บทบาทของไนตริกออกไซด์ต่อความไวในการตอบสนองต่อการกระตุ้นให้เกิดการตาย แบบอะพอพโทซิสของเซลล์มะเร็งปอดและเซลล์ปอดปกติ

นาย ปิติ จันทร์วรโชติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีเภสัชกรรม
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2548

ISBN 974-53-2777-8

THE ROLE OF NITRIC OXIDE IN APOPTOTIC SUSCEPTIBILITY OF HUMAN LUNG CANCER AND NORMAL LUNG CELLS

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Program in Pharmaceutical Technology
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2005

ISBN 974-53-2777-8

Thesis Title	THE ROLE OF NITRIC OXIDE IN APOPTOTIC SUSCEPTIBILITY OF HUMAN LUNG CANCER AND NORMAL LUNG CELLS
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ปิติ จันทร์วรโชติ: บทบาทของไนตริกออกไซด์ต่อความไวในการตอบสนองต่อการกระตุ้นให้เกิดการตายแบบอะพอพโทซิสของเซลล์มะเร็งปอดและเซลล์ปอดปกติ (THE ROLE OF NITRIC OXIDE IN APOPTOTIC SUSCEPTIBILITY OF HUMAN LUNG CANCER AND NORMAL LUNG CELLS) อ.ที่ปรึกษา: รศ.ดร. อุบลทิพย์ นิมมานนิตย์, อ.ที่ปรึกษาร่วม: Prof. Pattric S. Callery และ ผศ.ดร. บุญศรี องค์พิพัฒนกุล, 188 หน้า, ISBN 974-53-2777-8

การรบกวนความไวในการตอบสนองต่อการกระตุ้นให้เกิดการตายของเซลล์แบบอะพอพโทซิสคือสาเหตุสำคัญในการเจริญของโรคต่างๆโดยเฉพาะโรคมะเร็ง การดื้อยาของเซลล์มะเร็งต่อยาต้านมะเร็ง cisplatin ซึ่งเป็นยาที่กระตุ้นให้เกิดการตายของเซลล์แบบอะพอพโทซิสเป็นข้อจำกัดสำคัญที่มีผลต่อประสิทธิภาพในการรักษาที่พบมากในโรคมะเร็งปอด นอกเหนือจากนี้กลไกที่สำคัญซึ่งก่อให้เกิดการลุกลามของโรคมะเร็ง คือการดื้อต่อการกระตุ้นให้เกิดการตายของเซลล์แบบอะพอพโทซิสโดย Fas ligand (FasL) ไนตริกออกไซด์เป็นตัวควบคุมการตายของเซลล์แบบอะพอพโทซิส แต่อย่างไรก็ตามบทบาทและกลไกของไนตริกออกไซด์ต่อการตายแบบอะพอพโทซิสที่ถูกกระตุ้นโดย cisplatin และ FasL ยังไม่ทราบแน่ชัด มีรายงานว่าการเพิ่มปริมาณของไนตริกออกไซด์ในภาวะบาดเจ็บของปอดและภาวะปอดอักเสบรวมทั้งมะเร็งปอดมีความสัมพันธ์กับการดื้อต่อยาต้านมะเร็ง การวิจัยนี้พบว่าไนตริกออกไซด์ลดการตายของเซลล์และเพิ่มการดื้อต่อ cisplatin และ FasL ในเซลล์มะเร็งปอด (H460) และเซลล์ปอดปกติ (BEAS) เมื่อเพิ่มปริมาณไนตริกออกไซด์โดยการให้ sodium nitroprusside (SNP) และ dipropylenetriamine (DPTA) NONOate สามารถยับยั้งการตายที่ถูกกระตุ้นโดย cisplatin และ FasLได้ ในขณะที่เมื่อลดปริมาณไนตริกออกไซด์โดยการให้ Aminoguanidine (AG) and 2-(4-carboxyphenyl)-4,4,5,5-tetra-methylimidazoline-1-oxyl-3-oxide (PTIO) ส่งผลตรงกันข้าม ในการศึกษานี้พบว่าไนตริกออกไซด์ยั้งยั้งกระบวนการ ubiquitin-proteasome ทำให้การทำลาย Bcl-2 ลดลง ส่งผลให้ปริมาณ Bcl-2ในเซลล์เพิ่มขึ้น ซึ่งการเพิ่มขึ้นของ Bcl-2 นี้ทำให้เซลล์ H460 ดื้อต่อยา cisplatin โดยปกติยา cisplatin จะกระตุ้นให้เกิด dephosphorylation ของ Bcl-2 โดยการเพิ่มปริมาณ reactive oxygen species (ROS) ซึ่ง dephosphorylation นี้นำไปสู่การทำลาย Bcl-2 แต่ในการทดลองนี้พบว่าการเพิ่มปริมาณของไนตริกออกไซด์ไม่มีผลกระทบต่อภาวการณ์เกิด phosphorylation ของ Bcl-2 แต่ส่งผลทำให้เกิด S-nitrosylation ซึ่งมีผลในการยับยั้งกระบวนการทำลาย Bcl-2 การวิจัยนี้ยังพบว่าไนตริกออกไซด์มีผลควบคุมการกระตุ้นให้เกิดการตายโดย FasL ในเซลล์ BEAS การเพิ่มปริมาณไนตริกออกไซด์ทำให้เกิดกระบวนการ S-nitrosylation ของ FLICE inhibitory protein (FLIP) ซึ่งการเกิด S-nitrosylation นี้จะส่งผลให้ FLIP ดื้อต่อการทำลายโดยกระบวนการ ubiquitin-proteasome จากการทำ deletion และ mutation analysis ทำให้ทราบว่า cysteine ที่ตำแหน่ง 254 และ 259 ซึ่งอยู่ในส่วนของ caspase-like domain ในโมเลกุลของ FLIP เป็นจุดสำคัญที่เกิด S-nitrosylation ผลการวิจัยทั้งหมดนี้ทำให้ทราบกลไกของไนตริกออกไซด์ในการควบคุมปริมาณโปรตีน Bcl-2 และการดื้อต่อยา cisplatin ซึ่งมีผลต่อการพัฒนายาต้านมะเร็ง นอกจากนี้การวิจัยนี้ยังอธิบายถึงกลไกของไนตริกออกไซด์ในการควบคุมปริมาณโปรตีน FLIP ซึ่งนำไปสู่ความเข้าใจในกลไกการตายของเซลล์แบบอะพอพโทซิส

สาขาวิชา....เทคโนโลยีเภสัชกรรม.....
ปีการศึกษา.....2548.....

ลายมือชื่อนิสิต.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

#4576962933: MAJOR PHARMACEUTICAL TECHNOLOGY (INTERNATIONAL)
KEYWORD : NITRIC OXIDE/ APOPTOSIS/ CISPLATIN/ FAS/ BCL-2/ FLIP/ UBIQUITINATION/ S-NITROSYLATION

PITHI CHANVORACHOTE: THE ROLE OF NITRIC OXIDE IN APOPTOTIC SUSCEPTIBILITY OF HUMAN LUNG CANCER AND NORMAL LUNG CELLS, THESIS ADVISOR: ASSOC. PROF. UBONTHIP NIMMANNIT, Ph.D., THESIS CO-ADVISOR: PROF. PATTRIC S. CALLERY, Ph.D., ASSIST. PROF. BOONSRI ONGPIPATTANAKUL, Ph.D. 188 PAGES. ISBN 974-53-2777-8

Interfering apoptotic susceptibility is important in progression of many diseases, in particular cancers. Cancer cell-acquired resistance to cisplatin-induced apoptosis is a major limitation for efficient therapy, as frequently observed in human lung cancer. Moreover, escaping from Fas ligand (FasL)-induced apoptosis is a potential mechanism of cancer progression. Nitric oxide (NO) is a key regulator of apoptosis, but its role in cisplatin- and FasL-induced cell death and the underlying mechanism are largely unknown. Evidences indicated that increased NO production in lung injury, lung inflammation, and lung carcinomas, correlated with the incidence of chemotherapeutic resistance. The present study showed that NO impaired the apoptotic function of cells and increased their resistance to cisplatin- and FasL-induced apoptosis in both human lung carcinoma H460 cells and human lung epithelial BEAS cells. The NO donors sodium nitroprusside (SNP) and dipropylenetriamine (DPTA) NONOate were able to inhibit cisplatin- and FasL-induced cell death, whereas the NO inhibitors Aminoguanidine (AG) and 2-(4-carboxyphenyl)-4,4,5,5-tetra-methylimidazoline-1-oxyl-3-oxide (PTIO) had opposite effect. Cisplatin resistance in H460 cells was mediated by Bcl-2, and NO upregulated its expression by preventing the degradation of Bcl-2 via ubiquitin-proteasome pathway. Cisplatin-induced generation of reactive oxygen species (ROS) caused dephosphorylation and degradation of Bcl-2. In contrast, elevation of NO had no effect on Bcl-2 phosphorylation but induced S-nitrosylation of the protein, which inhibited its ubiquitination and subsequent proteasomal degradation. Also, this study revealed the mechanism of NO in regulating FasL-induced apoptosis in BEAS cells. FasL-induced downregulation of FLICE inhibitory protein (FLIP) was mediated by a ubiquitin-proteasome pathway that was negatively regulated by NO. S-nitrosylation of FLIP was an important mechanism rendering FLIP resistant to ubiquitination and proteasomal degradation. Deletion analysis showed that the caspase-like domain of FLIP was a key target for S-nitrosylation by NO, and mutation of its cysteine 254 and cysteine 259 residues completely inhibited S-nitrosylation, leading to increase ubiquitination and proteasomal degradation of FLIP. These findings indicated a novel pathway for NO regulation of Bcl-2 which provided a key mechanism for cisplatin resistance and its potential modulation for improved cancer chemotherapy. Also, this study revealed a novel pathway for NO regulation of FLIP that provided a key mechanism for apoptosis regulation and a potential new target for intervention in death receptor-associated diseases.

Field of study....Pharmaceutical.Technology.... Student's signature...Pithi Chanvorachote.....
Academic year.....2005..... Advisor's signature...Ubonthip Nimmannit.....
Co-advisor's signature...Boonsri Ongpipattanakul

ACKNOWLEDGEMENTS

My sincere and heartfelt thanks to my advisor, Associate Professor Ubonthip Nimmannit, my co-advisor Assistant Professor Boonsri Ongpipatanakul and Professor Pattric Callery who, from the very first day of my study in Pharmaceutical Technology program have given me the invaluable advices, supervision, encouragement throughout this study.

I would like to express my deep appreciate to Professor Yongyuth Rojanasakul of the Department of Pharmaceutical Sciences, West Virginia University, for giving a chance to perform experiments at West Virginia University, USA. His excellent advice, guidance, encouragement, understanding and prompt action which have made this study a reality in time, are highly regarded and appreciated. I am deeply honored for his keen interest, dedication and deep enthusiasms shown in my study.

I am very much indebted to Associate Professor Christian Stehlik of Mary Babb Randolph Cancer Center, West Virginia University for giving advices, and providing all plasmids used in this study. His kindness and helpfulness are also appreciated.

My special thanks go to Dr. Liying Wang of Pathology and Physiology Research Branch, National Institute for Occupational Safety and Health (NIOSH), USA for giving a change to perform experiment at NIOSH, and her help during my experimental study in USA.

I would like to thank to the personals of the Department of Pharmaceutical Sciences, West Virginia University, and Pharmaceutical Technology Program, Chulalongkorn University.

I am very much obliged and honored to the members of the Thesis Committee for their supportive attitude, constructive criticisms and for their invaluable discussions over my dissertation.

It would not be completed without expressing my heartfelt gratitude to my parents and to my sister and brother for their love, understanding, moral support and tremendous encouragements throughout my life.

The present work was partly supported by NIH Grant HL-071545 and TRF grant 5.Q.CU.46/A.1.

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LIST OF ABBREVIATIONS

%	percentage
°C	degree Celsius (centigrade)
µg	microgram (s)
µl	microlitre (s)
AG	aminoguanidine
ANOVA	analysis of variance
Apaf-1	the initiating apoptosis protease activating factor 1
Bax	a pro-apoptotic protein in Bcl-2 family
Bcl-2	an anti-apoptotic protein inBcl-2 family
Bcl-XL	an anti-apoptotic protein in Bcl-2 family
BID	a pro-apoptotic protein in Bcl-2 family
CO_2	carbon dioxide
CP	cisplatin
Cys	Cysteine
d	day
DAF-DA	4,5-diamino-fluorescein diacetate
DCF-DA	2', 7' –dichlorodihydrofluorescein diacetate
DED	death effector domain
DISC	death-inducing signaling complex
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DPTA	dipropylenetriamine
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
eNOS	endothelial nitric oxide synthase
et al.	et alii, and others
FADD	Fas-associated death domain
Fas	Fas receptor

FasL	Fas ligand
FLIP	FLICE-inhibitory protein
g	gram
GSH	glutathione
h	hour
H_2O_2	hydrogen peroxide
IFN-γ	interferon gamma
IL-1β	interlukin-1-beta
iNOS	inducible nitric oxide synthase
L-NAME	NG-nitro-L-arginine methyl ester
LPS	lipopolysaccheride
MAPK	mitogen-activated protein kinase
mg	milligram (s)
min	minute (s)
mM	millimolar
mm	millimeter (s)
NAC	N-acetylcysteine
NaCl	sodium chloride
NADH	reduced nicotinamide adenosine dinucleotide
NaF	sodium fluoride
nM	nanomolar
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate-buffered saline
pH	the negative logarithm of hydrogen ion concentration
PTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-immidazoline-1-oxyl-3-oxide
PVDF	polyvinylidene fluoride
RPMI	Roswell Park Memorial Institute’s medium
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM	standard effort of mean
Ser	Serine
SNP	sodium nitroprusside
TBST	Tris-buffered saline, 0.1 % Tween 20
TNFR	tumor necrosis factor receptor; TNFR-associated factor-2, TRAF-2
TNF	tumor necrosis facter
TNF-α	tumor necrosis factor alpha
UV	ultraviolet
z-VAD-fmk	benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone

CHAPTER I

INTRODUCTION

The aim of cancer research is for a better understanding of the molecular basis of cancer, with the expectation that this will result in therapeutic advances and improved outcomes for patients. The discovery of apoptosis has contributed much to the understanding of the mechanisms of cell death, in both normal and cancer cells.

Apoptosis, the most common and well-defined form of programmed cell death, is essential for the maintenance of homeostasis as well as pathological processes (Reed, 2000; Mullauer et al., 2001). Apoptosis has been known to play an important role in regulating carcinogenesis, cancer progression, and the development of chemotherapeutic resistance (Mullauer et al., 2001). Cell apoptosis can be triggered by the tumor suppressor proteins in response to DNA-damaged signal, resulting in the elimination of the defected cells and preventing such a DNA-defect from being passed on to the next generation of cells (Reed, 2000; Mullauer et al., 2001). The suppression of carcinogenesis is thus highly dependent on the functioning of cell apoptosis. In addition, apoptosis initiated by immune-based stimuli is the natural mechanism to prevent the cancer progression (Arase et al., 1995; Arase et al., 1998; Kagi et al., 1994; Melief et al., 1997; and O'Connell et al., 1996). In terms of cancer treatment, the important part of the efficacy of chemotherapy is due to the ability to induce apoptosis (Reed, 2000; and Mullauer et al., 2001). Moreover, defects in apoptotic mechanisms have been associated with the development of chemotherapy resistance (Hu and Kavanagh, 2003). Modulating cell susceptibility to apoptosis thus potentially affects the course of carcinogenesis, cancer progression, and chemotherapeutic resistance. The ability to mitigate or accelerate cell apoptotic process requires that key molecular signals involved must be lineated. One of the likely central signals being studied at the moment is nitric oxide.

Endogenously produced nitric oxide (NO) synthesized from L-arginine by NO synthase is a mediator of a variety of physiological and pathological processes (Moncada et al., 1991; and Nathan, 1992). NO has been shown to possess both pro- and anti-apoptotic functions in various cell types (Moncada and Erusalimsky, 2002; Davis et al.,

2001; and Kolb, 2002). The apoptosis-inducing effect of NO was attributed to its ability to induce oxidative stress and caspase activation (Cui et al., 1994; Farias-Eisner et al., 1994; Gansauge et al., 1998; Klein and Ackerman, 2003; and Chlichlia et al., 1998). Nonetheless, endogenous NO production or exposure to appropriate amounts of NO has been reported to inhibit apoptosis in several cell types (Chung et al., 2001; and Chun et al., 1995). Furthermore, NO has been shown to inhibit cell death induced by a variety of agents including chemotherapeutic agents, viral infections, and death ligands (Kolb, 2002; Mannick et al., 1994; and Chen et al., 2005).

Recent studies have shown that inflammation in many lung cells is accompanied by upregulation of an inducible isoform of nitric oxide synthase (iNOS) enzyme resulting in high NO production (Hibbs et al., 1988; Lamas et al., 1991; Nakayama et al., 1992; Nathan, 1992; Kolls et al., 1994; Robbins et al., 1994; Ermert et al., 2002; and Mehta, 2005). Since chronic inflammation has been recognized as being associated with increased risk for many cancers including lung cancer, it is highly anticipated that elevated NO level in response to lung inflammation might play an anti-apoptotic role and stimulate the generation and the progression of cancerous cells by rendering traumatic cell resistant to immune-mediated apoptosis.

Elevated NO production has also been observed in either lung or other cancer cells, particularly in comparison to the surrounding normal cells (Liu et al., 1998; Arias-Diaz et al., 1994; and Fujimoto et al., 1997). Combining with the evidences indicating high chemotherapeutic resistance in lung cancer, it is interesting to investigate whether elevated NO level in lung cancer plays an anti-apoptotic role and induces the cancer cell resistant to chemotherapy.

It is generally known that apoptosis is mediated through two major mechanisms, namely, death receptor and mitochondrial pathways. Although the detailed mechanism by which NO regulates apoptosis are not well understood, both mitochondrial and death receptor pathways of apoptosis could strongly be involved (Borutaite and Brown, 2003; and Fukuo et al., 1996). Attempt to clarify the roles of NO in each apoptotic pathway and the role of NO on the regulation of cell susceptibility to apoptosis may be succeeded by examining the influence of NO under the presence of specific apoptotic inducers for either pathways.

Fas/FasL-mediated apoptosis, considered as one of the most important members of death receptor pathway, plays a crucial role in immune-mediated cancer cell apoptosis (Nagata, 1997; and Medema et al., 1999). Since Fas/FasL mediated apoptosis is an important immune mechanism for eliminating cancer cells, using Fas/FasL-mediated apoptosis as a model for investigating the role of NO in the death receptor pathway might lead to the understanding on the role of NO in death receptor-mediated apoptosis and on the cell resistance to immune-mediated apoptosis.

In addition, the platinum coordination complex *cis*-diamminedichloroplatinum or cisplatin is known to induce apoptosis via mitochondrial pathway. Cisplatin is widely prescribed as chemotherapeutic agent against lung cancers (Loehrer et al., 1984; Wong et al., 1999; and Cohen et al., 2001); however, cancer cell-acquired resistance to cisplatin-induced apoptosis is a major limitation for efficient therapy (Wong et al., 1999). Using cisplatin-mediated apoptosis as a model for investigating the role of NO on mitochondrial apoptosis might lead to the understanding on the role of NO in mitochondrial apoptosis pathway and provide knowledge of cell resistance to cisplatin-mediated apoptosis.

Since, the role of NO on cisplatin- and FasL-mediated apoptosis has not been investigated in lung cells. The present study thus investigated the role of NO in cisplatin- and FasL- induced apoptosis in normal lung epithelial (BEAS) cells and cancer lung epithelial (H460) cells. In addition, the present study use pharmacological and genetic manipulation approaches to elucidate the underlying mechanisms by which NO regulated cisplatin- and FasL-mediated apoptosis.

Lung cancer results in an enormous clinical burden for health care providers and possesses very high mortality rate. Moreover, many clinical studies indicated the high rate of chemotherapeutic resistances. Overcoming resistance to chemotherapy is a major goal of cancer research that could dramatically improve clinical outcomes for patients. This aim will most likely be achieved by rational targeted investigations based on a detailed knowledge of resistance mechanisms. This study provides the evidences supporting the significant effect of NO on cell susceptibility to apoptosis and reveals the underlying mechanisms of NO in controlling death receptor as well as mitochondrial mediated apoptosis. Understanding the mechanisms by which NO regulated apoptotic susceptibility might lead to the development of new therapeutic strategy for lung cancer treatment.

CHAPTER II

LITERATURE REVIEW

1. Nitric Oxide (NO)

1.1 Biochemistry of nitric oxide

Nitric oxide is a pleiotropic, ubiquitous modulator of several cellular functions. Since it is such a small and lipophilic molecule, NO is able to diffuse rapidly across cell membranes and, depending on the conditions, is able to diffuse distances of more than several hundred microns. The biological chemistry of NO hinges around its unpaired electron, which makes NO free, but stable radical (Wink and Mitchell, 1998; and Stamler, 1994). NO is synthesized by a family of Nitric Oxide Synthase (NOS) isoenzymes (Kim et al., 2002; and Nathan et al., 1994). These enzymes convert L-arginine into L-citrulline, producing NO in the process. Oxygen and NADPH are necessary co-factors (Ignarro et al., 1988; Palmer et al., 1988; and Miranda et al., 2000) (As shown in Figure 1).

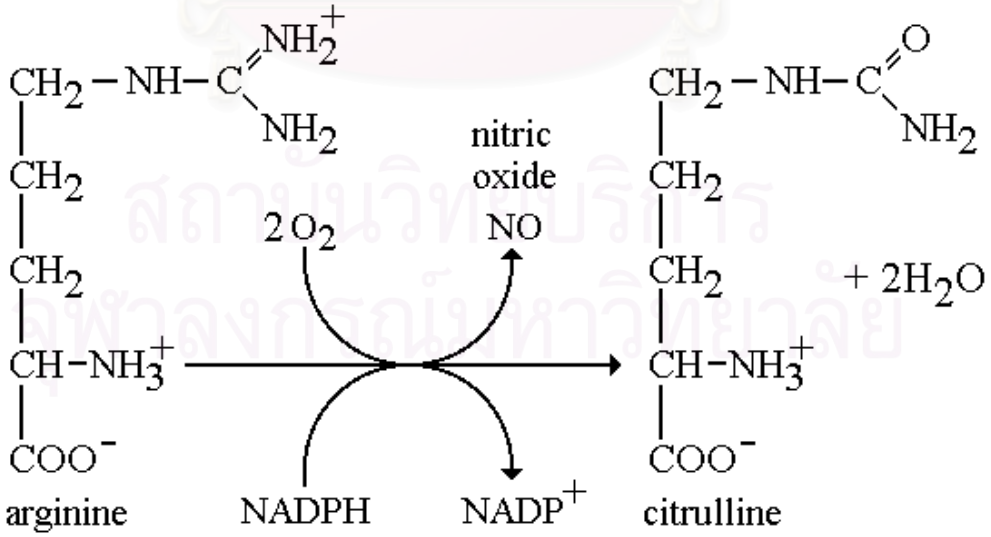

Figure 1 Nitric oxide synthesis (Nathan et al., 1994).

There are three isoforms of nitric oxide synthase (NOS) named according to their activity or the tissue type in which they were first described. The isoforms of NOS are neuronal NOS (or nNOS), endothelial NOS (or eNOS) and inducible NOS (or iNOS) (Michel and Feron, 1997; McDonald and Murad, 1996; and Alderton et al., 2001). An isoform named mtNOS has also been isolated in mitochondria (Tatoyan and Giulivi, 1998). These enzymes are also sometimes referred to by number, so that nNOS is known as NOS1, iNOS is known as NOS2 and eNOS is NOS3. Despite the names of these enzymes, all three isoforms can be found in a variety of tissues and cell types.

Two of the enzymes (nNOS and eNOS) are constitutively expressed in mammalian cells and synthesize NO in response to increases in intracellular calcium levels. In some cases, however, they are able to increase NO production independently of calcium levels in response to stimuli such as shear stress. Importantly, iNOS activity is independent of the level of calcium in the cell; however its activity - like all of the NOS isoforms is dependent on the binding of calmodulin. Increases in cellular calcium leads to increases in levels of calmodulin and the increased binding of calmodulin to eNOS and nNOS leads to a transient increase in NO production by these enzymes. By contrast iNOS is able to bind tightly to calmodulin even at very low cellular concentration of calcium. Consequently iNOS activity is not able to respond to changes in calcium levels in the cell. As a result the production of NO by iNOS lasts much longer than from the other isoforms of NOS, and tends to produce much higher concentrations of NO in the cell.

1.2 Physiological roles of NO

NO is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological processes. NO is able to induce vasodilation and a large number of other roles have been described for NO. It is also known to play a role in the immune system, the nervous system, in inflammation and in programmed cell death (apoptosis). NO has also been implicated in smooth muscle relaxation, pregnancy and blood vessel formation (angiogenesis) (Butler and Williams, 1993; Moncada et al., 1991; and Nathan, 1992).

1.2.1 Role in the immune system

NO can be produced by a number of cells involved in immune responses. In particular cytokine-activated macrophages can produce high concentrations of NO in order to kill target cells such as bacteria or tumor cells. NO-mediated cytotoxicity is often associated with the formation of nitrosyl-thiol complexes in enzymes within the target cell. NO has been shown to kill cells by disrupting enzymes involved in the Kreb's cycle, DNA synthesis and mitochondrial function (Nathan and Shiloh, 2000).

1.2.2 Role in inflammation

NO may act as a mediator of inflammatory process. It enhances the effect of cyclooxygenases and stimulates the production of pro-inflammatory eiconosoids. Furthermore, NO production can be induced through the upregulation of iNOS, by a number of factors involved in inflammation, including interleukins, interferon-gamma, TNF-alpha and LPS (Moncada et al., 1991).

1.2.3 Role in the nervous system

NO acts as a neurotransmitter in the central and peripheral nervous systems. It has also been shown to be involved in regulating apoptosis in neurons (Garthwaite, 1991).

1.2.4 Role in apoptosis

NO has also been demonstrated to be involved in the regulation of apoptosis. NO has been shown to be able to both induce apoptosis (known as pro-apoptotic effect) and to protect from apoptosis (known as anti-apoptotic effect) in different cell types. The effects of NO on apoptosis may vary depending upon the dose of NO, type of cell used, and redox condition of cells. Endogenous NO production or exposure to appropriate amounts of NO has been reported to inhibit apoptosis in several cell types (Chung et al., 2001; and Chun et al., 1995). Likewise, NO has been shown to inhibit cell death induced by a variety of agents including chemotherapeutic agents, viral infections, and death ligands (Kolb, 2002; Mannick et al., 1994; and Chen et al., 2005).

The anti-apoptotic effect of NO has been demonstrated in a number of cell types including leukocytes, hepatocytes, trophoblasts and endothelial cells. A number of mechanisms of the anti-apoptotic effects of NO have been proposed such as the nitrosylation and inactivation of many of the caspase including caspase 3, caspase 1 and caspase 8 (Heigold et a., 2002; Li et al., 2004). Other mechanisms include activating p53, upregulating heat shock protein 70 (and consequently blocking recruitment of pro-caspase 9 to the Apaf-1 apoptosome), inhibition of cytochrome c release from the mitochondria and activating cGMP signaling leading to activation of cGMP-dependent protein kinases and suppression of caspase activity. The effects of NO on apoptosis are generally classified as cGMP dependent or independent. Nitric oxide is able to activate cGMP signaling through the interaction of NO with the heme group of guanylate cyclase. The production of cGMP leads to the activation of cGMP-dependent protein kinases and possibly to increased expression of anti-apoptotic proteins (Kim, 2001; Chung, 2001; Kolb, 2000; Mannick et al., 1994; Estevez et al., 1998; Dimmeler et al., 1997; and Lievremont et al., 1999). However, in most cases, the protection of NO against apoptosis is independent of cGMP, suggesting alternate pathways of regulation. Although NO can upregulate several anti-apoptotic proteins such as heme oxygenase-1 and metallothionein (Spahl et al., 2003), the most effective way of NO-mediated inhibition of apoptosis is S-nitrosylation of key apoptotic proteins (Li et al., 1997; and Kim et al., 1997). *In vitro*, NO can also prevent apoptosis induced by cytostatic agents such as vincristine, paclitaxel (Ogura et al., 1998), flavone, and campothecin (Wenzel et al., 2003). In addition, the well-known mechanism of hypoxia-induced drug resistance appears to result, in part, from suppression of endogenous NO production (Matthews et al., 2001).

In contrast, the pro-apoptotic effect of NO has been demonstrated in many studies. NO has been reported to induce cell apoptosis in cancer cells of different histological origin (Cui et al., 1994; Farias-Eisner et al., 1994; Gansauge et al., 1998; and Chlichlia et al., 1998). NO either delivered by NO donors or endogenously produced by NOS enzymes induces apoptosis both *in vivo* (Nishikawa et al., 1998; and Donovan et al., 2001) and in several cell types *in vitro*, such as neuronal cells (Wei et al., 2000), macrophages (D'Acquisto et al., 2001), cardiac myocytes (Andreka et al., 2001), endothelial cells (Shen et al., 1998), lymphocytes and thymocytes (Okuda et al., 1996;

and Zhou et al., 2000). In human cell lines, Binder and colleagues reported that NO generation was essential for TNFα- mediated apoptosis. Inhibition of iNOS activity abolished the cytotoxicity of TNFα in MCF7 and other TNFα-susceptible cells (Binder et al., 1999). In a rat adenocarcinoma model, in which endothelial cells of the tumor vasculature expressed iNOS, treatment with iNOS inhibitor (NG-nitro-L-arginine methyl ester (L-NAME)) decrease NO production and result in tumor growth reduction (Peeyush and Chakraborty, 2001). Introduction of the exogenous NO (NO-producing isosorbide mononitrate and isosibide dinitrate) resulted in a significant decrease in the size of Lewis lung carcinoma cells in mice. In addition, iNOS transduction in a human colonic adenocarcinoma cell line led to stimulation of tumor growth in nude mice, which was abrogated by treatment with a selective iNOS inhibitor (1400W) (Peeyush and Chakraborty, 2001).

1.3 NO production in lung

In the lung, NOS is constitutively expressed in endothelial cell (eNOS) and in neurones (nNOS). Both eNOS and nNOS are important in pulmonary homeostasis, including mediating direct and neurogenic pulmonary vasodilatation, bronchodilation, and immune modulation (Scott and McCormack, 1999; Mehta and Drazen, 2000; and Scott et al., 1996). However, these NOSs have a lesser role in sepsis, lung inflammation, and lung injury, in which many studies have shown eNOS and nNOS are down-regulated (Razavi et al., 2002; Scott et al., 2002; and Ermert et al., 2002). In contrast, iNOS expression is induced in the majority of mammalian cell types upon exposure to inflammatory stimuli, including cytokines, bacteria, and bacterial products (e.g. lipopolysaccharide (LPS)) (Hibbs et al., 1988; Lamas et al., 1991; Nakayama et al., 1992; Nathan, 1992; Kolls et al., 1994; Robbins et al., 1994; and Ermert et al., 2002).

With regard to lung inflammation and lung injury, all of the key cellular participants (e.g. neutrophils, macrophages, endothelial cells, and epithelial cells) can express iNOS under inflammatory conditions (Robbins et al., 1993; Guo et al., 1995; and Nathan and Hibbs, 1991). Neutrophils and macrophages express high levels of iNOS and are important sources of iNOS-derived NO. However, NO can passive diffuse through the cell membrane, leading to the elevation of NO level in surrounding cells (Hibbs et al.,

1988; McCall et al., 1989; Munoz-Fernandez et al., 1992; Amin et al., 1995; Bratt and Gyllenhammar, 1995; MacMicking et al., 1995; Evans et al., 1996; Tsukahara et al., 1998; and Boyle et al., 2000; Evans et al., 1996; Nicholson et al., 1996; Wheeler et al., 1997; Tsukahara et al., 1998; Kobayashi et al., 1998; Annane et al., 2000; and Kooguchi et al., 2002). Moreover, elevation of NOS activity has been reported in human lung adenocarcinomas and increased tumor-associated NO production has been observed in lung cancer patients (Liu et al., 1998; Arias-Dias et al., 1994; and Fujimoto et al., 1997)

2. Apoptosis

Apoptosis is a highly organized process that is biochemically characterized by cell shrinkage, plasma and nuclear membrane blebbing, caspase activation, chromatin condensation, and DNA fragmentation (Thornberry and Lazeblik, 1998). Apoptosis is physiologically important process in growth and development as well as in the removal of excess, autoreactive, damaged or infected cells in development, immune reactions, and many other biological processes (Jacobson et al., 1997). Apoptosis can be triggered by two main pathways, which are death receptor and mitochondrial pathways.

2.1 Death receptor pathway

The death receptor apoptotic pathway is known as the extrinsic pathway, and requires binding of a ligand to a death receptor on the cell surface (Kiechle and Zhang, 2002). Death receptors are part of the tumor necrosis factor (TNF) gene superfamily and provide a rapid and efficient route to apoptosis. The characteristics of death receptors are cysteine-rich extracellular domains and an intracellular cytoplasmic sequence known as the ‘death domain’. The death receptors best described to date are listed in Table 1.

Table 1 Summary of the death receptors and their cognate ligands (Kumar et al., 2005)

Activating ligand	**Death receptor**
TNF	**TNFR1**/DR1/CD120a/p55
FasL/CD95L	**Fas**/CD95/Apo1/DR2
Apo3L/TWEAK	**DR3**/Apo3/WSL-1/TRAMP/LARD
TRAIL/Apo2L	**TRAIL-R1**/DR4
	TRAIL-R2/DR5/Apo-2/TRAILCK2/KILLER
TRADD	DR6

The mechanism of death-receptor pathway is that binding of death ligand induces trimerization of their receptors, recruitment of specialized adaptor proteins and activation of caspase cascades (as shown in figure 2). Caspases are a family of proteins that are one of the main effectors of apoptosis. The caspases are a group of cysteine proteases that exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis (Adrain et al., 2001; Earnshaw et al., 1999; and Kiechle and Zhang, 2002). This complex then activates caspase-8, which initiates the protease cascade, leading to apoptosis.

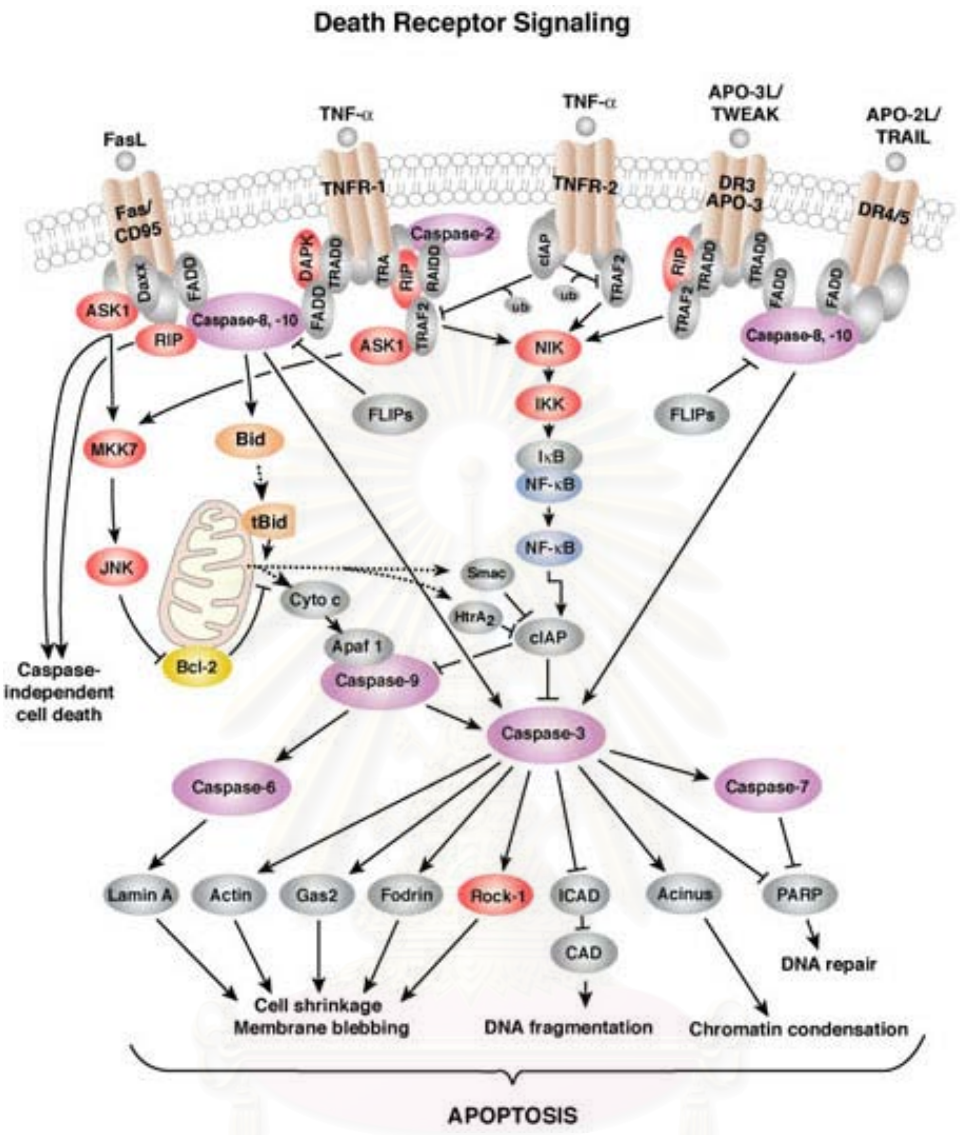

Figure 2 The death receptor apoptotic pathway (Ashkenazi et al., 1998; and Li et al., 1998)

2.1.1 Fas/FasL mediated apoptosis

In particular, Fas (CD95/Apo-1), one of the most important receptor of TNFR family, plays a crucial role in maintaining the immune system by inducing apoptosis of immune cells as well as in killing harmful cells such as cancerous cells and bacterial or viral infected cells (Nagata, 1997). Defects in the apoptosis regulatory mechanisms of the Fas/FasL system often result in lymphoproliferative disorders and autoimmune diseases (Takahashi et al., 1994; Chervonsky et al., 1997; and Hsu et al., 2001) as well as tumor outgrowth *in vivo* (Medema et al., 1999). In addition, the Fas/FasL system plays important roles in various apoptosis condition such as those evoked by anti-tumor agents, viral infection, and irradiation (Westendorp et al., 1995; Friesen et al., 1996; Muller et al., 1997; and Rehemtulla et al., 1997).

Activation of the Fas receptor by FasL triggers a complex cascade of intracellular events that require Fas-associated death domain (FADD) adapter protein and the formation of death-inducing signaling complex (DISC), leading to caspase-8 activation and apoptosis (Lenardo et al., 1997; and Nagata, 1999) (as shown in figure 3). Although FasL binding to its receptor is required for such activation, Fas surface expression does not necessarily render cells susceptible to FasL-induced cell death, indicating that inhibitors of the apoptosis signaling pathway exist and play a role (Nagata, 1997; and Takahashi et al., 1994). FLICE-inhibitory protein (FLIP) is a key apoptosis regulatory protein of the death receptor-mediated pathway. FLIP inhibits apoptotic signaling by interfering with the binding of caspase-8 to FADD at the DISC (Irmler et al., 1997; and Tschopp et al., 1997). FLIP is involved in renderings cells resistant to death receptor-mediated apoptosis in various cell types (Irmler et al., 1997; Tschopp et al., 1997; Abedini et al., 2004; Korkolopoulou et al., 2004; Lee et al., 2003; and Tanaka et al., 2002) and elevated expression of FLIP is associated with tumor cells that can escape from immune surveillance *in vivo* (Medema et al., 1999). Furthermore, downregulation of FLIP by cytotoxic agents has been shown to sensitize cells to Fas-mediated apoptosis (Kinoshita et al., 2000). The FLIP proteins exist as two splice variants, $FLIP_L$ and $FLIP_S$, with distinct roles in death receptor. Both FLIPs are spontaneously degraded by ubiquitin-proteasome pathway, the common pathway for intracellular protein degradation (Poukkula et al., 2005).

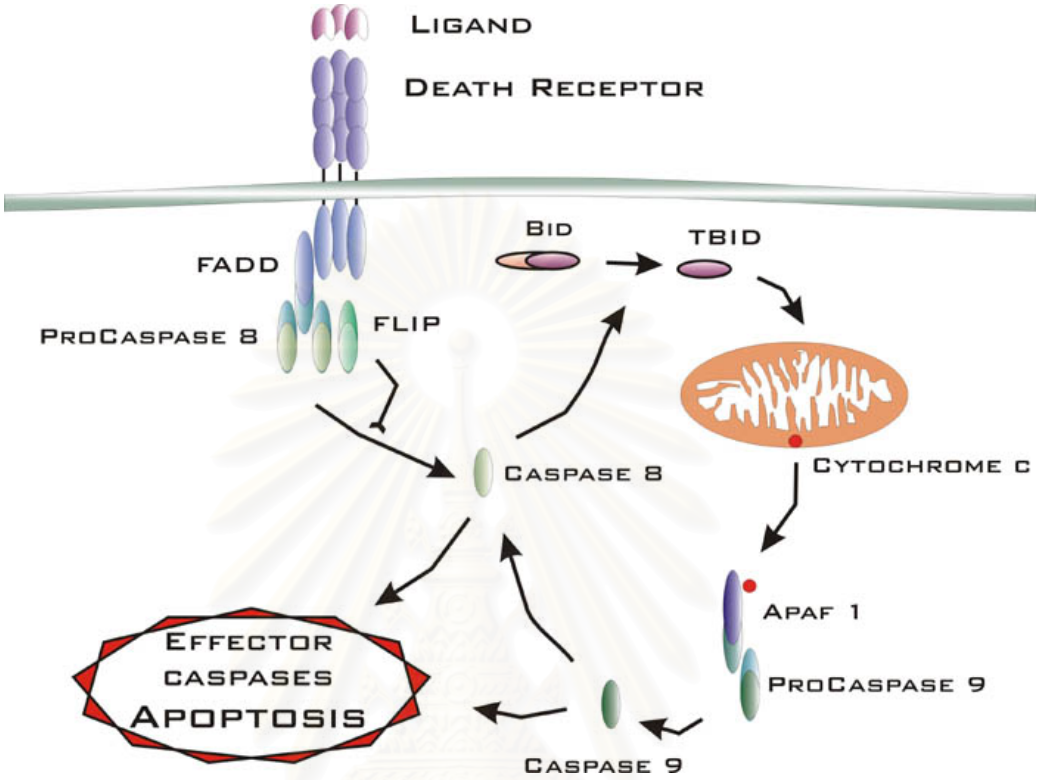

Figure 3 Diagram of Fas/FasL mediated apoptosis (Poukkula et al., 2005).

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2.1.2 Fas/FasL mediated apoptosis in lung

In the lungs, Fas receptor and Fas ligand expression is localized to the alveolar and airway epithelium, fibroblasts, and resident alveolar macrophages (Fine et al., 1997). Proximal lung epithelial cells and distal lung epithelial cells have been shown to express functional Fas receptor, and activation of Fas *in vivo* and *in vitro* can induce epithelial cell apoptosis (Fine et al., 1997; Xerri et al., 1997; Hamann et al., 1998; Hagimoto et al., 1999; and De Paepe et al., 2000). In addition, several studies indicate that the alveolar epithelium responds with apoptosis to Fas ligation, both *in vivo* and *in vitro* (Fine et al., 1997; Xerri et al., 1997; Hamann et al., 1998; Hagimoto et al., 1999; and De Paepe et al., 2000). In particular, proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interlukin (IL)-1β, and interferon gamma (IFN)-γ can modulate Fas-mediated apoptosis by regulating the expression of cell surface Fas receptor and intracellular apoptosis related proteins (Fine et al., 1997; and Xerri et al., 1997). However, the mechanisms that regulate apoptosis in lung cells and the factors that modulate the response of the lung cells to Fas activation remain unclear.

2.2 Mitochondrial pathway

The mitochondrial pathway is known as the intrinsic pathway. This pathway is mediated by the mitochondria, which integrates multiple stress signals, such as hypoxia, calcium, survival factor withdrawal and DNA damage from chemotherapeutic agents, UV-irradiation, and IR- irradiation (Kelekar et al., 1998; and Shimizu et al., 1999). In response to these stimuli, the death signaling cascades will be activated and begin the mitochondrial pathway (Kelekar et al., 1998; and Shimizu et al., 1999).

There are two groups of Bcl-2 family proteins which are pro-apoptotic proteins (the proteins that facilitate apoptosis by causing cytochrome c release) and anti-apoptotic proteins (the proteins that bind to the pro-apoptotic proteins and inhibit its functions), that play an important role in regulating mitochondrial apoptotic pathway. The Bcl-2 family of proteins regulates apoptosis by controlling mitochondrial permeability and the release of cytochrome c (Adrain and Martin, 2001).

