 細胞處理 1 μM NOx sloution，分別在 0hr、12hr、24hr 收細胞，細胞週期的 S 期(DNA 合成期)從原來的 12.99％ (0hr) 增加到後來的 28.03％ (24hr)。氣體性 NOx(1 μM)有促使人類肺纖維細胞(MRC-5)DNA 合成增加的作用(Fig.1)。我們更進一步的經由 MTT assay 來觀察發現低劑量的氣體性 NOx(1 μM)有促使人類肺纖維細胞(MRC-5)細胞有增加生長的現象 (Fig.2)。以 immunoblotting 偵測 phospho-ATF2、PCNA 及 RBBP 表現，結果 phospho-ATF2 在 3 小時，PCNA、RBBP 在 12 小時表現量最高。因為此三者都與細胞之增生有關之蛋白，因此證明低劑量的氣體性 NOx(1 μM)有促使人類肺纖維細胞(MRC-5)細胞增生的作用(Fig.3)。偵測 NF-κB、c-Jun 及 Fos 表現，結果 c-Fos 在 24 小時，NF-κB、c-Jun 在 48 小時表現量最高(Fig.4)。偵測 iNOS 表現，結果 iNOS 在處理 NOx 氣體飽合溶液，48 小時後有最大表現量 (Fig.5)。偵測 Raf 與 ERK 表現，發現 MRC-5 細胞不表現 Raf 與 ERK (Fig.6)；偵測 MEKK1/JNK1/p38MAPK 表現，發現 MEKK1 與 JNK1 在處理 NOx 氣體飽合溶液 12 小時後有最大表現量 (Fig.7A、B)；p38MAPK 則是在 24 小時有最大表現量(Fig.7C)。觀察 NAME(iNOS 抑制劑)對 MRC-5 cells 之 phospho-JNK 及 phospho-MKK4 表現的影響，結果發現 1 μM NOx 促使 MRC-5 細胞之 phospho-JNK 表現明顯部分受到抑制(Fig.8A)；而在 phospho-MKK4 方面則完全被抑制(Fig.8B)。SB203580(p38MAPK 抑制劑)對氣體性 NOx 活化 MRC-5 細胞 p38 表現的影響可由 flow cytometry 看出，以氣體性 1 μM NOx 活化的 MRC-5 細胞 S phase，在加入 3 μM SB203580 之後可以部分抑制細胞的 DNA 合成(Fig.9)。

偵測 PI-3-K、Akt 及 phospho-Bad 表現，結果 PI-3-K 與 phospho-Bad 在 24 小時，Akt 在 48 小時表現量最高 (Fig.10)。在加入 10nM Wortmannin 之後可以完全抑制細胞的 DNA 合成(Fig.11)。處理含 1μM 的 NOx 氣體飽合溶液及 100μM L-NAME(iNOS 抑制劑)偵測 PI-3-K、Akt 及 phospho-Bad 表現，結果 PI-3-K、Akt、phospho-Bad 隨著時間增加，與之前 Fig.10 相比較下，發現都有抑制表現的情況(Fig.12)。偵測 phospho-Rb 與 Rb 表現，結果發現 Rb 隨著時間增長其表現量有遞減的趨勢，而 phospho-Rb 則隨著時間增長其表現量有遞增的趨勢(Fig.13)。偵測 cyclinA、cyclinB、cyclinD1、cyclinE 表現，結果發現隨著時間增長，所有 cyclins 表現量都有遞增的趨勢，cyclinA 與 cyclinB 在 24hr 時表現量達到最高，cyclinD1 在 9hr 時表現量最高，而 cyclinE 則是在 12hr 時表現量達最高(Fig.14)。偵測 cdk1/cdc2、cdk2、cdk4 表現，結果發現隨著時間增長，所有 cdks 表現量都有遞增的趨勢，而且在 9hr 時表現量達到最高(Fig.15)。偵測 p27、p21、p16 表現，結果發現隨著時間增長，p27、p21、p16 表現量都有遞減的趨勢，而且在 24hr 時抑制量達最低(Fig.16)。偵測 IAP、cytochrome c、caspase 3 表現，結果發現抗細胞凋亡蛋白 IAP 的表現量在 24hr 時表現量最高，而在細胞凋亡蛋白 cytochrome c 與 caspase 3 的表現上則有相對減少的趨勢(Fig.17)。本篇實驗所得到的結果綜合於 Fig.18，NOx 可造成細胞增生、抗凋謝死亡及促進細胞週期，這些作用可能與氣體性 NOx 致肺病變有關。氣體性氮氧化物可以刺激人類肺纖維細胞生長，而這與肺病變中的肺囊胞性纖維症(cystic fibrosis)之間也許有正相關。在 1998 年 Greening 等人提出，肺囊胞性纖維症病人身上發現其 NO_2 是增加的(129)；而在 1999 年 Smith 等人提出 NO 與肺囊胞性纖維症有關(130)；同樣在 1999 年 Beck 等人也發現，在肺囊胞性纖維症病人身上可以發現微量的 NO 存在(131)。此外在 2000 年 Downey 等人發現，肺囊胞性纖維症病人體內發現 iNOS