The anti-apoptotic proteins Bcl-2 and Bcl-XL reside in the outer mitochondrial wall and inhibit cytochrome c release. The pro-apoptotic Bcl-2 proteins Bad, Bid, Bax and Bim reside in the cytosol but translocate to mitochondria following death signaling. Bax and Bim translocate to mitochondria in response to death stimuli, including survival factor withdrawal. p53, activated following DNA damage and then increase mitochondrial permeability causing cytochrome c release. Moreover, the death signaling induces the transcription of Bax. In addition, this pathway can be activated by caspase 8 from death receptor pathway. Cytosolic BID is cleaved by caspase 8 following signaling through the death receptor-signaling pathway: its active fragment (tBid) translocates to mitochondria and cause cytochrome c release (Kelekar et al., 1998; and Shimizu et al., 1999). Once cytochrome c is present in the cytosol, it drives the assembly of a high molecular weight caspase activating complex termed the apoptosome complex. This complex composes of released cytochrome c, Apoptosis Activation Factor 1 (Apaf-1), and procaspase-9. This complex cleaves off caspase-9 precursor's domain. This allows the molecule to change conformation, and bind to another cleaved caspase-9 precursor, forming a homodimer, the active caspase 9. Then caspase 9 will acts as the initiator for activating downstream caspases, caspase 3 and procaspase 9, resulting in cell apoptosis as show in figure 4. (Gross et al., 1999; Kelekar et al., 1998; and Shimizu et al., 1999)

2.2.1 Anti-apoptotic Bcl-2 protein

The expression of Bcl-2 is regulated by several mechanisms, including transcription, posttranslational modifications, dimerization, and degradation (Li et al., 2004; Raspollini et al., 2004; Yang et al., 2004; and Kausch et al., 2005). Increasing evidence suggest that Bcl-2 expression is mainly regulated at the post-transcriptional level by protein stability (Li et al., 2004; Dimmeler et al., 1999; Britschopf et al., 2000; Basu and Haldar, 2002). Numerous stimuli can induced the degradation of Bcl-2, including tumor necrosis factor (TNF)-α, ROS, lipopolysaccharide, β-amyloid, and ischemia (Li et al., 2004; Krajewski et al., 1995; Haendeler et al., 1996; Paradis et al., 1996; Dimmeler et al., 1999; and Breitschopf et al., 2000). Degradation of Bcl-2 is mainly mediated by the ubiquitin-dependent proteasome complex upon the covalent attachment of ubiquitin (Coux et al., 1996; and Hochstrasser et al., 1996). Bcl-2 stability

is mainly regulated by phosphorylation with the serine 87 (Ser87) phosphorylation playing a dominant role in this process (Dimmeler et al., 1999; and Breitschopf et al., 2000). Apoptotic stimuli such as TNF-α and ROS induce dephosphorylation of Ser87 which promotes proteasome degradation of Bcl-2 (Li et al., 2004; Dimmeler et al., 1999; and Breitschopf et al., 2000)

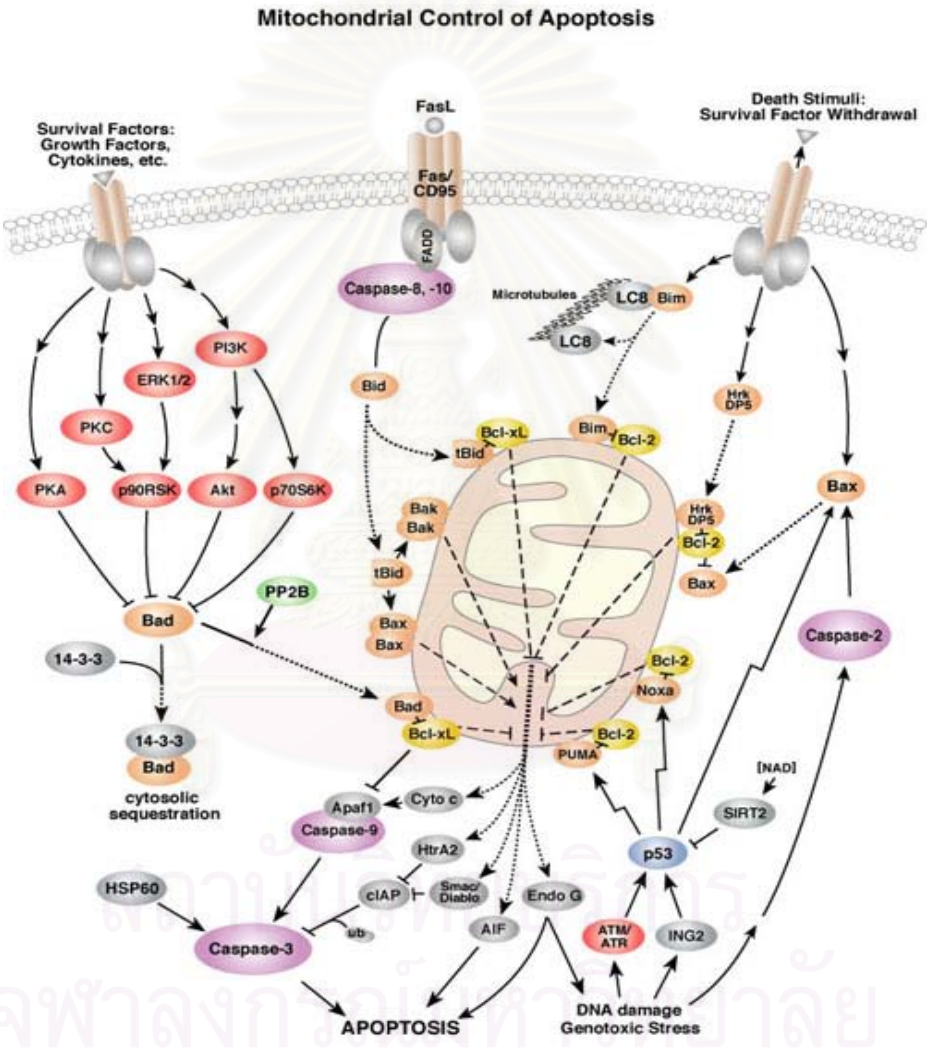


Figure 4 The mitochondrial apoptotic pathway (Kelekar et al., 1998; and Shimizu et al., 1999).

2.2.2 Cisplatin-induced apoptosis

Cisplatin is one of the most effective chemotherapeutic agents. It is generally used alone or in combination with other therapeutic agents for the treatment of solid tumors such as testicular, ovarian, bladder, cervical head and neck and small cell and non-small cell lung cancer (Loehrer and Einhorn, 1984; Wong and Giandomenico, 1999; Broch et al., 1987; and Cohen and Lippard, 2001). Cisplatin is known to induce cell apoptosis via mitochondrial pathway (Park et al., 2002; Lee et al, 2001; and Jiang et al., 1999).

The cytotoxic mode of action of cisplatin is suggested that the interaction of this compound with DNA forms DNA adducts, primarily intra-strand crosslink adducts, which activate several signal transduction pathways, including ATR, p53, p73, and MAPK, and culminate in the activation of cell apoptosis (Jamieson and Lippard, 1999). Although, the mechanism of cisplatin-mediated apoptosis is still controversial, most evidences indicated the involvement of mitochondrial apoptotic pathway. In addition, Bcl-2 the key regulator of mitochondrial apoptotic pathway, plays a crucial role in cisplatin-induced apoptosis and is a key determining factor of cisplatin resistance (Sartorius and Krammer, 2002; Sasaki et al., 2003; Li et al., 2004; Raspollini et al., 2004; Yang et al., 2004; and Kausch et al., 2005). Cisplatin-mediated cell death is frequently impaired and is a major limitation of cisplatin-based chemotherapy (Wong and Giandomenico, 1999; and Seve and Dumontet, 2005).

Multiple mechanisms of cisplatin resistance in tumor cells have been proposed, including impaired cellular uptake and increased cellular efflux of cisplatin (Katalou and Essigmann, 2001), increased DNA lesion repair (Dabholkar et al., 1994; and Koberle et al., 1999), and defects in mismatch repair that fail to trigger cell death (Aebi et al., 1996; Drummond et al., 1996; Brown et al., 1997; and Fink et al., 1996). Intracellular cisplatin inactivation by redox reactions has also been proposed as a mechanism of cisplatin resistance (Ishikawa et al., 1993; Eastman, 1987; and Rudin et al., 2003). Increased ROS generation by glutathione depletion or by superoxide dismutase antisense transfection has been shown to prevent Bcl-2-mediated cisplatin resistance (Li et al., 2004; and Rudin et al., 2003). Conversely, inhibition of ROS by antioxidants has

been shown to increase cisplatin resistance (Yang et al., 2000). Increasing evidence has also shown that NO plays a role in apoptosis regulation through its ability to modulate ROS, i.e., NO can interact with superoxide anion to form peroxynitrite (Heigold et al., 2002; and Borutaite and Brown, 2003), and to modify key apoptosis-regulatory proteins through *S*-nitrosylation (Li et al., 1997; and Kim et al., 1997).

3. S-nitrosylation

NO is a free radical gas that provides many biological regulation. One of the mechanisms by which NO regulates biological processes including apoptosis is S-nitrosylation of the proteins (Stamler et. al., 2001; Li et al., 1997; and Kim et al., 1997). Endogenous *S*- nitrosothiols (SNOs) are naturally occurring moieties on proteins in which a sulfur atom from cysteine or homocysteine reacts with nitric oxide to form an *S*- NO bond. Within mammalian tissues, the concentration of SNOs can vary from nM to µM levels (Gaston, 1999; Gaston et al., 1993; and Kluge et al., 1997), and thiol *S*-nitrosylation and NO transfer reactions (transnitrosation reactions) are involved in virtually all classes of cell signaling, ranging from regulation of ion channels and G-protein coupled reactions to receptor stimulation and activation of nuclear regulatory proteins. Furthermore, it is now apparent that the synthesis, transport, activation, and catabolism of SNOs are regulated.

S-nitrosylation, a posttranslational modification, involves the transfer of an NO^+ group to an active site thiol on cysteine amino acids, if the cysteine residues are in the reduced (free sulfhydryl) state. NO can combine directly with local electron-accepting species like oxygen, transition metal ions, or superoxide radicals to generate potent S-nitrosylating species, such as N2O3, NO+, and ONOO-, that engage in nitrosylation of sulfer-containing molecules. NO- in its singlet or high-energy state can also react with a thiol group (Lipton, 1994). Formation of such S-nitrosylated protein, or S-nitrosothiols is an important, posttranslational modification of protein structure that can modulate protein function. Example includes the activation or inhibition of several enzymes that are involves with numerous cellular processes including apoptosis. S-nitrosylation by NO has become an increasingly identified posttranslational modification of important intracellular proteins including Ras, ornithine decarboxylase, transglutaminase, c-Jun N-

terminal kinase/stress-activated protein kinase, and especially caspases (Teng, 1999; Bauer, 1999; Bernassola, 1999; and Park, 2000). S-nitrosylation of these and other proteins may regulate their activity and apoptotic potential.

4. Ubiquitin-proteasome degradation.

Proteasomes are large multi-subunit complexes, localized in the nucleus and cytosol that selectively degrade intracellular proteins. The ubiquitin-proteasome pathway plays a major role in the degradation of many proteins involved in cell cycle, proliferation, and apoptosis including Bcl-2 family proteins and FLIP. Proteasomes also breakdown abnormal proteins that result from oxidative stress and mutations that might otherwise disrupt normal cellular homeostasis. This pathway has been implicated in several forms of malignancy, in the pathogenesis of several genetic diseases, and in the pathology of muscle wasting. It is also involved in the destruction of proteins that participate in cell cycle progression, transcription control, signal transduction, and metabolic regulation. A protein marked for degradation is covalently attached to multiple molecules of ubiquitin (Ub), a highly conserved 76-amino acid (8.6 kDa) protein, which escorts it for rapid hydrolysis to the multi-component enzymatic complex known as the 26S proteasome. The proteolytic core of this complex, the 20S proteasome, contains multiple peptidase activities and functions as the catalytic machine. This core is composed of 28 subunits arranged in four heptameric, tightly stacked, rings (α7, β7, β7, α7) to form a cylindrical structure. The α-subunits make up the two outer and the β-subunits the two inner rings of the stack. The entrance of substrate proteins to the active site of the complex is guarded by the α-subunits that allow access only to unfolded and extended polypeptides. The proteolytic activity is confined to the β-subunits.

In ubiquitin-proteasome pathway, ubiquitin functions as a degradation marker for ATP-dependent proteolysis of cellular proteins by the 26S proteasome (Hershko et al., 2000). Degradation of a protein via the ubiquitin/proteasome pathway proceeds in two discrete and successive steps: (i) covalent attachment of multiple ubiquitin molecules to the protein substrate with help of ubiquitination enzymes and (ii) degradation of the multiubiquitinated target protein by the 26S proteasome complex, scattered in the cytosol, with the release of free and reusable ubiquitin .

The ubiquitination process is first activated by an E1 enzyme (also known as activating enzyme), forming a high-energy thiol ester bond between cysteine residue in its active site and glycine 76 of ubiquitin in the presence of ATP. The activated ubiquitin is then transferred to an E2 (conjugating enzyme), forming a similar thiol ester linkage between ubiquitin and an E2. In some cases, E2 directly transfers ubiquitin to the target proteins, but the reaction often requires the participation of an E3 (ligating enzyme, and thus referred as ubiquitin-protein ligase) (as shown in figure 5). Through a cascade of enzymatic reactions, ubiquitin is covalently attached through its C-terminal glycine residue to the ε-NH_2 group of the lysine residue on the target proteins. Finally, a polyubiquitin chain is formed by repeated reactions through which another ubiquitin links a lysine residue at position 48 within one ubiquitin associated with the target protein. Ubiquitin has seven lysine residues, which are all used for polymerization catalyzed by this ubiquitin-modifying system (Pickart, 2001; Peng, 2003), but a polyubiquitin chain formed via lysine at 48 functions mainly becomes a marker for proteolytic attack by the 26S proteasome (a eukaryotic ATP-dependent 2.5-MDa multisubunit complex) (Coux et al., 1996; Baumeister et al., 1998; and for review see Yang and Yu, 2003).

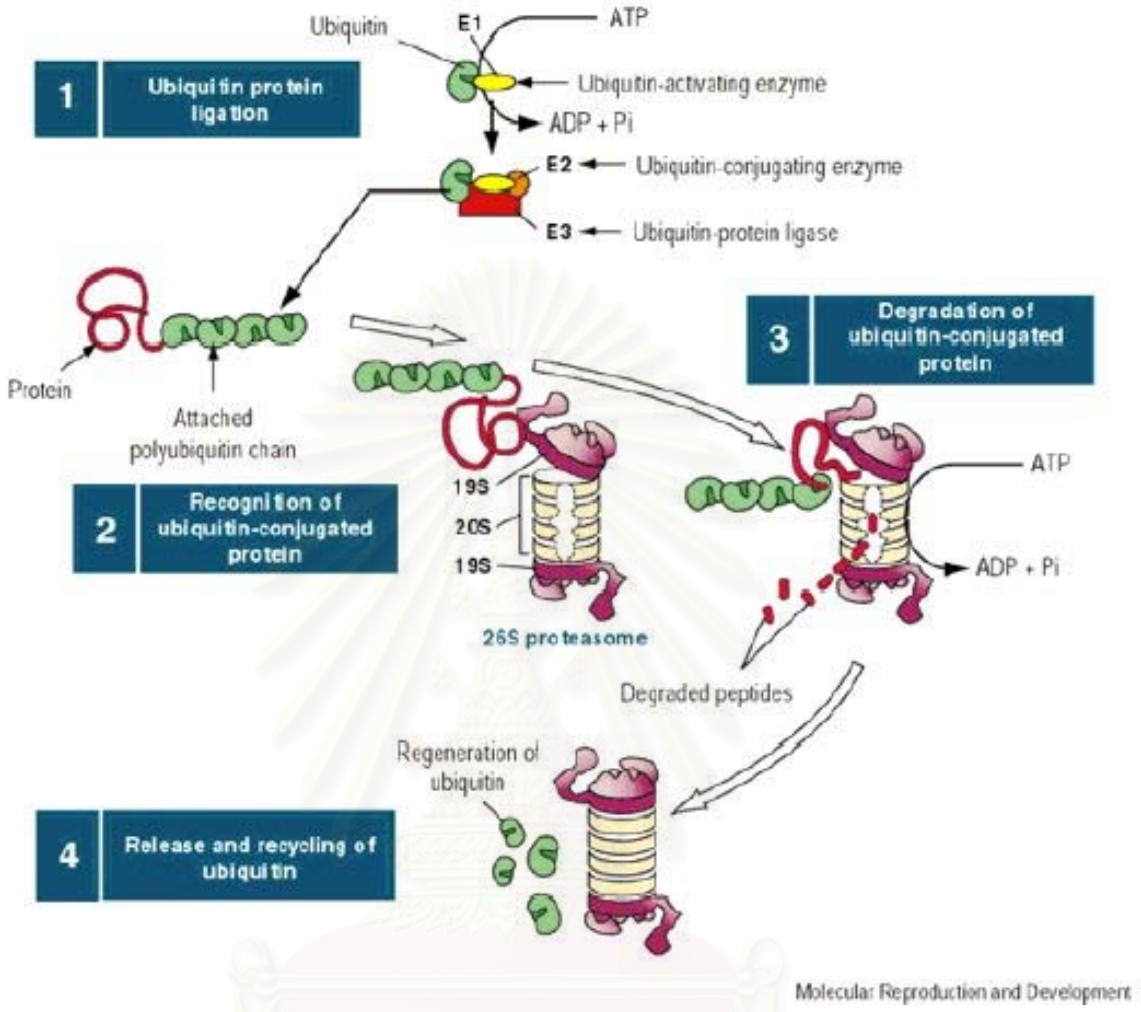


Figure 5 Diagram of ubiquitination (Yang and Yu, 2003).

CHAPTER III

MATERIALS AND METHODS

Materials

Sodium nitroprusside (SNP), aminoguanidine (AG), 2-(4-carboxy-phenyl)-4,4,5,5 tetramethylimidazoline-1-oxy-3-oxide (PTIO), N-acetylcysteine (NAC), dithiothreitol (DTT), benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (zVAD-fmk), and cisplatin were purchased from Sigma Chemical Inc. (St. Louis, MO). Dipropylenetriamine (DPTA) NONOate was obtained from Alexis Biochem. (San Diego, CA). Diaminofluorescein diacetate (DAF-DA), dichlorofluorescein diacetate (DCF-DA), and Hoechst 33342 were obtained from Molecular Probes Inc. (Eugene, OR). Recombinant FasL (SuperFasL), monoclonal antibody against FLIP (Dave-2) were purchased from Alexis Biochem. (San Diego, CA). Antibodies for Fas, FADD, Bcl-2, phospho-Bcl-2 (Ser87), myc, and peroxidase-labeled secondary antibodies to IgG and protein A-agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for ubiquitin, S-nitrosocysteine, and β-actin were purchased from Sigma (St. Louis, MO). The transfecting agent Lipofectamine was obtained from Invitrogen (Carlsbad, CA). The caspase-8 fluorometric substrate IETD-AMC was from Biovision Inc. (Mountain View, CA). The human bronchial epithelial cell line BEAS-2B (BEAS cells) and the NCI-H460 cells (H460 cells) were obtained from the American Type Culture Collection (Rockville, MD).

Methods

1. Cell culture

The BEAS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum, 2 mM L-glutamine, 20 mM 4-(2-hydroxy ethyl)-1-piperazine ethane-sulfonic acid (HEPES), 100 U/ml

penicillin, and 100 µg/ml streptomycin in a 5% CO_2 environment at 37ºC. The H460 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin in a 5% CO_2 environment at 37ºC.

2. Plasmid construction and transfection

The pcDNA3-$FLIP_L$, Bcl-2, and ubiquitin plasmids were generously provided by Dr. C. Stehlik (West Virginia University Cancer Center, Morgantown, WV). The open reading frame of $FLIP_L$, Bcl-2, and ubiquitin were amplified by high-fidelity PCR (Stratagene) from corresponding expressed sequence tags (ESTs) and cloned into pcDNA3 expression vectors containing N-terminal myc epitope tag. Myc-tagged $FLIP_{Cys/Ala}$ was generated using the QuickChange mutagenesis kit (Stratagene). Authenticity of all constructs was verified by DNA sequencing. Transient transfection was performed using Lipofectamine Plus (Invitrogen) according to the manufacture's instructions at 80-90% confluency. Thus, 8 µg of each plasmids and the plus reagent (10 µl) were mixed in medium (without serum) (85 µl) in a small, sterile tube. After immediate mixing with a Vortex mixer and standing at room temperature for 15 min, the Lipofectamine reagent (4 µl) in medium (without serum) (100 µl) was added, and the mixture was left at room temperature for 15 min. Then, 0.8 ml of medium was added to generate transfection complex. Serum containing medium was removed, then cells were wash with sterile PBS for three times. Then serum-free medium was added with the transfection complex (the total volume of which was 1 ml), and incubated for 5 h at 37°C. After incubation, the medium was change to serum containing medium. The amount of DNA was normalized in all transfection experiments with pcDNA3. Expression of proteins was verified by Western blotting or immunoprecipitation.

3. Generation of stable Bcl-2 transfectant

Stable transfectant of Bcl-2 was generated by culturing H460 cells in a 6-well plate until they reached 80-90% confluency. One microgram of cytomegalovirus-neo vector and 15 µl of Lipofectamine reagent with 2 µg of myc-tagged Bcl-2 plasmid were used to transfect the cells in each well in the absence of serum. After 10 h, the medium

was replaced with culture medium containing 5% fetal bovine serum. Approximately 36 h after the beginning of the transfection, cells were trypsinized and the cell suspensions were plated onto 75-ml culture flasks and cultured for 24-28 d with G418 selection (400 μg/ml). The pooled stable transfectant was identified by Western blot analysis of Bcl-2 and was cultured in G418-free RPMI medium for at least two passages before each experiment.

4. Caspase-8 activity and apoptosis assays

Caspase-8 activity was determined by fluorometric assay using the substrate IETD-AMC which is specifically cleaved by the enzyme at the Asp residue to release the fluorescent leaving group, amino-4-methyl coumarin (AMC). After treatment, treated cells were washed with ice-cold PBS three times. Cell extracts then were performed by incubating the cells in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a commercial protease inhibitor mixture (Roche) for 20 min on ice. After insoluble debris was pelletted by centrifugation at 14,000g for 15 min at 4^{o}C, the supernatants were collected and determined for protein content using the Bradford method (Bio-Rad). Cell extracts containing 50 μg protein were incubated with 100 mM HEPES, pH 7.4, containing 10% sucrose, 0.1% 3-[(3-cholamidiopropyl)-1] propane sulfonate (CHAPS), 10 mM dithiothreitol (DTT), and 50 μM of caspase substrate in a total reaction volume of 0.25 ml. The reaction mixture was incubated for 60 min at 37ºC. At the end of incubation, the liberated fluorescent group AMC was determined fluorometrically at the excitation and emission wavelengths of 380 nm and 460 nm, respectively.

Apoptosis was determined by Hoechst 33342 assay. Hoechst 33342 is a non-cytotoxic DNA dye that preferentially binds to triplet adenine and thymine base pairs in the minor groove outside of the double helix. Nuclear morphological changes of apoptotic cells using the fluorescent dye Hoechst 33342 were determined (McKeague et al., 2003). Cells were seeded onto 24-well plates and treated as described in experimental design. After treatment the cells were incubated with 10 μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR) for 30 min in dark. The cells were visualized and

photographed under UV microscope and scoring the percentage of cells having intensely condensed chromatin and/or fragmented nuclei using a pixera software (Leica, Germany). At least three replicate wells were analyzed for each treatment of five random fields in each well.

5. NO detection

The production of NO was assessed by flow cytometry using a NO-specific fluorescent probe, 4,5-diaminofluorescein diacetate (DAF-DA) (Molecular Probes, Eugene, OR), according to the manufacturer's instruction. After treatment, cells were washed three times with ice-cold phosphate-buffered saline (pH 7.4). The cells were then incubated with 10 μM DAF-DA for 30 min at 37^{o}C, after which they were thoroughly washed three times with PBS to remove the extracellular DAF-DA, trypsinized, resuspended in PBS (1x10^{6} cells/ml), and immediately analyzed by flow cytometry. A FACSort (Becton-Dickinson, Rutherford, NJ) flow cytometer, equipped with a 488-nm argon ion laser and supplied with the Cell Quest software, was applied to measure NO levels in the cells. Signals were obtained using a 538-nm bandpass filter. Each determination is based on mean fluorescence intensity of 5,000 cells. The relative amount of intracellular NO production was expressed as the fluorescence ratio of the treatment to control.

6. ROS detection

Intracellular ROS production was determined by flow cytometry using the oxidative probe dichlorofluorescein diacetate (DCF-DA) (Molecular Probes), according to the manufacturer's instructions and the procedure described previously (Pae et al., 2003). After treatment, cells were washed three times with ice-cold phosphate-buffered saline (PBS) (pH 7.4). The cells were then incubated with the probe (10 μM) for 30 min at 37ºC, after which they were washed three times with PBS, trypsinized, resuspended in PBS (1x10^{6} cells/ml), and immediately analyzed by flow cytometry using a 488 nm excitation beam and a 525 nm bandpass filter (FACSort, Becton-Dickinson, Rutherford, NJ) with CellQuest software (Becton-Dickinson).

7. Western blot analysis

After treatment, treated cells were washed with ice-cold PBS three times. Cell extracts then were performed by incubating the cells in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 1 mM phenylmetylsulfonyl fluoride (PMSF), and a commercial protease inhibitor mixture (Roche) for 20 min on ice. After insoluble debris was pelletted by centrifugation at 14,000g for 15 min at 4^{o}C, the supernatants were collected and determined for protein content using the Bradford method (Bio-Rad). Whole cells extracts were mixed with Laemmli loading buffer (225 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 9% 2-mercaptoethanol, and 0.009% bromphenol blue), boiled for 5 min. Equal amounts of proteins (40 μg) were resolved on a reducing 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for 1 h in 5% non-fat dry milk in TBST (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.01% Tween-20) and incubated with the appropriate primary antibodies at 4°C overnight. Membranes were washed three times with TBST for 10 min, incubated with horseradish peroxidase-coupled isotype-specific secondary antibodies for 1 h at room temperature. The immune complexes were detected by enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) and quantified using analyst/PC densitometry software (Bio-Rad). Mean densitometry data from independent experiments were normalized to result in cells in the control. The data were presented as the mean ± S.D. and analyzed by the Student's t test.

8. Immunoprecipitation

Cells are washed after treatments with ice-cold PBS and lysed in lysis buffer (containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 1 mM phenylmetylsulfonyl fluoride (PMSF), and a commercial protease inhibitor mixture (Roche)) at 4°C for 30 min. After centrifugation at 14,000 g for 15 min at 4^{o}C, the supernatants were collected and determined for protein content. Protein content was determined by the Bradford method (Bio-Rad). Cell lysates containing 60 μg proteins were incubated with 12 μl of anti-myc agarose bead (Santa Cruz Biotechnology)

diluted with 12 µl sepharose for 6 h at 4^{o}C. The immune complexes were then washed 3 times with 20 volumes of lysis buffer, resuspended in 2x Laemmli sample buffer and boiled at 95^{o}C for 5 min. Immunoprecipitates containing ~20 µg protein equivalent are separated by 10% SDS-PAGE and analyzed by Western blot as described.

9. Statistics

Values were expressed as means ± S.D. The reproducibility of the results was confirmed in at least three independent sets of experiments. Data shown in figures were from a representative set of experiments. All data represent at least three independent experiments and were expressed as the mean ± S.D. unless otherwise indicated. Differences between groups were compared using ANOVA for repeated measures and a post hoc Bonferroni test to correct for multiple comparisons. Values of $P < 0.05$ were regarded to be statistically significant.

10. Experimental design

The methods were designed as the followings. Apoptotic cell death was detected by fluorescence microscopic assay staining with Hoechst 33342. Intracellular NO oxide and ROS levels were determined by flow cytometry using DAF-DA and DCF-DA, respectively. Caspase-8 activity was determined by fluorometric assay using the substrate IETD-AMC and flow cytometry. Mechanistic study of the Bcl-2, Phosphorylated Bcl-2 (Ser87), FLIP, FADD, and Fas were determined by Western blot analysis. Mechanistic study of the ubiquitin-Bcl-2 complex, ubiquitin-FLIP complex, S-nitrosylated Bcl-2, and S-nitrosylated FLIP were investigated by immunoprecipitation and Western blot analysis.

10.1 Different NO levels in BEAS versus H460 cells under basal condition and apoptotic condition.

In an attempt to investigate the correlation between NO levels and susceptibility to apoptosis, the basal NO levels and NO levels in apoptotic condition were determined.

10.1.1 Different basal NO levels between BEAS and H460 cells.

To examine the role of NO in susceptibility to apoptosis in normal and cancer cells. This study first characterized basal NO level in BEAS versus H460 cells. The cellular NO level in BEAS and H460 cells were determined by flow cytometry using NO-specific probe DAF-DA. After seeding in 60-mm disc (at a concentration of 5×10^5 cells/ml), BEAS or H460 cells were cultivated in growth medium for further 24 h. Then the cells were collected and subjected to flow cytometer detected with DAF-DA fluorescence dye.

10.1.2 Different NO levels in apoptotic conditions.

NO level was determined in apoptotic conditions using two apoptosis inducing agents, which were cisplatin and FasL.

10.1.2.1 NO levels in cisplatin-induced apoptosis.

NO levels were determined in the function of time and dose. For time dependent study, subconfluent (90%) monolayers of BEAS and H460 cells in 60 mm-disc were treated with 100 μM cisplatin at various incubation times (0, 0.5, 1, 2, 3, 6, and 9h) in serum-free medium. For dose-dependent study, subconfluent (90%) monolayers of BEAS and H460 cells were treated with various concentrations of cisplatin (0, 50, 100, 250, and 500 μM) for 2 h in serum-free medium. Then the intracellular NO levels were determined using flow cytometer with DAF-DA as described.

10.1.2.2 NO levels in FasL-induced apoptosis.

For time-dependent study, subconfluent (90%) monolayers of BEAS and H460 cells in 60 mm-disc were treated with 100 ng/ml FasL for various incubation times (0.5, 1, 2, 3, 6, and 9 h). For dose-dependent study, the cells were treated at various doses of FasL (0, 50, 100, and 250 ng/ml) for 1 h. Then cellular levels of NO were determined using flow cytometer and DAF-DA as described in materials and methods.

10.2 Cisplatin- and FasL-induced apoptosis.

In order to investigate the role of NO on susceptibility to cisplatin- and FasL-induced apoptosis, this study characterized the apoptotic cell death responding to these stimuli.

10.2.1 Cisplatin-induced apoptosis.

For time-dependent study, subconfluent (90%) monolayers of BEAS or H460 cells were exposed to 100 μM of cisplatin for 0, 6, 9, 12, 16, and 24 h. Then the apoptotic cells were detected by Hoechst 33342 assay. For dose-dependent study, cells were treated with various concentrations of cisplatin (0, 50, 100, 250, 500 μM) for 16 h. Then, cell apoptosis was analyzed by Hoechst 33342 assay.

10.2.2 FasL-induced apoptosis.

For time-dependent study, Subconfluent (90%) monolayers of BEAS or H460 cells were exposed to 100 ng/ml of FasL for 0, 6, 9, 12, 16, and 24 h.

For dose-dependent study, cells were treated with various concentrations of FasL (0, 50, 100, and 250 ng/ml) for 16 h. Then, cell apoptosis was analyzed by Hoechst 33342 assay.

10.3 Effect of basal NO levels on apoptotic susceptibility.

NO inhibitors have been used by many studies, showing the effective effect on decreasing intracellular NO level (Sokolov et al., 2005). In an attempt to investigate the correlation between basal NO level and susceptibility to apoptosis, NO inhibitors were used to reduce basal NO level in BEAS and H460 cells

10.3.1 Effect of NO inhibitors on NO levels.

This study first characterized effect of NO inhibitors, which are iNOS inhibitor (Aminoguanidine, AG) and NO scavenger (c-PTIO), on intracellular NO level in BEAS and H460 cells.

Subconfluent (90%) monolayers of BEAS and H460 cells were treated with iNOS inhibitor (300 μM AG) or NO scavenger (300 μM c-PTIO) for 1 h. Then, cells were collected and cellular NO levels were determined.

10.3.2 Effect of NO inhibitors on cisplatin- and FasL-induced apoptosis.

In order to study the effect of basal NO levels on susceptibility to apoptosis in BEAS and H460 cells, the Hoechst 33342 nuclear staining was used to evaluate the apoptotic cells.

10.3.2.1 Effect of NO inhibitors on cisplatin-induced apoptosis.

Subconfluent (90%) monolayers of BEAS or H460 cells were pretreated with the NO inhibitors AG (300 μM) or PTIO (300 μM) for 1 h. The cells were then treated with cisplatin (100 μM) for 16 h and analyzed for apoptosis by Hoechst 33342 assay.

10.3.2.2 Effect of NO inhibitors on FasL-induced apoptosis.

This study further investigated the effect of NO inhibitors on FasL-induced apoptosis. Subconfluent (90%) monolayers of BEAS or H460 cells were pretreated with the NO inhibitors AG (300 μM) or PTIO (300 μM) for 1 h. The cells were then treated with FasL (100 ng/ml) for 16 h and analyzed for apoptosis by Hoechst assay.

10.4 Effect of elevated NO levels on apoptotic susceptibility.

In order to investigate the effect of elevated NO levels on apoptotic susceptibility, NO donors SNP and DPTA NONOate were used to increase the intracellular NO level.

10.4.1 Effect of NO donors on NO levels.

Subconfluent (90%) monolayers of BEAS and H460 cells were pretreated with SNP (300 μg/ml) or DPTA NONOate (200 μM) for 1 h and then treated

with 100 μM cisplatin or 100 ng/ml FasL for 1 h. NO level was determined by flow cytometer with DAF-DA.

10.4.2 Effect of NO donors on cisplatin- and FasL-induced apoptosis.

In order to study the effect of elevated NO levels on susceptibility to apoptosis in BEAS and H460 cells, the Hoechst 33342 nuclear staining was used to evaluate the apoptotic cells.

10.4.2.1 Effect of NO donors on cisplatin-induced apoptosis.

Subconfluent (90%) monolayers of BEAS and H460 cells were pretreated with SNP (300 μg/ml) or DPTA NONOate (200 μM) for 1 h. Then treated with 100 μM cisplatin for further 16 h. Apoptotic cells were analyzed by Hoechst 33342 assay.

10.4.2.2 Effect of NO donors on FasL-induced apoptosis.

Subconfluent (90%) monolayers of BEAS and H460 cells were pretreated with SNP (300 μg/ml) or DPTA NONOate (200 μM) for 1 h. Then the cells were treated with 100 ng/ml FasL for further 16 h. Apoptotic cells were analyzed by Hoechst 33342 assay.

10.5 Effect of varying NO levels on susceptibility to apoptosis.

This study investigated whether in this cell system, dose of NO played a role in controlling susceptibility to apoptosis.

10.5.1 Effect of various NO donor concentrations on NO levels.

The intracellular NO levels in response to various concentrations of NO donor SNP were investigate by flow cytometer with DAF-DA fluorescence dye. Subconfluent (90%) monolayers of BEAS or H460 cells were pretreated with various doses of SNP (0, 50, 100, 300, and 500 μg/ml) for 1 h. The cells were then treated with

cisplatin (100 μM) or FasL (100 ng/ml) for further 1 h and analyzed for NO levels by flow cytometry with DAF-DA as described.

10.5.2 Effect of elevated NO levels on susceptibility to apoptosis.

10.5.2.1 Effect of elevated NO levels on cisplatin-induced apoptosis.

Cells were pretreated with different concentration of SNP (0, 50, 100, 300 and 500 μg/ml). Then cells were exposed to cisplatin (100 μM) for 16 h. Apoptotic cells were analyzed by Hoechst 33342 nuclear staining method as described.

10.5.2.2 Effect of elevated NO levels on FasL-induced apoptosis.

Cells were pretreated with different concentration of SNP (0, 50, 100, 300 and 500 μg/ml). Then cells were exposed to FasL (100 ng/ml) for 16 h. Apoptotic cells were analyzed by Hoechst 33342 nuclear staining method as described.

10.6 The mechanisms of NO in controlling cisplatin- and FasL- induced apoptosis.

This study investigated the mechanisms of NO in cisplatin-induced apoptosis in H460 cells, and FasL-induced apoptosis in BEAS cells.

10.6.1 The mechanism of NO in controlling cisplatin-induced H460 cells apoptosis.

Since, NO is considered as a key regulator for apoptosis, but role in cisplatin-induced cell death and the underlying mechanism are largely unknown. To study the role of NO in cisplatin-induced apoptosis and investigate the mechanism of cisplatin-resistance, this study used the cancer H460 cells as a model.

10.6.1.1 Cisplatin-induced H460 cells apoptosis.

This study first characterized cell death response to cisplatin treatment. Subconfluent (90%) monolayers of H460 cells were treated with various

concentrations of cisplatin (0, 50, 100, 250, and 500 μM) and apoptosis was determined after 12 h by Hoechst 33342 assay.

10.6.1.2 Effects of NO modulators and antioxidant on cisplatin-induced H460 cells apoptosis.

To provide a relationship between the apoptotic response and NO or ROS modulation, the effect of NO inhibitors, NO donors, and antioxidant on cisplatin-induced apoptosis were examined. The subconfluent (90%) monolayers of H460 cells were pretreated with NO inhibitors (300 μM AG or 300 μM PTIO), NO donors (500 μg/ml SNP or 200 μM DPTA), or antioxidant (1mM NAC) for 1 h. Then the cells were exposed to 100 μM cisplatin for further 12 h. Apoptotic cells were detected by Hoechst 33342 assay.

Also, this study treated the cells with NO modulators or NAC alone and investigated their effects on apoptosis. The H460 cells were pretreated with NO inhibitors (300 μM AG or 300 μM PTIO), NO donors (500 μg/ml SNP or 200 μM DPTA), or antioxidant (1mM NAC) for 12 h. Then cell apoptosis was determined by Hoechst 33342 assay.

10.6.1.3 Effects of NO modulators and antioxidant on NO and ROS levels in H460 cells

To provide a relationship between the apoptotic response and NO or ROS modulation by the test agents, this study pretreated the cells with NO inhibitors 300 μM AG, NO donors 500 μg/ml SNP, or antioxidant (1mM NAC) for 1 h. Then the cells were exposed to 100 μM cisplatin for further 1 h. Then intracellular NO and ROS levels were determined by flow cytometry using the specific NO fluorescent probe DAF-DA and specific ROS probe DCF-DA as described.

10.6.1.4 Effect of Bcl-2 overexpression on cell death resistance to cisplatin in H460 cells

To test whether Bcl-2 is involved in apoptosis resistance to cisplatin in H460 cells, this study stably transfected the cells with Bcl-2 or control plasmid (mock transfection) as described and their effect on cisplatin-induced apoptosis was determined by Hoechst 33342 assay. The transfected cells were treated with increasing dose of cisplatin (0, 50, 100, 250, and 500 μM) for 12 h and apoptosis was determined by Hoechst 33342 as described.

To confirm whether transfection was sufficient, the mock transfected cells and Bcl-2 transfected cells were lyzed and the cell lysates were prepared and subjected to Western blot analysis probing with anti-Bcl-2 and anti-myc antibodies. Also, the blot was reprobed with anti-β-actin antibody to confirm the equal amount of loaded proteins.

10.6.1.5 Effects of NO modulators and proteasome inhibitors on Bcl-2 expression in H-460 cells

To demonstrate the potential regulation of Bcl-2 expression by NO in H-460 cells, Bcl-2 protein was determined by Western blotting using antibody specific for Bcl-2. Cells were pretreated with 300 μM AG, 300 μM PTIO, 300 μg/ml SNP, or 200 μM DPTA NONOate for 1 h. Then cells were treated with 200 μM cisplatin, and Bcl-2 expression was determined by immunoblotting after 12 h.

To test whether ubiquitin-proteasome degradation and ROS might be involved in cisplatin-induced Bcl-2 degradation in H-460 cells, this study pretreated the cells with 20 μM lactacystin, a highly specific proteasome inhibitor, or antioxidant 1mM antioxidant NAC for 1 h. Then the cells were incubated with 200 μM cisplatin for further 12 h. The effect on cisplatin-induced Bcl-2 downregulation was examined by immunoblotting detecting with Bcl-2 antibody. To test whether enzymatic cleavage by caspase-3 was involved in cisplatin induced Bcl-2 degradation in H460 cells, the cells were co-treated with caspase inhibitor, zVAD-fmk (10μM), and cisplatin (200μM) for 12 h. Then Bcl-2 protein expression was determined by Western blot

analysis. Also, the blots were reprobed with β-actin antibody for confirm amount of loaded proteins and density normalization.

10.6.1.6 Effects of NO modulators and NAC on Bcl-2 phosphorylation at Ser87

Dephosphorylation at Ser87 is required for ROS induced Bcl-2 degradation (Dimmeler et al., 1999; and Breitschopf et al., 2000). To investigate the potential mechanism of NO regulation of Bcl-2, this study tested the effect of NO modulators and antioxidant on Bcl-2 phosphorylation (Ser87) in cisplatin-treated cells. The H460 cells were pretreated with 300 μM AG, 300 μM PTIO, 300 μg/ml SNP, 200 μM DPTA NONOate, or 1mM NAC for 1 h. Then cells were treated with 200 μM cisplatin, and Phosphorylated Bcl-2 (Ser87) protein was determined by immunoblotting after 12 h using anti-phospho-Bcl-2 (Ser87) antibody. Also, the blot was reprobed with β-actin antibody for confirm amount of loaded proteins and density normalization.

10.6.1.7 Effect of NO modulators on cisplatin-induced ubiquitination of Bcl-2.

To further investigate the mechanism by which NO protects cisplatin-induced Bcl-2 degradation, this study analyzed ubiquitination of Bcl-2 in response to cisplatin treatment by immunoprecipitation. Cells were co-transfected with myc-tagged Bcl-2 and ubiquitin expression plasmids as described, and 36 hours later they were pretreated with 300 μM AG, 300 μM PTIO, 300 μg/ml SNP, or 200 μM DPTA NONOate for 1 h. Then cells were treated with 100, 200, and 500 μM cisplatin in the present of 10 μM lactacystin to prevent proteasomal degradation of Bcl-2. After 2 h, cell lysates were prepared and immunoprecipitated using an anti-myc antibody as described. The resulting immune complexes were then analyzed for ubiquitin by Western blot using an anti-ubiquitin antibody.

10.6.1.8 Effect of NO modulators on Bcl-2 S-nitrosylation and ubiquitination.

Increasing evidence indicates that NO plays an important role in apoptosis through S-nitrosylation of key apoptosis regulatory proteins (Li et al., 1997; and Kim et al., 1997). To determine whether NO could nitrosylate Bcl-2, which has not been demonstrated, and whether this process could affect Bcl-2 stability, this study performed immunoprecipitation experiments evaluating the effect NO on Bcl-2 S-nitrosylation.

Cells were co-transfected with myc-tagged Bcl-2 and ubiquitin expression plasmids as described, and 36 hours later they were pretreated with 300 μM AG, 300 μg/ml SNP, or 200 μM DPTA NONOate for 1 h. Then cells were treated with 200 μM cisplatin for 2 h. Cell lysates were immunoprecipitated with anti-myc antibody and analyzed by Western blot using an anti-S-nitrosocysteine antibody. Also, the same cell lysates were analyzed by Western blot analysis (in parallel with immunoprecipitation). The blots were detected with anti-Bcl-2 antibody to confirm the equal amount of loaded proteins to the immunoprecipitation process.

In order to confirm the NO induced Bcl-2 S-nitrosylation, the effect of dithiothreitol (DTT), the S-nitrosylation inhibitor, on NO induced Bcl-2 S-nitrosylation was investigated. Cells were pretreated with 300 μg/ml SNP or 200 μM DPTA NONOate in the presence or absence of DTT (10 mM) for 1 h. Then cells were treated with 200 μM cisplatin for 2 h. Cell lysates were immunoprecipitated with anti-myc antibody and analyzed by Western blot using an anti-S-nitrosocysteine antibody.

To investigate the effect of NO modulators on Bcl-2 ubiquitination, the same procedures as described above were preformed. After immunoprecipitation, the lysates were analyzed by Western blot analysis using anti-ubiquitin antibody for ubiquitin investigation.

10.6.2 The mechanism of NO in controlling FasL-induced BEAS cells apoptosis.

To study the role of NO in FasL-induced apoptosis and investigate the underlying mechanism, the BEAS cells were used as a model.

10.6.2.1 FasL-induced BEAS cells apoptosis.

To study the role of NO in Fas-mediated apoptosis, this study first characterized cell death response to FasL treatment in lung epithelial BEAS cells using Hoechst 33342 nuclear staining assay. Subconfluent (90%) monolayers of BEAS cells were treated with various doses of FasL (0, 50, 100, and 250 ng/ml) for 16 h and analyzed for apoptosis by Hoechst 33342 assay.

10.6.2.2 FasL-induced caspase-8 activation in BEAS cells.

FasL induces cell apoptosis through caspase-8 activation; this study investigated the effect of FasL treatment on caspase-8 activity in BEAS cells. The BEAS cells were treated with various concentrations of FasL (0, 50, 100, and 250 ng/ml) for 3 h, and cell lysates were prepared as described in material and method. Cell lysates (50 μg protein) were prepared and determined for caspase-8 activity using the fluorometric substrate IETD-AMC.

10.6.2.3 Effect of NO modulators on NO levels, FasL-induced apoptosis and caspase-8 activation in BEAS cells.

Subconfluent (90%) monolayers of BEAS cells were pretreated with the NO donor SNP (300 μg/ml) or DPTA NONOate (200 μM), or with the NO inhibitor AG (300 μM) or PTIO (300 μM) for 1 h. The cells were then treated with FasL (100 ng/ml) for 16 h and analyzed for apoptosis by Hoechst 33342 assay.

For caspase-8 activity investigation, Subconfluent (90%) monolayers of BEAS cells were pretreated with the NO donor SNP (300 μg/ml) or DPTA NONOate (200 μM), or with the NO inhibitor AG (300 μM) or PTIO (300 μM) for 1 h.

The cells were then treated with FasL (100 ng/ml) for 3 h. Then cell lysates (50 μg protein) were prepared and determined for caspase-8 activity using the fluorometric substrate IETD-AMC as described.

For intracellular NO level determination, the cells were pretreated with the NO donor SNP (300 μg/ml) or DPTA NONOate (200 μM), or with the NO inhibitor AG (300 μM) or PTIO (300 μM) for 1 h. Then the cells were treated with 100 ng/ml FasL for 1 h, and intracellular NO was determine by flow cytometer with DAF-DA.

Also, this study investigated the effect of NO modulators alone on cell apoptosis and caspase-8 activity. The cells were pretreated with the NO donor SNP (300 μg/ml) or DPTA NONOate (200 μM), or with the NO inhibitor AG (300 μM) or PTIO (300 μM). Caspase-8 activity was determined at 3 h after treatment, and cell apoptosis was detected at 16 h.

10.6.2.4 Effects of FasL and proteasome inhibitors on Fas, FADD, and FLIP expression.

This study examined by immunoblotting, the expression levels of key proteins known to be relevant to the mechanisms of Fas signaling, including the Fas death receptor, the adapter protein FADD, and the anti-apoptotic FLIP in response to FasL treatment. Cells were treated with different concentration of FasL (0, 50, 100 ng/ml) in the presence or absence of 10 μM lactacystin for 12 h. The cells were then washed with ice-cold PBS and extracted with SDS sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with antibodies specific for Fas, FADD, and FLIP. Also, the blot was reprobed with β-actin antibody for confirm amount of loaded proteins and density normalization.

This study investigated whether the downregulation of FLIP by FasL is mediated by the ubiquitin-proteasome pathway. Cells were treated with FasL (100 ng/ml) in the present and absent of lactacystin (10 μM) and MG132 (5 μM), highly specific proteasome inhibitors, for 12h. Then cell lysates were prepared and subjected to

Western blot analysis detecting by anti-FLIP antibody. Also, the blot was reprobed with anti-β-actin antibody to confirm equal loaded proteins.

10.6.2.5 Effect of NO modulators on FasL-induced FLIP downregulation.

The effect of NO donors or inhibitors on FLIP expression levels was determined by immunoblotting. Subconfluent (90%) monolayers of BEAS cells were pretreated with the NO donor SNP (300 μg/ml) or DPTA NONOate (200 μM), or with the NO inhibitor AG (300 μM) or PTIO (300 μM), for 1 h, after which they were treated with FasL (100 ng/ml) for 12 h and analyzed for FLIP by Western blot using FLIP antibody. Blots were reprobed with β-actin antibody to confirm equal loading of samples.

10.6.2.6 Effect of NO modulators on FLIP ubiquitination.

Due to the proteasome acts on proteins destined to be degraded by ubiquitination, this study investigated whether FLIP is inducibly ubiquitinated by FasL. Cells were transiently co-transfected with myc-tagged FLIP and ubiquitin plasmids as described. After 24 h, the transfected cells were then treated with FasL (100 ng/ml) for 0, 1, 2, and 3 h in the presence or absence of SNP (300 μg/ml), DPTA NONOate (200 μM), AG (300 μM), or PTIO (300 μM). Lysates were prepared and immunoprecipitated by incubation with 12 μl of anti-myc agarose bead diluted with 12 μl sepharose for 6 h at 4^{o}C. The beads were washed, boiled, and subjected to 10% polyacrylamide gel electrophoresis. The separated proteins were analyzed by Western blot with antibody against ubiquitin. Also, the same lysates were subjected to Western blot analysis detecting by anti-myc antibody to confirm the equal loading of proteins to immunoprecipitation.

10.6.2.7 Effect of NO modulators on FLIP S-nitrosylation.

To determine whether NO could nitrosylate FLIP which has not previously been demonstrated, the immunoprecipitation experiments were performed to evaluating the effect of NO on S-nitrosylation of FLIP. Cells were transiently

transfected with myc-tagged FLIP plasmid as described. Cells expressing ectopic myc-FLIP were pretreated with SNP (300 μg/ml), DPTA NONOate (200 μM), AG (300 μM), or PTIO (300 μM) for 1 h. Then cells were treated with FasL (100 ng/ml) for further 2 h. Lysates were immunoprecipitated using anti-myc antibody and analyzed by Western blot using S-nitrosocysteine antibody.

10.6.2.8 Effect of NO on S-nitrosylation of FLIP deletion series.

To study the mechanism of S-nitrosylation of FLIP, this study first determined which domain(s) of FLIP is responsible for its nitrosylation. The series of FLIP deletion plasmids (Δ1–Δ3, as shown in figure 47) were constructed by Dr. C. Stehlik (West Virginia University Cancer center, Morgantown, WV, USA).

Each group of cells was transiently transfected with each of the FLIP deletion series (Δ1–Δ3). After 24 h, the transfected cells were pretreated with SNP (300 μg/ml) for 1 h. Then cells were treated with FasL (100 ng/ml) for further 2 h. Lysates were immunoprecipitated using anti-myc antibody and analyzed by Western blot using S-nitrosocysteine antibody.

Also, the effect on FLIP ubiquitination was investigated by immunoprecipitation and Western blot analysis. The cells were transiently co-transfected with the FLIP deletion series (Δ1–Δ3) and ubiquitin plasmids. After 24 h, the transfected cells were pretreated with SNP (300 μg/ml) for 1 h. Then cells were treated with FasL (100 ng/ml) for further 2 h. Lysates were immunoprecipitated using anti-myc antibody and analyzed by Western blot using ubiquitin antibody.

10.6.2.9 Effect of cysteines 254 and 259 mutations on FLIP S-nitrosylation and FLIP ubiquitination.

Since, the results suggested that the S-nitrosylation site was in the amino acid sequence difference between Δ1 and Δ2 (or the amino acid sequence 233-328 of the caspase-like domain), this study thus investigated the mutation of two cysteine (Cys 254 and Cys 259) which locates in that region. Also, the mutated FLIP plasmid (mutated the Cys 254 and 259 to alanine, Δ4) was constructed by Dr. C. Stehlik and its

effect on FLIP S-nitrosylation and FLIP ubiquitination were investigated. The cells were transiently transfected with the mutated FLIP (Δ4) plasmid. 24 h later, the transfected cells were pretreated with SNP (300 μg/ml) for 1 h. Then cells were treated with FasL (100 ng/ml) for further 2 h. Lysates were immunoprecipitated using anti-myc antibody and analyzed by Western blot using S-nitrosocysteine antibody. Also, the effect of cysteines 254 and 259 mutation on FLIP ubiquitination was investigated by immunoprecipitation and Western blot analysis. The cells were transiently co-transfected with the mutated FLIP (Δ4) and ubiquitin plasmids. After 24 h, the transfected cells were pretreated with SNP (300 μg/ml) for 1 h. Then cells were treated with FasL (100 ng/ml) for further 2 h. Lysates were immunoprecipitated using anti-myc antibody and analyzed by Western blot using ubiquitin antibody.

10.6.2.10 Effect of cysteines 254 and 259 mutations on FasL-induced FLIP degradation.

The cells were transiently transfected with the mutated FLIP (Δ4) or myc-FLIP (wild-type) plasmids. After 24 h, the transfected cells were pretreated with SNP (300 μg/ml) for 1 h. Then cells were treated with FasL (100 ng/ml) for further 12 h. Lysates were prepared and analyzed by Western blot using anti-FLIP antibody.

CHAPTER IV

RESULTS

1. Different NO levels in BEAS versus H460 cells under basal condition and apoptotic condition.

1.1 Different basal NO levels between BEAS and H460 cells.

To examine the role of NO in susceptibility to apoptosis in normal and cancer cells. This study first characterized basal NO levels in BEAS versus H460 cells. The fluorometric results clearly showed that the basal NO level in H460 cells was approximately 2.5 folds (2.62 ± 0.13) higher than basal NO level in BEAS cells (as shown in figure 6).

1.2 Different NO levels in apoptotic conditions.

In apoptotic condition, it is likely that NO level might change. NO levels were determined in apoptotic conditions using two apoptosis inducing agents, which are cisplatin and FasL.

1.2.1 NO levels in cisplatin-induced apoptosis.

To investigate the involvement of NO in regulating mitochondrial apoptotic pathway, cisplatin was used as an apoptosis stimulating agent. The alteration of NO level in response to cisplatin treatment was characterized.

Time-dependent study clearly showed that in BEAS cells intracellular NO level was decreased as early as 0.5 h after 100 μM cisplatin treatment (0.45 ± 0.08 fold) (figure 7A), whereas the significant decrease of NO level in H460 cells was observed at 1h after 100 μM cisplatin treatment (0.76 ± 0.01 fold) (figure 7B). The effect of cisplatin on NO depletion lasted until 3 h (0.43 ± 0.02 fold) in BEAS cells and until 6 h (0.76 ± 0.03 fold) in H460 cells. The decreased NO levels were reversed to basal level at 6 h and 9 h in BEAS and H460 cells, respectively.

For dose-dependent study, intracellular NO levels were decreased in response to cisplatin treatment in both cells. In BEAS cells, NO level was decreased in dose-dependent manner from 0.65 ± 0.11 to 0.27 ± 0.09 fold in response to cisplatin treatment (50 – 500 μM) (figure 8A). In H460 cells, NO level was decreased from 0.67 ± 0.03 to 0.22 ± 0.02 fold in response to cisplatin treatment (50 - 250 μM), and for higher dose (500 μM) there was no further NO reduction (0.24 ± 0.04) (figure 8B).

1.2.2 NO levels in FasL-induced apoptosis.

To investigate the involvement of NO in regulating death receptor pathway, FasL was used as an apoptosis stimulating agent. The alteration of NO level in response to FasL treatment was characterized.

The NO levels were determined in the functions of time and dose. In both BEAS and H460 cells, NO levels were increased in time-dependent manner in response to FasL treatment (as shown in figure 9 and 10).

In BEAS cells, intracellular NO level was increased as fast as 0.5 h in response to FasL (100 ng/ml) treatment (1.60 ± 0.16 folds, figure 9A) and peaked at 1h. NO level was increased approximately 2 folds (2.06 ± 0.11) comparing to basal level at the peak time and it was decreased back to the basal level at 6 h (1.06 ± 0.13 folds) as shown in figure 9A.

In H460 cells, intracellular NO level was significantly increased at 1 h in response to FasL (100 ng/ml) treatment (2.08 ± 0.10 folds, figure 9B). NO level also peaked at 1 h and it was approximately 2 folds (2.08 ± 0.10 folds) comparing to basal level, the NO level was reversed to the basal level at 9 h (figure 9B).

Dose-dependent study revealed that NO levels were increased in dose-dependency in response to FasL treatment in both cells. In BEAS cells, NO level was increased from 1.55 ± 0.08 to 3.42 ± 0.21 folds in response to FasL concentrations of 50 – 250 ng/ml in dose-dependent manner (figure 10A).

In H460 cells, NO level was increased from 1.58 ± 0.08 to 1.94 ± 0.05 folds in response to FasL concentrations of 50 - 100 ng/ml, and for higher dose of FasL (250 ng/ml) there was no more significantly induction (1.96 ± 0.07) (figure 10B).

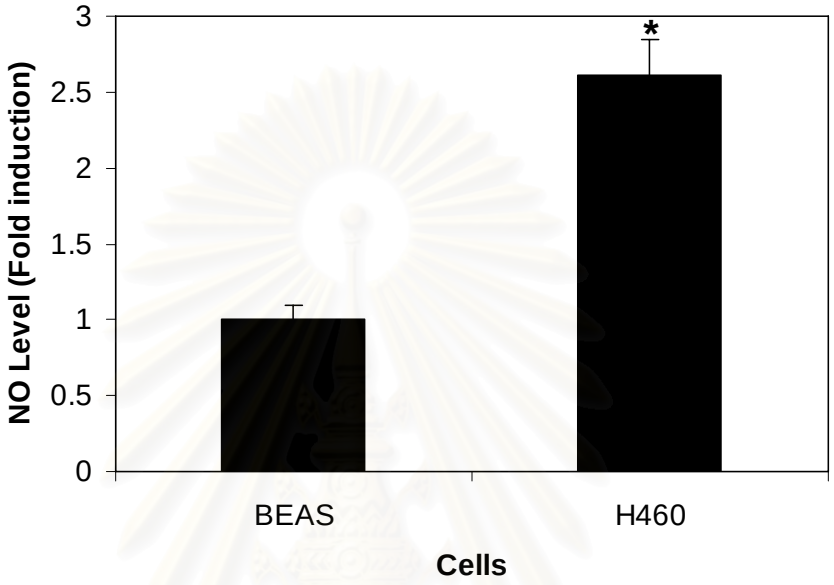
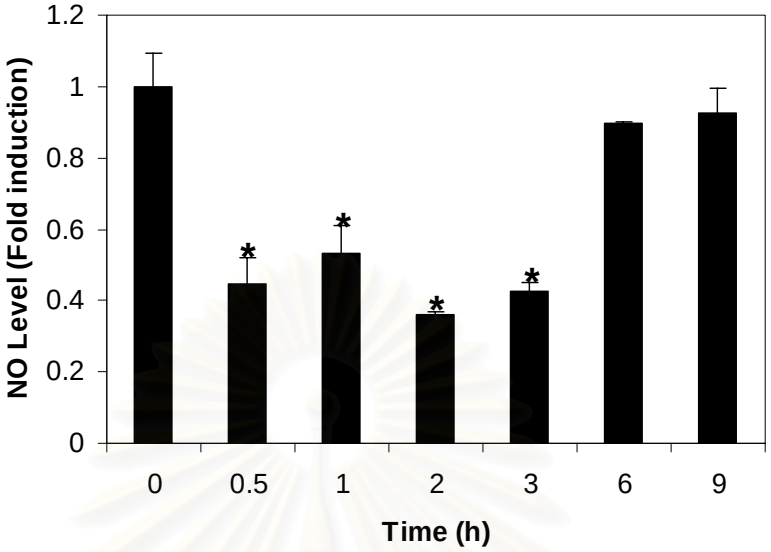


Figure 6 Basal NO levels in BEAS versus H460 cells. The basal NO levels were detected by flow cytometry with DAF-DA. Quantification of the relative fluorescence intensity from six independent experiments was performed, and results were expressed as the mean ± S.D. * $P < 0.05$ compared to the basal NO level of BEAS cells.

(A)



(B)

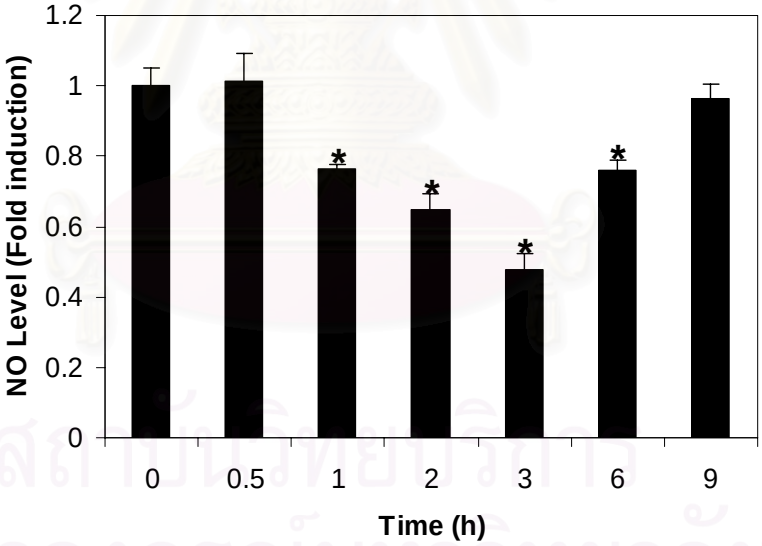
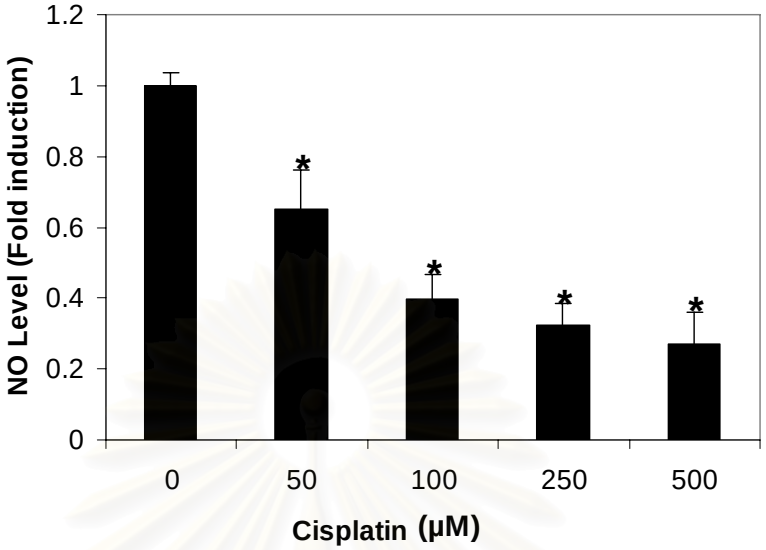


Figure 7 Effect of cisplatin on NO levels in BEAS (A), and H460 (B) as a function of time. Cells were treated with 100 μM of cisplatin at various incubation times (0, 0.5, 1, 2, 3, 6, and 9 h) and detected by flow cytometry with DAF-DA. Values are relative DAF fluorescence change over control level. Plots are mean ± S.D. (n=4). * $P < 0.05$ versus non-treated control.

(A)



(B)

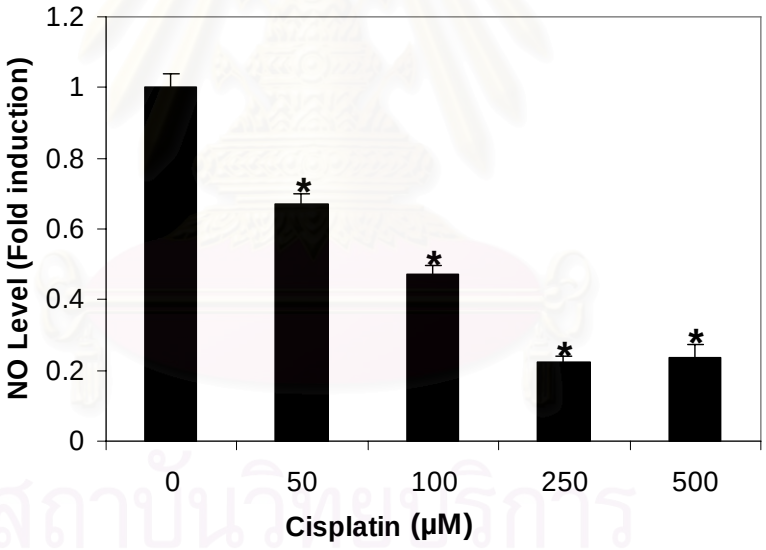
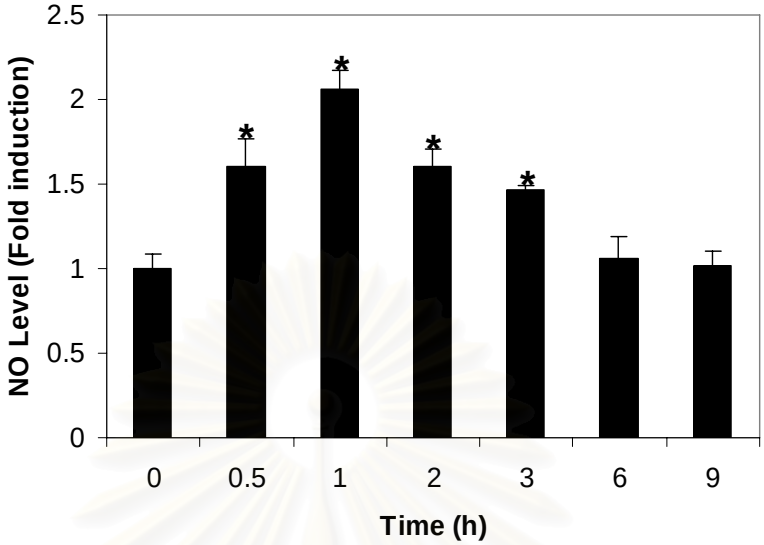


Figure 8 Effect of cisplatin on NO levels in BEAS (A), and H460 (B) as a function of dose. Cells were treated with various doses of cisplatin (0, 50, 100, 250, and 500 μM) for 2 h and detected by flow cytometry with DAF-DA. Values are relative DAF fluorescence change over control level. Plots are mean ± S.D. (n=5). * $P < 0.05$ versus non-treated control.

(A)



(B)

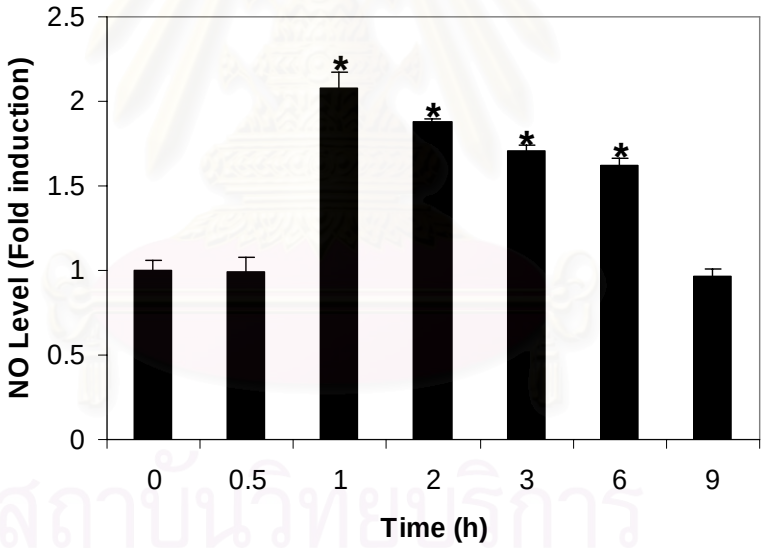
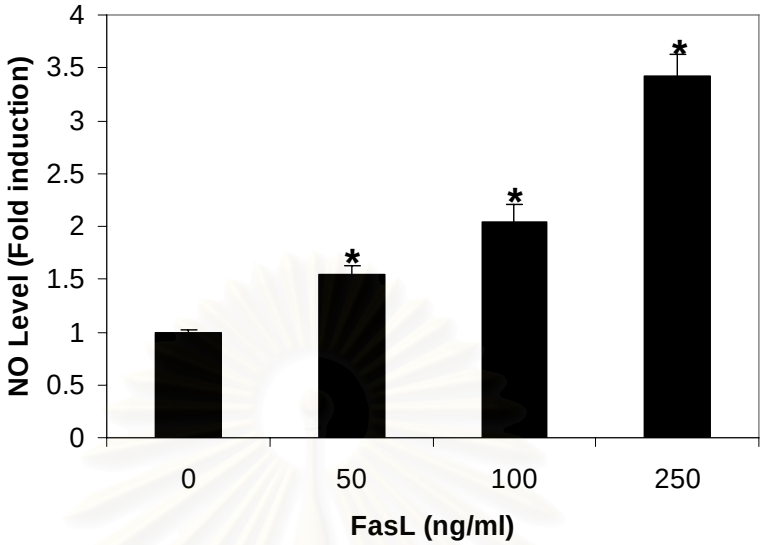


Figure 9 Effect of FasL on NO levels in BEAS (A), and H460 (B) as a function of time. Cells were treated with 100 ng/ml of FasL at various incubation times (0, 0.5, 1, 2, 3, 6, and 9 h) and detected by flow cytometry with DAF-DA. Values are relative DAF fluorescence change over control level. Plots are mean ± S.D. (n=5). * $P < 0.05$ versus non-treated control.

(A)



(B)

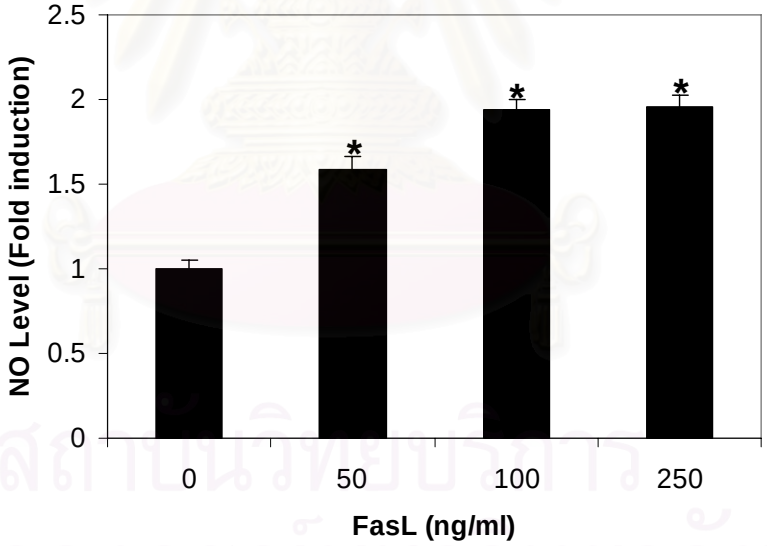


Figure 10 Effect of FasL on NO levels in BEAS (A), and H460 (B) as a function of dose. Cells were treated with various doses of FasL (0, 50, 100, and 250 ng/ml) and detected by flow cytometry with DAF-DA. Values are relative DAF fluorescence change over control level. Plots are mean ± S.D. (n=5). * $P < 0.05$ versus non-treated control.

2. Cisplatin- and FasL-induced apoptosis in BEAS and H460 cells.

In order to study the effect of NO on cell susceptibility to apoptosis, this study first characterized the cisplatin- and FasL-induced apoptosis in both BEAS and H460 cells. Hoechst 33342 nuclear staining was used to evaluate the apoptotic cells.

2.1 Cisplatin-induced apoptosis.

The control cells exhibited the normal morphology of living cells (figures 11C and D) and very few dead cells were seen. In contrast, apoptotic cells exhibited the typical morphological changes, including nuclear condensation, fragmented chromatin accumulation to the inside of nucleolus membrane, nuclear cleavage and apoptotic bodies (figures 11C and D), were detected in response to 100 μM cisplatin treatment. Cisplatin treatment caused time-dependent increase in cell apoptosis over control in both BEAS and H460 cells (figure 11A).

In BEAS cells, approximately 14% (14.00 ± 2.00 %) of the cisplatin-treated cells showed apoptotic nuclear morphology which was observed as early as 6 h and peaked at about 16 h (48.00 ± 2.00 %) post-treatment (figure 11A). In H460 cells, very slightly apoptotic cells were detected at 6 h after cisplatin treatment. The significant apoptosis response was detected after 9 h (8.00 ± 1.00 %) and peaked at 16 h (19.67 ± 2.51 %) (figure 11A). Since, time-dependent study showed that the apoptotic response peaked at 16 h in both cells, apoptotic cells in dose-response study would be detected at 16 h.

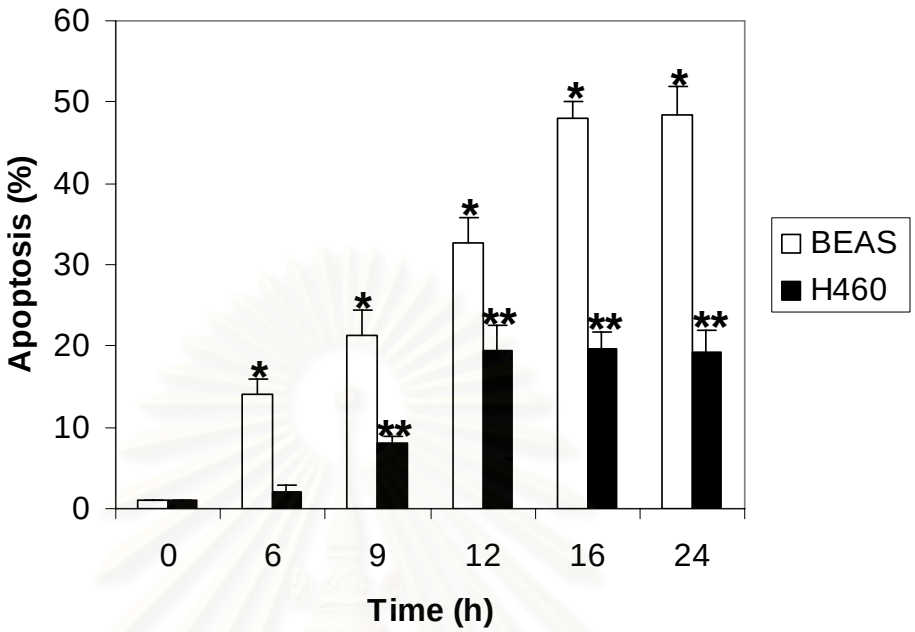
For dose-dependent study, the treated BEAS cells showed apoptotic nuclear morphology of 23.50 ± 1.80 % at the cisplatin concentration of 50 μM with the cell death response exceeding 83.00 ± 2.00 % at 500 μM of cisplatin (figure 11B). In H460 cells, apoptotic cells were increased in dose-dependent manner from 8.33 ± 1.52 % to 53.67 ± 3.05 % in response to cisplatin 50 μM and 500 μM, respectively (figure 11B).

2.2 FasL-induced apoptosis.

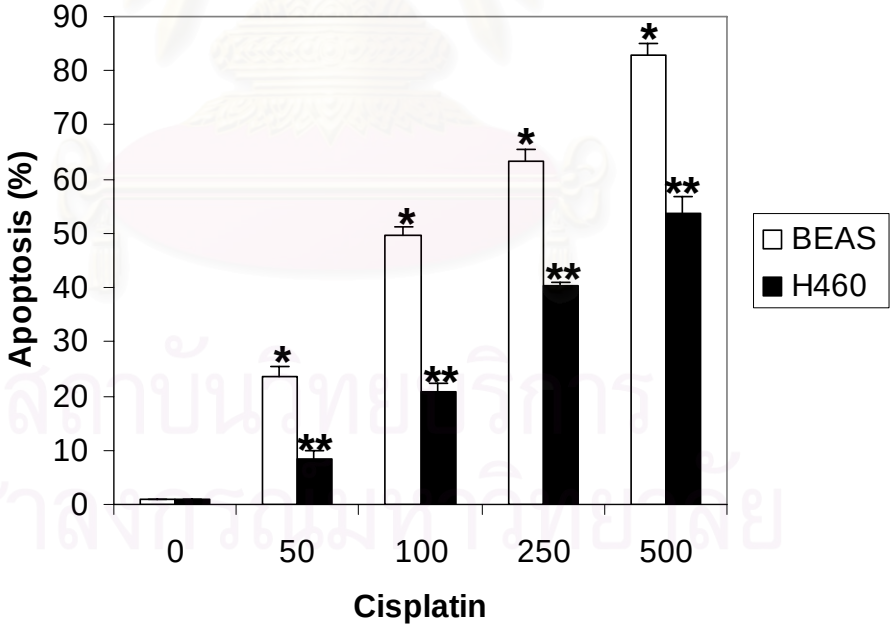
In BEAS cells, 6.26 ± 0.41 % of the FasL (100 ng/ml) -treated cells showed apoptotic nuclear morphology which was observed as early as 6 h and peaked at about 16 h (19.33 ± 0.52 %) post-treatment (figure 12A). Whereas, very slightly apoptotic cells were detected at 6 h in H460 cells, significant increase of apoptotic cells was detected at 9 h (4.00 ± 1.00 %) and peaked at 16 h (7.33 ± 0.65 %) (figure 12A). Since, time-dependent study showed that the apoptotic response peaked at 16 h, apoptotic cells in dose-response study would be detected at 16 h.

For dose-dependent study, the treated BEAS cells showed apoptotic nuclear morphology of 9.47 ± 1.04 % at the FasL concentration of 50 ng/ml with the cell death response exceeding 27.16 ± 1.75 % at 250 ng/ml (figures 12B and C). In H460 cells, apoptotic cells were increased in dose-dependent manner from 6.50 ± 1.50 % to 17.66 ± 2.51 % of cells in response to FasL concentrations of 100 ng/ml and 250 ng/ml, respectively (figure 12B). The normal and apoptotic nuclear morphology were shown in figure 12 C, D.

(A)



(B)



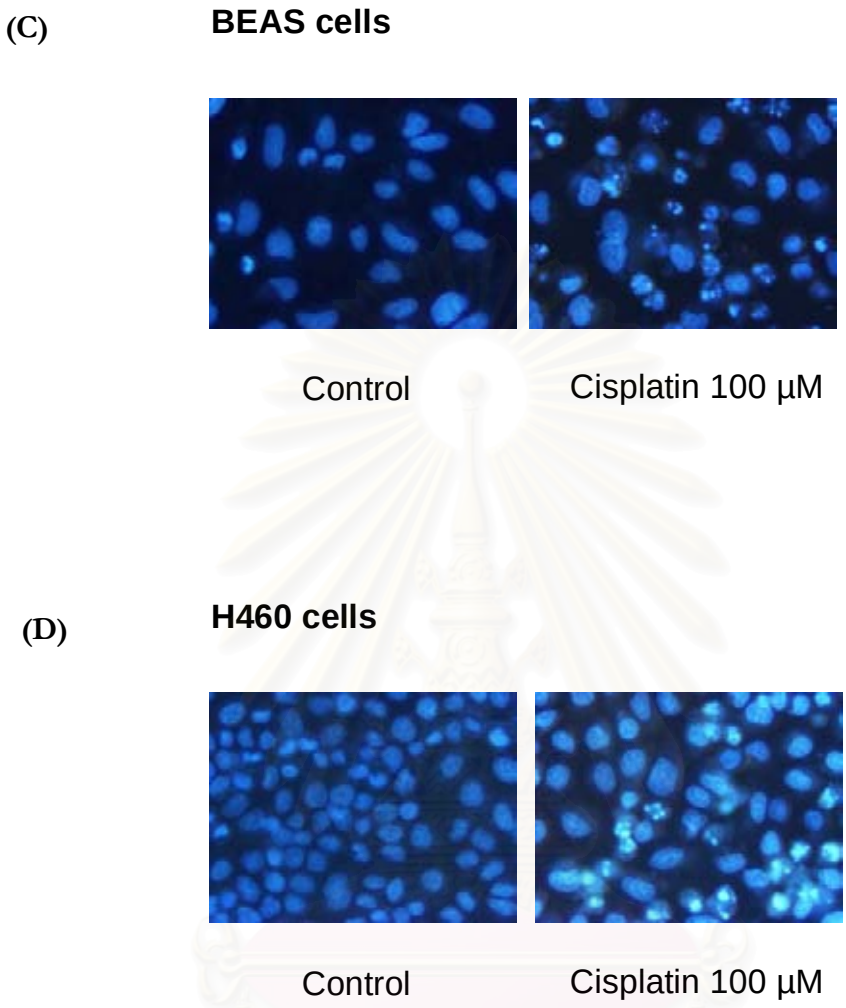
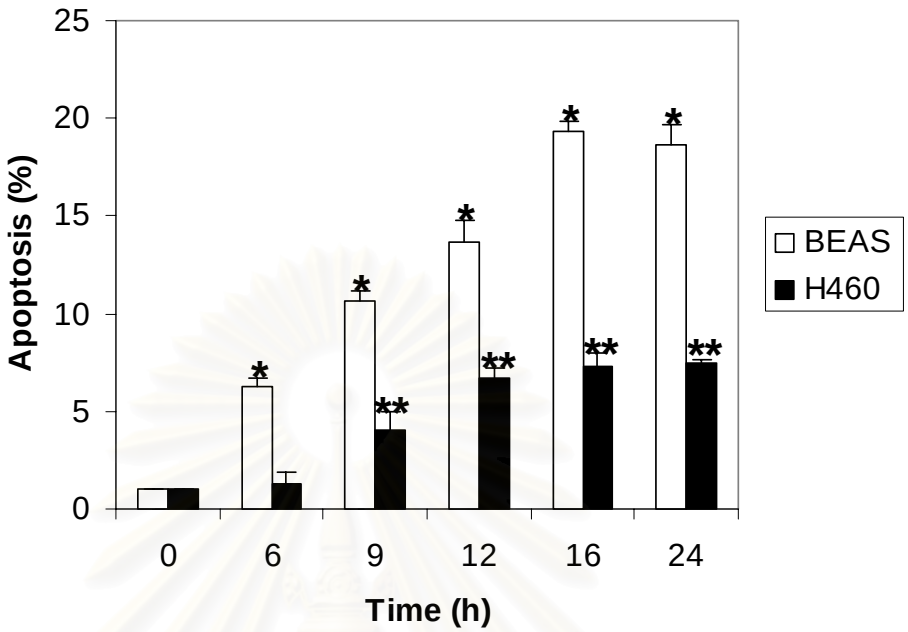
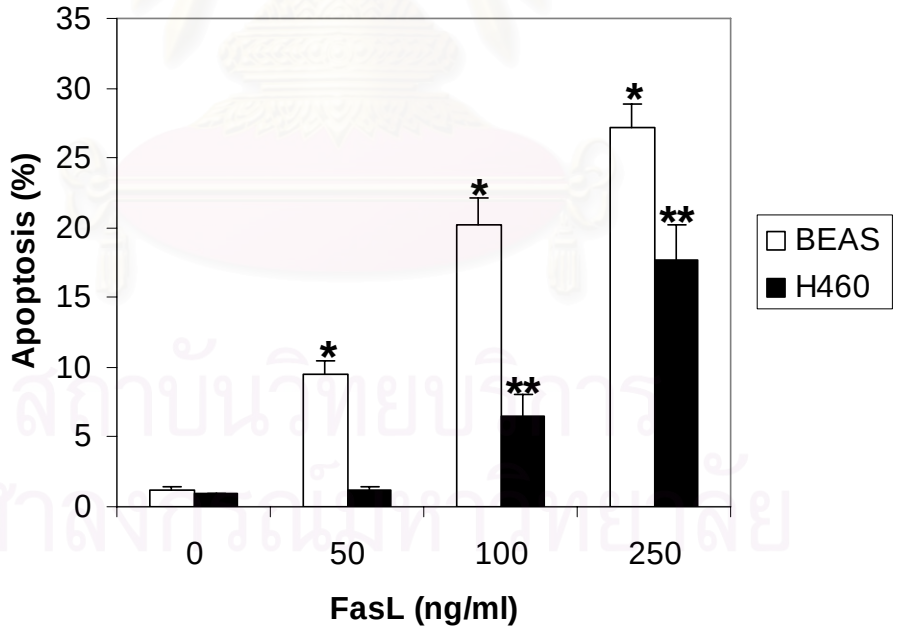


Figure 11 Cisplatin induced apoptosis in BEAS versus H460 cells in time-dependent (A), and dose-dependent (B) manners. (A) Subconfluent (90%) monolayer of BEAS or H460 cells were exposed to 100 μM of cisplatin at various incubation times (0, 6, 9, 12, 16, and 24 h) and analyzed for apoptosis by Hoechst 33342 assay. (B) Cells were exposed to cisplatin (0, 50, 100, 250, and 500 μM) for 16 h and analyzed for apoptosis by Hoechst 33342 assay. Values are relative % apoptosis change over control level. Plots are mean ± S.D. (n=3). * $P < 0.05$ versus non-treated BEAS cells, and ** $P < 0.05$ versus non-treated H460 cells. (C), (D) Morphologic analysis of apoptosis by Hoechst 33342 assay. Apoptotic cells exhibited shrunken nuclei with bright nuclear fluorescence. (Original magnification, X400)

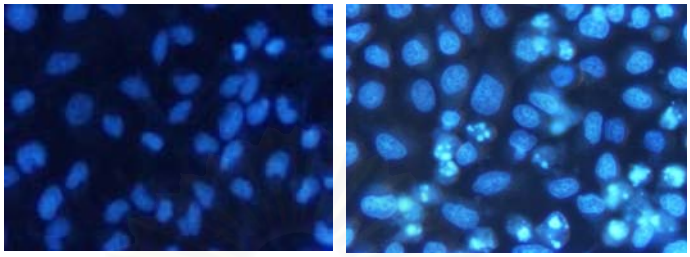
(A)



(B)

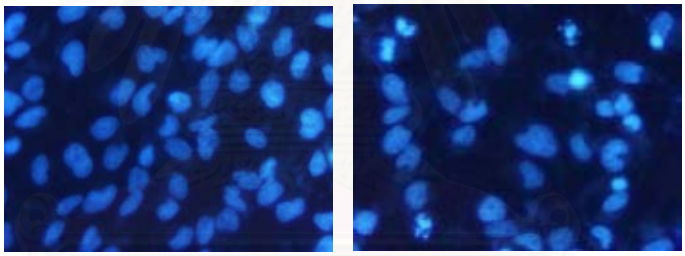


(C) **BEAS cells**



Control FasL 100 ng/ml

(D) **H460 cells**



Control FasL 100 ng/ml

Figure 12 FasL induced apoptosis in BEAS versus H460 cells in time-dependent (A), and dose-dependent (B) manners. (A) Subconfluent (90%) monolayer of BEAS or H460 cells were expose to 100 ng/ml of FasL at various incubation times (0, 6, 9, 12, 16, and 24 h) and analyzed for apoptosis by Hoechst 33342 assay. (B) Cells were exposed to FasL (0, 50, 100, and 250 ng/ml) for 16 h and analyzed for apoptosis by Hoechst 33342 assay. Plots are mean ± S.D. (n=3). * $P < 0.05$ versus non-treated BEAS cells, and ** $P < 0.05$ versus non-treated H460 cells. (C), (D) Morphologic analysis of apoptosis by Hoechst 33342 assay. Apoptotic cells exhibited shrunken nuclei with bright nuclear fluorescence. (Original magnification, X400)

3. Effect of basal NO levels on apoptotic susceptibility.

In an attempt to investigate the role of basal NO level in these cells, NO inhibitors were used to reduce NO level.

3.1 Effect of NO inhibitors on NO levels.

NO levels were significantly decreased in response to NO inhibitors treatment in both BEAS and H460 cells.

In BEAS cells, NO level was decreased to 0.59 ± 0.05 fold in response to treatment with 300 μM AG (figure 13A). Also, NO level was significantly decreased to 0.41 ± 0.07 fold in response to 300 μM PTIO treatment (figure 13A). In H460 cells, NO level was decreased to 0.18 ± 0.02 fold in response to treatment with 300 μM AG (figure 13B). Also, NO level was significantly decreased to 0.13 ± 0.03 fold in H460 cells in response to 300 μM PTIO treatment (figure 13 B).

3.2 Effect of NO inhibitors on cisplatin- and FasL-induced apoptosis.

3.2.1 Effect of NO inhibitors on cisplatin-induced apoptosis.

Results clearly showed that cisplatin-mediated apoptosis was enhanced by pretreatment with NO inhibitors (AG and PTIO) in both BEAS and H460 cells.

In BEAS cells, non-treated cells showed very low apoptotic morphology and cisplatin (100 μM) induced significant increase in apoptotic cells (49.33 ± 4.04 %) (figure 14B). Pretreatment with NO inhibitor AG promoted cisplatin-induced apoptosis up to 79.00 ± 3.27 %. Also, pretreatment with PTIO promoted cisplatin-induced apoptosis up to 77.66 ± 2.84 % (figure 14).

In H460 cells, non-treated cells showed very low apoptotic morphology and cisplatin (100 μM) induced significant increase in apoptotic cells (19.50 ± 2.29 %) (figure 15B). Pretreatment with NO inhibitor AG promoted cisplatin-induced apoptosis up to 31.5 ± 2.78 %. Also, pretreatment with PTIO promoted cisplatin-induced apoptosis

up to 35.43 ± 1.20 % (figure 15). The doses of AG and PTIO used in this study did not cause significant increase in apoptotic cells (data not shown).