有表現量增加的趨勢(132)。也因此本實驗結果或許能提供解釋造成肺病變疾病發生的分子機轉。而氣體性 NOx 是否會造成肺癌發生？1988 年知道 NO_2 與肺癌之間有關(133)；在許多癌症病患也都發現 iNOS 的表現有明顯的增加，如皮膚癌(134)、乳癌(135)及大腸癌(136)等等。所以我們研究證實，氣體性氮氧化物可以刺激人類肺纖維細胞生長，而這與肺病變中的肺囊胞性纖維症也許有關，至於氣體性氮氧化物能否有致癌效果？致癌作用是直接或間接？這都是未來我們要更詳細加以探討的方向。

四、 計畫成果自評

1. 本計畫成果發現氣體性 NOx 可促進肺纖維細胞增生，抑制凋謝死亡及促進細胞週期等作用。
2. 發現上述作用的分子機轉。
3. 這些作用可能與肺病變及肺癌佔有重要角色。

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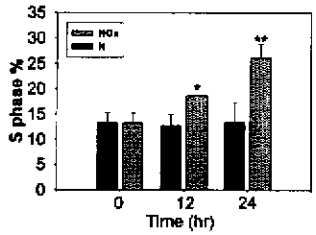
Fig.1



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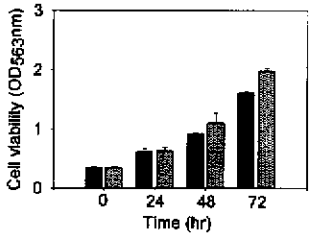

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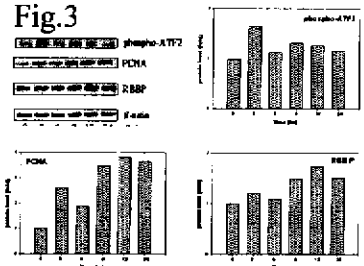

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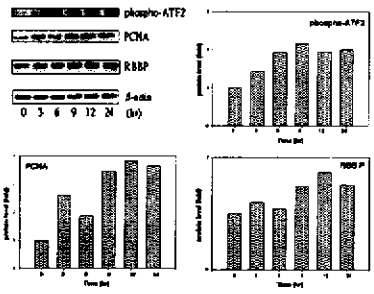

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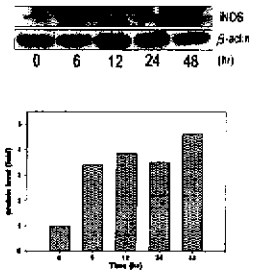

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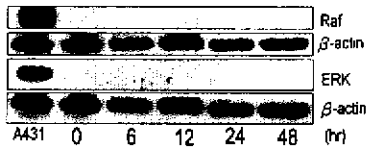

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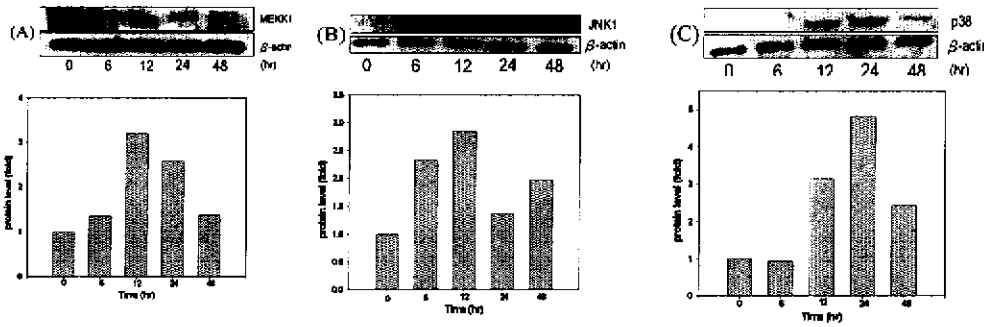