The nuclear morphological analysis of control and cisplatin-treated BEAS cells were shown in figure 14B, and the nuclear morphological analysis of control and cisplatin-treated H460 cells were shown in figure 15B.

3.2.2 Effect of NO inhibitors on FasL-induced apoptosis.

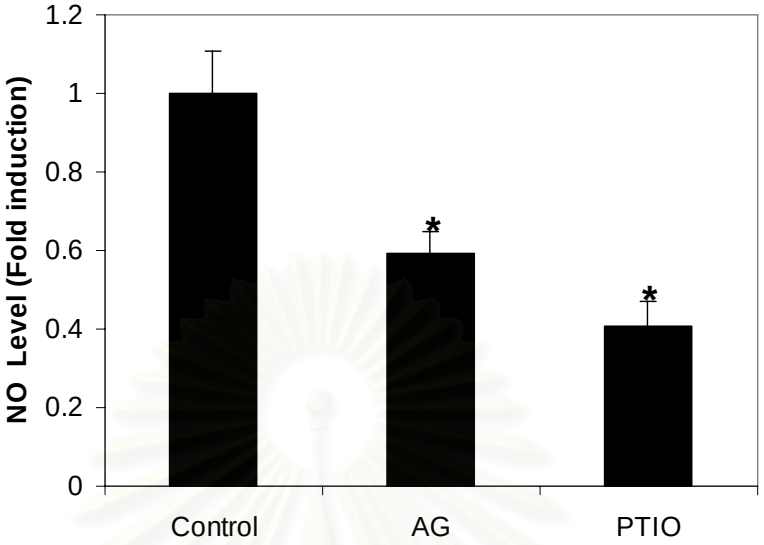
Consistent with the results from cisplatin-induced apoptosis, FasL-mediated apoptosis was enhanced by pretreatment with NO inhibitors (AG and PTIO) in both BEAS and H460 cells.

In BEAS cells, non-treated cells showed very low apoptotic morphology. Apoptotic cells were significantly increased (19.66 ± 2.08 %) in response to FasL (100 ng/ml). Pretreatment with NO inhibitor AG promoted FasL-induced apoptosis up to 33.50 ± 1.32 %. Also, pretreatment with PTIO promoted FasL-induced apoptosis up to 29.00 ± 1.50 % (figure 16).

In H460 cells, apoptotic cells significantly increased (6.00 ± 0.50 %) in response to treatment with FasL (100 ng/ml) comparing to control. Pretreatment with NO inhibitor AG promoted FasL-induced apoptosis up to 11.16 ± 1.04 %. Also, pretreatment with PTIO promoted FasL-induced apoptosis up to 10.16 ± 1.02 % (figure 17). The doses of AG and PTIO used in this study did not cause significant increase in apoptotic cells in preliminary study (data not shown).

The nuclear morphological analysis of control and FasL-treated BEAS cells were shown in figure 16B, and the nuclear morphological analysis of control and FasL-treated H460 cells were shown in figure 17B.

(A)



(B)

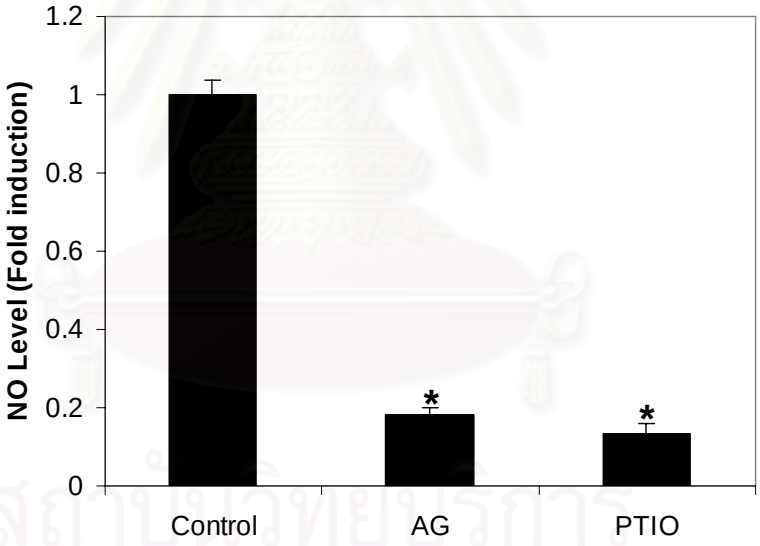
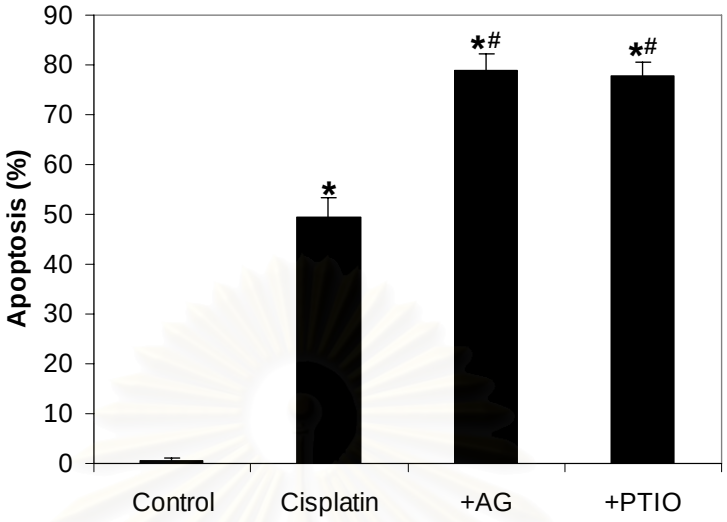


Figure 13 Effect of NO inhibitors on NO levels in BEAS (A), and H460 (B). Cells were treated with NO inhibitor AG (300 μM) or c-PTIO (300 μM) for 1 h. Then NO level was detected by flow cytometry with DAF-DA. Values are relative DAF fluorescence change over control level. Plots are mean ± S.D. (n=3). * $P < 0.05$ versus non-treated control.

(A)



(B)

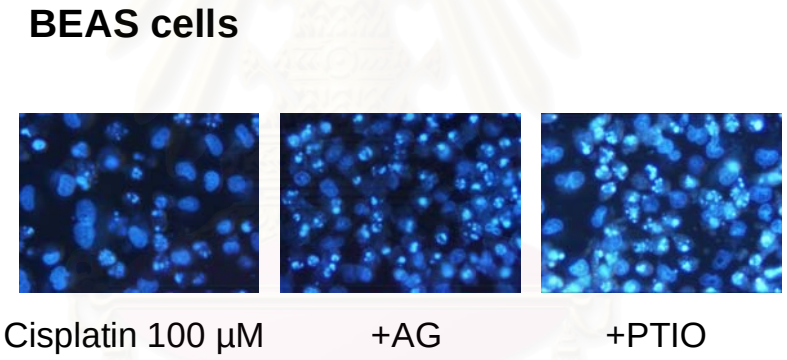


Figure 14 Effect of NO inhibitors on cisplatin-induced apoptosis in BEAS cells. (A) Subconfluent (90%) monolayer of BEAS cells were pretreated with the NO inhibitors AG (300 µM) or PTIO (300 µM) for 1 h. The cells were then treated with cisplatin (100 µM) for 16 h and analyzed for apoptosis by Hoechst assay. Plots are mean ± S.D. (n=3). * $P < 0.05$ versus non-treated BEAS cells, and # $P < 0.05$ versus cisplatin-treated control. (B) Morphologic analysis of apoptosis by Hoechst 33342 assay. (Original magnification, X400)

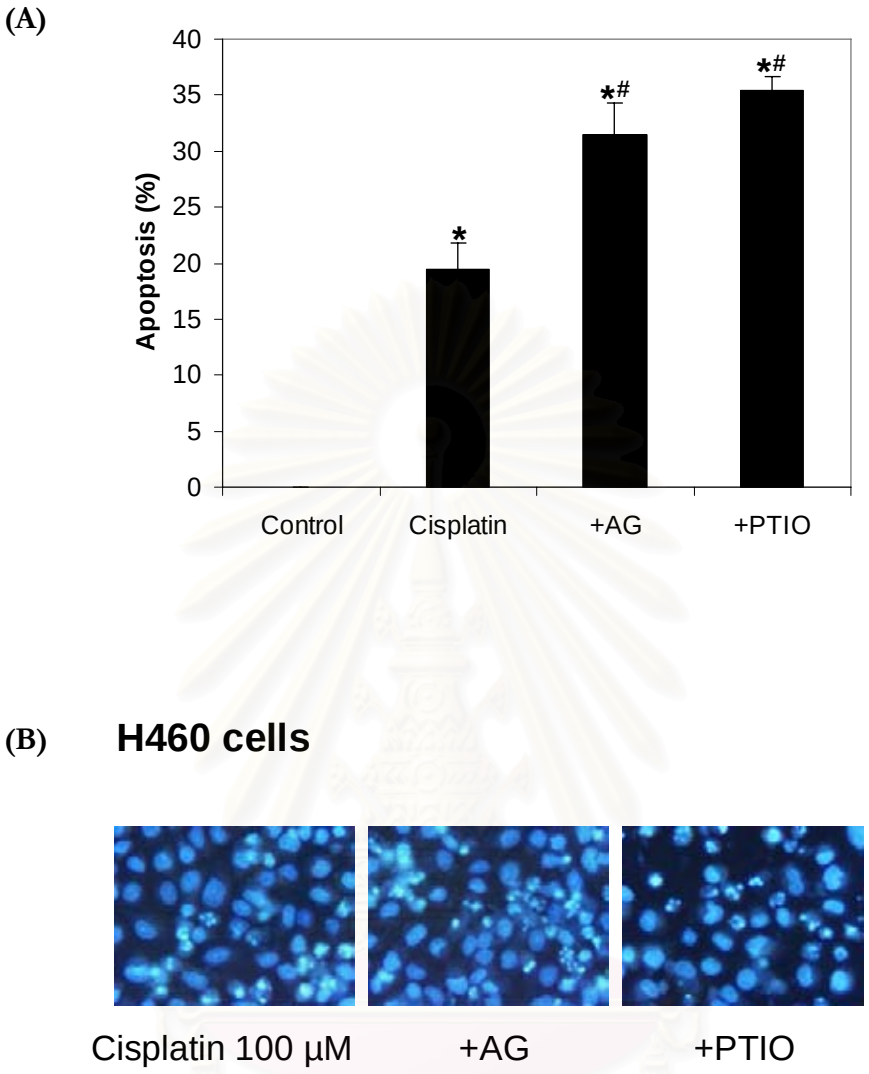


Figure 15 Effect of NO inhibitors on cisplatin-induced apoptosis in H460 cells. (A) Subconfluent (90%) monolayer of H460 cells were pretreated with the NO inhibitors AG (300 μM) or PTIO (300 μM) for 1 h. The cells were then treated with cisplatin (100 μM) for 16 h and analyzed for apoptosis by Hoechst assay. Plots are mean ± S.D. (n=3). * $P < 0.05$ versus non-treated H460 cells, and # $P < 0.05$ versus cisplatin-treated control. (B) Morphologic analysis of apoptosis by Hoechst 33342 assay. (Original magnification, X400)

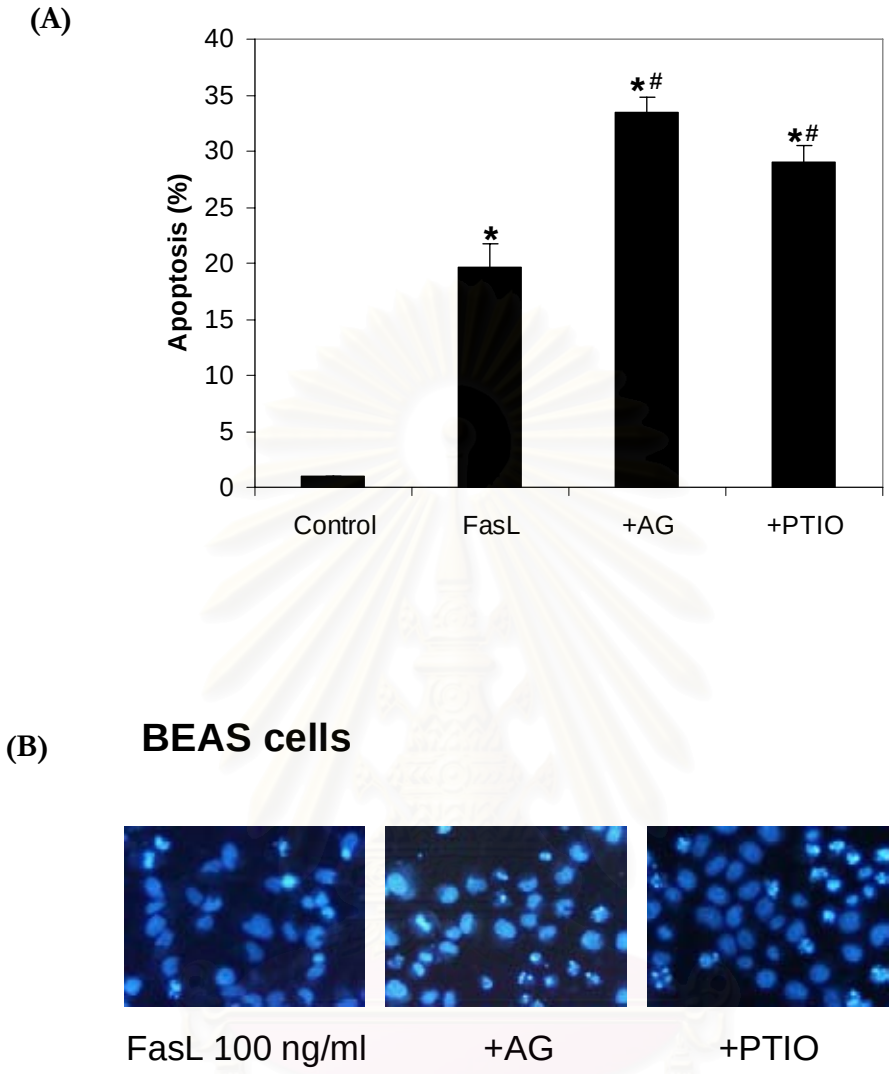


Figure 16 Effect of NO inhibitors on FasL-induced apoptosis in BEAS cells. (A) Subconfluent (90%) monolayer of BEAS cells were pretreated with the NO inhibitors AG (300 μM) or PTIO (300 μM) for 1 h. The cells were then treated with FasL (100 ng/ml) for 16 h and analyzed for apoptosis by Hoechst assay. Plots are mean ± S.D. (n=3). * $P < 0.05$ versus non-treated BEAS cells, and # $P < 0.05$ versus FasL-treated control. (B) Morphologic analysis of apoptosis by Hoechst 33342 assay. (Original magnification, X400)

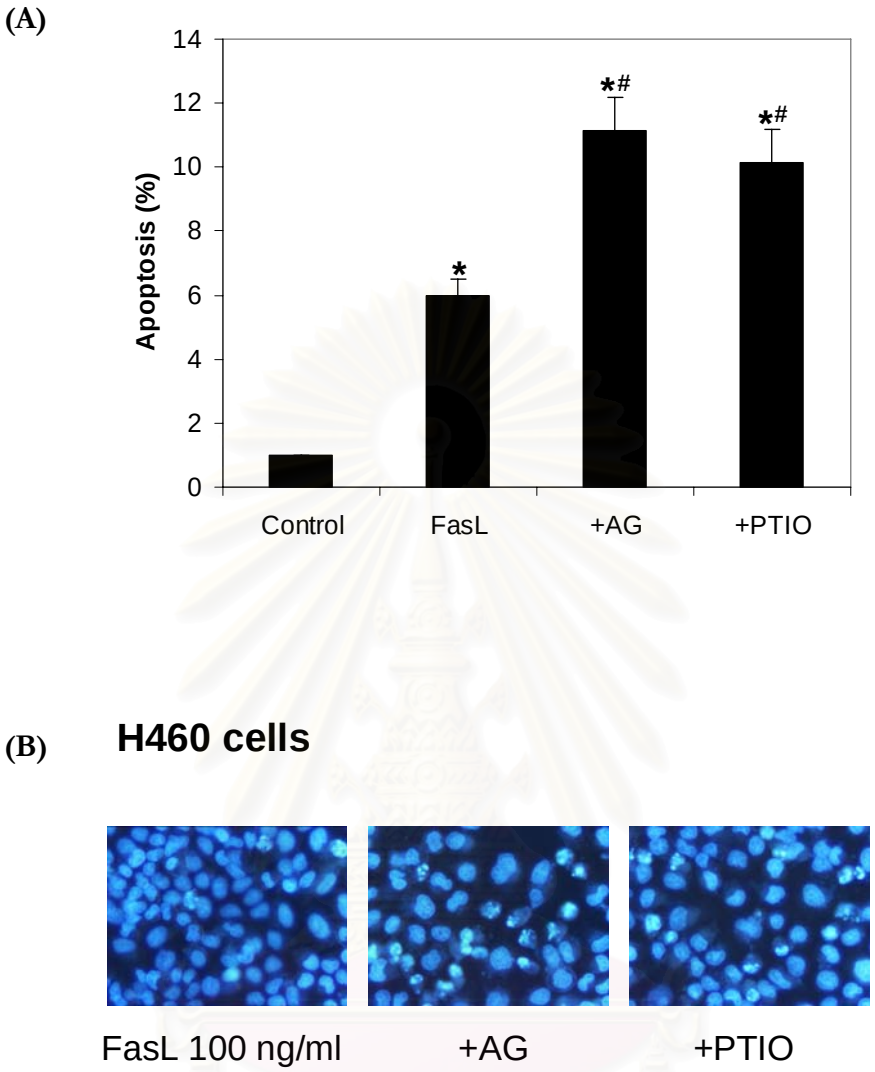


Figure 17 Effect of NO inhibitors on FasL-induced apoptosis in H460 cells. (A) Subconfluent (90%) monolayer of H460 cells were pretreated with the NO inhibitors AG (300 μM) or PTIO (300 μM) for 1 h. The cells were then treated with FasL (100 ng/ml) for 16 h and analyzed for apoptosis by Hoechst assay. Plots are mean ± S.D. (n=3). * $P < 0.05$ versus non-treated H460 cells, and # $P < 0.05$ versus FasL-treated control. (B) Morphologic analysis of apoptosis by Hoechst 33342 assay. (Original magnification, X400)

4. Effect of elevated NO levels on apoptotic susceptibility.

In an attempt to investigate the effect of elevated NO levels on susceptibility to apoptosis, NO donors were used to increase NO level.

4.1 Effect of NO donors on NO levels.

The results from DAF-DA and flow cytometry indicated that both SNP and DPTA NONOate effectively increased NO level in BEAS and H460 cells (figure 18)

In BEAS cells, NO level was decreased to 0.33 fold (0.33 ± 0.11) in response to 100 μM cisplatin treatment (figure 18A). Co-treatment with 100 μM cisplatin and 300 μg/ml SNP increased NO level 6.82 folds (6.82 ± 0.34) comparing to controls. Also, co-treatment with 100 μM cisplatin and 200 μM DPTA NONOate increased NO level 5.95 folds (5.95 ± 0.33) comparing to control (figure 18A).

In H460 cells, NO level was decreased to 0.50 fold (0.50 ± 0.05) in response to 100 μM cisplatin treatment. Co-treatment with 100 μM cisplatin and 300 μg/ml SNP increased NO level 6.42 folds (6.42 ± 0.08) comparing to controls. Co-treatment with 100 μM cisplatin and 200 μM DPTA NONOate increased NO level 6.66 folds (6.66 ± 0.11) comparing to control (figure 18B).

NO level in BEAS cells was increased 2.25 folds (2.25 ± 0.08) in response to FasL treatment. Additional of SNP and DPTA NONOate further increased NO levels to 6.46 ± 0.52 and 6.63 ± 0.32 folds, respectively (figure 18C). In H460 cells, NO level was increased 2.18 folds (2.18 ± 0.05) in response to FasL treatment. Additional of SNP and DPTA NONOate further increased NO level to 5.83 ± 0.10 and 5.81 ± 0.07 folds, respectively (figure 18D).

4.2 Effect of NO donors on cisplatin- and FasL-induced apoptosis.

4.2.1 Effect of NO donors on cisplatin-induced apoptosis.

The results clearly indicated that pretreatment with NO donors resulted in inhibition of cisplatin-induced apoptosis. In BEAS cells, pretreatment with SNP (300 μg/ml) clearly protected cells from cisplatin (100 μM)- induced apoptosis (3.67 ± 1.52 %

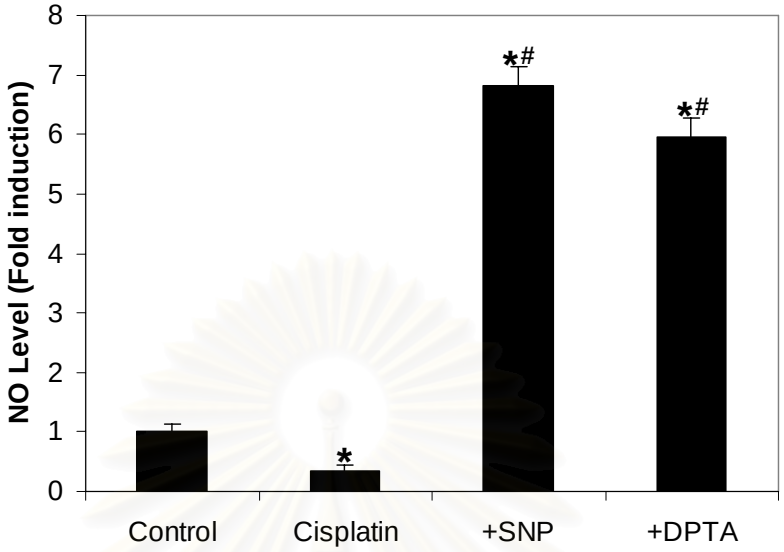
comparing to 49.33 ± 4.04 % in cisplatin-treated control) (figure 19). Also, pretreatment with DPTA NONOate (200 μM) inhibited cisplatin-induced apoptosis (3.33 ± 1.52 % comparing to 49.33 ± 4.04 % in cisplatin-treated control) (figure 19).

In H460 cells, pretreatment with SNP (300 μg/ml) completely protected cells from cisplatin (100 μM) - induced apoptosis (2.00 ± 1.00 % comparing to 19.50 ± 2.29 %) (figure 20). Also, pretreatment with DPTA NONOate (200 μM) completely inhibited cisplatin-induced apoptosis (1.33 ± 0.57 %) (figure 20).

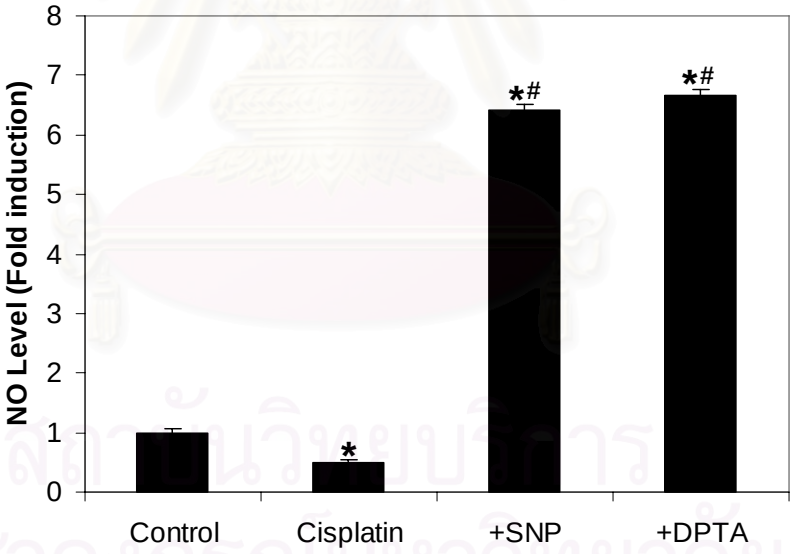
4.2.2 Effect of NO donors on FasL-induced apoptosis.

For FasL-induced apoptosis, the consistent results indicated the protective effect of NO. In BEAS cells, apoptotic cells were decreased from 17.50 ± 2.29 % in FasL-treated control to 2.00 ± 1.00 % and 1.67 ± 1.15 % in SNP- and DPTA NONOate-pretreated cells, respectively (figure 21). In H460 cells, addition of NO donors completely inhibited FasL-induced apoptosis from 7.33 ± 0.33 % (FasL-treated control) to 1.66 ± 0.57 % and 1.33 ± 0.54 % in SNP- and DPTA NONOate-pretreated cells, respectively (figure 22).

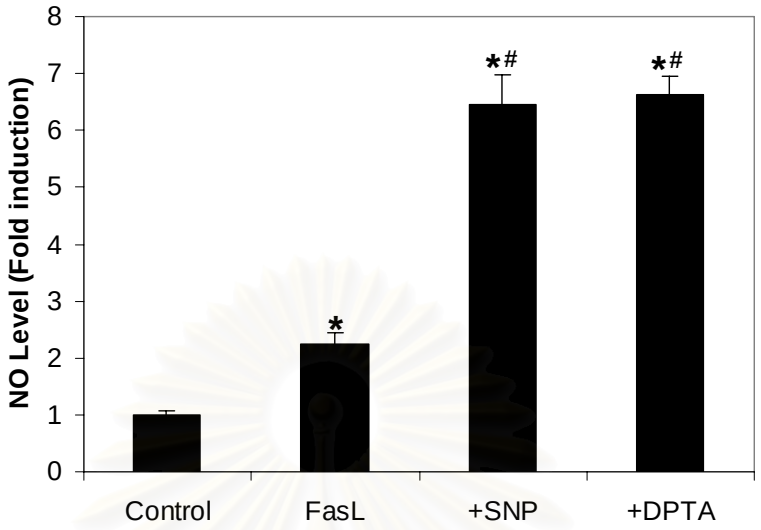
(A)



(B)



(C)



(D)

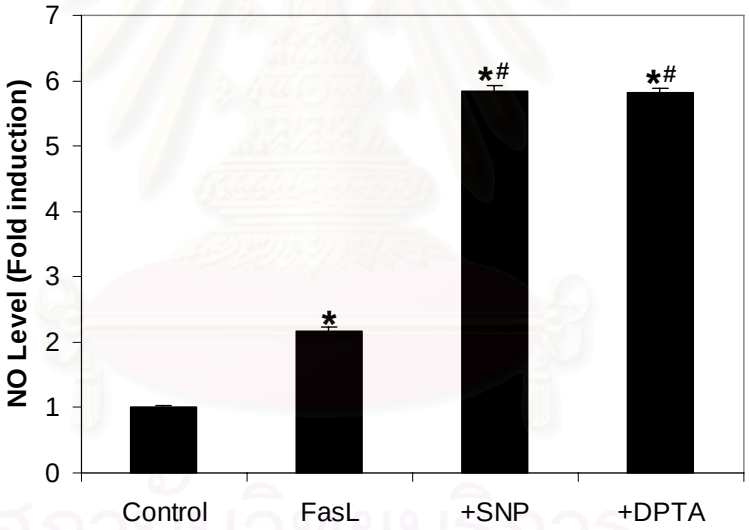


Figure 18 Effect of NO donors (SNP and DPTA NONOate) on NO levels in cisplatin-treated BEAS (A) and H460 (B) cells, and in FasL-treated BEAS (C) and H460 cells (D). (A-D) cells were pretreated with SNP (300 μg/ml) or DPTA NONOate (200 μM) and then treated with 100 μM cisplatin or 100 ng/ml FasL for 1 h. NO level was determined by flow cytometer with DAF-DA. Values are relative DAF fluorescence change over control level. Plots are mean ± S.D. (n=3). * $P < 0.01$ versus non-treated control and # $P < 0.01$ versus cisplatin-treated control (A-B) or FasL-treated control (C-D).

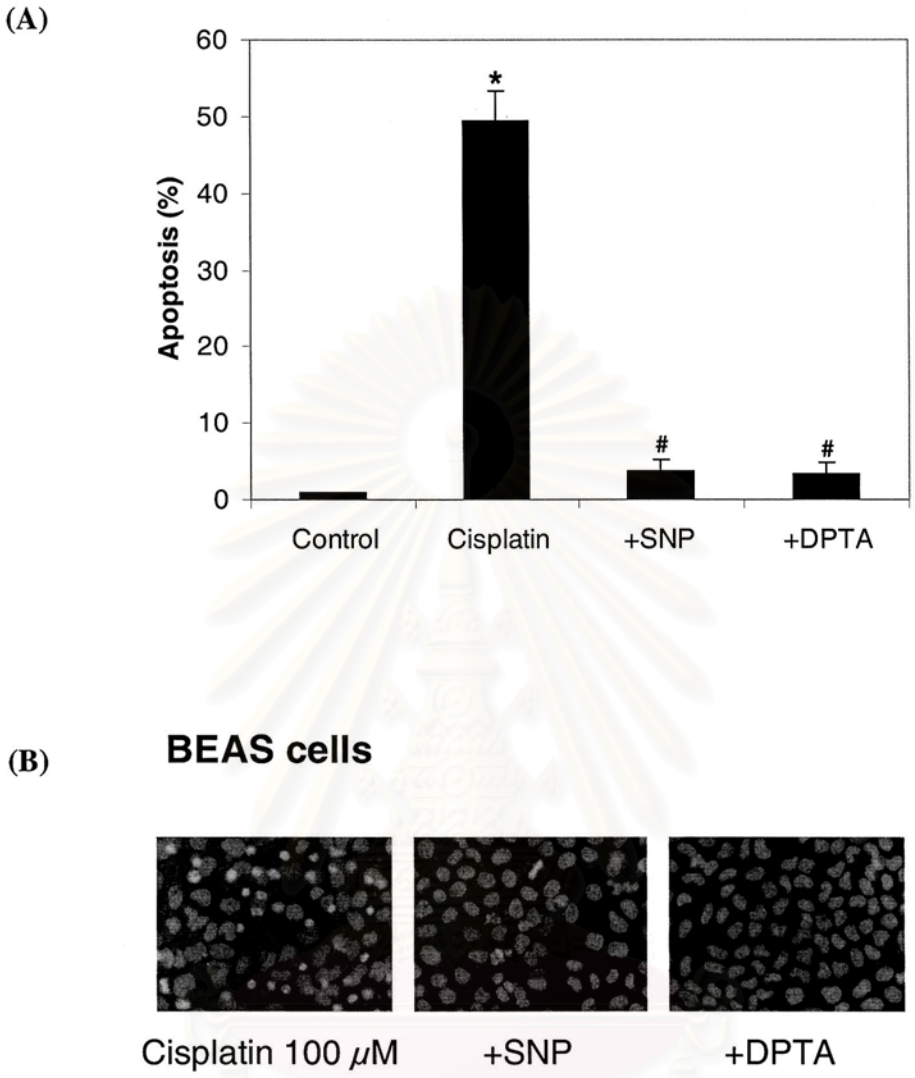
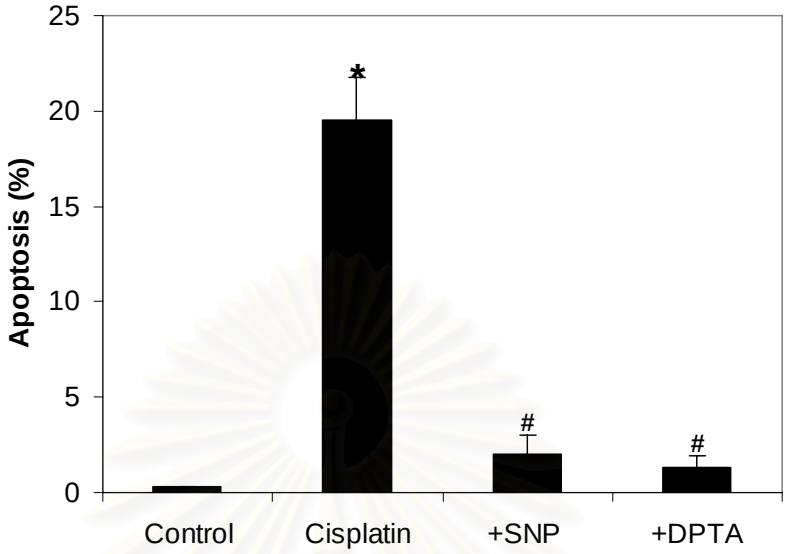


Figure 19 Effect of NO donors on cisplatin-induced apoptosis in BEAS cells. (A) Cells were pretreated with SNP (300 μg/ml) or DPTA NONOate (200 μM) and then treated with 100 μM cisplatin for 16 h. Apoptotic cells were determined by Hoechst 33342 analysis. Plots are mean ± S.D. (n=3). * $P < 0.01$ versus non-treated control and # $P < 0.01$ versus cisplatin-treated control. (B) Morphologic analysis of apoptosis by Hoechst 33342 assay. (Original magnification, X400)

(A)



(B)

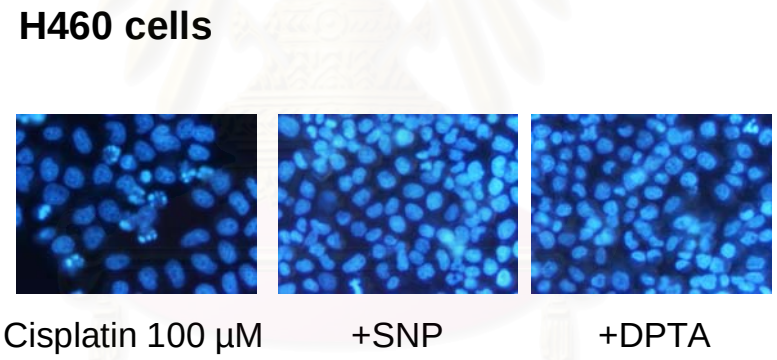
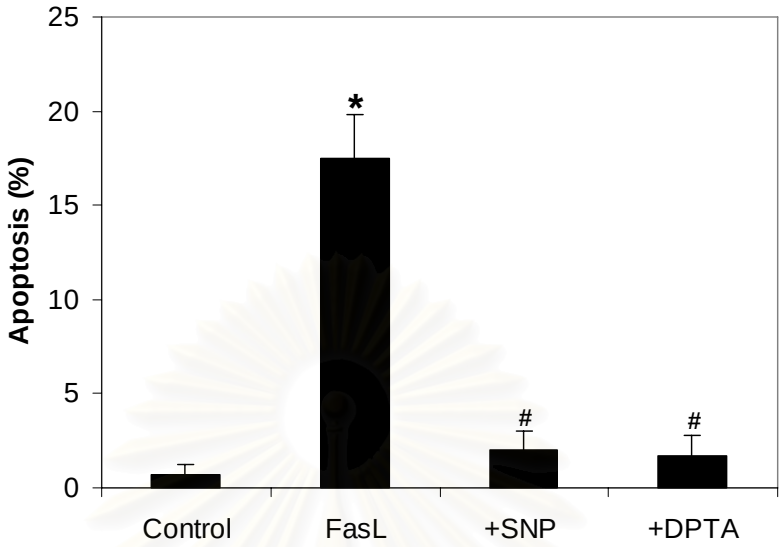


Figure 20 Effect of NO donors on cisplatin-induced apoptosis in H460 cells. (A) Cells were pretreated with SNP (300 μg/ml) or DPTA NONOate (200 μM) and then treated with 100 μM cisplatin for 16 h. Apoptotic cells were determined by Hoechst 33342 analysis. Plots are mean ± S.D. (n=3). * $P < 0.01$ versus non-treated control and # $P < 0.01$ versus cisplatin-treated control. (B) Morphologic analysis of apoptosis by Hoechst 33342 assay. (Original magnification, X400)

(A)



(B)

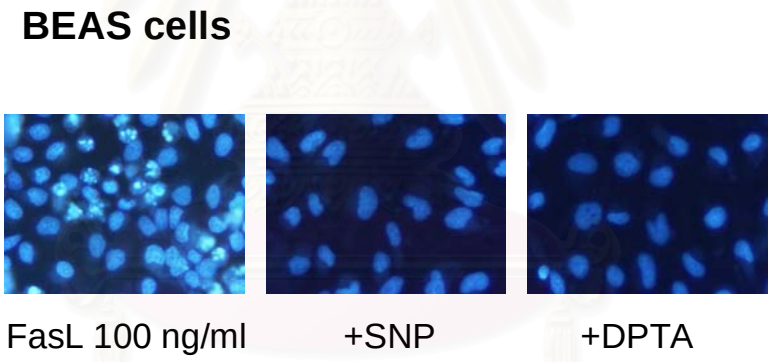
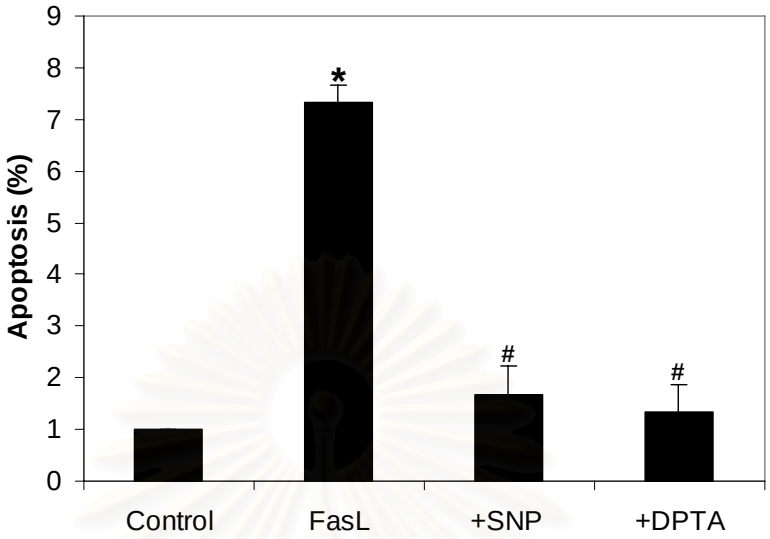


Figure 21 Effect of NO donors on FasL-induced apoptosis in BEAS cells. (A) Cells were pretreated with SNP (300 μg/ml) or DPTA NONOate (200 μM) and then treated with 100 ng/ml FasL for 16 h. Apoptotic cells were determined by Hoechst 33342 analysis. Plots are mean ± S.D. (n=3). * $P < 0.01$ versus non-treated control and # $P < 0.01$ versus FasL-treated control. (B) Morphologic analysis of apoptosis by Hoechst 33342 assay. (Original magnification, X400)

(A)



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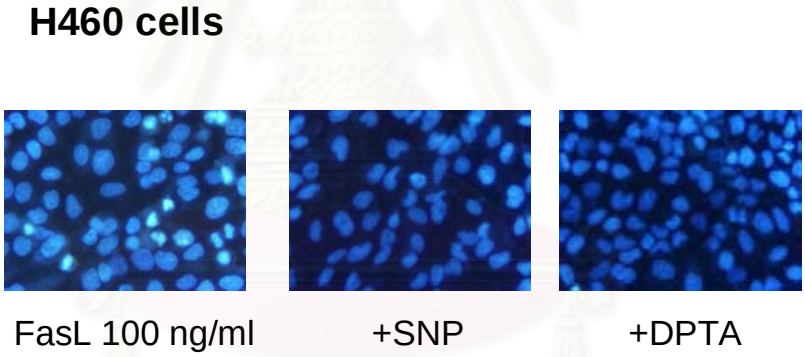


Figure 22 Effect of NO donors on FasL-induced apoptosis in H460 cells. (A) Cells were pretreated with SNP (300 μg/ml) or DPTA NONOate (200 μM) and then treated with 100 ng/ml FasL for 16 h. Apoptotic cells were determined by Hoechst 33342 analysis. Plots are mean ± S.D. (n=3). * $P < 0.01$ versus non-treated control and # $P < 0.01$ versus FasL-treated control. (B) Morphologic analysis of apoptosis by Hoechst 33342 assay. (Original magnification, X400)

5. Effect of different NO levels on susceptibility to apoptosis.

5.1 Effect of various NO donor concentrations on NO levels.

The results showed that increasing concentrations of NO donor (SNP) effectively increased the level of intracellular NO in dose-dependent manner.

In cisplatin-induced apoptosis, addition of increasing concentrations of SNP (50-500 μg/ml) increased NO level in BEAS cells from 2.19 ± 0.30 at the SNP concentration of 50 μg/ml with the increase of NO level exceeding 9.63 ± 0.13 folds at 500 μg/ml of SNP (figure 23A). In H460 cells, pretreatment with SNP (50-500 μg/ml) increased NO level from 1.83 ± 0.08 at the SNP concentration of 50 μg/ml with the increase of NO level exceeding 4.68 ± 0.22 folds at 500 μg/ml of SNP (figure 23B).

In FasL-induced apoptosis condition, pretreatment with SNP (50-500 μg/ml) increased NO level in BEAS cells from 1.83 ± 0.05 at the SNP concentration of 50 μg/ml with the increase of NO level exceeding 7.80 ± 0.30 folds at 500 μg/ml of SNP (figure 24A). In H460 cells, pretreatment with SNP (50-500 μg/ml) increased NO level from 1.78 ± 0.01 at the SNP concentration of 50 μg/ml with the increase of NO level exceeding 5.67 ± 0.25 folds at 500 μg/ml of SNP (figure 24B).

5.2 Effect of different NO levels on susceptibility to apoptosis.

5.2.1 Effect of different NO levels on cisplatin-induced apoptosis.

The results showed that increased NO levels caused by addition of SNP, significantly inhibited cisplatin-induced apoptosis in dose-dependent manner. In BEAS cells, apoptotic cells induced by cisplatin decreased from 50.00 ± 3.60 % in cisplatin-treated control to 37.67 ± 5.13 %, 12.67 ± 2.08 %, 2.67 ± 2.08 %, and 1.66 ± 1.12 % in response to SNP concentrations of 50, 100, 300 and 500 μg/ml, respectively (figure 25A).

In H460 cells, pretreatment of SNP decreased cell apoptosis from 19.08 ± 0.38 % in cisplatin-treated control to 9.05 ± 1.58 %, 5.67 ± 1.54 %, 0.67 ± 0.58 %, and 0.33

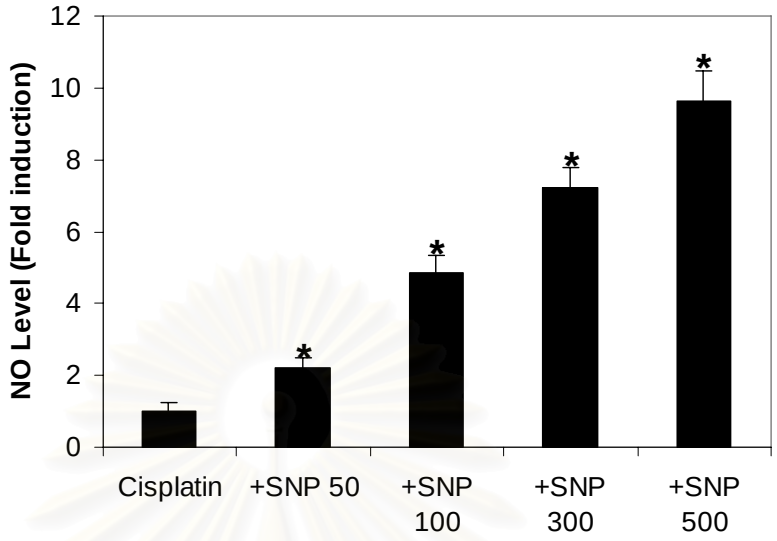
± 0.27 % in response to SNP concentrations of 50, 100, 300 and 500 μg/ml, respectively (figure 25B).

5.2.2 Effect of different NO levels on FasL-induced apoptosis.

In BEAS cells, pretreatment with SNP significantly decreased cell apoptosis from 17.39 ± 3.10 % in FasL-treated control to 10.25 ± 1.64 %, 7.00 ± 2.00 %, 0.67 ± 0.58 %, and 0.33 ± 0.27 % in response to SNP concentrations of 50, 100, 300 and 500 μg/ml, respectively (figure 26A).

In H460 cells, pretreatment with SNP decreased cell apoptosis from 8.20 ± 1.04 % in FasL-treated control to 4.12 ± 1.13 %, 0.33 ± 0.58 %, 0.33 ± 0.58 %, and 0.33 ± 0.00 % in response to SNP concentrations of 50, 100, 300 and 500 μg/ml, respectively (figure 26B).

(A)



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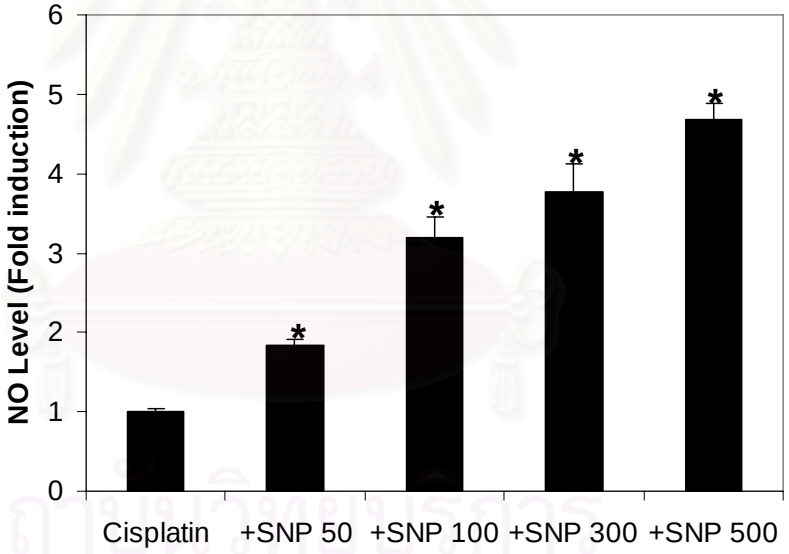
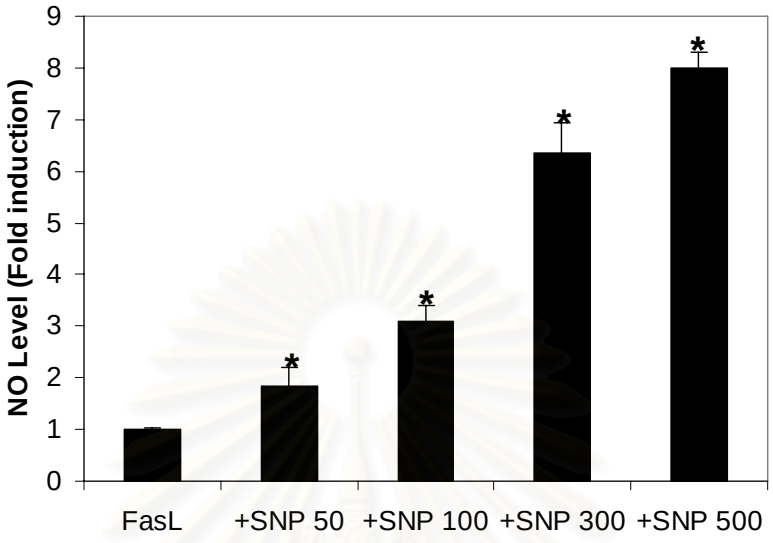


Figure 23 Effect of various concentrations of NO donor (SNP) on NO levels in cisplatin-treated BEAS (A), and H460 cells (B). Cells were pretreated with SNP (0, 50, 100, 300, and 500 μg/ml) and then treated with 100 μM cisplatin for 1 h. NO levels were detected by flow cytometry with DAF-DA. Values are relative DAF fluorescence change over control level. Plots are mean ± S.D. (n=3). * $P < 0.05$ versus cisplatin-treated control.

(A)



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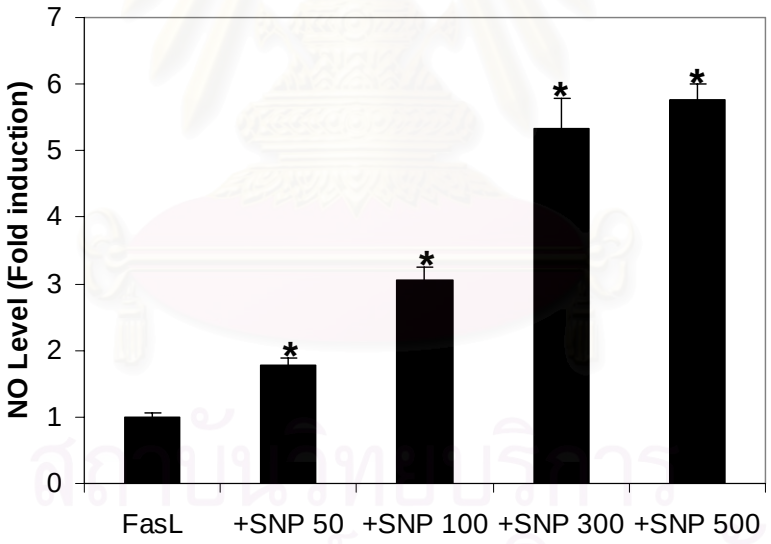
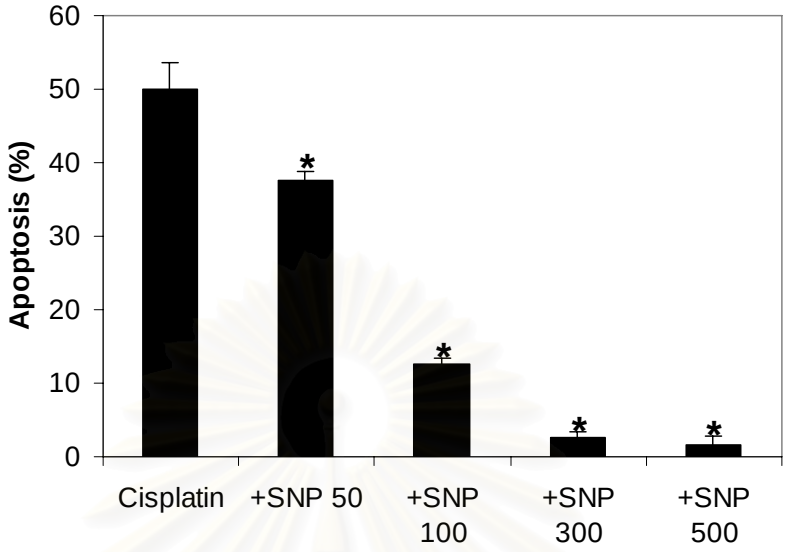


Figure 24 Effect of various concentrations of NO donor (SNP) on NO levels in FasL-treated BEAS (A), and H460 cells (B). Cells were pretreated with SNP (0, 50, 100, 300, and 500 g/ml) and then treated with 100 ng/ml FasL for 1 h. NO levels were detected by flow cytometry with DAF-DA. Values are relative DAF fluorescence change over control level. Plots are mean ± S.D. (n=3). * $P < 0.05$ versus FasL-treated control.

(A)



(B)

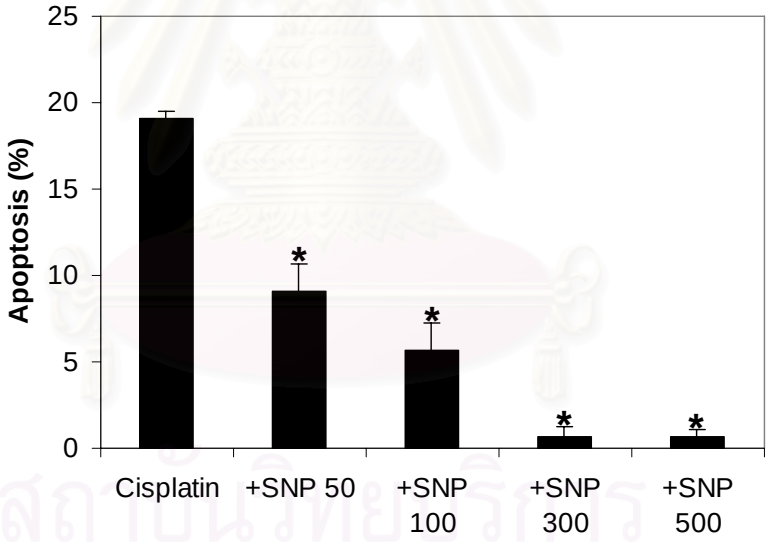


Figure 25 Effect of NO levels on cisplatin-induced BEAS (A), and H460 cells (B) apoptosis. Cells were pretreated with SNP (0, 50, 100, 300 and 500 μg/ml) and then treated with 100 μM cisplatin for 16 h. Apoptotic cells were determined by Hoechst 33342 analysis. Plots are mean ± S.D. (n=3). * $P < 0.05$ versus cisplatin-treated control.

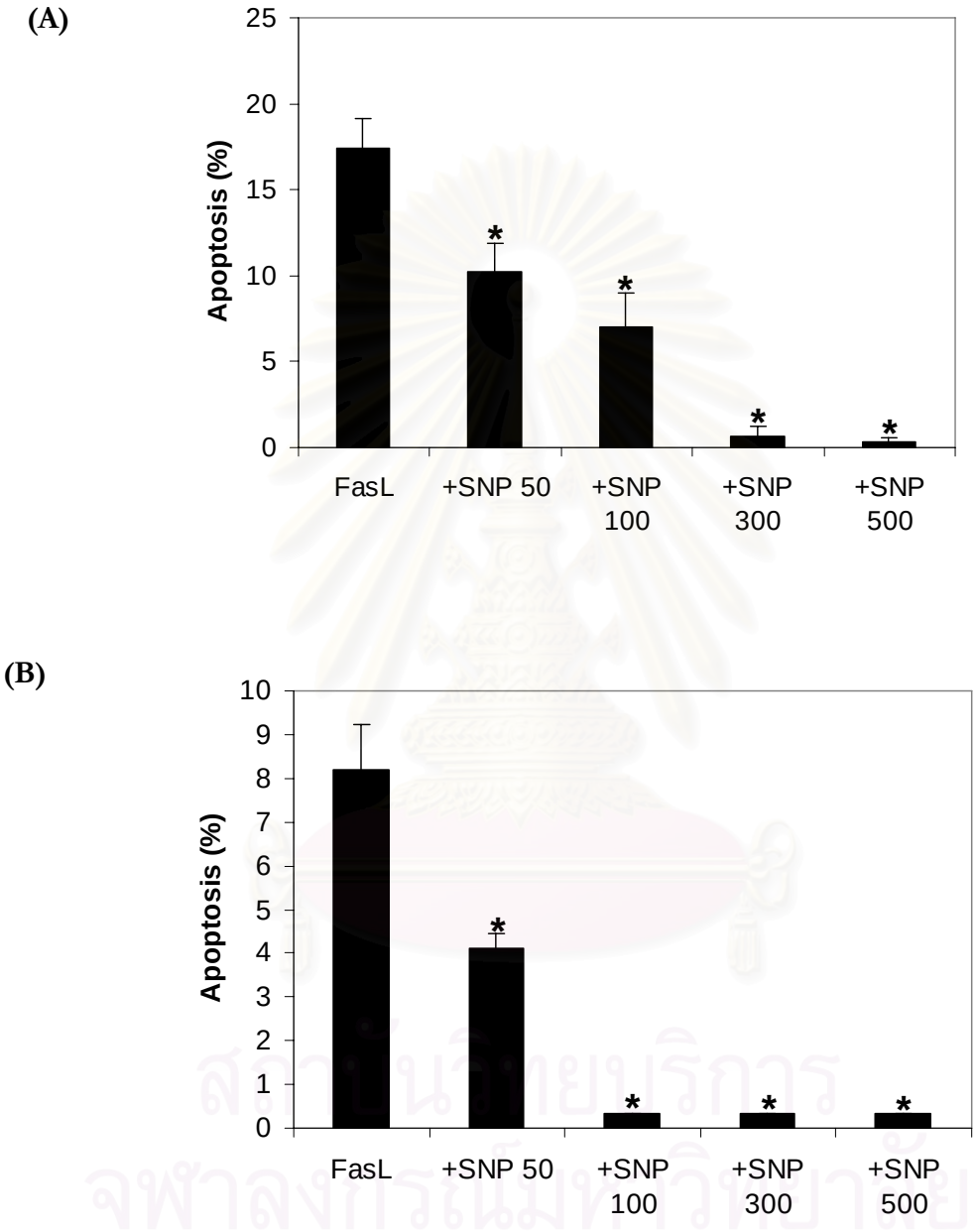


Figure 26 Effect of NO levels on FasL-induced BEAS (A), and H460 cells (B) apoptosis. Cells were pretreated with SNP (0, 50, 100, 300 and 500 μg/ml) and then treated with 100 μg/ml FasL for 16 h. Apoptotic cells were determined by Hoechst 33342 analysis. Plots are mean ± S.D. (n=3). * $P < 0.05$ versus FasL-treated control.

6. Mechanisms of NO in controlling cisplatin- and FasL- induced apoptosis.

6.1 Mechanism of NO in controlling cisplatin-induced H460 cells apoptosis.

To study the role of NO in cisplatin-induced apoptosis and investigate the mechanism of cisplatin-resistance in cancer cells, the H460 cells were used.

6.1.1 Cisplatin-induced H460 cells apoptosis.

This study first characterized cell death response to cisplatin treatment in human lung epithelial carcinoma H460 cells.

Cisplatin induced H460 cells apoptosis in dose-dependent manner. Very few apoptotic cells were seen in the untreated control (figure 27). Treatment of the cells with increasing cisplatin concentrations caused a dose-dependent increase in cell apoptosis over control level, as indicated by the increased nuclear fluorescence and chromatin condensation and fragmentation (figure 27). At the treatment dose of 50 μM, approximately 10% (10.13 ± 0.85 %) of the treated cells showed apoptotic nuclear morphology with the cell death response approaching 60% (58.75 ± 3.50 %) at the treatment dose of 500 μM (figure 27).

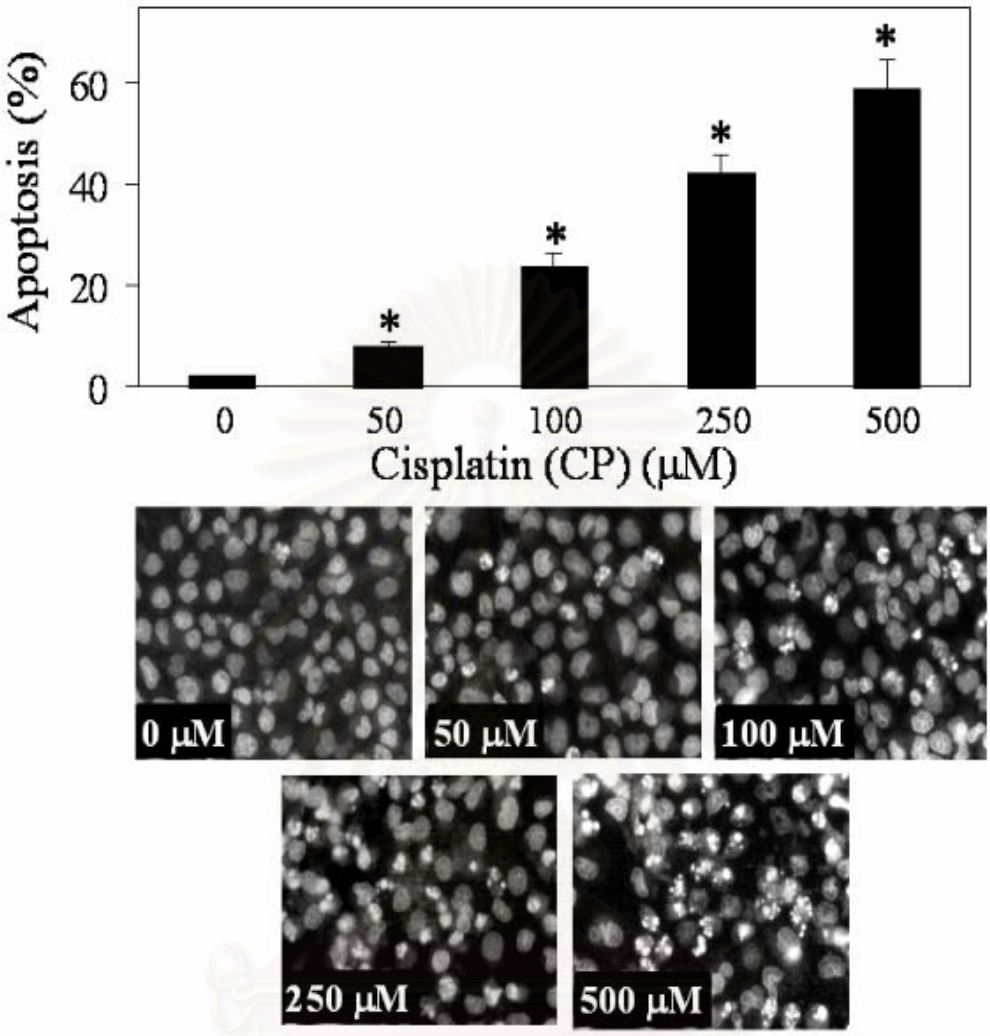


Figure 27 Cisplatin-induced apoptosis in H460 cells. Subconfluent (90%) monolayers of H460 cells were treated with various concentrations of cisplatin (0-500 μM) for 12 h and apoptosis was determined by nuclear morphology using Hoechst 33342 assay. Data are mean ± S.D. (n = 4). *$P < 0.05$ versus non-treated control.

6.1.2 Effects of NO modulators and antioxidant on cisplatin-induced H460 cells apoptosis.

To investigate the potential role of NO in the regulation of cisplatin-induced apoptosis, cells were pretreated with various NO inhibitors (AG and PTIO) and donors (SNP and DPTA NONOate) followed by cisplatin treatment. The NO synthase inhibitor AG and the NO scavenger PTIO effectively increased the cellular response to cisplatin-induced cell death, whereas the NO donors SNP and DPTA NONOate had opposite effect (figure 28).

In non-treated control, very few apoptotic cells were detected (2.88 ± 0.25 %). Co-treatment with cisplatin and AG significantly increased cell apoptosis to approximately 33% (32.75 ± 3.77 %) comparing to cisplatin-treated control (22.50 ± 1.92 %). Correspondingly, co-treatment with cisplatin and PTIO significantly enhanced cell apoptosis up to 34% (34.38 ± 4.31 %). Whereas, pretreatment with SNP and DPTA NONOate clearly inhibited cisplatin-induced apoptosis (2.86 ± 0.28 % and 2.88 ± 0.28 %, respectively) (figure 28).

Since previous studies have shown that cisplatin induces cell death via an ROS-dependent mechanism (Li et al., 2004; and Wu et al., 2005), this study also tested whether ROS inhibition by the antioxidant NAC could prevent cisplatin-induced apoptosis in our cell system. Figure 28 showed that addition of NAC completely blocked cisplatin-induced apoptosis (2.84 ± 0.33 %). The NO modulating agents and NAC, when used alone at the indicated concentrations, had no significant effect on cell apoptosis (figure 29).

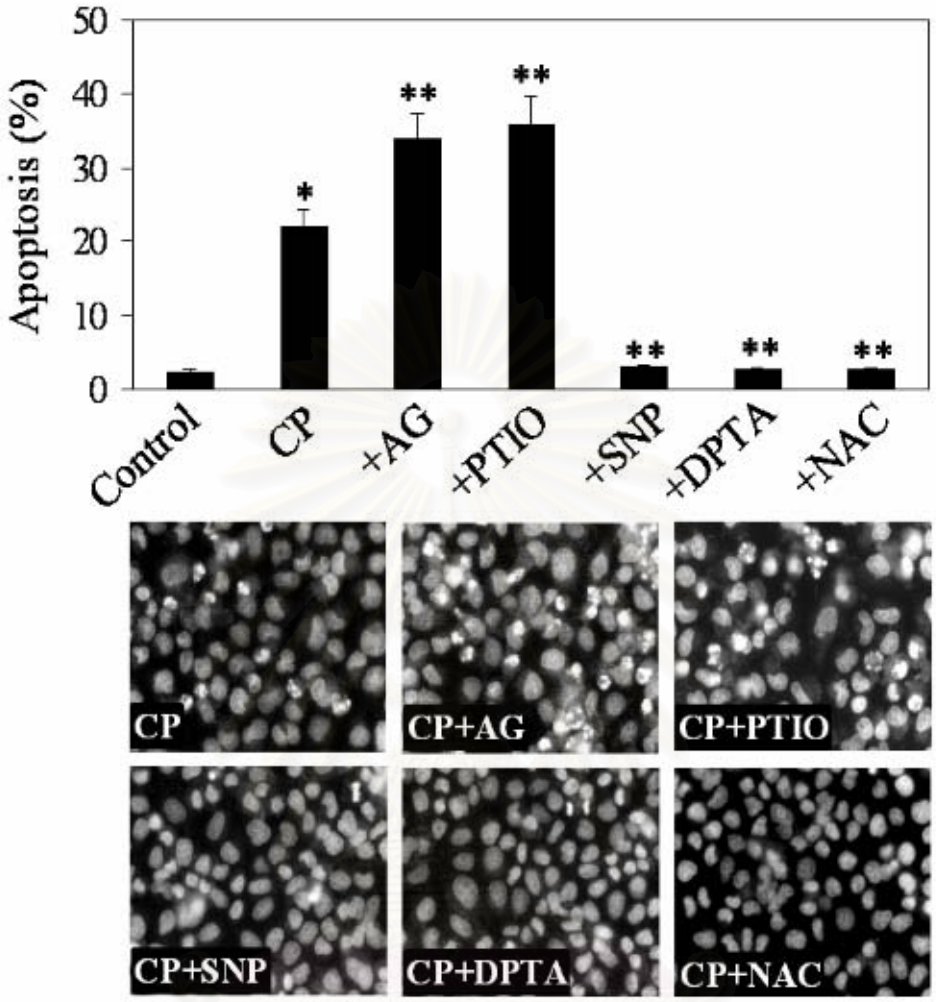


Figure 28 Effects of NO and ROS modulators on cisplatin-induced apoptosis. H460 cells were pretreated with the NO donor SNP (500 μg/ml) or DPTA NONOate (200 μM), the NO inhibitor AG (300 μM) or PTIO (300 μM), or with the ROS scavenger NAC (1 mM) for 1 h. The cells were then treated with cisplatin (100 μM) for 12 h and apoptosis was determined. Data are mean ± S.D. (n = 4). *$P < 0.05$ versus non-treated control. **$P < 0.05$ versus cisplatin-treated control.

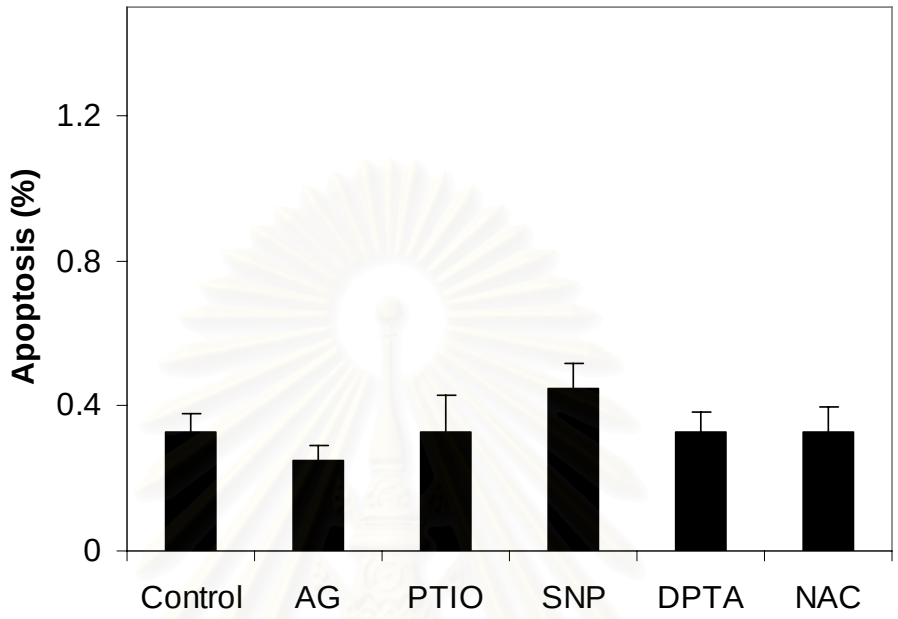


Figure 29 Effects of NO and ROS modulators on cell apoptosis. H460 cells were treated with the NO donor SNP (500 μg/ml) or DPTA NONOate (200 μM), the NO inhibitor AG (300 μM) or PTIO (300 μM), or with the ROS scavenger NAC (1 mM) for 12 h and apoptosis was determined by Hoechst 33342 nuclear staining method. Data are mean ± S.D. (n = 4).

6.1.3 Effects of NO modulators and antioxidant on NO and ROS levels in H460 cells

To provide a relationship between the apoptotic response and NO or ROS modulation by the test agents, the cells were treated with cisplatin and NO or ROS modulators, and their effects on intracellular NO and ROS levels were determined by flow cytometry using the specific NO fluorescent probe DAF-DA and specific ROS probe DCF-DA. The NO donor SNP significantly increased the cellular level of NO whereas the NO inhibitor AG inhibited it, as indicated by the corresponding changes in DAF fluorescence intensity. Treatment with cisplatin decreased NO level to 0.49 fold (0.49 ± 0.08) comparing to the non-treated control. Addition of SNP significantly increased NO level up to 4.12 folds (4.12 ± 0.27 folds). Whereas addition of AG significantly decreased NO level to 0.17 ± 0.02 fold (figure 30).

The antioxidant NAC completely inhibited cisplatin-induced ROS generation as demonstrated by the decreased DCF fluorescence intensity (figure 31). Treatment of the cells with cisplatin increased the ROS level over non-treated controls (7.56 ± 0.72 folds). Addition of NAC completely blocked cisplatin-induced ROS generation to 0.26 fold (0.26 ± 0.00 fold) (figure 31).

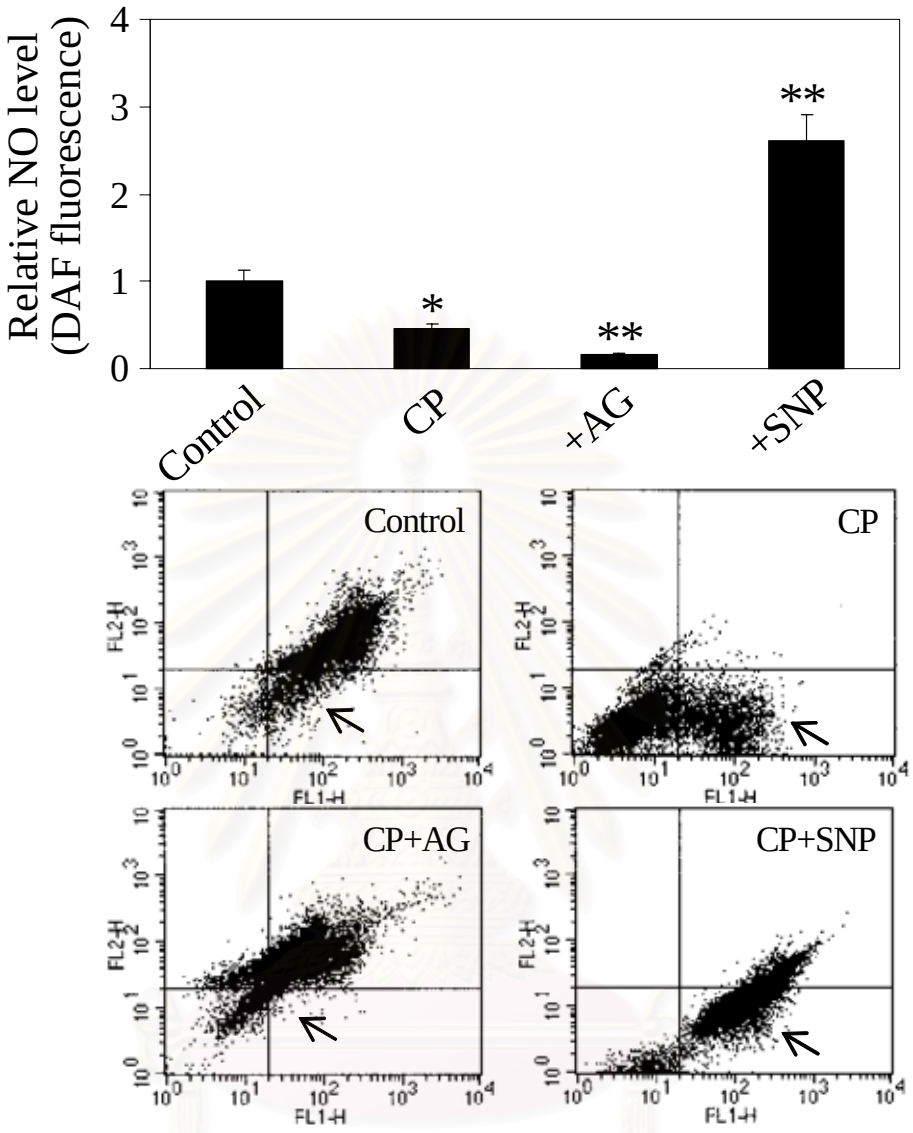


Figure 30 Effects of cisplatin and NO modulators on intracellular NO levels. Subconfluent (90%) monolayers of H460 cells were either left untreated or pretreated with the NO inhibitor AG (300 μM) or NO donor SNP (500 μg/ml) for 1 h, after which they were treated with cisplatin (200 μM) and analyzed for NO levels by flow cytometry using the fluorescent probe DAF-DA as described in the methods section. The plots show peak NO response at 1 h post-cisplatin treatment. Representative results are shown with the corresponding region indicated by an arrow. Data are mean ± S.D. (n = 4). *P < 0.05 versus non-treated control. **P < 0.05 versus cisplatin-treated control.

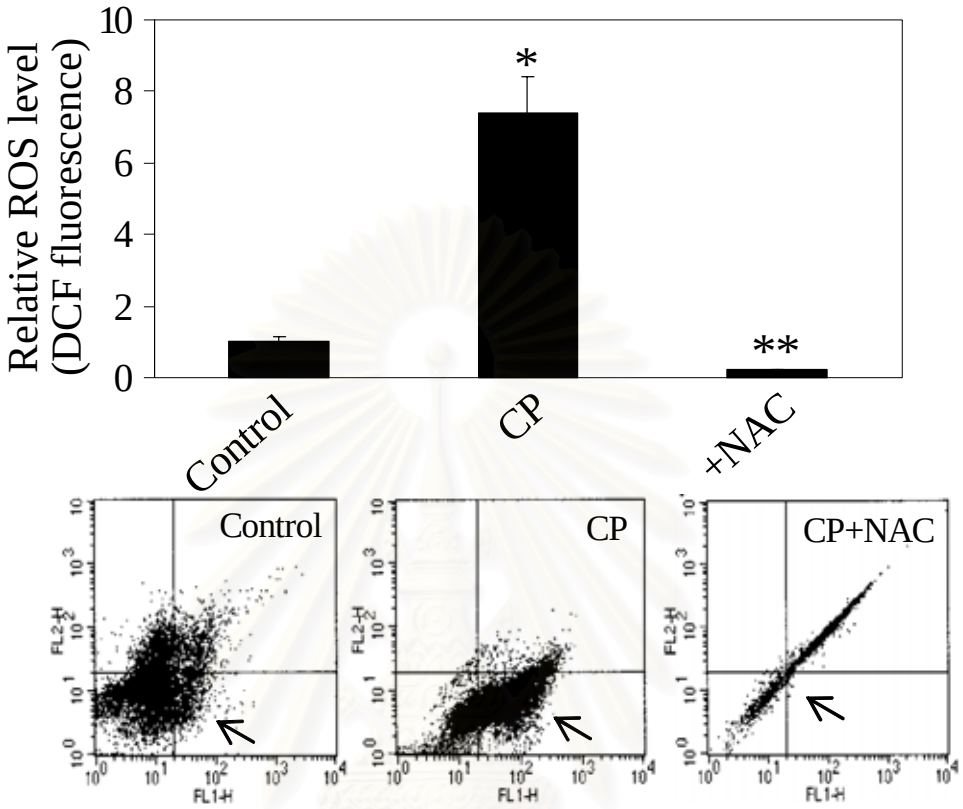


Figure 31 Effects of cisplatin and antioxidant NAC on intracellular ROS levels. Subconfluent (90%) monolayer of cells were pretreated with the ROS scavenger NAC (1 mM) for 1 h and then exposed to cisplatin (200 μM) for 1 h. ROS production was determined by flow cytometry using the fluorescent oxidative probe DCF-DA. Data are mean ± S.D. (n = 4). *P < 0.05 versus non-treated control. **P < 0.05 versus cisplatin-treated control.

6.1.4 Effect of Bcl-2 overexpression on cell death resistance to cisplatin in H460 cells

Anti-apoptotic Bcl-2 overexpression has been shown to mediate cisplatin resistance in many cell types (Li et al., 2004; Raspollini et al., 2004; Yang et al., 2004; and Kausch et al., 2005). To investigate whether Bcl-2 is involved in apoptosis resistance to cisplatin in H460 cells, the cells stably transfected with Bcl-2 or control plasmid (mock transfection) as described in materials and methods and their effect on cisplatin-induced apoptosis was determined by Hoechst 33342 assay.

Figure 32A showed that overexpression of Bcl-2 significantly inhibited apoptosis over a wide concentration range of cisplatin treatment as compared to mock transfection controls. In cisplatin concentration of 50 μM, the Bcl-2 overexpresed cells exhibited apoptotic cells only 3% (3.12 ± 0.25 %) comparing to 10 % in control cells. At the highest cisplatin concentration (500 μM), the Bcl-2 overexpresed cells exhibited apoptotic cells only 22% (21.98 ± 3.02 %) comparing to approximately 59 % in control cells.

The stable Bcl-2 transfected H460 cells exhibited the Bcl-2 overexpression over the control cells (mock transfected cells). Western blot analysis of Bcl-2 showed an increased expression of the protein in Bcl-2-transfected cells but not in mock transfected cells. Lane 1 of figure 32B showed the normal Bcl-2 level in mock transfection (control plasmid transfection). Dramatically increased in Bcl-2 level was occurred in Bcl-2 transfected cells (Lane 2 of figure 32B). Also, the blot was reprobed with anti-β-actin and anti-myc antibody to confirm the equal amount of loaded proteins and transfection efficiency, respectively.

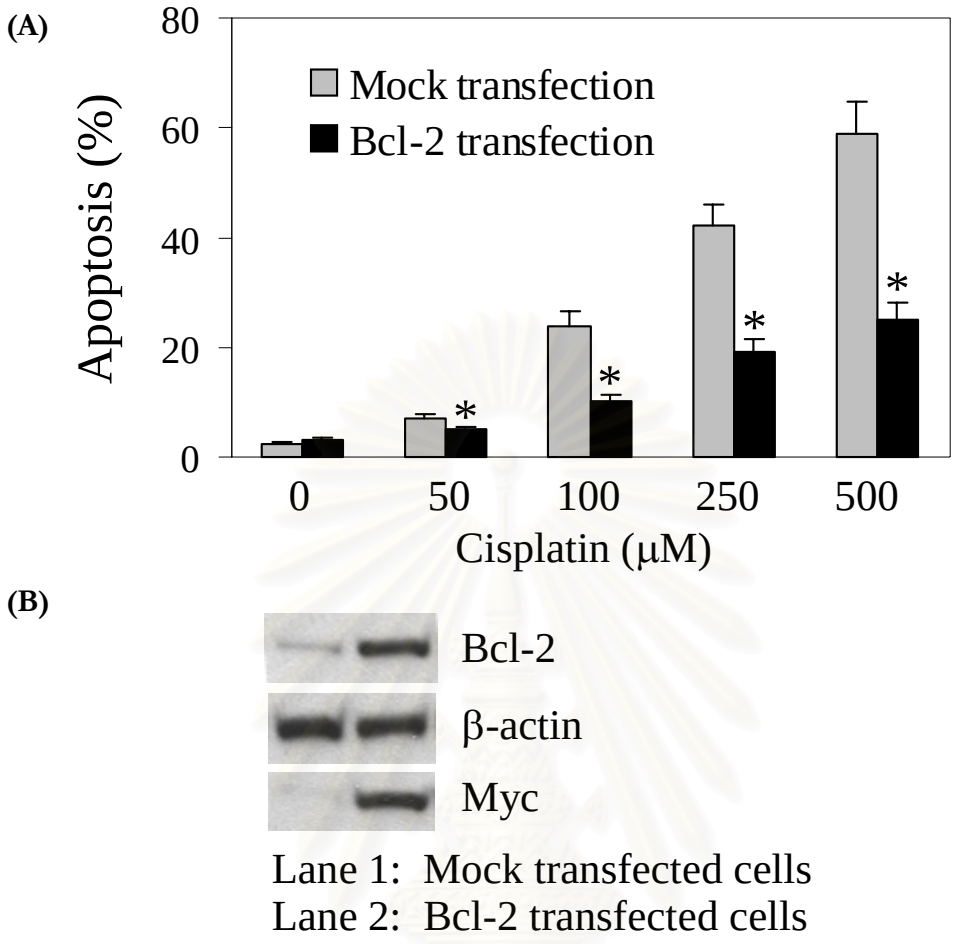


Figure 32 Effect of Bcl-2 overexpression on cisplatin-induced cell apoptosis. (A) H460 cells were stably transfected with a myc-tagged Bcl-2 plasmid or a control pcDNA3 plasmid as described in the Methods section. Transfected cells were treated with increasing doses of cisplatin (0, 50, 100, 250, 500 μM) for 12 h and apoptosis was determined by Hoechst 33342 assay. *$P < 0.05$ versus mock transfection. (B) Western blot analysis of Bcl-2 expression in mock and Bcl-2 transfected H460 cells. Cell extracts were prepared and separated on 10% polyacrylamide-SDS gels, transferred, and probed with Bcl-2 and myc antibodies. β-actin was used as a loading control.

6.1.5 Effects of NO modulators and proteasome inhibitors on Bcl-2 expression in H460 cells

The potential regulation of Bcl-2 by NO in H460 cells were further investigated. Cells were pretreated with cisplatin in the presence or absence of NO modulators, and Bcl-2 expression was determined by Western blot analysis.

Cisplatin treatment caused a significant decease in Bcl-2 expression (0.48 ± 0.17 fold reduction) comparing to the non-treated control. Co-treatment with cisplatin and NO inhibitors (AG and PTIO) enhanced cisplatin-induced Bcl-2 downregulation (0.17 and 0.14 fold in response to AG and PTIO treatments, respectively). In contrast, pretreatment with the NO donor SNP or DPTA NONOate completely inhibited cisplatin-induced Bcl-2 downregulation and further increased the Bcl-2 expression over non-treated control level (1.68 ± 0.39 and 1.97 ± 0.41 folds in response to SNP and DPTA NONOate treatments, respectively). (figure 33)

Importantly, the results showed that addition of the proteasome inhibitors lactacystin completely inhibited Bcl-2 downregulation induced by cisplatin (1.71 ± 0.34 folds). Since ROS has been demonstrated to be involved in Bcl-2 degradation processes, this study also investigated the effect of antioxidant NAC on Bcl-2 expression. Figure 34 showed that the antioxidant NAC was able to inhibit Bcl-2 downregulation by cisplatin (1.9 ± 0.38 folds).

In addition, Bcl-2 has also been reported to be enzymatically cleaved by caspase-3 in some cells (Zhang et al., 1999). To investigate whether this process might be involved in cisplatin-induced Bcl-2 degradation in H460 cells, the cells were treated with caspase inhibitor, zVAD-fmk, and studied its effect on Bcl-2 expression by immunoblotting. Figure 34 shows that the caspase inhibitor was unable to inhibit Bcl-2 downregulation induced by cisplatin (0.41 ± 0.03 folds).

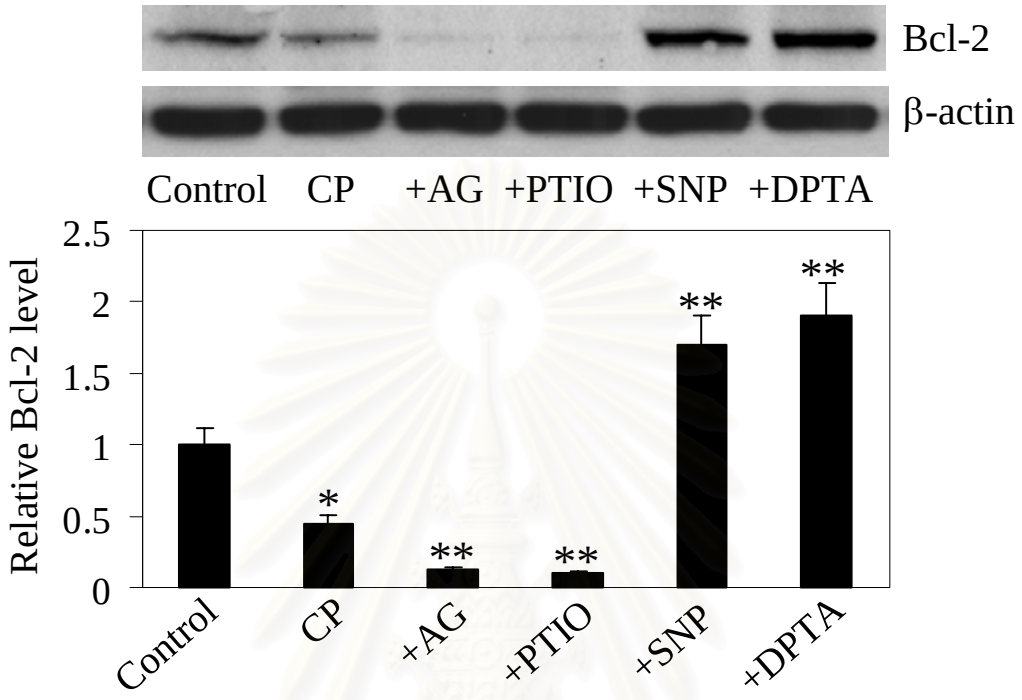


Figure 33 Effect of NO modulators on cisplatin-induced Bcl-2 downregulation. Subconfluent (90%) monolayers of H460 cells were pretreated for 1 h with the NO inhibitor AG (300 μM) or PTIO (300 μM), or with the NO donor SNP (500 μg/ml) or DPTA NONOate (200 μM). The cells were then treated with cisplatin (200 μM) for 12 h and cell extracts were prepared and analyzed for Bcl-2 by immunoblotting. Blots were reprobed with β-actin antibody to confirm equal loading of samples. The immunoblot signals were quantified by densitometry and mean data from independent experiments were normalized to the result obtained in cells in the absence of cisplatin (control). Plots are mean ± S.D. (n = 4). *P < 0.05 versus non-treated control, **P < 0.05 versus cisplatin-treated control.

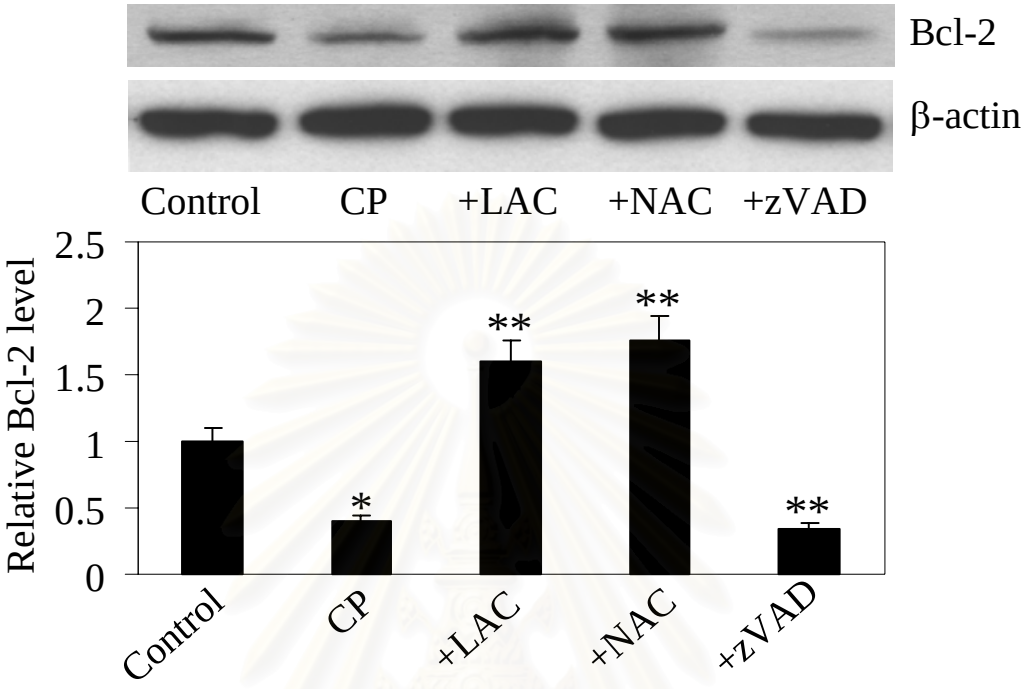


Figure 34 Effect of proteasome inhibitor lactacystin and antioxidant NAC on cisplatin-induced Bcl-2 downregulation. Cells were pretreated with the proteasome inhibitor lactacystin (20 μM), antioxidant NAC (1 mM), or caspase inhibitor zVAD-fmk (10 μM) for 1 h, followed by cisplatin treatment (200 μM) for 12 h. Cell lysates were prepared and analyzed for Bcl-2. Plots are mean ± S.D. (n = 4). *P < 0.05 versus non-treated control, **P < 0.05 versus cisplatin-treated control.