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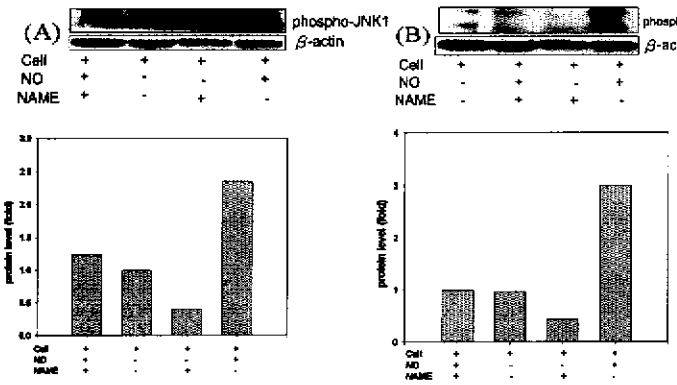

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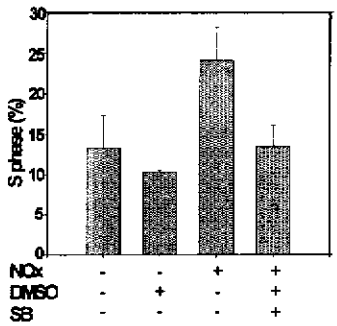

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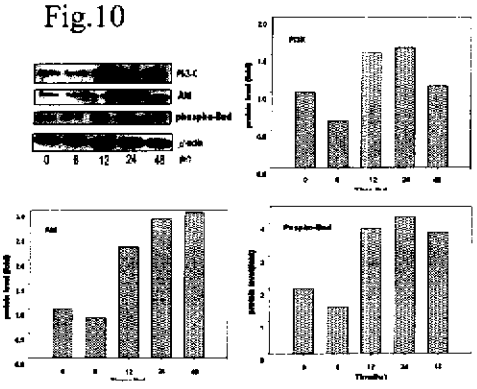

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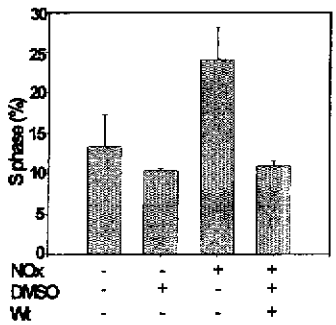

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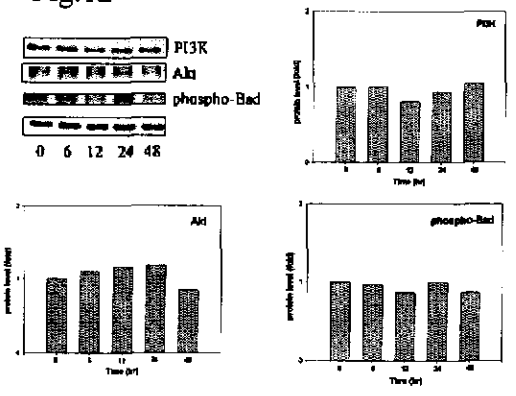

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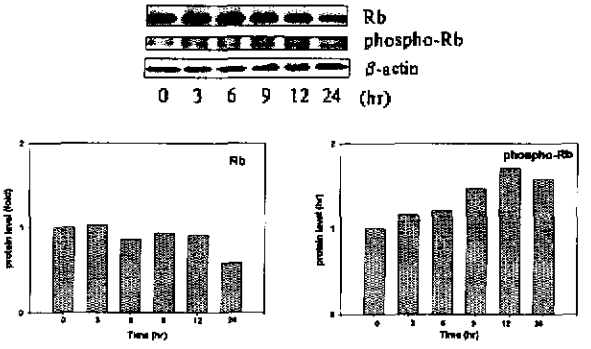