6.1.6 Effects of NO modulators and antioxidant on Bcl-2 phosphorylation at Ser87

Previous studies had shown that the Ser 87 phosphorylation of Bcl-2 inhibited Bcl-2 ubiquitin-proteasomal degradation, and dephosphorylation at this Ser 87 was required for Bcl-2 degradation process (Dimmeler et al., 1999; and Breitschopf et al., 2000). To investigate the potential mechanism of NO regulation of Bcl-2, the effect of NO modulators on Bcl-2 phosphorylation in cisplatin-treated cells were determined.

As control, cells were treated with the ROS scavenger NAC and its effect on Bcl-2 phosphorylation was determined by Western blotting with anti-phospho-Bcl-2 (Ser 87) antibody. The results showed that cisplatin strongly induced Bcl-2 dephosphorylation at Ser 87 (0.17 ± 0.02 fold reduction) and NAC completely inhibited this dephosphorylation to approximately 1.42 ± 0.36 folds (figure 35). In contrast, neither of the NO donors (SNP and DPTA NONOate) nor NO inhibitors (AG and PTIO) was able to significantly affect this dephosphorylation (figure 35).

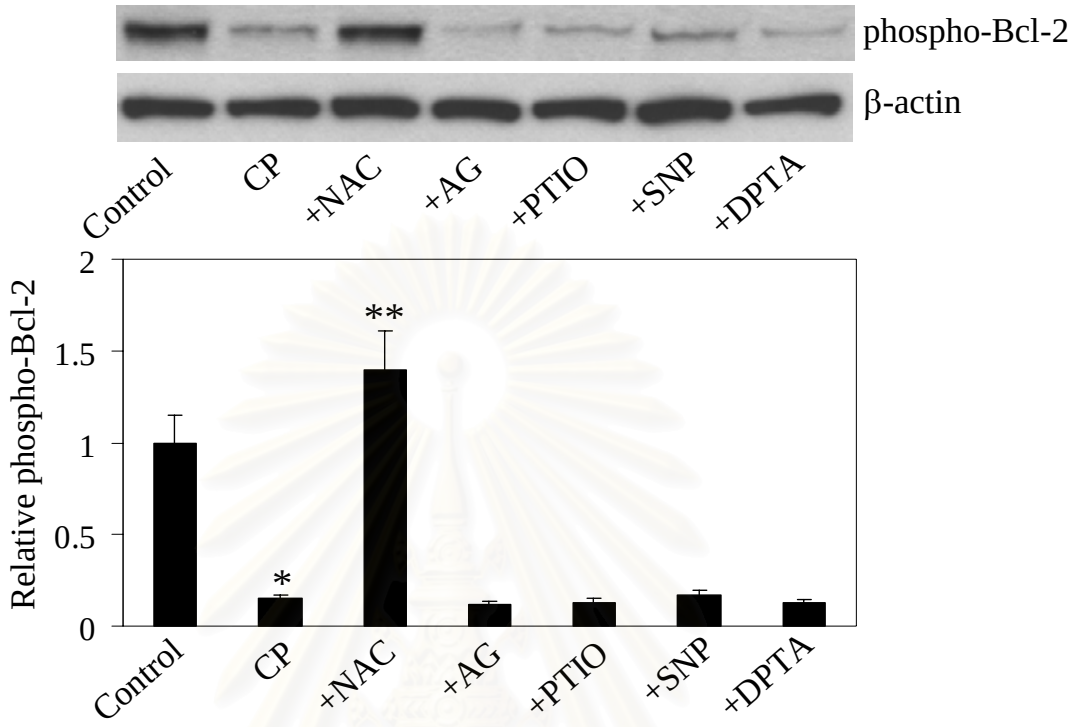


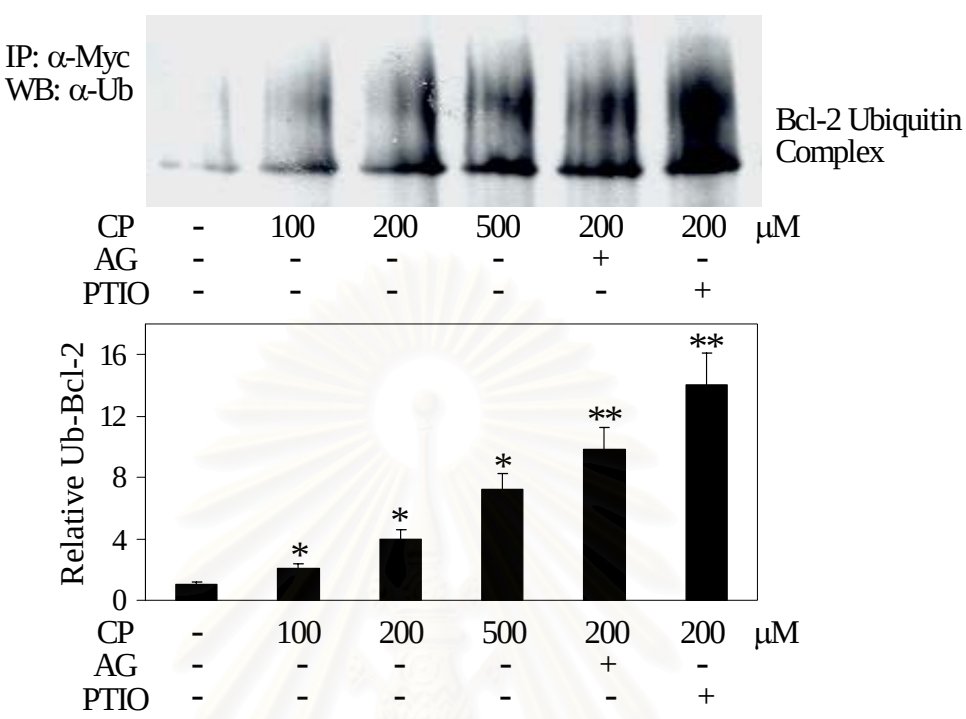
Figure 35 Effects of NO and ROS Modulators on Bcl-2 phosphorylation (Ser87) in H460 cells. Subconfluent (90%) monolayers of H460 cells were pretreated for 1 h with one of the following agents: SNP (500 μg/ml), DPTA NONOate (200 μM), AG (300 μM), PTIO (300 μM), and NAC (1 mM). The cells were then treated with cisplatin (200 μM) for 12 h and cell lysates were prepared and analyzed for Bcl-2 phosphorylation using phospho-specific Bcl-2 (Ser87) antibody. Densitometry was performed to determine the relative levels of Bcl-2 phosphorylation after reprobing with β-actin antibody. Plots are mean ± S.D. (n = 4). *$P < 0.05$ versus non-treated control, **$P < 0.05$ versus cisplatin-treated control.

6.1.7 Effect of NO modulators on cisplatin-induced ubiquitination of Bcl-2.

To further investigate the mechanism by which NO protects cisplatin-induced Bcl-2 degradation, this study investigate the effect of NO on ubiquitination of Bcl-2 in response to cisplatin treatment.

The results demonstrated that cisplatin (0, 100, 200, and 500 μM) were able to induce ubiquitination of Bcl-2 in a dose-dependent manner (1.91 ± 0.23, 4.09 ± 0.45, and 7.67 ± 0.98 folds in response to cisplatin concentration 100, 200, and 500 μM, respectively) and that the NO inhibitors AG and PTIO promoted ubiquitination of Bcl-2 to 10.78 ± 1.21 and 14.13 ± 2.08, respectively (figure 36A). In contrast, the NO donors SNP and DPTA NONOate completely inhibited ubiquitination of Bcl-2 to 0.92 ± 0.18 and 0.91 ± 0.16 fold comparing to cisplatin-treated control 3.82 ± 0.42 folds (figure 36B).

(A)


IP: α-Myc
WB: α-Ub
Bcl-2 Ubiquitin Complex
CP - 100 200 500 200 200 μM
AG - - - - + -
PTIO - - - - - +
Relative Ub-Bcl-2
0
4
8
12
16
*
*
*
**
**
CP - 100 200 500 200 200 μM
AG - - - - + -
PTIO - - - - - +

(B)

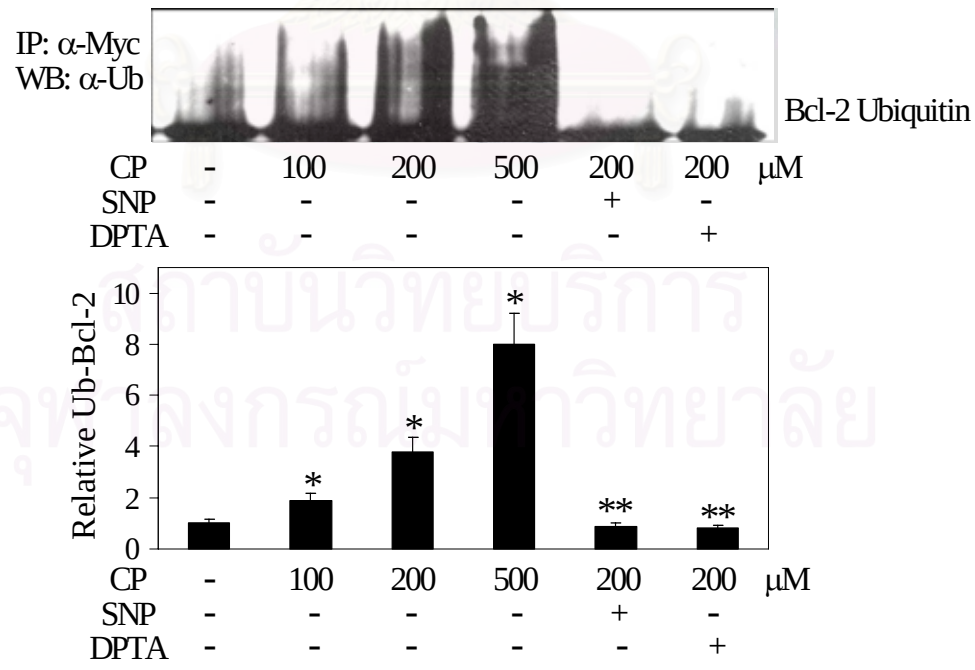

IP: α-Myc
WB: α-Ub
Bcl-2 Ubiquitin
CP - 100 200 500 200 200 μM
SNP - - - - + -
DPTA - - - - - +
Relative Ub-Bcl-2
0
2
4
6
8
10
*
*
*
**
**
CP - 100 200 500 200 200 μM
SNP - - - - + -
DPTA - - - - - +

Figure 36 Effect of NO modulators on cisplatin-induced ubiquitination of Bcl-2. Subconfluent (90%) monolayers of H460 cells were transiently transfected with ubiquitin and myc-tagged Bcl-2 plasmids. Thirty six hours later, the cells were treated with the NO inhibitor AG (300 μM) or PTIO (300 μM), or with the NO donor SNP (500 μg/ml) or DPTA NONOate (200 μM) for 1 h. Subsequently, the cells were treated with cisplatin (100-500 μM) in the presence of lactacystin (10 μM) to prevent proteasome-mediated degradation of Bcl-2. Cell lysates were immunoprecipitated with anti-myc antibody and the immune complexes were analyzed for ubiquitin by Western blotting. Analysis of ubiquitin was performed at 2 h post-cisplatin treatment where ubiquitination was found to be maximum. Data are mean ± S.D. (n = 4). *$P < 0.05$ versus non-treated control. **$P < 0.05$ versus cisplatin-treated control.

6.1.8 Effect of NO modulators on Bcl-2 S-nitrosylation and ubiquitination.

Increasing evidence indicates that NO plays an important role in apoptosis through S-nitrosylation of key apoptosis regulatory proteins (Li et al., 1997; and Kim et al., 1997). To determine whether NO could nitrosylate Bcl-2, which has not been demonstrated, and whether this process could affect Bcl-2 stability, the immunoprecipitation experiment was performed to evaluate the effect of NO on Bcl-2 S-nitrosylation.

Cells expressing ectopic myc-Bcl-2 were treated with cisplatin and NO modulators as described, and cell lysates were immunoprecipitated and analyzed by Western blot using an anti-S-nitrosocysteine antibody.

Figure 37 showed that treatment of the cells with cisplatin resulted in a reduction in S-nitrosylated Bcl-2 levels and co-treatment of the cells with the NO donor SNP or DPTA NONOate reversed this effect. Treatment with cisplatin decreased S-nitrosylated Bcl-2 to 0.32 ± 0.03 fold, and addition of SNP or DPTA NONOate reversed this effect up to 1.22 ± 0.34 and 1.19 ± 0.33 folds, respectively. Since S-nitrosylation by NO has been reported to be inhibited by strong reducing agents such as dithiothreitol (DTT) (Ryua et al., 2004; and Moon et al., 2005), this study further tested whether DTT could prevent the effect of NO on Bcl-2 S-nitrosylation. The results showed that treatment with DTT together with NO donors significantly decreased S-nitrosylation level.

Moreover, the same cell lysates were immunoprecipitated and analyzed by Western blot using anti-ubiquitin antibody for ubiquitination investigation. The results showed that treatment with cisplatin dramatically increased Bcl-2 ubiquitination (3.5 ± 0.33 folds) comparing to the non-treated control (figure 38). Addition of NO donors SNP or DPTA NONOate clearly decreased cisplatin-induced ubiquitination (1.12 ± 0.03 and 0.87 ± 0.28 folds, respectively). Importantly, DTT was able to reverse the effect of NO donors SNP and DPTA NONOate in cisplatin-treated cells (3.88 ± 0.36 and 2.97 ± 0.32 folds, respectively as shown in figure 38).

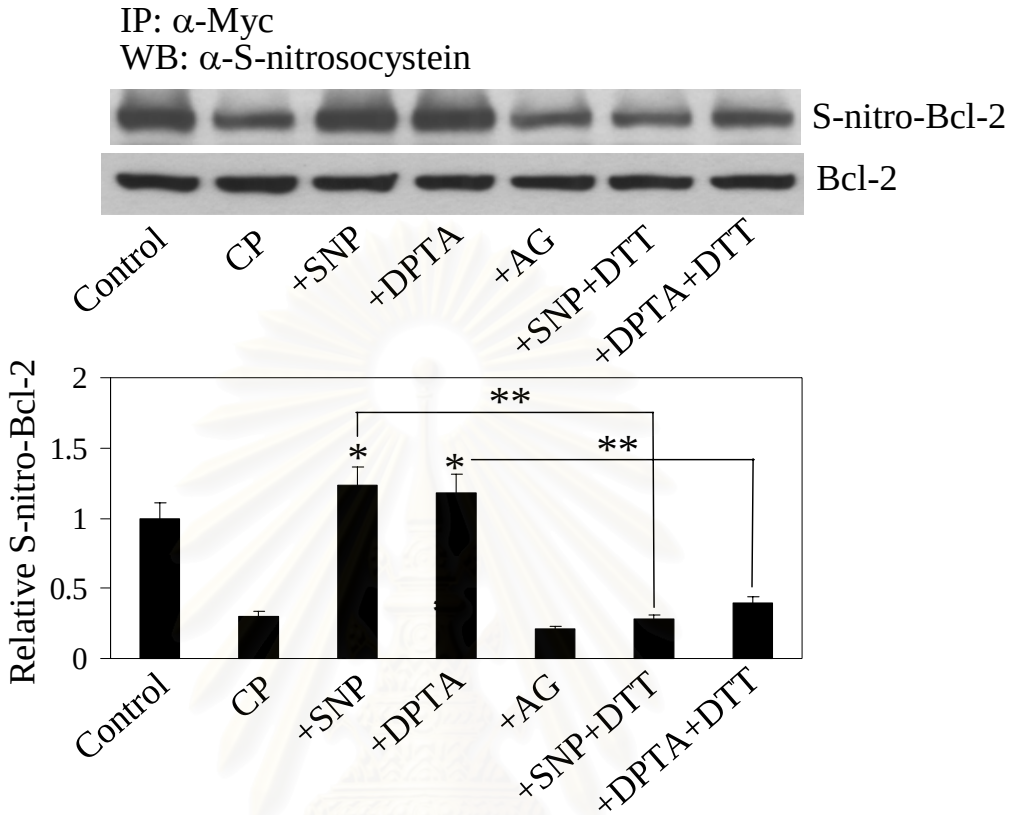


Figure 37 Effect of NO modulators on cisplatin-induced S-nitrosylation of Bcl-2. H460 cells were transiently transfected with myc-tagged Bcl-2 plasmid. Thirty six hours later, the cells were pretreated with SNP (500 μg/ml), DPTA NONOate (200 μM), or AG (300 μM) for 1 h. The cells were then treated with cisplatin (200 μM) for 2 h and cell lysates were prepared for immunoprecipitation using anti-myc antibody. The resulting immune complexes were analyzed for S-nitrosocysteine by Western blotting. The same cell lysates were subjected to Western blot analysis detected with Bcl-2 antibody to confirm the equal amount of proteins loaded to immunoprecipitation. Data are mean ± S.D. (n = 4). *$P < 0.05$ versus cisplatin-treated control. **$P < 0.05$ versus NO-modulated controls.

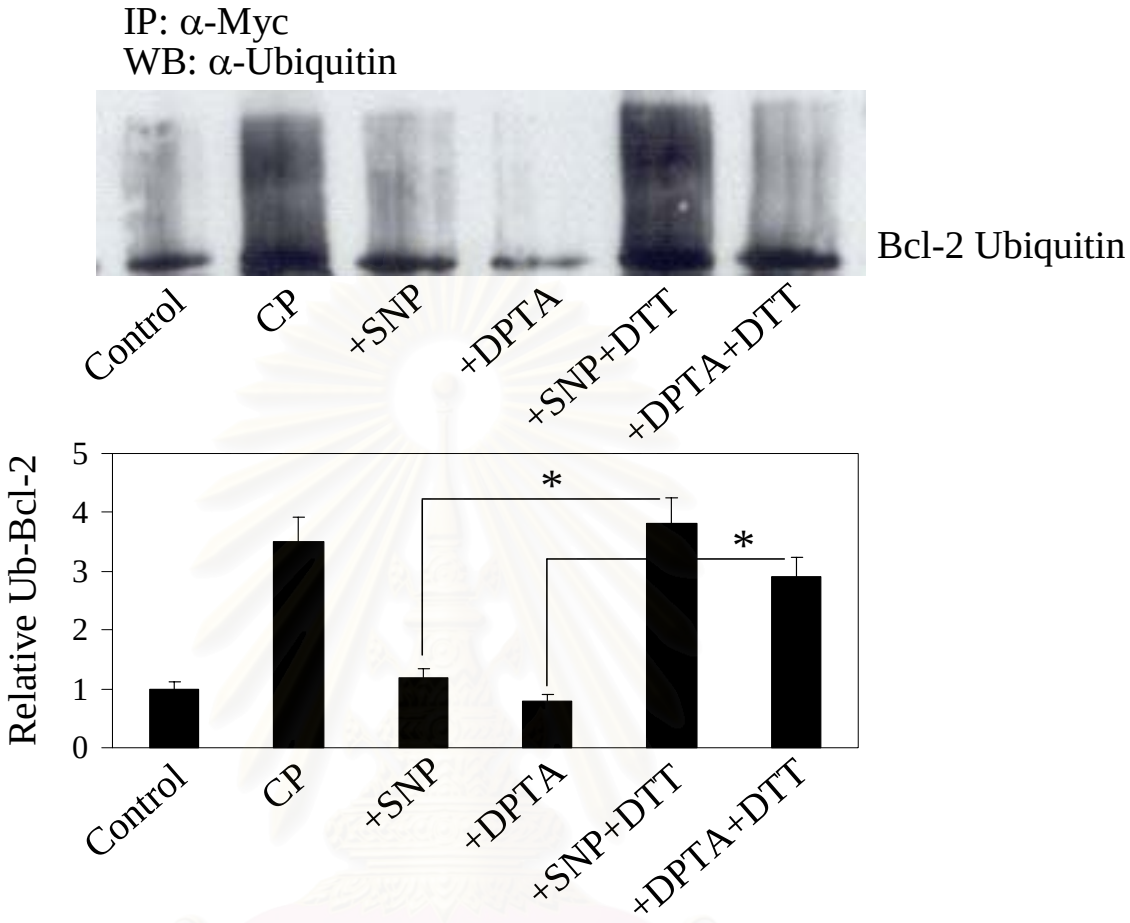


Figure 38 Effects of NO donors and DTT on cisplatin-induced Bcl-2 ubiquitination. Cells were co-transfected with ubiquitin and myc-Bcl-2 plasmids. Thirty six hours later, they were pretreated with SNP (500 μg/ml) or DPTA NONOate (200 μM) in the presence or abence of DTT (10 mM) for 1 h. The cells were then treated with cisplatin (200 μM) for 2 h. Cell lysates were then immunoprecipitated with anti-myc antibody and the immune complexes were analyzed for ubiquitin. Data are mean ± S.D. (n = 4). *P < 0.05 versus cisplatin-treated control. **P< 0.05 versus NO-modulated controls.

6.2 Mechanism of NO in controlling FasL-induced BEAS cells apoptosis.

6.2.1 FasL-induced BEAS cells apoptosis.

FasL has been reported to induce apoptosis in sensitive cells, including bronchial epithelial and lung epithelial cells (Fine et al., 1997; Xerri et al., 1997; Hamann et al., 1998; Hagimoto et al., 1999; and De Paepe et al., 2000). To study the role of NO in FasL-mediated apoptosis, cell death response to FasL treatment in lung epithelial BEAS cells was characterized using Hoechst 33342 nuclear staining assay.

Treatment of the cells with FasL caused a dose-dependent increase in cell apoptosis over control level, as indicated by increased nuclear fluorescence and chromatin condensation and fragmentation (figures 39 C, D, E, and F). Approximately 9% (8.75 ± 0.97 %) of the treated cells showed apoptotic nuclear morphology at the FasL concentration of 50 ng/ml with the cell death response exceeding 26% (26.12 ± 3.09 %) at 250 ng/ml (figure 39A).

6.2.2 FasL induces caspase-8 activation in BEAS cells.

As FasL is known to induce apoptosis through caspase-8-dependent pathway (Nagata, 1997; and Takahashi et al., 1994), the effect of FasL treatment on caspase-8 activity was investigated using fluorogenic caspase-8 substrate IETD-AMC.

The results indicated that FasL was able to increase the activity of caspase-8 in BEAS cells in a dose-dependent manner (Fig. 39B). Treatment with FasL (50 ng/ml) increased caspase-8 activity approximately 2 folds (1.98 ± 0.23 folds) comparing to the control. As increasing FasL concentration (100 and 250 ng/ml), the caspase-8 activities increased up to 4.62 ± 0.64 and 5.89 ± 0.60 folds, respectively.

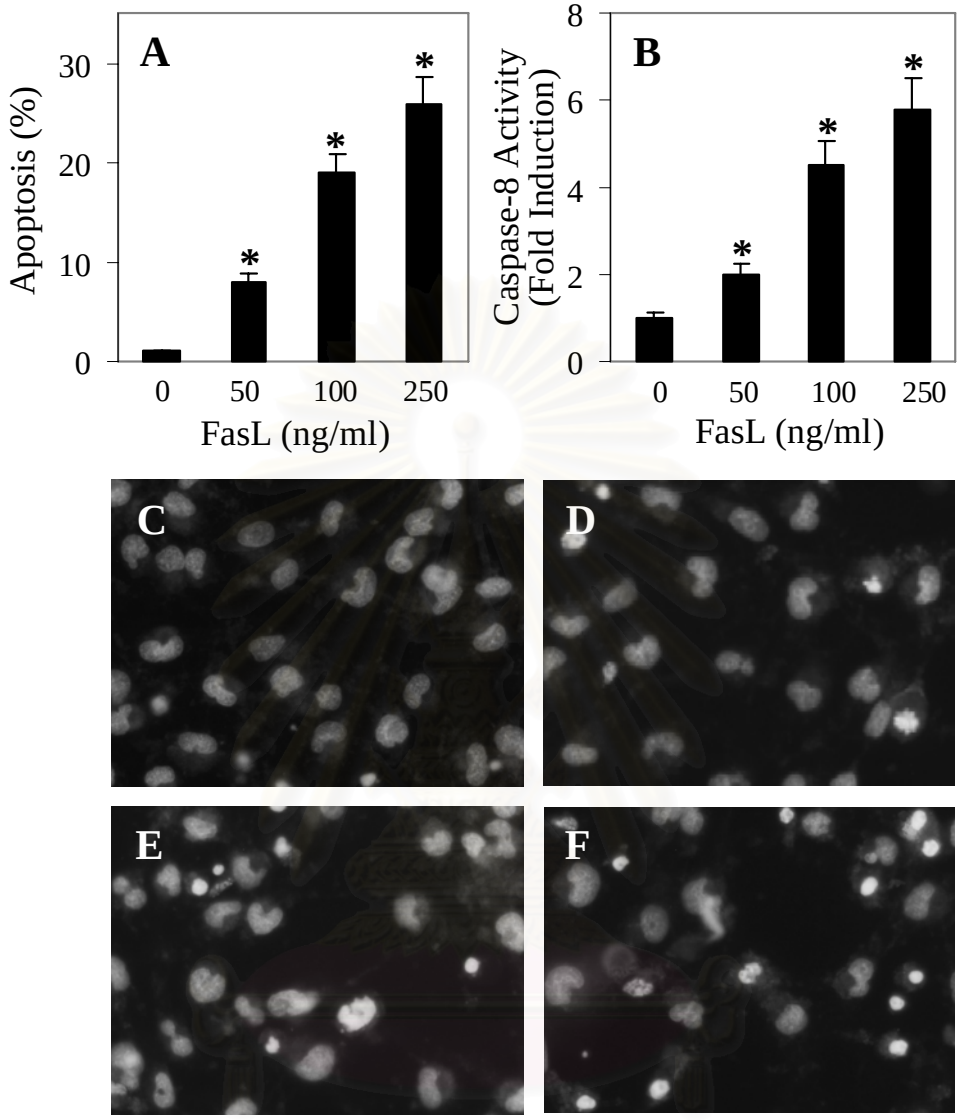


Figure 39 FasL induces apoptosis and caspase-8 activation in human lung epithelial BEAS cells. A, Subconfluent (90%) monolayers of BEAS cells were exposed to FasL (0-250 ng/ml) for 16 h and analyzed for apoptosis by Hoechst 33342 assay. B, Fluorometric assay of caspase-8 activity in cells treated with FasL (0-250 ng/ml) for 3 h. Cell lysates (50 μg protein) were prepared and determined for caspase-8 activity using the fluorometric substrate IETD-AMC. Data are mean ± S.D. (n = 4). *$P < 0.05$ versus non-treated control. C-F, Morphologic analysis of apoptosis by Hoechst assay. Cells were treated with 0, 50, 100, and 250 ng/ml of FasL, respectively, for 16 h. Apoptotic cells exhibit shrunken nuclei with bright nuclear fluorescence. (Original magnification, x400)

6.2.3 Effect of NO modulators on NO levels, FasL-induced apoptosis and caspase-8 activation in BEAS cells.

The effect of NO on FasL-induced apoptosis and caspase-8 activation was further investigated. Cell apoptosis was increased up to 18.33 ± 1.67 % in response to FasL treatment. Co-treatment of the cells with FasL and NO donor, SNP or DPTA NONOate, significantly inhibited apoptosis compared to the FasL-treated control (2.33 ± 0.23% and 1.67 ± 0.35 %, in response to SNP and DPTA NONOate treatment, respectively) (Fig. 40A). In contrast, the NO inhibitor AG or PTIO promoted this effect (31.65 ± 3.51% and 29.67 ± 3.33 % in response to AG and PTIO treatment, respectively) (Fig. 40A). Treatment with NO modulators alone at the indicated concentrations, had no significant effect on cell apoptosis and caspase-8 activity (figure 41 A and B).

To test whether the apoptosis-modulating effect of NO was mediated through the death receptor signaling pathway, cells were similarly treated with FasL and NO modulators, and cell lysates were prepared and analyzed for caspase-8 activity.

Figure 40B showed that the NO donors, SNP and DPTA NONOate, were able to inhibit caspase-8 activation by FasL, whereas the NO inhibitors, AG and PTIO, promoted the FasL effect. Treatment with FasL increased caspase-8 activity to 4.13 ± 0.52 folds comparing to non-treated control (1.00 ± 0.08). Addition of DPTA NONOate and SNP decreased caspase-8 activity to 1.16 ± 0.12 and 1.08 ± 0.10 folds. Whereas, addition of AG and PTIO increased caspase-8 activity up to 6.60 ± 0.64 and 6.53 ± 0.64 folds, respectively.

To test the effect of FasL and to determine the NO modulating effect of test agents, BEAS cells were treated with FasL in the presence or absence of various NO donors and inhibitors, and their effect on cellular NO levels was determined by flow cytometry using NO-specific probe DAF-DA. Clearly, FasL was able to increase DAF fluorescence intensity over control level (2.50 ± 0.22 folds), and the NO inhibitors AG and PTIO suppressed this signal (0.87 ± 0.05 and 0.95 ± 0.07 fold, respectively) (figure. 40C). In contrast, the NO donors SNP and DPTA NONOate promoted the NO-inducing effect of FasL (6.82 ± 0.79 and 6.05 ± 0.80 folds, respectively).

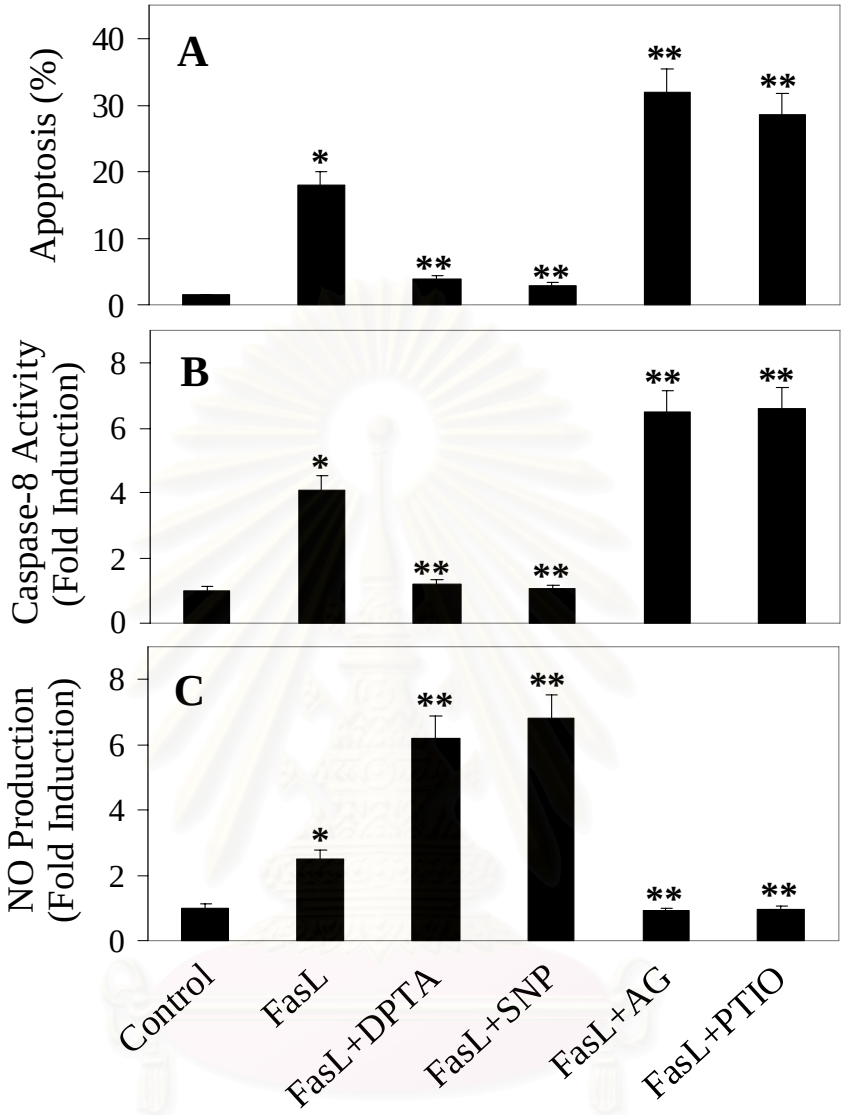
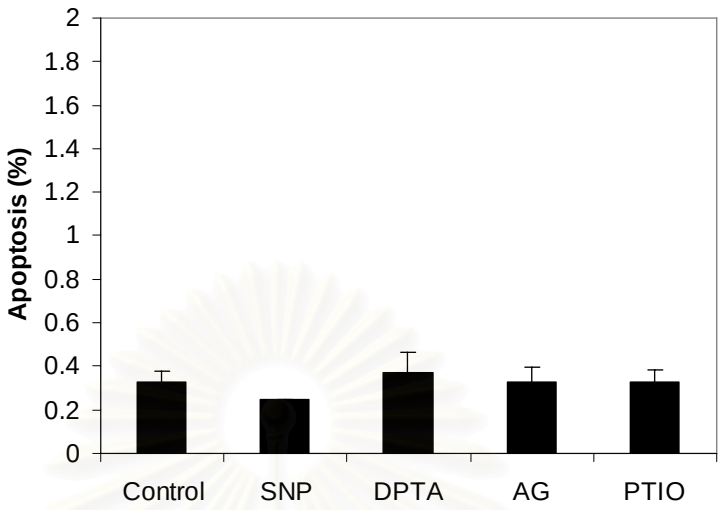


Figure 40 Effect of NO modulators on FasL-induced apoptosis, caspase-8 activation, and NO level. A, Subconfluent (90%) monolayers of BEAS cells were pretreated with the NO donor SNP (300 μg/ml) or DPTA NONOate (200 μM), or with the NO inhibitor AG (300 μM) or PTIO (300 μM) for 1 h. The cells were then treated with FasL (100 ng/ml) for 16 h and analyzed for apoptosis by Hoechst assay. B, Fluorometric analysis of caspase-8 activity determined at 3 h after FasL treatment. C, Flow cytometric analysis of NO production by DAF fluorescence at 1 h after FasL treatment. Cells were treated with FasL and NO modulators as described. Values are relative DAF fluorescence increase over control level. Plots are mean ± S.D. (n = 3). *$P < 0.05$ versus non-treated control, **$P < 0.05$ versus FasL-treated control.

(A)



(B)

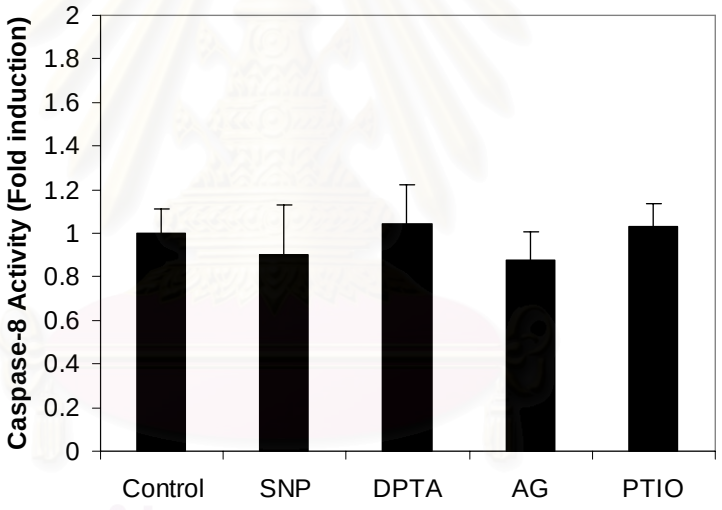


Figure 41 Effect of NO modulators on apoptosis and caspase-8 activation. (A), Subconfluent (90%) monolayers of BEAS cells were pretreated with the NO donor SNP (300 μg/ml) or DPTA NONOate (200 μM), or with the NO inhibitor AG (300 μM) or PTIO (300 μM) for 16 h. The cells were then analyzed for apoptosis by Hoechst assay. (B), Fluorometric analysis of caspase-8 activity determined at 3 h after treatment with NO modulators. Plots are mean ± S.D. (n = 3).

6.2.4 Effects of FasL and proteasome inhibitors on Fas, FADD, and FLIP expressions.

In searching for a mechanism that might explain the apoptotic regulatory effect of NO on FasL-induced cell death, this study examined by immunoblotting the expression levels of key proteins known to be relevant to the mechanisms of Fas signaling, including the Fas death receptor, the adapter protein FADD, and the anti-apoptotic FLIP which is known to bind caspase 8 and FADD and suppress apoptosis induction by death ligands (Irmler et al., 1997; and Tschopp et al., 1998).

The levels of Fas, FADD, and FLIP in responding to FasL treatment were determined by Western blot analysis using anti-Fas, anti-FADD, and anti-FLIP, respectively. Among these, only the level of FLIP was affected by the FasL treatment at the concentrations shown to induce apoptosis (figure 42A). The levels of both Fas and FADD were not significantly altered in responding to FasL treatment. Importantly, FasL caused the dose-dependent down-regulation of FLIP. Treatment of FasL at the concentration of 50 ng/ml decreased FLIP level to 0.42 ± 0.37 fold, comparing to the non-treated control (1.00 ± 0.67). FLIP level decreased to 0.21 ± 0.11 % in response to 100ng/ml FasL treatment.

Since FLIP has been reported to be downregulated via ubiquitin-proteasome pathway (Fukazawa et al., 2001; Kim et al., 2002; Perez and White, 2003; and Poukkula et al., 2005), this study investigated whether the downregulation of FLIP by FasL is also mediated by this pathway.

Figures 42 and43 showed that treatment with highly specific proteasome inhibitor lactacystin was able to inhibit FLIP downregulation (0.82 ± 0.58 fold in figure 42). The result was confirmed by the fact that another proteasome inhibitor, MG132, also inhibited the decrease in FLIP expression caused by FasL (figure 43).

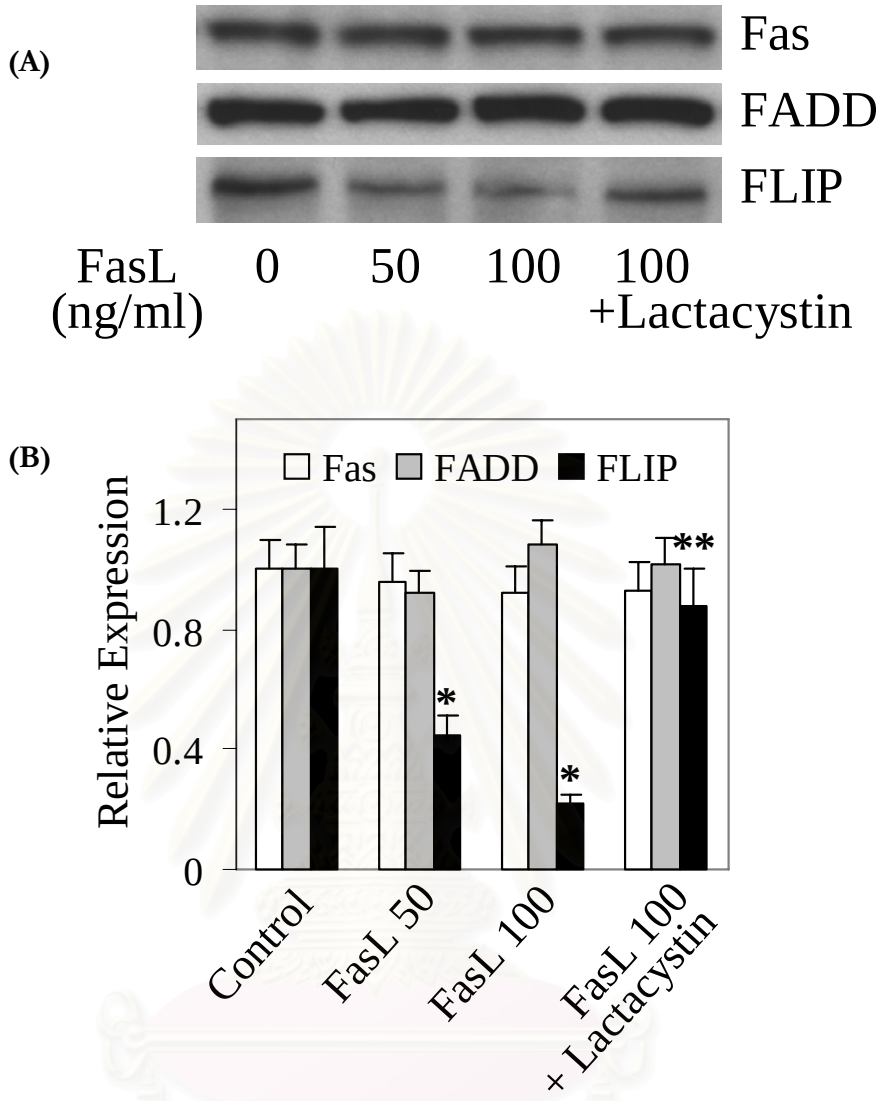


Figure 42 Effects of FasL and proteasome inhibitors on Fas, FADD, and FLIP expressions. (A), Western blot analysis of FLIP in response to FasL treatment. Subconfluent (90%) monolayers of BEAS cells were treated with FasL (0-100 ng/ml) in the presence or absence lactacystin (10 μM) for 12 h. The cells were then washed with ice-cold PBS and extracted with SDS sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with antibodies specific for Fas, FADD, and FLIP. Blots were reprobed with β-actin antibody to confirm equal loading of samples. (B), The immunoblot signals were quantified by densitometry and mean data from independent experiments (one of which is shown here) were normalized to the result obtained in cells in the absence of FasL (control). Plots are mean ± S.D. (n = 3). *$P < 0.05$ versus control, **$P < 0.05$ versus FasL-treated control.

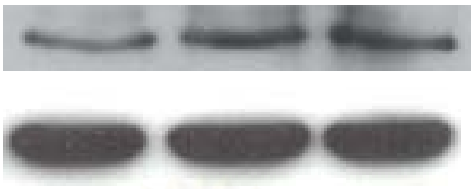


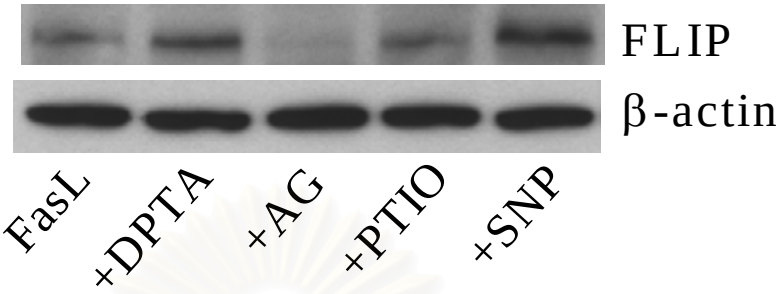
Figure 43 Effect of proteasome inhibitors on FasL-induced FLIP down-regulation. Subconfluent (90%) monolayers of BEAS cells were treated with FasL (100 ng/ml) in the presence or absence of lactacystin (10 μM) or MG132 (5 μM) for 12 h. Lysates were analyzed by Western blot using antibody against FLIP.

6.2.5 Effect of NO modulators on FasL-induced FLIP downregulation.

To test whether NO might modulate Fas signaling through FLIP, cells were treated with FasL in the presence or absence of NO donors or inhibitors, and their effect on FLIP expression levels was determined by immunoblotting.

Figure 44 showed that addition of the NO donor SNP or DPTA NONOate strongly increased FLIP levels to 1.89 ± 0.42 and 1.21 ± 0.27 folds, respectively, over FasL-treated control (0.22 ± 0.02 fold), whereas treatment with NO inhibitors AG significantly decreased FLIP levels (0.08 ± 0.01fold). Treatment with NO scavenger PTIO only slightly decreased FLIP levels (0.18 ± 0.02 fold).

(A)



(B)

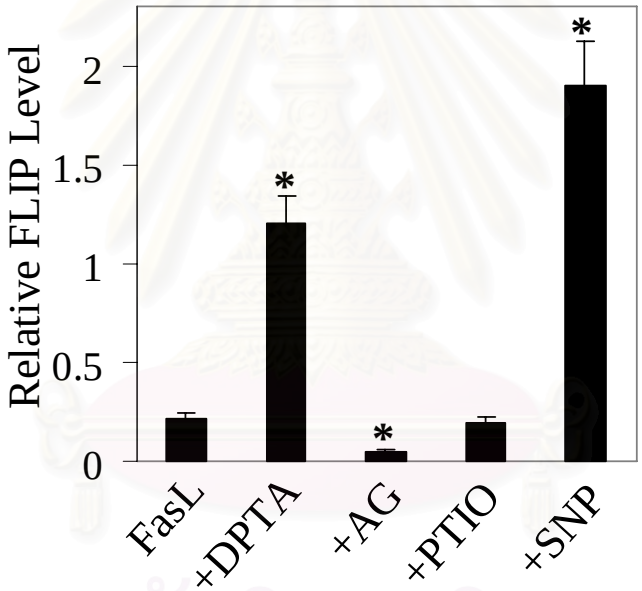


Figure 44 Effect of NO modulating agents on FasL-induced FLIP down-regulation. (A), Effect of NO modulators on FasL-induced FLIP downregulation. Cells were pretreated with the NO donor SNP (300 μg/ml) or DPTA NONOate (200 μM), or with the NO inhibitor AG (300 μM) or PTIO (300 μM), for 1 h, after which they were treated with FasL (100 ng/ml) for 12 h and analyzed for FLIP by Western blot using FLIP antibody. Blots were reprobed with antibody against β-actin (loading control). (B), The immunoblot signals were quantified by densitometry and mean ± S.D. from independent experiments $*P < 0.05$ *versus* FasL-treated control (n =3).