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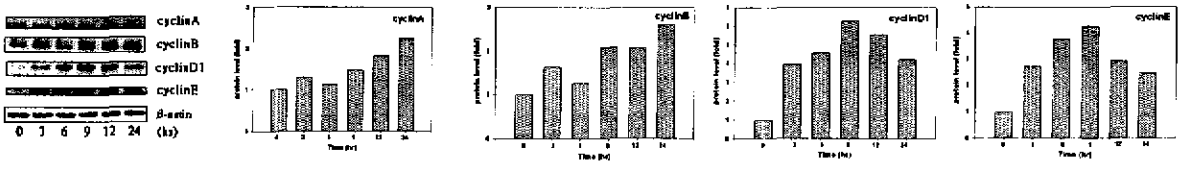

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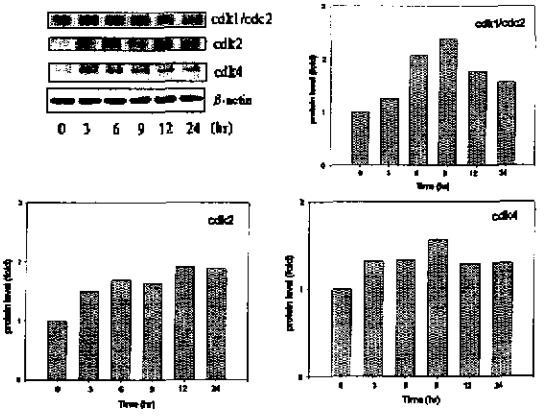

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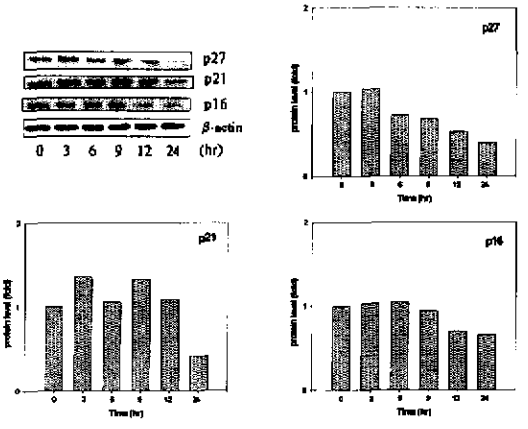

Fig.17

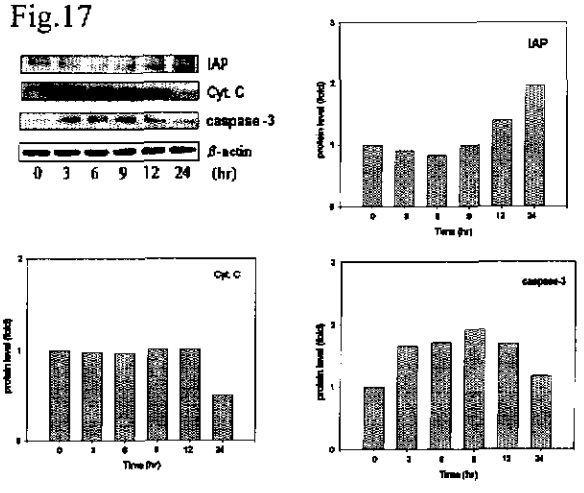

Fig.1 Effect of gaseous NOx induced cell cycle progress in MRC-5 cells. Culture cells were treated with NOx solution 1 μ M as indicated time. Cell cycle was analyzed by flow cytometry.

Fig.2 Time course of gaseous NOx treatment on MRC-5 cells. Culture cells were treated with NO gas-saturated solution 1 μ M as indicated time. Cell viability was analyzed by MTT assay. *p<0.05, **p<0.0005

Fig.3 Time course of gaseous NOx treatment on phospho-ATF2, PCNA, RBBP protein expression in MRC-5 cells. Culture cells were treated with NO gas-saturated solution 1 μ M as indicated time. The phospho-ATF2, PCNA, RBBP protein was analyzed by the immunoblotting.

Fig.4 Time course of gaseous NOx treatment on NF-κ B, c-Jun, c-Fos protein expression in MRC-5 cells. Culture cells were treated with NO gas-saturated solution 1 μ M as indicated time. The NF-κ B, c-Jun, c-Fos protein was analyzed by the immunoblotting.