6.2.6 Effect of NO modulators on FLIP ubiquitination.

Because the proteasome acts on proteins destined to be degraded by ubiquitination, this study investigated whether FLIP is inducibly ubiquitinated by FasL. Immunoprecipitation studies were performed in cells transiently transfected with plasmids encoding ubiquitin and myc-tagged FLIP. The resulting immune complexes were analyzed by SDS-PAGE immunoblotting using anti-ubiquitin antibody.

Figure 45 showed that in the absence of FasL stimulation (control), minimum ubiquitinated FLIP was produced. Upon FasL treatment, the ubiquitin-FLIP conjugate was greatly increased in a time dependent manner (figure 45). The ubiquitin-FLIP conjugate could be observed as early as 1 h, peaked at 2 h, and started to decrease at 3 h after FasL treatment.

Pre-treatment of the cells with NO donors, SNP or DPTA NONOate, potently inhibited FasL-induced ubiquitination of FLIP, whereas the NO inhibitors AG and PTIO enhanced this ubiquitination process (figure 45).

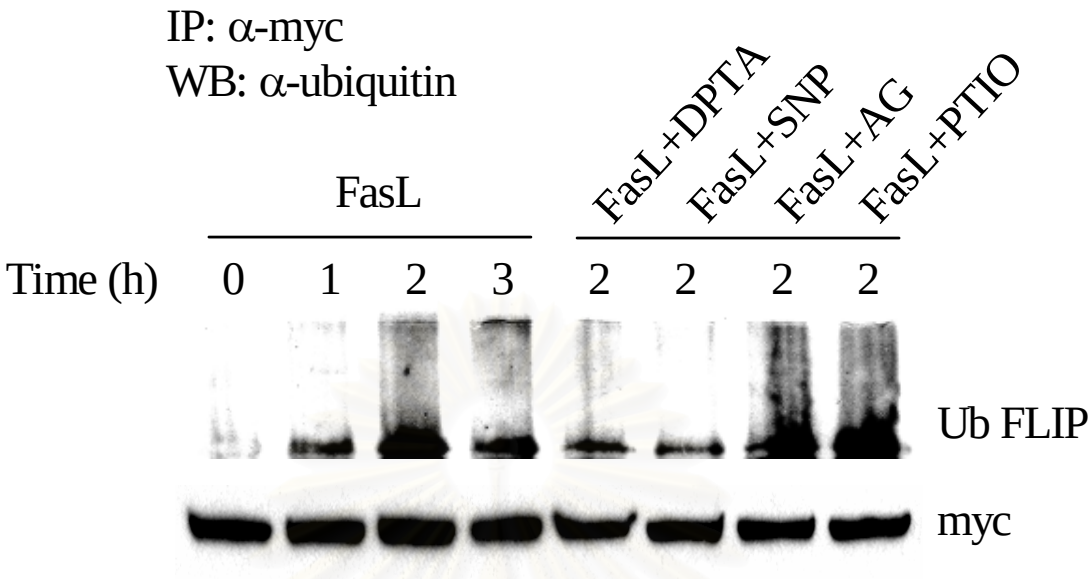


Figure 45 Effect of NO modulators on FLIP ubiquitination. BEAS cells were transiently transfected with myc-FLIP and ubiquitin plasmids and then treated 1 day later with FasL (100 ng/ml) for the indicated times in the presence or absence of SNP (300 μg/ml), DPTA NONOate (200 μM), AG (300 μM), or PTIO (300 μM). Lysates were immunoprecipitated by incubation with 12 μl of anti-myc agarose bead diluted with 12 μl sepharose for 6 h at 4^{o}C. The beads were washed, boiled, and subjected to 10% polyacrylamide gel electrophoresis. The separated proteins were analyzed by Western blot with antibody against ubiquitin. Blots were also probed with myc antibody to confirm equal loading of samples.

6.2.7 Effect of NO modulators on FLIP S-nitrosylation.

Increasing evidence indicate that NO plays an important role in apoptosis through S-nitrosylation of several key apoptosis-regulatory proteins (Li et al., 1997; and Kim et al., 19972). To determine whether NO could nitrosylate FLIP which has not previously been demonstrated, we performed immunoprecipitation experiments evaluating the effect NO on S-nitrosylation of FLIP.

Cells expressing ectopic myc-FLIP were treated with FasL and NO modulators, and cell lysates were immunoprecipitated and analyzed by Western blot using anti-S-nitrosocysteine antibody. Figure 46 showed that in the absence of NO modulators, FasL had minimal effect on S-nitrosylation of FLIP. However, upon addition of the NO donors, DPTA NONOate or SNP, the nitrosylated level of FLIP was strongly increased (1.81 ± 0.27 and 3.58 ± 0.38 folds in response to DPTA NONOate and SNP treatments, respectively). In contrast, the NO inhibitors, AG or PTIO, inhibited this nitrosylation (0.59 ± 0.05 and 0.61 ± 0.03 fold, respectively).

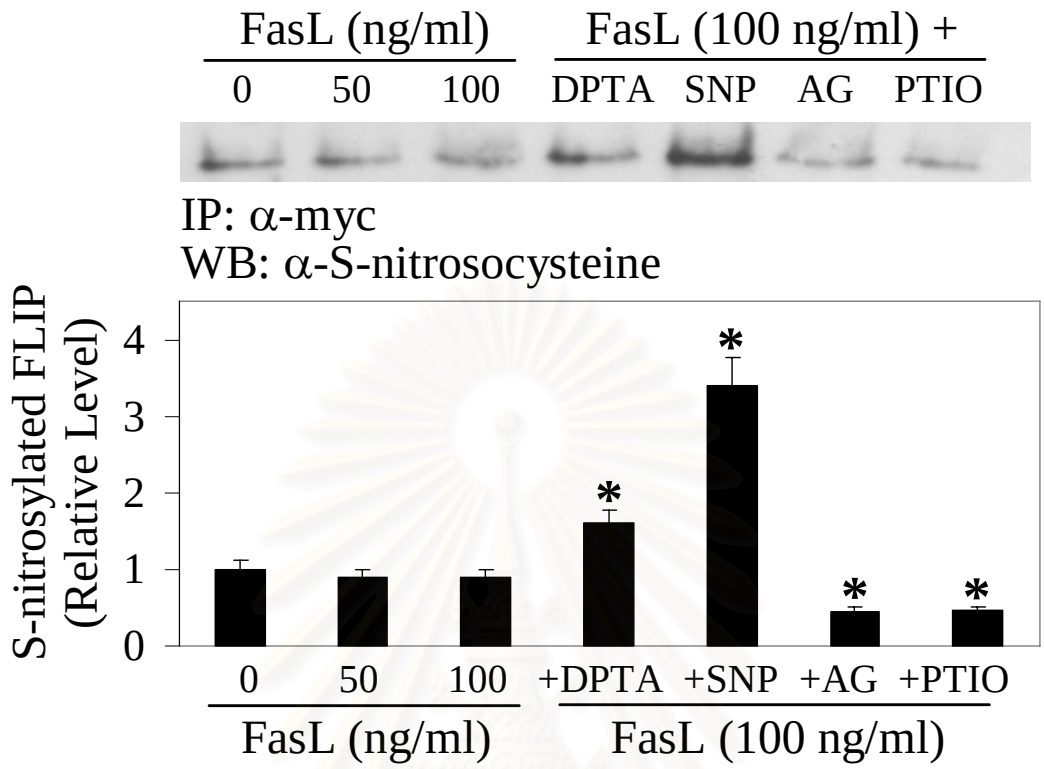


Figure 46 Effect of NO modulators on FLIP S-nitrosylation. BEAS cells were transiently transfected with myc-FLIP plasmid and treated 1 day later with FasL (0-100 ng/ml) in the presence or absence of SNP (300 μg/ml), DPTA NONOate (200 μM), AG (300 μM), or PTIO (300 μM) for 2 h. Lysates were immunoprecipitated using myc antibody and analyzed by Western blot using S-nitrosocysteine antibody. The density of S-nitrosylated FLIP bands was determined by densitometry and normalized against non-treated control band. Plots are mean ± S.D. (n = 3). *$P < 0.05$ *versus* FasL-treated control.

6.2.8 Effect of NO on S-nitrosylation and ubiquitination of FLIP deletion series.

To study the mechanism of S-nitrosylation of FLIP, this study further determined which domain(s) of FLIP was responsible for its nitrosylation. The present study constructed a series of FLIP deletion (Δ1–Δ3, as shown in figure 47) and tested their effect on S-nitrosylation by NO (figure 48).

In myc-FLIP (the wild-type FLIP) transfected cells, co-treatment with FasL and NO donor SNP significantly increased FLIP S-nitrosylation, comparing to the FasL-treated control. Partial deletion of the caspase-like domain of FLIP (amino acids 329-480, Δ1) had no effect on S-nitrosylation induced by the NO donor SNP, whereas complete deletion of the caspase-like domain (Δ2) as well as the DED2 domain (Δ3) strongly inhibited this effect when the plasmids were transfected into cells and subsequently treated with FasL (figure 48).

Immunoprecipitation and ubiquitination studies also showed that the NO donor SNP was able to inhibit FasL-induced ubiquitination of FLIP and its Δı mutant, but not the Δ2 and Δ3 mutants (figure 49), indicating the protective effect of S-nitrosylation on FLIP ubiquitination. The results of this study also suggest that the amino acid sequence difference between Δ1 and Δ2 (or the amino acid sequence 233-328 of the caspase-like domain) is essential for S-nitrosylation of FLIP.

6.2.9 Effect of cysteines 254 and 259 mutations on FLIP S-nitrosylation and FLIP ubiquitination.

To determine the target site(s) for S-nitrosylation of FLIP, we examined the amino acid sequence in the target region of caspase-like domain. Since S-nitrosylation involves the transfer of NO^+ group to an active site on cysteine residues, this study examined the presence of these residues in the target region and found two at position 254 and 259. The present study mutated the two residues (Δ4) in order to determine whether the mutations interfere with the S-nitrosylation of FLIP (figure 47).

As shown in figure 48, mutations of these cysteine residues (to alanine) resulted in a complete inhibition of FLIP nitrosylation. In myc-FLIP transfected cells, addition of NO donor SNP significantly increased FLIP S-nitrosylation. Whereas, in mutated FLIP (Δ4) transfected cells, addition of SNP showed no effect on S-nitrosylation, comparing to Fas-treated control.

Figure 49 also showed that such mutations effectively inhibited the protective effect of NO on FLIP ubiquitination. In myc-FLIP transfected cells, addition of SNP significantly inhibited FasL-induced FLIP ubiquitination, comparing to the FasL-treated control. In mutated FLIP transfected cells, addition of SNP showed no inhibition effect on FasL-induced FLIP ubiquitination.

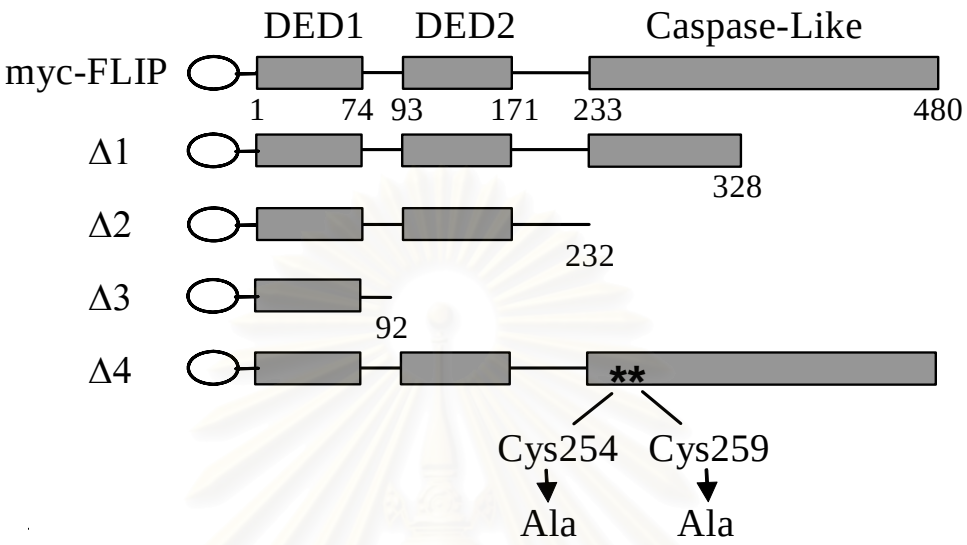


Figure 47 Schematic structures of FLIP and various constructs (Δ1-Δ4used in this study. The myc-FLIP is a wild-type FLIP tagged with myc. DED stands for death effector domain. Amino acids present in each construct are labeled. Asterisks indicate Cys254 and Cys259 to alanine mutations in the caspase-like domain.

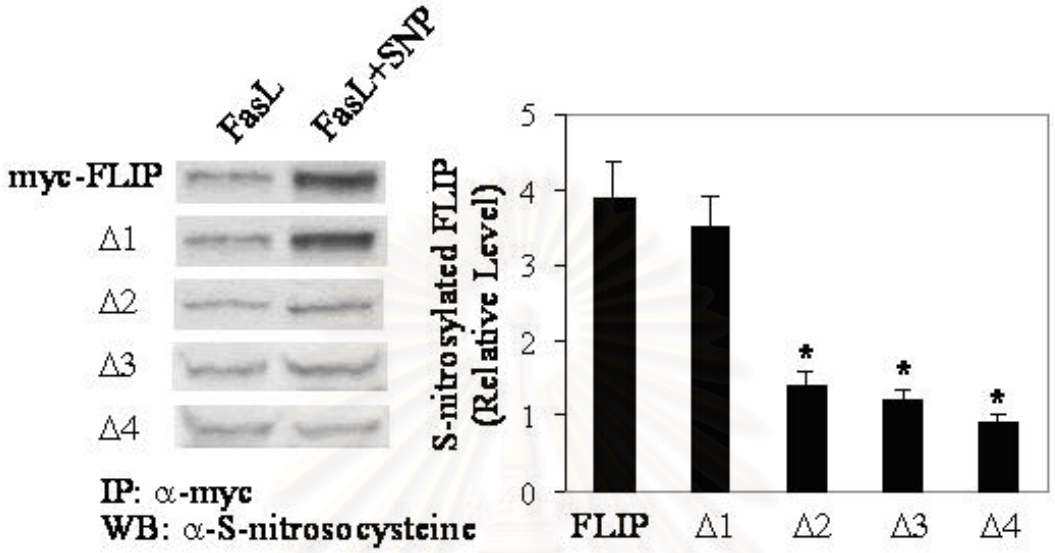


Figure 48 Effect of NO on S-nitrosylation of FLIP deletion series and mutated FLIP. S-nitrosylation of FLIP and its mutants was analyzed by transient transfection and immunoprecipitation with myc antibody as described. The density of S-nitrosylated bands was determined by densitometry and normalized against FasL-treated control bands. Plots are mean ± S.D. (n = 3). *P< 0.05 *versus* myc-FLIP-transfected control.

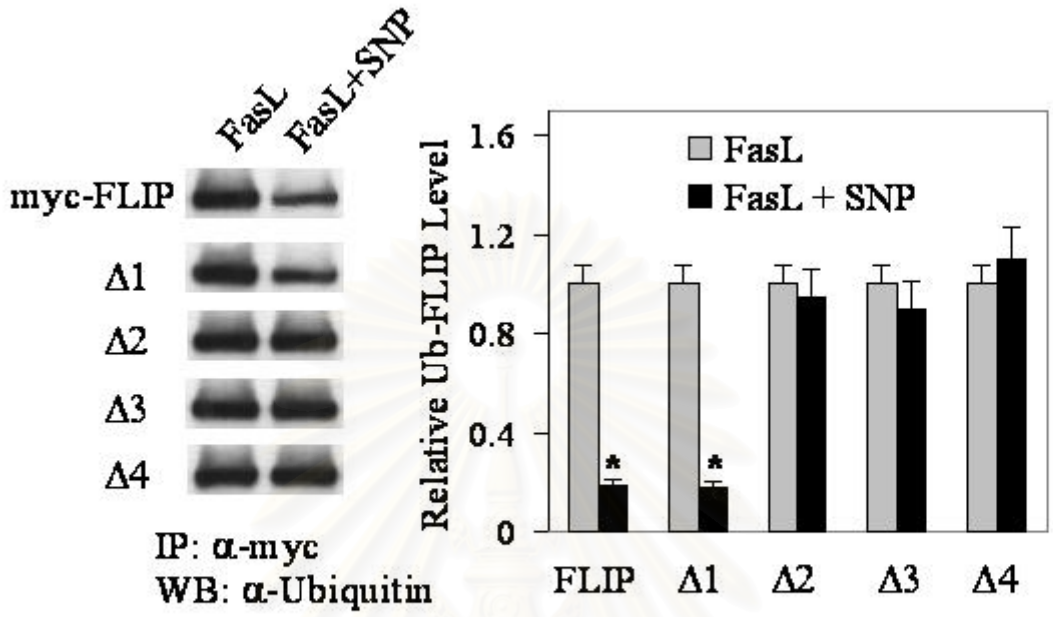


Figure 49 Effect of NO on ubiquitination of FLIP-deletion series and mutated FLIP. BEAS cells were transiently transfected with ubiquitin and myc-FLIP or its mutant plasmids. One day after the transfection, cells were treated with FasL (100 ng/ml) in the presence or absence of SNP (300 μg/ml) for 2 h and cell lysates were prepared for immunoprecipitation using myc antibody. The immunoprecipitated proteins were analyzed by Western blot with antibody against ubiquitin. Band densities were normalized against FasL-treated controls. Plots are mean ± S.D. (n = 3). **P* < 0.05 *versus* FasL-treated controls.

6.2.10 Effect of cysteines 254 and 259 mutation on FasL-Induced FLIP Degradation.

Inhibition of FLIP ubiquitination by NO should lead to a decrease in proteasomal degradation of FLIP, and likewise, failure to inhibit this ubiquitination in the FLIP Cys/Ala mutant should lead to its increased degradation. As expected, the results showed that the NO donor SNP was able to prevent FLIP degradation by FasL as indicated by its increased expression level (figure 50). On the other hand, the NO donor had no protective effect on the FLIP mutant, suggesting that S-nitrosylation of FLIP.

FasL treatment significantly downregulated FLIP levels in both wild-type FLIP and mutated FLIP (Δ4) transfected cells. Pretreatment with NO donor SNP only inhibited FasL-induced FLIP downregulation in the wild-type cells, whereas SNP could not protect FasL-induced FLIP downregulation in the mutated cells.

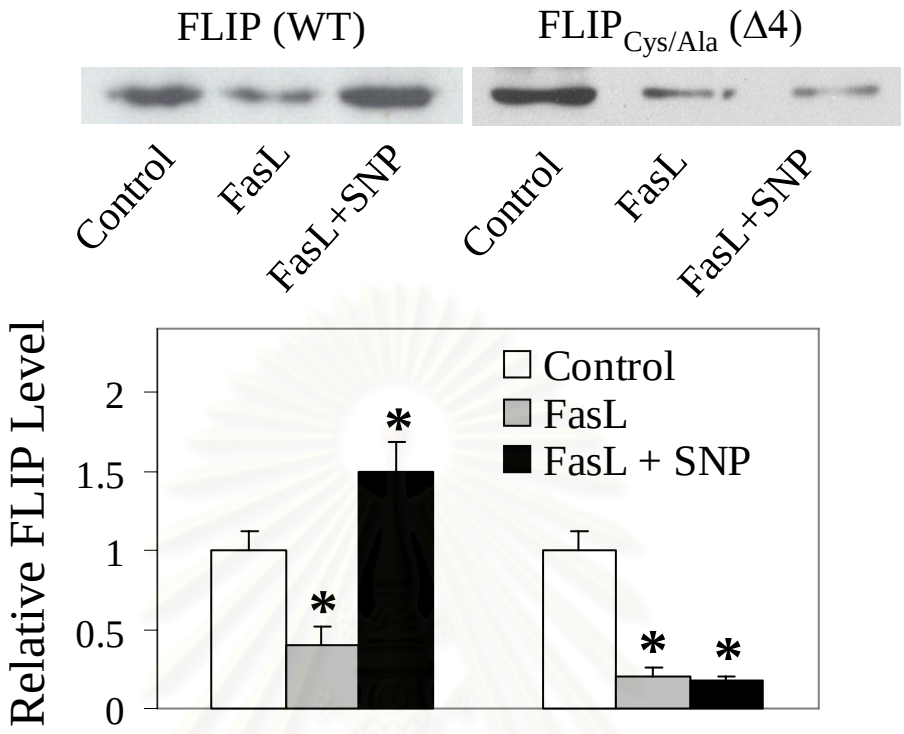


Figure 50 Effect of cysteine 254 and 259 mutations on FasL-induced FLIP degradation. BEAS cells were transiently transfected with myc-FLIP or Δ4mutant plasmid and then treated 1 day later with FasL (100 ng/ml) in the presence or absence of SNP (300 μg/ml) for 12 h. Lysates were immunoprecipitated with myc antibody and analyzed by Western blot using antibody against FLIP. Band densities were normalized against non-treated controls. Plots are mean ± S.D. (n = 3). *$P < 0.05$ *versus* non-treated controls.

CHAPTER V

DISCUSSION AND CONCLUSION

1. NO levels and cellular susceptibility to apoptosis in BEAS and H460 cells.

Previous studies showed that NO levels were elevated in lung cancer cells relative to surrounding normal cells (Liu et al., 1998; Arias-Dias et al., 1994; and Fujimoto et al., 1997). Modification of NO levels in cancer cells may be regarded as a potential means by which cancer cells escape from apoptosis and become resistant to chemotherapy. However, whether or not NO plays a role in rendering cancer cells resistant to apoptosis has not been clearly demonstrated.

In this study, the basal production of NO in cancerous H460 cells was found to be higher than that in normal BEAS cells (figure 6). Apoptosis assays revealed that H460 cells were more resistant to apoptosis induction by cisplatin compared to BEAS cells (figures 11 and 12). These findings led to the hypothesis that NO may play an anti-apoptotic role and that the observed higher level of NO in H460 cells might render these cells resistant to apoptotic cell death.

Treatment of the cells with NO inhibitors (AG and PTIO) significantly reduced the basal levels of NO in both BEAS and H460 cells (figure 13). This reduction in NO levels was associated with an increase in apoptotic cell death induced by cisplatin and FasL in both BEAS and H460 cells (figures 14-17), supporting the anti-apoptotic role of basal NO in these cells. In accordance with these results, a previous report indicated that depletion of basal NO level enhanced cisplatin-induced apoptosis in melanoma cells (Tang and Grimm, 2004).

Under physiological and pathological conditions, NO levels have been shown to be elevated in response to various stimuli, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (INF-γ) (Taylor et al., 1998; Adler et al., 1994; and Robbins et al., 1997). In particular, elevated NO levels have been demonstrated in

many lung pathologic conditions such as lung cancer, lung inflammation, and lung injury (Liu et al., 1998; Arias-Dias et al., 1994; Fujimoto et al., 1997; and Amin et al., 1995). Once NO in the microenvironment increases, it can freely diffuse across cell membrane due to its gaseous nature and increases the intracellular level of NO in the surrounding cells. To evaluate the effect of NO on cell apoptosis, specific NO donors including SNP and DPTA NONOate were used to increase the intracellular NO level and their effect on cisplatin- and FasL-induced apoptosis was determined. Indeed, elevated NO levels were detected in both BEAS and H460 cells upon treatment with the NO donors (figure 18). Such elevation was also accompanied by a decrease in cellular apoptotic response to cisplatin and FasL treatment. These results clearly demonstrated that NO was able to increase cell resistance to apoptotic cell death by cisplatin and FasL. The findings therefore confirm the anti-apoptotic role of NO and indicate the correlation between elevated NO levels and apoptosis resistance in BEAS and H460 cells.

NO has been shown to exhibit both pro- and anti-apoptotic effects, depending on the dose of NO and cell type. The possible explanation for this dual effect could be that at low concentrations, NO may serve as a suppressor of apoptosis, whereas at high concentrations, it is cytotoxic. To test this possibility, various concentrations of NO produced by the addition of NO donor (SNP) were exposed to the cells and their effect on cell apoptosis was determined. At all concentrations of the NO donor tested, only anti-apoptotic effect was observed. The highest concentration of SNP that caused no apoptotic effect when used alone was 500 µg/ml. The induction of cellular NO by this concentration of SNP was approximately 7-fold over non-treated control level in BEAS cells and 6-fold in H460 cells (figure 19 and 21). This induction of NO levels completely inhibited FasL-induced apoptosis in both cell types. Consistent with this finding, the results in cisplatin-treated cells similarly showed that cellular NO levels were induced by approximately 6.5-fold in BEAS cells and 5.5-fold in H460 cells, and that these elevated NO levels completely blocked cisplatin-induced apoptosis (figures 18 and 20).

In summary, this study provided evidence that the basal and inducible levels of NO play an anti-apoptotic role in cisplatin- and FasL-induced apoptosis in both BEAS and H460 cells. In addition, once the NO level in BEAS cells was elevated by the addition of NO donor to emulate the basal NO level in H460 cells, the BEAS cells

became resistant to apoptosis similar to the H460 cells, suggesting that the higher basal level of NO in H460 cells plays a role in rendering these cells resistant to cisplatin- and FasL-induced apoptosis. Thus, it is possible that lung abnormalities leading to an increase in NO levels may promote cells' ability to escape from FasL-induced apoptosis. The results of this study also supported the role of elevated NO levels in lung carcinogenesis and apoptosis resistance to cisplatin.

Intracellular NO levels have been shown to be altered in response to various stimuli (Taylor et al., 1998; Adler et al., 1994; and Robbins et al, 1997). Moreover, cellular NO level is strongly related to oxidative status of the cells (Heigold et al., 2002; and Borulite and Brown, 2003). Apoptotic signals may change the oxidative condition of the cells which could affect NO levels. In the present study, it was found that cisplatin treatment caused a dose- and time-dependent reduction in cellular NO levels (figures 7-8). Since cisplatin has been reported to induce ROS generation in various cell types (Godbout et al., 2002; Miyajima et al., 1997; and Miyajima et al., 1999), it is possible that ROS may interact with NO leading to a decrease in cellular NO level. Indeed, ROS such as superoxide anion (O_2^-) has been shown to interact with NO to form peroxynitrite (ONOO) [$O_2^{-\cdot} + NO \rightarrow ONOO^-$] (Heigold et al., 2002; and Borutaite and Brown, 2003).

It is known that O_2^- can be converted to hydrogen peroxide (H_2O_2) by the enzymatic reaction catalyzed by superoxide dismutase (SOD). The generated H_2O_2 can further be converted to hydroxyl radical ($OH^{\cdot}$) via a Fenton reaction. (figure 51).

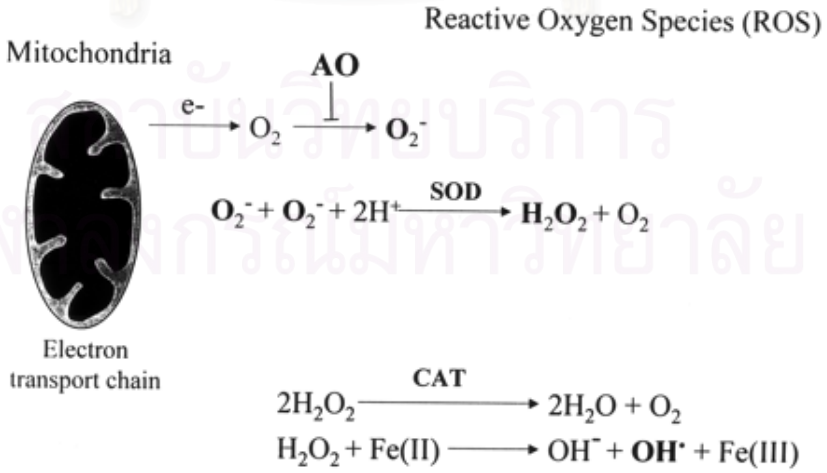


Figure 51 ROS generation and detoxification. Abbreviation: antioxidant (AO), superoxide dismutase (SOD), and catalase (CAT) (Heigold et al., 2002).

To confirm ROS generation in the tested cell system, electron spin resonance (ESR) spectroscopy with the aid of the spin trapping agent 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was used to detect $OH^{\cdot}$ formation. DMPO-$OH^{\cdot}$ adducts were formed as early as 10 min in H460 cells after cisplatin (100 μM) treatment (figure 52, Appendix C). Cisplatin-induced ROS generation was also determined by flow cytometry using the fluorescent oxidative probe DCF-DA. Treatment of the cells with cisplatin (200 μM) for 1 h significantly increased intracellular DAF fluorescence (figure 31). These results indicate that ROS are generated following cisplatin treatment and that this ROS generation may be responsible for the observed depletion of NO in the treated cells.

Other mechanisms that may be involved in the depletion of NO include iNOS downregulation, NO-protein interactions, and other NO-ROS interactions (Beckman and Koppenol, 1996; and Wink et al., 1994). In contrast to cisplatin, FasL increased the intracellular levels of NO in both BEAS and H460 cells. ROS levels were unaffected by the FasL treatment (data not shown). The mechanism by which FasL induced NO elevation is unclear and further research is needed. The elevation of NO in response to FasL treatment may have an anti-apoptotic effect on cell apoptosis. However, this elevation was relatively low and transient in nature, as compared to the effect caused by NO donors. Thus, the elevated NO level caused by FasL may attenuate the apoptotic effect of FasL but may not be high enough to inhibit this process.

In conclusion, this study provided evidence in support of the anti-apoptotic role of NO in cisplatin- and FasL-induced apoptosis. In both normal BEAS and cancerous H460 cells, NO exhibited the same anti-apoptotic effect, indicating its role in cell death resistance against cisplatin and FasL.

2. The mechanisms of NO in controlling apoptosis susceptibility.

In an attempt to elucidate the regulatory mechanisms of NO in cisplatin- and FasL-induced apoptosis, additional pharmacological and gene manipulation approaches were used. Having demonstrated that NO played a similar role in protecting BEAS and H460 cells from apoptosis, it was hypothesized that the same mechanism of NO

protection against cell death exists in the two cell types. In addition, preliminary results indicated the same pattern of anti-apoptotic protein expression in both BEAS and H460 cells in response to NO modulation (figures 53-54, Appendix C). Therefore, only either BEAS or H460 cells were used for subsequent mechanistic studies.

Cisplatin has been used for the treatment lung cancer and its resistance has been demonstrated to coincide with increased NO levels. Thus, the human cancer H460 cells were used as a model system to study the effect of NO on cisplatin resistance and its underlying mechanism. Elucidating such mechanism should be beneficial to a more effective treatment of this deadly disease.

For FasL experiments, BEAS cells were used due to its greater sensitivity to apoptosis induction by FasL. FasL-induced apoptosis mainly eliminates infected cells and cancer cells. Thus, investigations of the mechanism of NO-mediated resistance to FasL-induced apoptosis in BEAS cells may provide new insights into the escape mechanisms of infected and cancer cells escape from elimination.

2.1 Mechanism of NO protection against cisplatin-induced apoptosis in H460 cells.

The present study showed that treatment of the cells with cisplatin induced a dose-dependent increase in cell apoptosis. Pretreatment of the cells with NO inhibitors (AG and PTIO) significantly enhanced the apoptosis-inducing effect of cisplatin, whereas pretreatment with NO donors (SNP and DPTA NONOate) inhibited this effect (figure 28). These results indicated a negative regulatory role of NO in cisplatin-induced apoptosis.

Previous studies showed that cisplatin induced cell death via an ROS-dependent mechanism (Li et al., 2004; and Wu et al., 2005). Using the antioxidant NAC to inhibit cellular ROS, the results of this study substantiated this previous finding (figure 28). These results along with the NO results further indicated that both ROS and NO played important roles in regulating cisplatin-induced apoptosis. However, ROS served as a positive regulator whereas NO was a negative regulator of cisplatin-induced apoptosis.

Aberrant expression of the anti-apoptotic Bcl-2 protein has been shown to mediate cisplatin resistance in many cell types (Li et al., 2004; Raspollini et al., 2004; Yang et al., 2004; and Kausch et al., 2005). To test whether Bcl-2 was involved in the apoptosis resistance to cisplatin, H460 cells were stably transfected with Bcl-2 or control plasmid (mock transfection) and their effect on cisplatin-induced apoptosis was determined. The results showed that overexpression of Bcl-2 significantly inhibited apoptosis over a wide concentration range of cisplatin treatment as compared to mock transfection control. These results strongly indicated the role of Bcl-2 in cell death resistance to cisplatin. Western blot analysis of Bcl-2 showed an increased expression of the protein in Bcl-2-transfected cells but not in mock transfected cells, confirming the differential effect of gene transfection and the protective role of Bcl-2 (figure 32). Since Bcl-2-overexpressed cells were highly resistant to cisplatin-induced apoptosis, this study provided additional evidence supporting the role of Bcl-2 in cisplatin resistance.

Cisplatin treatment caused a significant decrease in Bcl-2 expression which was further decreased upon treatment with of NO inhibitors (AG and PTIO). In contrast, the NO donors SNP and DPTA NONOate completely inhibited cisplatin-induced downregulation of Bcl-2 and further increased its expression over non-treated control level (figure 33). These results suggested that NO mediated cisplatin resistance via Bcl-2 upregulation.

Post-translational modifications such as ubiquitination and phosphorylation have emerged as important regulators of Bcl-2 function (Li et al., 2004; Dimmeler et al., 1999; Britschopf et al., 2000; Basu and Haldar, 2002). Moreover, previous studies have shown that Bcl-2 was rapidly downregulated by proteasomal degradation via an ROS-dependent mechanism (Li et al., 2004; and Breitschopf et al., 2000). However, the effect of NO on Bcl-2 regulation and in particular those relevant to cisplatin resistance have not been demonstrated. It was found in this study that Bcl-2 downregulation by cisplatin was mediated by the proteasomal pathway. Treatment of the cells with the proteasome inhibitor lactacystin completely inhibited Bcl-2 downregulation induced by cisplatin, indicating the dominant role of the proteasomal pathway in cisplatin-induced Bcl-2 downregulation (figure 34). Furthermore, the antioxidant NAC was able to inhibit Bcl-2

downregulation by cisplatin, supporting the role of ROS in the proteasomal degradation process.

Apoptotic stimuli such as ROS and TNF-α have been shown to induce dephosphorylation of Bcl-2 at Ser87 position, which promotes its ubiquitination and subsequent degradation (Li et al., 2004; Dimmeler et al., 1999; and Breitschopf et al., 2000). The results on the inhibitory effects of ROS scavenger (NAC) on Bcl-2 dephosphorylation and degradation (figures 35 and 34) supported the earlier finding and further indicated the involvement of ROS in cisplatin-induced Bcl-2 degradation. However, whether or not NO can regulate Bcl-2 phosphorylation and stability has not been demonstrated. This study reported for the first time the effect of NO on Bcl-2 ubiquitination and degradation, and the lack of its effect on Bcl-2 phosphorylation (figure 35). The NO inhibitors AG and PTIO significantly promoted Bcl-2 ubiquitination (figure 36A), whereas the NO donors (SNP and DPTA NONOate) inhibited this effect (figure 36B). These results along with the phosphorylation data indicated that NO inhibited ubiquitination of Bcl-2 independent of its dephosphorylation. Thus, dephosphorylation of Bcl-2 might not be a necessary event for the triggering of ubiquitination as previously believed, and that NO and ROS may regulate Bcl-2 stability via different mechanisms.

Increasing evidences have also shown that NO can modify protein functions through S-nitrosylation process (Li et al., 1997; and Kim et al., 1997). However, whether or not NO can nitrosylate Bcl-2 protein and whether it plays a role in cisplatin resistance have not been determined. An important finding of this study is that NO can nitrosylate Bcl-2, which inhibits its degradation by the ubiquitin-proteasomal pathway. Addition of the NO donors SNP and DPTA NONOate significantly increased Bcl-2 S-nitrosylation (figure 37), decreased its ubiquitination and proteasomal degradation (figure 36B), and increased apoptosis resistance to cisplatin (figure 28). In contrast, the NO inhibitors AG and PTIO showed opposite effects, thus confirming the role of NO in Bcl-2-mediated cisplatin resistance.

S-nitrosylation of proteins by NO has been reported to be inhibited by strong reducing agent such as dithiothreitol (DTT) (Ryua et al., 2004; and Moon et al., 2005). In this study, the addition of DTT was shown to have a reversal effect on Bcl-2 S-

nitrosylation and ubiquitination induced by the NO donors (figures 37 and 38). These results indicated that S-nitrosylation might be a key mechanism utilized by NO to regulate ubiquitination and proteasomal degradation of Bcl-2.

The mechanism by which S-nitrosylation prevents ubiquitination of Bcl-2 is unclear but may involve conformational change of the S-nitrosylated Bcl-2 protein which could prevent its recognition and subsequent attachment of ubiquitin by the enzyme ubiquitin ligases. Conformational changes of Bcl-2 by phosphorylation have been reported to affect its ubiquitination and stability (Ling et al., 2002).

Bcl-2 has also been reported to be cleaved enzymatically by caspase-3 in some cell systems (Zhang et al., 1999). However, caspase inhibition analysis of H460 cells failed to detect the inhibitory effect of caspase inhibitor (zVAD-fmk) on Bcl-2 expression (figure 34). The likely explanation for the observed discrepancy between this and previous studies may be the difference in experimental conditions and cell type used. In Jurkat cells and prostate cancer PC-3 cells, caspase-3 inhibitor was also unable to prevent Bcl-2 cleavage, whereas proteasome inhibitors were able to prevent Bcl-2 degradation (Basu and Haldar, 2002). In H460 cells, caspase-mediated Bcl-2 cleavage was observed but only after 12 h of drug treatment, while proteasome-mediated cleavage was observed at earlier times (Ling et al, 2002). It was suggested that during the early apoptosis process, caspase activity was still low and insufficient to affect Bcl-2 stability, whereas in late apoptosis caspase activity was elevated and was able to induce Bcl-2 cleavage. Our results on the ubiquitination and proteasomal degradation of Bcl-2, and the lack of caspase-mediated cleavage at the early time period (12 h), supported the previous finding and indicated proteasomal degradation as a key early event in controlling Bcl-2 stability.

In summary, the results of this study indicated an important role of NO in the regulation of apoptosis resistance to cisplatin. Cisplatin induced downregulation of Bcl-2 through proteasome-mediated degradation. NO negatively regulated this process through its ability to S-nitrosylate the protein and inhibited its ubiquitination. In demonstrating the S-nitrosylation of Bcl-2, this study documented a novel layer of regulation that linked NO signaling with Bcl-2-mediated apoptosis resistance, which represents an important mechanism in the control of tumor development and progression. Since increased NO

production and Bcl-2 expression have been associated with several human tumors, NO may be one of the key regulators of cell death resistance and tumor growth through S-nitrosylation. This finding on the novel function of NO in Bcl-2 regulation may have important implications in cancer chemotherapy and prevention.

2.2 Mechanism of NO protection against FasL-induced apoptosis in BEAS cells.

FasL-induced apoptosis is an important mechanism that the body's immune system uses to eliminate infected and cancer cells. Increasing evidence have shown that NO plays an important role in regulating many key proteins in the death receptor pathway (Chen et al., 2005; Fukuo et al., 1996; and Li et al., 2004). In Fas-mediated apoptosis, NO has been reported to have anti-apoptotic effect (Chen et al., 2005; and Mannick et al., 1997), but its underlying mechanisms of regulation are unclear. Consistent with the previous reports in other cell systems (Chen et al., 2005; and Mannick et al., 1997), this study showed that NO exhibited an anti-apoptotic effect on FasL signaling in epithelial BEAS cells (figure 40). The results also showed that FasL treatment induced caspase-8 activation and co-administration of FasL with NO donors (SNP and DPTA NONOate) attenuated this effect. In contrast, the NO inhibitors AG and PTIO enhanced caspase-8 activation induced by FasL (figure 40). Since FasL is known to induce apoptosis through caspase-8-dependent pathway (Nagata, 1997; and Takahashi et al., 1994), these data therefore suggested that NO mediated its anti-apoptotic effect against FasL through the inhibition of caspase-8 activation.

The FLIP protein is a key regulatory protein of the Fas death pathway. Its ability to inhibit FasL-induced caspase-8 activation has led to the hypothesis that NO might regulate Fas death signaling through FLIP. Indeed, the results of this study showed that FasL treatment caused a downregulation of FLIP without significant effect on Fas or FADD expression (figure 42). Co-administration of FasL with NO donor (SNP or DPTA NONOate) inhibited FasL-induced FLIP downregulation (figure 44), whereas the NO inhibitors had opposite effect, thus supporting our hypothesis on the role NO on FLIP regulation.

The anti-apoptotic function of FLIP is tightly associated with its expression levels and downregulation of FLIP is an important mechanism to sensitize cells to receptor-mediated apoptosis (Krueger et al., 2001). Post-translational modifications of FLIP such as ubiquitination and phosphorylation have been reported (Holmstrom and Eriksson, 2000; and Jesenberger and Jentsch, 2002). Ubiquitin-mediated degradation of FLIP by the proteasome has also been implicated under different conditions, including PPAR ligand and p53 activation, chemotherapeutic administration, and viral infection (Fukazawa et al., 2001; Kim et al., 2002; Perez and White, 2003; and Poukkla et al., 2005). However, the effect of NO on FLIP degradation and its association with FasL-induced apoptosis has not been demonstrated.

Inhibition of proteasomal degradation by the proteasome inhibitor lactacystin and MG132 potently inhibited FasL-induced FLIP downregulation, the effect that was also observed with the NO donors (figures 42 and 43). These results along with the earlier finding on the protective effect of NO on FasL-induced apoptosis (figure 40) further indicated that NO may mediate its anti-apoptotic effect by interfering with FLIP degradation.

The proteasome acts on proteins destined to be degraded by ubiquitination. FasL treatment caused FLIP ubiquitination (figure 45) and concomitant decrease in FLIP level (figure 42). These results along with the observation that proteasome inhibitors inhibited FLIP downregulation indicated that FasL induced downregulation of FLIP via ubiquitin-proteasomal degradation. The ability of the NO donors and inhibitors to respectively inhibit and enhance FLIP ubiquitination (figure 45) further indicated that NO regulated FLIP downregulation by interfering with its ubiquitination.

The present study demonstrated for the first time that NO can nitrosylate FLIP (figure 46) and prevent its degradation via the ubiquitin-proteasome pathway. Gene deletion analysis revealed the importance of the caspase-like domain of FLIP in the nitrosylation process. Partial deletion of the caspase-like domain of FLIP (Δ1) had no effect on NO-mediated S-nitrosylation, whereas complete deletion of this domain (Δ2) as well as the DED2 domain (Δ3) strongly inhibited this effect, suggesting that the amino acid sequence difference between Δ1 and Δ2 (amino acid sequence 233-328) is essential

for S-nitrosylation of FLIP. To determine the precise amino acid(s) that may be responsible for this nitrosylation, the target amino acid sequence (233-328) of FLIP was further examined. Since S-nitrosylation involves the transfer of NO^+ group to an active site on cysteine residues, this study examined the presence of cysteine in the target region and found two at position 254 and 259. Mutational analysis further showed that Cys254 and Cys259 of FLIP were responsible for the nitrosylation. Figure 49 showed that SNP treatment could not increase S-nitrosylation of the mutated FLIP (mutation of Cys 254 and Cys 259 to alanine). Also, the addition of SNP could not inhibit FasL-induced FLIP ubiquitination (figure 49). Together, these results supported the role of S-nitrosylation in the ubiquitination process and demonstrated the requirement of Cys254 and Cys259 in FLIP S-nitrosylation. SNP also inhibited FasL-induced wild-type FLIP downregulation but had no protective effect on the mutated FLIP, suggesting that S-nitrosylation of FLIP, in addition to preventing ubiquitination, also protected this molecule from FasL-induced proteasomal degradation.

While S-nitrosylation of cysteine residues was shown to be important in preventing FLIP ubiquitination, it is not absolutely required for this inhibition since FLIP lacking the two cysteine residues ($\Delta 2$ and $\Delta 3$) can still be nitrosylated to some degree, indicating that the ubiquitination and S-nitrosylation processes are functionally separated and differently regulated by NO.

For proteins whose levels are regulated by ubiquitin-proteasomal degradation, ubiquitination is induced by binding of the proteins to E3 ubiquitin ligases (Ciechanover, 1998). In this regard, FLIP has been shown to bind and activate TNFR-associated factor (TRAF)-2, which contains a RING finger domain known to possess E3 ligase activity (Kataoka et al., 2001 and Kataoka et al., 2001). It is therefore conceivable that during Fas stimulation TRAF-2 is recruited to the death signaling complex along with FLIP and exerts its ligase activity leading to FLIP ubiquitination and degradation. Several other mechanisms, including upregulation of TRAF-2 or other types of FLIP-binding E3 ubiquitin ligases could also be envisioned.

It has been suggested that NO might inhibit FasL-mediated apoptosis through a decrease in Fas receptor expression (Jee et al., 2003). However, our Western blot studies

failed to detect the inhibitory effect of SNP on Fas expression at the concentrations that inhibited FasL-induced apoptosis. A similar finding was also observed in granulosa cells (Chen et al., 2005), although FLIP expression was not detected in that study. The likely explanation for the observed discrepancy may be the difference in experimental design and cell type used. It might be possible that other mechanisms of NO regulation such as induction of heat shock proteins (Yoon et al., 2002), upregulation of Bcl-2 (Jee et al., 2003), and suppression of Bax expression (Yoon et al., 2002) may also be involved in this process. The established importance of FLIP in death receptor signaling, however, supports the role of this molecule and its regulation by NO in FasL-induced apoptosis.

Although FLIP has been perceived primarily as an inhibitor of apoptosis, increasing evidence also suggest that this protein plays an additional role in cell survival and proliferation. For examples, overexpression of FLIP has been shown to activate NF-κB (Chaudhary et al., 2000; Hu et al., 2000; and Dohrman et al., 2005) and inhibition of this pathway by dominant expression of its inhibitory subunit IκB decreased cell survival (Lu et al., 2002). FLIP has also been shown to activate NF-κB through its ability to recruit key adapter proteins such as TRAF-2 and RIP-1 to the death signaling complex (Kataoka et al., 2000; and Dohrman et al., 2005). Since TRAF-2 may have a role in ubiquitination of FLIP as earlier described and since NO can modulate this ubiquitination process, it is likely that NO may also play a role in cell survival regulation through NF-κB signaling. Thus, NO may be a key regulator of death and survival signaling in the death receptor pathway and it can not only determine whether the apoptosis pathway is turned on or off but also allows the cell to switch between cell death and survival.

In conclusion, this study provided evidence that FasL can induce downregulation of FLIP through proteasome-mediated degradation. NO negatively regulated this process through its ability to inhibit ubiquitination. It is also worth noting that this regulation occured via S-nitrosylation of FLIP which interfered with the ubiquitination process. This novel function of NO in the death receptor pathway of apoptosis may have important implications in cell death resistance and pathogenesis of apoptosis-related disorders.

With regards to cisplatin studies, the results of this study revealed a novel role of NO in cisplatin resistance. This finding suggests that elevated NO levels, i.e., during lung inflammation and carcinogenic exposure, may have an anti-apoptotic effect on the cells and promote their progression to cancerous cells by rendering them resistant to immune-mediated cell death. Moreover, the elevated NO levels may induce cancer cell resistance to chemotherapy. The mechanism by which NO mediates apoptosis resistance involves S-nitrosylation of anti-apoptotic proteins such as FLIP and Bcl-2, which prevents them from ubiquitin-proteasomal degradation. These anti-apoptotic proteins then accumulate in the cells resulting in cell death resistance. NO represents a potential new target for intervention in apoptosis-related diseases such as cancer and neurodegenerative disorders.

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APPENDICES

APPENDIX A

PREPARATION OF REAGENTS

Acrylamide gel

Solution A	0.8% methylene bis acrylamide, 30% acrylamide
Solution B	1.5 M Tris HCl (pH 8.8)
Solution C	10% SDS
Solution D	0.5 M Tris HCl (pH 6.8)
APS (ammonium persulfate)	10% APS in DDW
TEMED (N, N, N′, N′- tetramethylenediamine)	

Solution A, B, APS, and TEMED	stored at 4 °C
Solution C	stored at room temperature
APS	freshly prepared

The gel apparatus and spacers (1.5 mm thick) were assembly.

1. Preparation of separating gel

To make two plates of 10% acrylamide gel, the ingredients of separating gel are

solution A	6.7 ml
solution B	5.0 ml
solution C	0.2 ml
DDW	7.9 ml

All of the ingredients were thoroughly mixed. The mixture was supplemented with 200 µl APS and 8 µl TEMED, then gentle mixed, and immediately pour the gel between the glass plates. Before gel polymerization was

complete, 0.1% SDS in DDW was layered on the top of the separating gel (5 mm thick). Wait until the gel completely polymerized (approximately 20-30 min).

2. Preparation of stacking gel

Once the separating gel has completely polymerized, 0.1% SDS was removed from the top of the gel. To make stacking gel, the ingredients are

solution A	1.3 ml
solution C	34 μl
solution D	2.5 ml
DDW	6.1 ml

All of the ingredients were thoroughly mixed. The mixture was supplemented with 50 μl APS and 10 μl TEMED, then thoroughly mixed, and immediately pour the gel between the glass plates.

The combs were inserted between the two glass plates of two sets of gel apparatus. The gels were leaved for approximately 30-40 min to polymerize.

3. Application of samples

Once the stacking gel has completely solidated, the combs were gently removed. The wells were flushed out thoroughly with running buffer. The clips and sealing tapes were removed and set up the gel chamber. The air bubbles between layers were removed by gently rolling the chamber.

Running buffer

To make 1 liter of 5X running buffer (250 mM Tris, 1.92 M glycine, and 0.5 % SDS) for stock solution, the ingredients are

Tris	30.2 g
Glycine	144.13 g
SDS	5 g

All ingredients were dissolved in DDW with continuously stirring. The solution was adjusted volume to 1000 ml. Before use, the solution was diluted to 1X running buffer (50 mM Tris, 0.384 M glycine, and 0.1 % SDS) with DDW, 5X running buffer : DDW = 4 : 1.

Transfer buffer

To make 1 liter of 10 X transfer buffer (1 M Tris, and 1.92 M glycine) for stock solution, the ingredients are

Tris	121.14 g
Glycine	144.13 g

All ingredients were dissolved in DDW with continuously stirring. The solution was adjusted volume to 1000 ml. Before use, the solution was diluted to 1000 ml of 1X running buffer (0.1 M Tris, 0.192 M glycine) supplemented with 5% methanol, 10X transfer buffer : methanol : DDW = 100 ml : 50 ml : 850 ml.

Tris-buffered saline, 0.1 % Tween 20 (TBST)

To make 1 liter of 10 X TBST (100 mM Tris, 1 M NaCl, and 0.1 % Tween 20) for stock solution, the ingredients are

Tris	12.114 g
NaCl	58.44 g
Tween 20	10 ml

All ingredients were dissolved in DDW with continuously stirring. The solution was adjusted volume to 1000 ml. Before use, the solution was diluted to 1 X TBST (10 mM Tris, 100 mM NaCl, and 0.01 % Tween 20) with DDW, 10X TBST : DDW = 9 : 1.

Sample buffer

To make 50 ml of 3X sample buffer (225 mM Tris HCl (pH 6.8), 6 % SDS, 30% glycerol, 9 % 2-mercaptoethanol, and 0.009 % bromphenol blue) for stock solution, the ingredients are

Tris HCl	22.5 of 0.5 M
SDS	3 g
Glycerol	10 ml
2-mercaptoethanol	4.5 ml
bromphenol blue	225 μl of 2 %

All ingredients were dissolved in DDW with continuously stirring. The solution was adjusted volume to 50 ml. 3X sample buffer was aliquot into 1 ml/tube and stored at –20 °C. Before use, the solution was diluted to 1 X sample buffer (75 mM Tris HCl, 2% SDS, 10 % glycerol, 3 % 2-mercaptoethanol, and 0.003 % bromphenol blue) with DDW, 3X sample buffer : DDW = 2 : 1.

Lysis buffer

To make 50 ml of lysis buffer (50 mM Tris HCl (pH 7.5), 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 1 % Triton X-100, 1 mM phenylmethanesulfonyl fluoride (PMSF) and a commercial protease inhibitor mixture (Roche)) the ingredients are

Tris HCl	2.5 ml of 1M (pH 7.5)
NaCl	0.4383 g
EDTA	0.3722 g
NaF	2.5 ml of 1 M
Triton X-100	0.51 ml

All ingredients were dissolved in DDW 40 ml. The lysis buffer was aliquot into 1 ml/tube and stored at 4 °C. Before use, the solution was supplemented with 200

μl of 50 mM PMSF and a commercial protease inhibitor mixture (1 tablet/50ml) and the volume was adjusted to 10 ml.

APPENDIX B

TABLES OF EXPERIMENTAL RESULTS

Table 2. The DAF-DA fluorescence intensity in BEAS and H460 cells quantitated by a flow cytometry, in basal condition (no treatment).

Cell type	Fluorescence intensity
BEAS	24.00 ± 2.34
H460	62.76 ± 5.63*

Each value represents the mean ± S.D. of six independent experiments. Asterisks refer significant difference from the control group (BEAS cells): * $P < 0.05$ determined by One-way ANOVA.

Table 3 The DAF-DA fluorescence intensity in BEAS cells quantitated by a flow cytometry, in response to 100 μM cisplatin treatment at various time points (time dependency).

Time (h)	Fluorescence intensity
0	22.19 ± 2.08
0.5	9.88 ± 1.67*
1	11.83 ± 1.69*
2	8.00 ± 0.16*
3	9.47 ± 0.50*
6	19.89 ± 0.10
9	20.52 ± 1.52

Each value represents the mean ± S.D. of four independent experiments. Asterisks refer significant difference from the control group (time = 0): * $P < 0.05$ determined by One-way ANOVA.

Table 4 The DAF-DA fluorescence intensity in H460 cells quantitated by a flow cytometry, in response to 100 μM cisplatin treatment at various time points (time dependency).

Time (h)	Fluorescence intensity
0	53.03 ± 2.60
0.5	53.70 ± 4.14
1	40.48 ± 0.69*
2	34.39 ± 2.38*
3	25.38 ± 2.28*
6	40.29 ± 1.63*
9	51.15 ± 2.10

Each value represents the mean ± S.D. of four independent experiments. Asterisks refer significant difference from the control group (time = 0): * $P < 0.05$ determined by One-way ANOVA.

Table 5 The DAF-DA fluorescence intensity in BEAS cells quantitated by a flow cytometry, in response to various cisplatin treatments at indicated concentrations for 1 h (dose dependency).

Cisplatin (μM)	Fluorescence intensity
0	20.45 ± 0.78
50	13.33 ± 2.28*
100	8.13 ± 1.45*
250	6.64 ± 1.23*
500	5.53 ± 1.83*

Each value represents the mean ± S.D. of five independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 6 The DAF-DA fluorescence intensity in H460 cells quantitated by a flow cytometry, in response to various cisplatin treatments at indicated concentrations for 1 h (dose dependency).

Cisplatin (μM)	Fluorescence intensity
0	64.15 ± 2.38
50	42.92 ± 2.01*
100	30.36 ± 1.56*
250	14.29 ± 1.05*
500	15.17 ± 2.45*

Each value represents the mean ± S.D. of five independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 7 The DAF-DA fluorescence intensity in BEAS cells quantitated by a flow cytometry, in response to 100 ng/ml FasL treatment at various time points (time dependency).

Time (h)	Fluorescence intensity
0	21.48 ± 1.82
0.5	34.37 ± 3.53*
1	44.26 ± 2.40*
2	34.45 ± 2.18*
3	31.42 ± 0.65*
6	22.73 ± 2.76
9	21.88 ± 1.78

Each value represents the mean ± S.D. of five independent experiments. Asterisks refer significant difference from the control group (time = 0): * $P < 0.05$ determined by One-way ANOVA.

Table 8 The DAF-DA fluorescence intensity in H460 cells quantitated by a flow cytometry, in response to 100 ng/ml FasL treatment at various time points (time dependency).

Time (h)	Fluorescence intensity
0	54.02 ± 3.28
0.5	53.78 ± 4.23
1	112.14 ± 5.23*
2	101.40 ± 0.98*
3	92.11 ± 2.13*
6	87.66 ± 2.21*
9	52.34 ± 2.32

Each value represents the mean ± S.D. of five independent experiments. Asterisks refer significant difference from the control group (time = 0): * $P < 0.05$ determined by One-way ANOVA.

Table 9 The DAF-DA fluorescence intensity in BEAS cells quantitated by a flow cytometry, in response to various FasL treatments at indicated concentrations for 1 h (dose dependency).

FasL (ng/ml)	Fluorescence intensity
0	21.87 ± 0.46
50	33.92 ± 1.68*
100	44.56 ± 3.66*
250	74.78 ± 4.68*

Each value represents the mean ± S.D. of five independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 10 The DAF-DA fluorescence intensity in H460 cells quantitated by a flow cytometry, in response to various FasL treatments at indicated concentrations for 1 h (dose dependency).

FasL (ng/ml)	Fluorescence intensity
0	62.48 ± 3.48
50	98.77 ± 5.16*
100	121.35 ± 3.46*
250	122.14 ± 4.55*

Each value represents the mean ± S.D. of five independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 11 The percentage of apoptotic BEAS (A) and H460 (B) cells, detected by Hoechst 33342 assay induced by 100 μM cisplatin treatment at various time points (time dependency).

(A)

Time (h)	Apoptosis(%)
0	1.00 ± 0.00
6	14.00 ± 2.00*
9	21.33 ± 3.05*
12	32.66 ± 3.05*
16	48.00 ± 2.00*
24	48.33 ± 3.51*

(B)

Time (h)	Apoptosis(%)
0	1.00 ± 0.00
6	2.00 ± 1.03
9	8.00 ± 1.08**
12	19.56 ± 3.05**
16	19.66 ± 2.08**
24	19.33 ± 2.51**

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ versus non-treated BEAS cells and ** $P < 0.05$ versus non-treated H460 cells determined by One-way ANOVA.

Table 12 The percentage of apoptotic BEAS (A) and H460 (B) cells, detected by Hoechst 33342 assay induced by various cisplatin treatments at indicated concentrations for 16 h and 12 h in BEAS and H460 cells, respectively (dose dependency).

(A)

Cisplatin (μM)	Apoptosis(%)
0	1.00 ± 0.00
50	23.50 ± 1.80*
100	49.67 ± 1.52*
250	63.33 ± 2.08*
500	83.00 ± 2.00*

(B)

Cisplatin (μM)	Apoptosis(%)
0	1.00 ± 0.00
50	8.33 ± 1.52**
100	20.67 ± 1.52**
250	40.33 ± 0.57**
500	53.66 ± 3.05**

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ versus non-treated BEAS cells and ** $P < 0.05$ versus non-treated H460 cells determined by One-way ANOVA.

Table 13 The percentage of apoptotic BEAS (A) and H460 (B) cells, detected by Hoechst 33342 assay induced by 100 ng/ml FasL treatment at various time points (time dependency).

(A)

Time (h)	Apoptosis(%)
0	1.00 ± 0.00
6	6.26 ± 0.41*
9	10.66 ± 0.52*
12	15.66 ± 1.08*
16	19.33 ± 0.52*
24	18.66 ± 1.00*

(B)

Time (h)	Apoptosis(%)
0	1.00 ± 0.00
6	1.33 ± 0.57
9	4.05 ± 1.09**
12	6.66 ± 0.57**
16	7.33 ± 0.65**
24	7.33 ± 0.13**

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ versus non-treated BEAS cells and ** $P < 0.05$ versus non-treated H460 cells determined by One-way ANOVA.

Table 14 The percentage of apoptotic BEAS (A) and H460 (B) cells, detected by Hoechst 33342 assay induced by various FasL treatments at indicated concentrations for 16 h (dose dependency).

(A)

FasL (ng/ml)	Apoptosis (%)
0	1.16 ± 0.28
50	9.47 ± 1.04*
100	20.16 ± 2.02*
250	27.16 ± 1.75*

(B)

FasL (ng/ml)	Apoptosis(%)
0	1.00 ± 0.00
50	1.16 ± 0.28
100	6.50 ± 1.50**
250	17.66 ± 2.51**

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ versus non-treated BEAS cells and ** $P < 0.05$ versus non-treated H460 cells determined by One-way ANOVA.

Table 15 The DAF-DA fluorescence intensity in BEAS cells quantitated by a flow cytometry, in response to NO inhibitor treatments, 300 μM AG or 300 μM c-PTIO at for 1 h.

Treatment	Fluorescence intensity
control	20.68 ± 2.25
AG	12.29 ± 1.13*
PTIO	8.40 ± 1.36*

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 16 The DAF-DA fluorescence intensity in H460 cells quantitated by a flow cytometry, in response to NO inhibitor treatments, 300 µM AG or 300 µM c-PTIO for 1 h.

Treatment	Fluorescence intensity
control	65.02 ± 2.45
AG	11.80 ± 1.14*
PTIO	8.61 ± 1.69*

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 17 The percentage of apoptotic BEAS cells, detected by Hoechst 33342 assay induced by 100 µM cisplatin in the presence or absence NO inhibitors, 300 µM AG or 300 µM c-PTIO for 16 h.

Treatment	Apoptosis(%)
control	0.66 ± 0.57
cisplatin	49.33 ± 4.04*
cisplatin + AG	79.00 ± 3.27*#
cisplatin + PTIO	77.66 ± 2.84*#

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ and # $P < 0.05$ comparing with cisplatin-treated control determined by One-way ANOVA.

Table 18 The percentage of apoptotic H460 cells, detected by Hoechst 33342 assay induced by 100 μM cisplatin in the presence or absence NO inhibitors, 300 μM AG or 300 μM c-PTIO for 12 h.

Treatment	Apoptosis(%)
control	0.00 ± 0.00
cisplatin	19.50 ± 2.29*
cisplatin + AG	31.50 ± 2.78*#
cisplatin + PTIO	35.43 ± 1.20*#

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ and # $P < 0.05$ comparing with cisplatin-treated control determined by One-way ANOVA.

Table 19 The percentage of apoptotic BEAS cells, detected by Hoechst 33342 assay induced by 100 ng/ml FasL in the presence or absence NO inhibitors, 300 μM AG or 300 μM c-PTIO for 16 h.

Treatment	Apoptosis(%)
control	1.00 ± 0.00
FasL	19.66 ± 2.08*
FasL + AG	33.50 ± 1.32*#
FasL + PTIO	29.00 ± 1.50*#

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ and # $P < 0.05$ comparing with FasL-treated control determined by One-way ANOVA.

Table 20 The percentage of apoptotic H460 cells, detected by Hoechst 33342 assay induced by 100 ng/ml FasL in the presence or absence NO inhibitors, 300 µM AG or 300 µM c-PTIO for 16 h.

Treatment	Apoptosis(%)
control	1.00 ± 0.00
FasL	6.00 ± 0.50*
FasL + AG	11.16 ± 1.04*#
FasL + PTIO	10.16 ± 1.02*#

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ and # $P < 0.05$ comparing with FasL-treated control determined by One-way ANOVA.

Table 21 The DAF-DA fluorescence intensity in BEAS cells quantitated by a flow cytometry, in response to 100 µM cisplatin in the presence or absence NO donors, SNP (300 µg/ml) or DPTA NONOate (200 µM) (detected at 16 h).

Treatment	Fluorescence intensity
Control	25.11 ± 3.40
cisplatin 100 µM	8.50 ± 2.79*
cisplatin 100 µM + SNP 300 µg/ml	171.33 ± 8.14*#
cisplatin 100 µM + DTPA 200 µM	149.40 ± 8.40*#

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.01$ and # $P < 0.01$ comparing with cisplatin-treated control determined by One-way ANOVA.

Table 22 The DAF-DA fluorescence intensity in H460 cells quantitated by a flow cytometry, in response to 100 µM cisplatin in the presence or absence NO donors, SNP (300 µg/ml) or DPTA NONOate (200 µM) (detected at 16 h).

Treatment	Fluorescence intensity
control	60.33 ± 4.51
cisplatin 100 µM	30.24 ± 3.21*
cisplatin 100 µM + SNP 300 µg/ml	387.26 ± 5.02*#
cisplatin 100 µM + DTPA 200 µM	401.67 ± 6.51*#

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.01$ and # $P < 0.01$ comparing with cisplatin-treated control determined by One-way ANOVA.

Table 23 The DAF-DA fluorescence intensity in BEAS cells quantitated by a flow cytometry, in response to 100 µg/ml FasL in the presence or absence NO donors, SNP (300 µg/ml) or DPTA NONOate (200 µM) (detected at 16 h).

Treatment	Fluorescence intensity
control	23.87 ± 1.62
FasL 100 ng/ml	53.67 ± 4.50*
FasL 100 ng/ml + SNP 300 µg/ml	154.33 ± 12.52*#
FasL 100 ng/ml + DTPA 200 µM	158.33 ± 7.58*#

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.01$ and # $P < 0.01$ comparing with FasL-treated control determined by One-way ANOVA.

Table 24 The DAF-DA fluorescence intensity in H460 cells quantitated by a flow cytometry, in response to 100 µg/ml FasL in the presence or absence NO donors, SNP (300 µg/ml) or DPTA NONOate (200 µM) (detected at 16 h).

Treatment	Fluorescence intensity
control	63.67 ± 2.30
FasL 100 ng/ml	138.67 ± 3.50*
FasL 100 ng/ml + SNP 300 µg/ml	371.33 ± 6.50*#
FasL 100 ng/ml + DTPA 200 µM	370.00 ± 4.58*#

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.01$ and # $P < 0.01$ comparing with FasL-treated control determined by One-way ANOVA.

Table 25 The percentage of apoptotic BEAS cells, detected by Hoechst 33342 assay induced by 100 µM cisplatin in the presence or absence NO donors, SNP (300 µg/ml) or DPTA NONOate (200 µM) (detected at 16 h).

Treatment	Apoptosis (%)
control	1.00 ± 0.00
cisplatin 100 µM	49.33 ± 4.04*
cisplatin 100 µM + SNP 300 µg/ml	3.67 ± 1.52#
cisplatin 100 µM + DTPA 200 µM	3.33 ± 1.52#

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.01$ and # $P < 0.01$ comparing with cisplatin-treated control determined by One-way ANOVA.

Table 26 The percentage of apoptotic H460 cells, detected by Hoechst 33342 assay induced by 100 μM cisplatin in the presence or absence NO donors, SNP (300 μg/ml) or DPTA NONOate (200 μM) (detected at 12 h).

Treatment	Apoptosis (%)
control	1.00 ± 0.00
cisplatin 100 μM	19.50 ± 2.29*
cisplatin 100 μM + SNP 300 μg/ml	2.00 ± 1.00#
cisplatin 100 μM + DTPA 200 μM	1.33 ± 0.57#

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.01$ and # $P < 0.01$ comparing with cisplatin-treated control determined by One-way ANOVA.

Table 27 The percentage of apoptotic BEAS cells, detected by Hoechst 33342 assay induced by 100 ng/ml FasL in the presence or absence NO donors, SNP (300 μg/ml) or DPTA NONOate (200 μM) (detected at 16 h).

Treatment	Apoptosis (%)
control	0.67 ± 0.05
FasL 100 ng/ml	17.50 ± 2.29*
FasL 100 ng/ml + SNP 300 μg/ml	2.00 ± 1.00#
FasL 100 ng/ml + DTPA 200 μM	1.67 ± 1.15#

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.01$ and # $P < 0.01$ comparing with FasL-treated control determined by One-way ANOVA.

Table 28 The percentage of apoptotic H460 cells, detected by Hoechst 33342 assay induced by 100 ng/ml FasL in the presence or absence NO donors, SNP (300 µg/ml) or DPTA NONOate (200 µM) (detected at 16 h).

Treatment	Apoptosis (%)
control	1.00 ± 0.00
FasL 100 ng/ml	7.33 ± 0.33*
FasL 100 ng/ml + SNP 300 µg/ml	1.67 ± 0.57#
FasL 100 ng/ml + DTPA 200 µM	1.33 ± 0.54#

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P <$ 0.01 and # $P <$ 0.01 comparing with FasL-treated control determined by One-way ANOVA.

Table 29 The DAF-DA fluorescence intensity in BEAS cells quantitated by a flow cytometry, in response to 100 µM cisplatin in the presence or absence NO donor, SNP at indicated concentrations.

Treatment	Fluorescence intensity
cisplatin 100 µM	11.67 ± 3.05
cisplatin 100 µM + SNP 50 µg/ml	25.56 ± 3.50*
cisplatin 100 µM + SNP 100 µg/ml	56.83 ± 5.46*
cisplatin 100 µM + SNP 300 µg/ml	84.33 ± 6.51*
cisplatin 100 µM + SNP 500 µg/ml	112.33 ± 9.78*

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P <$ 0.05 versus cisplatin-treated control determined by One-way ANOVA.

Table 30 The DAF-DA fluorescence intensity in H460 cells quantitated by a flow cytometry, in response to 100 µM cisplatin in the presence or absence NO donor, SNP at indicated concentrations.

Treatment	Fluorescence intensity
cisplatin 100 µM	45.65 ± 1.80
cisplatin 100 µM + SNP 50 µg/ml	83.65 ± 3.86*
cisplatin 100 µM + SNP 100 µg/ml	145.74 ± 12.34*
cisplatin 100 µM + SNP 300 µg/ml	172.14 ± 15.97*
cisplatin 100 µM + SNP 500 µg/ml	213.47 ± 9.87*

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ versus cisplatin-treated control determined by One-way ANOVA.

Table 31 The DAF-DA fluorescence intensity in BEAS cells quantitated by a flow cytometry, in response to 100 ng/ml FasL in the presence or absence NO donor, SNP at indicated concentrations.

Treatment	Fluorescence intensity
FasL 100 ng/ml	40.40 ± 0.81
FasL 100 ng/ml + SNP 50 µg/ml	73.82 ± 15.02*
FasL 100 ng/ml + SNP 100 µg/ml	125.00 ± 12.34*
FasL 100 ng/ml + SNP 300 µg/ml	256.33 ± 24.04*
FasL 100 ng/ml + SNP 500 µg/ml	323.33 ± 12.29*

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ versus FasL-treated control determined by One-way ANOVA.

Table 32 The DAF-DA fluorescence intensity in H460 cells quantitated by a flow cytometry, in response to 100 ng/ml FasL in the presence or absence NO donor, SNP at indicated concentrations.

Treatment	Fluorescence intensity
FasL 100 ng/ml	67.67 ± 3.51
FasL 100 ng/ml + SNP 50 µg/ml	120.26 ± 7.78*
FasL 100 ng/ml + SNP 100 µg/ml	206.33 ± 13.43*
FasL 100 ng/ml + SNP 300 µg/ml	360.93 ± 30.73*
FasL 100 ng/ml + SNP 500 µg/ml	389.95 ± 16.98*

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ versus FasL-treated control determined by One-way ANOVA.

Table 33 The percentage of apoptotic BEAS cells, detected by Hoechst 33342 assay induced by 100 µM cisplatin in the presence or absence NO donor, SNP at indicated concentrations (detected at 16 h).

Treatment	Apoptosis (%)
cisplatin 100 µM	50.00 ± 3.60
cisplatin 100 µM + SNP 50 µg/ml	37.67 ± 1.63*
cisplatin 100 µM + SNP 100 µg/ml	12.67 ± 0.67*
cisplatin 100 µM + SNP 300 µg/ml	2.67 ± 0.67*
cisplatin 100 µM + SNP 500 µg/ml	1.66 ± 1.12*

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ versus cisplatin-treated control determined by One-way ANOVA.

Table 34 The percentage of apoptotic H460 cells, detected by Hoechst 33342 assay induced by 100 µM cisplatin in the presence or absence NO donor, SNP at indicated concentrations (detected at 12 h).

Treatment	Apoptosis (%)
cisplatin 100 µM	19.08 ± 0.38
cisplatin 100 µM + SNP 50 µg/ml	9.05 ± 1.58*
cisplatin 100 µM + SNP 100 µg/ml	5.67 ± 1.54*
cisplatin 100 µM + SNP 300 µg/ml	0.67 ± 0.58*
cisplatin 100 µM + SNP 500 µg/ml	0.33 ± 0.27*

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 35 The percentage of apoptotic BEAS cells, detected by Hoechst 33342 assay induced by 100 ng/ml FasL in the presence or absence NO donor, SNP at indicated concentrations (detected at 16 h).

Treatment	Apoptosis (%)
FasL 100 ng/ml	17.39 ± 1.78
FasL 100 ng/ml + SNP 50 µg/ml	10.25 ± 1.64*
FasL 100 ng/ml + SNP 100 µg/ml	7.00 ± 2.00*
FasL 100 ng/ml + SNP 300 µg/ml	0.67 ± 0.58*
FasL 100 ng/ml + SNP 500 µg/ml	0.33 ± 0.27*

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 36 The percentage of apoptotic H460 cells, detected by Hoechst 33342 assay induced by 100 ng/ml FasL in the presence or absence NO donor, SNP at indicated concentrations (detected at 16 h).

Treatment	Apoptosis (%)
FasL 100 ng/ml	8.2 ± 2.04
FasL 100 ng/ml + SNP 50 μg/ml	4.12 ± 1.13*
FasL 100 ng/ml + SNP 100 μg/ml	0.33 ± 0.00*
FasL 100 ng/ml + SNP 300 μg/ml	0.33 ± 0.00*
FasL 100 ng/ml + SNP 500 μg/ml	0.33 ± 0.00*

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 37 The percentage of apoptotic H460 cells, detected by Hoechst 33342 assay, responding to cisplatin (0, 50, 100, 250, and 500 μM) (detected at 12 h).

Cisplatin (μM)	Apoptosis (%)
0	2.50 ± 0.00
50	10.13 ± 0.85*
100	25.38 ± 2.50*
200	43.75 ± 3.30*
500	58.75 ± 3.50*

Each value represents the mean ± S.D. of four independent experiments. * $P < 0.05$ versus non-treated control determined by One-way ANOVA.

Table 38 The percentage of apoptotic Bcl-2 overexpressed H460 cells, detected by Hoechst 33342 assay, responding to cisplatin (0, 50, 100, 250, and 500 μM) (detected at 12 h).

Cisplatin (μM)	Apoptosis (%)
0	2.78 ± 0.03
50	3.13 ± 0.25*
100	12.18 ± 1.65*
200	19.53 ± 2.67*
500	21.98 ± 3.02*

Each value represents the mean ± S.D. of four independent experiments. * $P < 0.05$ versus H460 cells control determined by One-way ANOVA.

Table 39 The percentage of apoptotic H460 cells, detected by Hoechst 33342 assay, Cells were pretreated with 300 μM AG, 300 μM PTIO, 500 μg/ml SNP, 200 μM DPTA NONOate, or 1mM NAC ,then treated with 100 μM for 12 h.

Treatment	Apoptosis (%)
control	2.88 ± 0.25
cisplatin	22.50 ± 1.92*
cisplatin+AG	32.75 ± 3.77**
cisplatin+PTIO	34.38 ± 4.31**
cisplatin+SNP	2.86 ± 0.28**
cisplatin+DPTA	2.88 ± 0.28**
cisplatin+NAC	2.84 ± 0.33**

Each value represents the mean ± S.D. of four independent experiments. * $P < 0.05$ versus non-treated control, ** $P < 0.05$ versus cisplatin-treated control determined by One-way ANOVA.

Table 40 The percentage of apoptotic H460 cells, detected by Hoechst 33342 assay, Cells were treated with 300 μM AG, 300 μM PTIO, 500 μg/ml SNP, 200 μM DPTA NONOate, or 1mM NAC for 12 h.

Treatment	Apoptosis (%)
control	0.33 ± 0.05
AG	0.25 ± 0.04
PTIO	0.33 ± 0.10
SNP	0.45 ± 0.07
DPTA	0.33 ± 0.05
NAC	0.33 ± 0.07

Each value represents the mean ± S.D. of three independent experiments.

Table 41 The DAF-DA fluorescence intensity in H460 cells quantitated by a flow cytometry, in response to cisplatin treatment (100 ng/ml) pretreated with the NO donor SNP (500 μg/ml) or the NO inhibitor AG (300 μM) (detected at 1 h after cisplatin treatment).

Treatment	Fluorescence intensity
control	61.62 ± 2.62
cisplatin	30.00 ± 5.10*
cisplatin+AG	10.50 ± 1.29**
cisplatin+SNP	253.75 ± 16.38**

Each value represents the mean ± S.D. of four independent experiments. * $P < 0.05$ versus non-treated control, ** $P < 0.05$ versus cisplatin-treated control determined by One-way ANOVA.

Table 42 The DCF-DA fluorescence intensity in H460 cells quantitated by a flow cytometry, in response to cisplatin treatment (100 ng/ml) pretreated with the antioxidant NAC (1mM) (detected at 1 h after cisplatin treatment).

Treatment	Fluorescence intensity
control	21.34 ± 2.12
cisplatin	161.33 ± 15.31*
cisplatin+NAC	5.56 ± 0.04**

Each value represents the mean ± S.D. of four independent experiments. * $P < 0.05$ versus non-treated control, ** $P < 0.05$ versus cisplatin-treated control determined by One-way ANOVA.

Table 43 The percentage of apoptotic BEAS cells, detected by Hoechst 33342 assay, responding to FasL treatment (0, 50, 100, 250 ng/ml) (detected at 16 h).

FasL (ng/ml)	Apoptosis (%)
0	1.16 ± 0.00
50	8.75 ± 0.97*
100	19.98 ± 2.02*
250	26.12 ± 3.09*

Each value represents the mean ± S.D. of four independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 44 Fluorometric assay of caspase-8 activity in BEAS cells using fluorogenic caspase-8 substrate IETD-AMC in response to FasL treatment (0, 50, 100, 250 ng/ml) (detected at 3h after treatment).

FasL (ng/ml)	Fluorescence intensity
0	187.94 ± 21.34
50	372.43 ± 43.56*
100	867.67 ± 120.17*
250	1107.56 ± 112.35*

Each value represents the mean ± S.D. of four independent experiments. Asterisks refer significant difference from the control group (non-treated control): * $P < 0.05$ determined by One-way ANOVA.

Table 45 The percentage of apoptotic BEAS cells, detected by Hoechst 33342 assay, responding to FasL treatment (100 ng/ml) pretreated with the NO donor SNP (300 µg/ml) or DPTA NONOate (200 µM), or with the NO inhibitor AG (300 µM) or PTIO (300 µM) (detected at 16 h).

Treatment	Apoptosis (%)
control	1.00 ± 0.00
FasL	18.33 ± 1.67*
FasL+DPTA	2.33 ± 0.23**
FasL+SNP	1.67 ± 0.35**
FasL+AG	31.65 ± 3.51**
FasL+PTIO	29.67 ± 3.33**

Each value represents the mean ± S.D. of three independent experiments. * $P < 0.05$ versus non-treated control, ** $P < 0.05$ versus FasL-treated control determined by One-way ANOVA.

Table 46 Fluorometric assay of caspase-8 activity in BEAS cells using fluorogenic caspase-8 substrate IETD-AMC in response to FasL treatment (100 ng/ml) pretreated with the NO donor SNP (300 µg/ml) or DPTA NONOate (200 µM), or with the NO inhibitor AG (300 µM) or PTIO (300 µM) (detected at 3 h).

Treatment	Fluorescence intensity
control	188.75 ± 14.33
FasL	779.83 ± 97.87*
FasL+DPTA	218.97 ± 23.43**
FasL+SNP	204.56 ± 20.19**
FasL+AG	1247.56 ± 119.89**
FasL+PTIO	1232.47 ± 121.33**

Each value represents the mean ± S.D. of three independent experiments. * $P < 0.05$ versus non-treated control, ** $P < 0.05$ versus FasL-treated control determined by One-way ANOVA.

Table 47 The DAF-DA fluorescence intensity in BEAS cells quantitated by a flow cytometry, in response to to FasL treatment (100 ng/ml) pretreated with the NO donor SNP (300 µg/ml) or DPTA NONOate (200 µM), or with the NO inhibitor AG (300 µM) or PTIO (300 µM) (detected at 1 h after FasL treatment).

Treatment	Fluorescence intensity
control	24.56 ± 2.23
FasL	61.33 ± 5.51*
FasL+DPTA	148.56 ± 19.56**
FasL+SNP	167.43 ± 19.48**
FasL+AG	21.33 ± 1.23**
FasL+PTIO	23.33 ± 1.67**

Each value represents the mean ± S.D. of three independent experiments. * $P < 0.05$ versus non-treated control, ** $P < 0.05$ versus FasL-treated control determined by One-way ANOVA.

Table 48 The percentage of apoptotic BEAS cells, detected by Hoechst 33342 assay, responding to treatment with the NO donor SNP (300 µg/ml) or DPTA NONOate (200 µM), or with the NO inhibitor AG (300 µM) or PTIO (300 µM) (detected at 16 h).

Treatment	Apoptosis (%)
control	0.33 ± 0.05
SNP	0.25 ± 0.00
DPTA	0.37 ± 0.10
AG	0.33 ± 0.07
PTIO	0.33 ± 0.05

Each value represents the mean ± S.D. of three independent experiments.

Table 49 Fluorometric assay of caspase-8 activity in BEAS cells using fluorogenic caspase-8 substrate IETD-AMC in response to treatment with the NO donor SNP (300 µg/ml) or DPTA NONOate (200 µM), or with the NO inhibitor AG (300 µM) or PTIO (300 µM) (detected at 16 h).

Treatment	Fluorescence intensity
control	188.64 ± 21.34
SNP	169.89 ± 43.56
DPTA	196.78 ± 34.21
AG	165.43 ± 24.67
PTIO	194.67 ± 19.36

Each value represents the mean ± S.D. of three independent experiments. * $P < 0.05$ versus non-treated control, ** $P < 0.05$ versus FasL-treated control determined by One-way ANOVA.

APPENDIX C

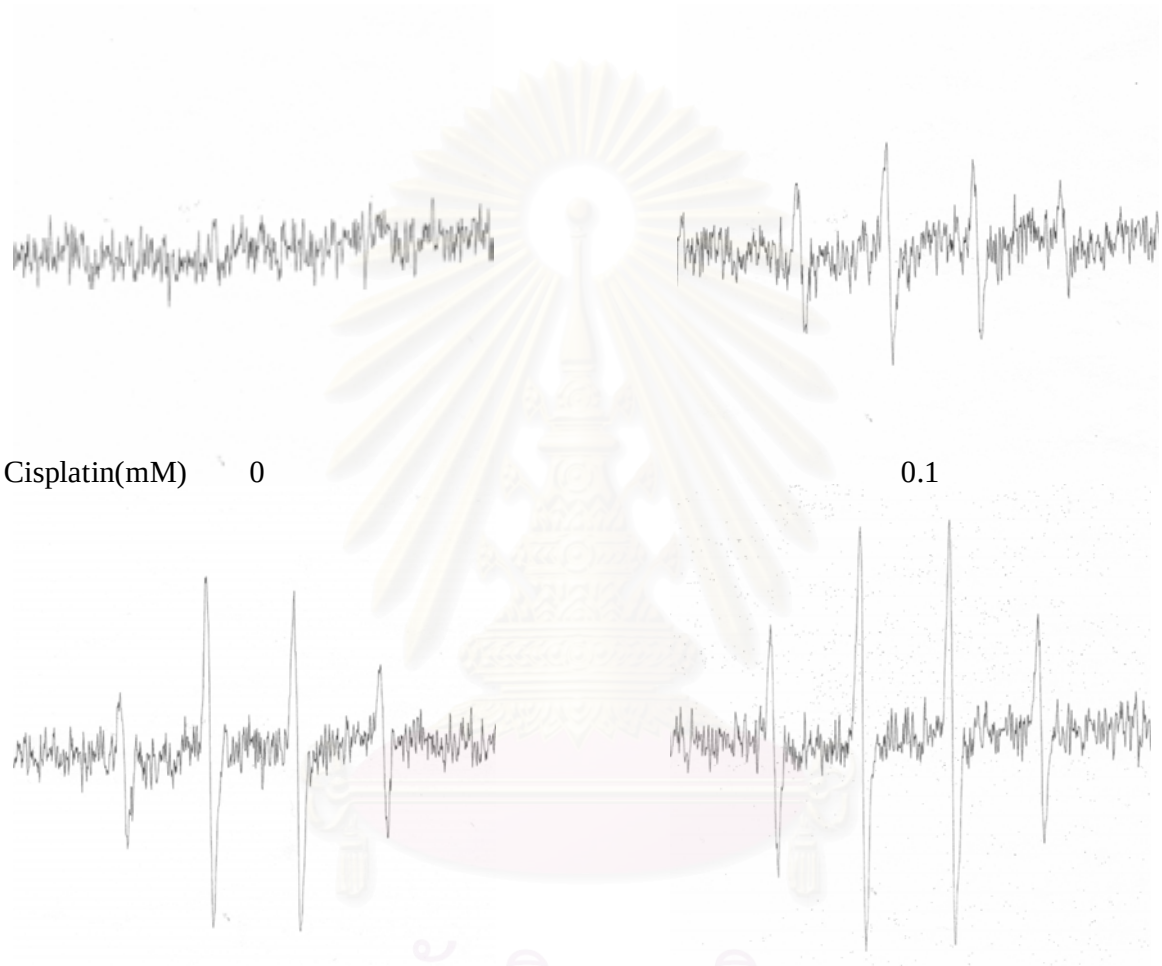

Figure 52 Specific hydroxyl radical peak (ratio 1:2:2:1) detected by ESR with DMPO. H460 cells were treated with various concentrations of cisplatin (0, 0.1, 0.5, 1.0 mM) for 10 min. Then the hydroxyl radical was detected by ESR with DMPO.

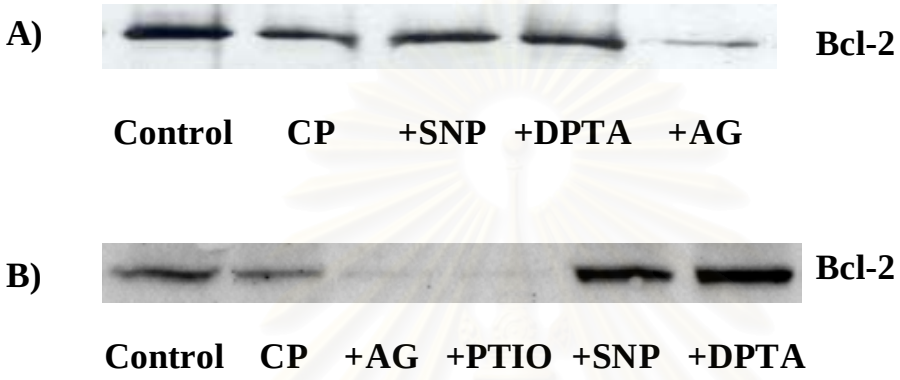

Figure 53 Effect of NO modulators on cisplatin-induced Bcl-2 dowmregulation in BEAS (A) or H460 (B) cells. The cells were pretreated for 1 h with the NO inhibitors AG (300 μM) or PTIO (300 μM), or with the NO donor SNP (500 μg/ml) or DPTA NONOate (200 μM). The cells were then treated with cisplatin (200 μM) for 12 h and cell extracts were prepared and analyzed for Bcl-2 by immunoblotting.

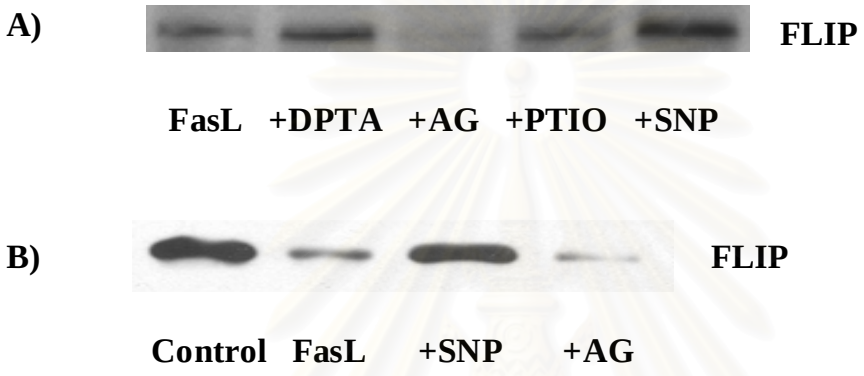

Figure 54 Effect of NO modulators on FasL-induced FLIP dowmregulation in BEAS (A) or H460 (B) cells. The cells were pretreated for 1 h with the NO inhibitors AG (300 μM) or PTIO (300 μM), or with the NO donor SNP (300 μg/ml) or DPTA NONOate (200 μM). The cells were then treated with FasL (100 ng/ml) for 12 h and cell extracts were prepared and analyzed for FLIP by immunoblotting.

Vita

Mr. Pithi Chanvorachote was born on March 18, 1980 in Bangkok. He received his B.Sc. in Pharmacy (1st honor) from the Faculty of Pharmaceutical Sciences, Chulalongkorn University in 2002.