Fig.5 Time course of gaseous NOx treatment on iNOS protein expression in MRC-5 cells. Culture cells were treated with NO gas-saturated solution 1 μ M as indicated time. The iNOS protein was analyzed by the immunoblotting.

Fig.6 Raf and Erk protein is not expressed in MRC-5 cells. Culture cells were treated with NO gas-saturated solution 1 μ M for indicated time. Raf and Erk proteins were analyzed by immunoblotting. A431 is positive control.

Fig.7(A) Time course of gaseous NOx treatment on MEKK1 protein expression in MRC-5 cells. Culture cells were treated with NO gas-saturated solution 1 μ M as indicated time. The MEKK1 protein was analyzed by the immunoblotting.

Fig.7(B) Time course of gaseous NOx treatment on JNK protein expression in MRC-5 cells. Culture cells were treated with NO gas-saturated solution 1 μ M as indicated time. The JNK protein was analyzed by the immunoblotting.

Fig.7(C) Time course of gaseous NOx treatment on p38 protein expression in MRC-5 cells. Culture cells were treated with NO gas-saturated solution 1 μ M as indicated time. The p38 protein was analyzed by the immunoblotting.

Fig.8(A) The inhibit effect of NAME treatment on phospho-JNK expression in MRC-5 cells. Culture cells were treated with NO gas-saturated solution 2 μ M and NAME 100 μ M for 24hr. phospho-JNK protein was analyzed by the immunoblotting.

Fig.8(B) The inhibit effect of NAME treatment on phospho-MKK4 expression in MRC-5 cells. Culture cells were treated with NOx solution 2 μ M and NAME 100 μ M for 12hr. phospho-MKK4 protein was analyzed by the immunoblotting.

Fig.9 Effect of gaseous NOx induced cell cycle progress in MRC-5 cells. Culture cells were treated with NOx solution 1 μ M(NOx) and 3 μ M SB203580(SB).24hrs latter, cell cycle was analyzed by flow cytometry. N is control, DMSO is solvent control.

Fig.10 Time course of gaseous NOx treatment on PI3K, Akt, phospho-Bad protein expression in MRC-5 cells. Culture cells were treated with NO gas-saturated solution 1 μ M as indicated time. The PI3K, Akt, phospho-Bad protein was analyzed by the immunoblotting.

Fig.11 Effect of gaseous NOx induced cell cycle progress in MRC-5 cells. Culture cells were treted with NOx solution 1 μ M(NOx) and 10nM Wortmannin(Wt).24hrs latter,cell cycle was analyzed by flow cytometry. N is control, DMSO is solvent control.

Fig.12 Time course of gaseous NOx treatment on PI3K, Akt, phospho-Bad protein expression in MRC-5 cells. Culture cells were treated with NO gas-saturated solution 1 μ M and iNOS inhibitor (L-NAME)100μM as indicated time. The PI3K, Akt, phospho-Bad protein was analyzed by the immunoblotting.

Fig.13 Time course of gaseous NOx treatment on Rb and phospho-Rb proteins expression in MRC-5 cells. Culture cells were treated with NO gas-saturated solution 1 μ M as indicated time.The Rb and phospho-Rb proteins were analyzed by the immunoblotting.

Fig.14 Time course of gaseous NOx treatment on cyclinA, cyclinB, cyclinD1, cyclinE protein expression in MRC-5 cells. Culture cells were treated with NO gas-saturated solution 1 μ M as indicated time. The cyclinA, cyclinB, cyclinD1, cyclinE protein was analyzed by the immunoblotting.

Fig.15 Time course of gaseous NOx treatment on cdk1/cdc2, cdk2, cdk4 protein expression in MRC-5 cells.Culture cells were treated with NO gas-saturated solution 1 μ M as indicated time. The cdk1/cdc2, cdk2, cdk4 protein was analyzed by the immunoblotting.

Fig.16 Time course of gaseous NOx treatment on p27, p21, p16 protein expression in MRC-5 cells. Culture cells were treated with NO gas-saturated solution 1 μ M as indicated time. The p27, p21, p16 protein was analyzed by the immunoblotting.

Fig.17 Time course of gaseous NOx treatment on IAP protein expression in MRC-5 cells. Culture cells were treated with NO gas-saturated solution 1 μ M as indicated time. The IAP protein was analyzed by the immunoblotting.