บทบาทของไนตริกออกไซด์ต่อการแสดงออกของอินทิกรินและการเคลื่อนที่ในเซลล์มะเร็งปอดของมนุษย์

นายวุฒิพงศ์ สายสงเคราะห์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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ROLE OF NITRIC OXIDE ON INTEGRIN EXPRESSION AND MIGRATION IN HUMAN LUNG
CANCER CELLS

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วุฒิพงศ์ สายสงเคราะห์ : บทบาทของไนตริกออกไซด์ต่อการแสดงออกของอินทิกรินและการเคลื่อนที่ในเซลล์มะเร็งปอดของมนุษย์ (ROLE OF NITRIC OXIDE ON INTEGRIN EXPRESSION AND MIGRATION IN HUMAN LUNG CANCER CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภก. ดร.ปิติ จันทร์วรโชติ, 72 หน้า.

ไนตริกออกไซด์ ก๊าซอนุมูลอิสระซึ่งนอกจากมีบทบาทควบคุมการขยายตัวของหลอดเลือดและกระบวนการอักเสบแล้วยังมีอิทธิพลต่อการแพร่กระจายของเซลล์มะเร็งอีกด้วย อย่างไรก็ตาม ผลของไนตริกออกไซด์ต่อการแสดงออกของอินทิกริน ซึ่งเป็นโปรตีนที่ควบคุมการยึดเกาะและการเคลื่อนที่ของเซลล์ยังคงไม่ทราบเป็นที่แน่ชัด เนื่องจากการเปลี่ยนแปลงรูปแบบ (การแสดงออก) ของอินทิกรินมีผลต่อการเคลื่อนที่ตลอดจนการแพร่กระกระจายของเซลล์มะเร็ง ดังนั้นในงานวิจัยนี้จึงศึกษาผลของไนตริกออกไซด์ต่อรูปแบบการของอินทิกริน ตลอดจนกลไกที่เกี่ยวข้องที่ใช้ในกระบวนการควบคุมการแสดงออกของอินทิกริน ผลการทดลองพบว่า การรับสัมผัสไนตริกออกไซด์ในขนาดที่ไม่เป็นเป็นพิษต่อเซลล์ (0-50 mM) มีผลเหนี่ยวนำให้เซลล์มะเร็งปอด (H460, H292 และ H23) มีความสามารถในการเคลื่อนที่ที่เพิ่มขึ้น โดยความสามารถในการเคลื่อนที่ของเซลล์จะมีความสอดคล้องกับระดับการแสดงออกของอินทิกรินชนิด α_v และ β_1 ตลอดจนโปรตีนที่ทำหน้าที่ควบคุมการเคลื่อนที่ของเซลล์ ได้แก่ FAK, Rho-GTP และ Cdc42-GTP อย่างมีนัยสำคัญ ในขณะที่เมื่อพิจารณารูปลักษณะของเซลล์พบว่า เซลล์มีการเปลี่ยนแปลงลักษณะทางสัณฐานวิทยา คือ มีจำนวนของ filopodia ซึ่งเป็นโครงสร้างแอคตินที่ใช้สำหรับการเคลื่อนที่ของเซลล์เพิ่มขึ้นเช่นกัน นอกจากนี้ในการศึกษาลำดับขั้นหรือกลไกที่ใช้ในการควบคุมการแสดงออกของอินทิกรินในทางเภสัชวิทยาพบว่าเมื่อยับยั้งการทำงานของโปรตีน AKT ซึ่งเป็นเอนไซม์ที่ถูกควบคุมโดยโปรตีน PKG พบว่าการเปลี่ยนแปลงลักษณะการแสดงออกของอินทิกรินถูกยับยั้งได้ด้วยตัวยับยั้งการทำงานของ AKT จากการเปลี่ยนแปลงลักษณะดังกล่าว บ่งชี้ให้เห็นว่าไนตริกออกไซด์ควบคุมการแสดงออกของอินทิกรินโดนผ่านวิถีสัญญาณ PKG-AKT โดยสรุปงานวิจัยนี้ได้แสดงให้เห็นถึงบทบาทใหม่ของไนตริกออกไซด์ในการควบคุมการแสดงออกของอินทิกรินและพฤติกรรมการเคลื่อนที่ของเซลล์มะเร็ง

ภาควิชา	เภสัชวิทยาและสรีรวิทยา	ลายมือชื่อนิสิต _____
สาขาวิชา	เภสัชวิทยาและพิษวิทยา	ลายมือชื่อ อ.ที่ปรึกษาหลัก _____
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VHUDHIPONG SAISONGKORH: ROLE OF NITRIC OXIDE ON INTEGRIN EXPRESSION AND MIGRATION IN HUMAN LUNG CANCER CELLS. ADVISOR: ASSOC. PROF. PITHI CHANVORACHOTE, Ph.D., 72 pp.

Nitric oxide (NO), a highly reactive free radical gas involving in vascular control and inflammatory response, has influenced on cancer metastasis, but the understanding of its regulation on integrin, an important protein functioning in adhesion and motility, is still largely unknown. Alteration of integrin pattern has been shown to have a dramatic impact on cancer cell migratory activities as well as the survival during dissemination. This study has revealed that the human lung cancer cell lines (H460, H292 and H23) exposed to non-toxic concentrations of NO (0-50 μM) had higher level of integrin αv and β1, and such an increase of the specific integrins correlated with augmented ability of cells to motile. The known migratory proteins downstream of integrins including focal adhesion kinase (FAK), active RhoA (Rho-GTP), active cell division control 42(Cdc42-GTP) were found to be significantly activated in response to NO. Besides, this study observed the augmented motile morphology of NO-treated cells indicated by the dramatic increase of filopodia (actin protrusion) per cell. In terms of underlying mechanism, this study also found that NO-treated cells exhibited increased active protein kinase G (PKG), protein kinase B (AKT) and FAK. Using pharmacological approach, as AKT is downstream target of PKG, the results proved that integrin modulating effect of NO was mediated through PKG/AKT-dependent mechanism as the change in integrin as mentioned were completely diminished by AKT inhibitor, but not FAK inhibitor. These findings indicate a novel role of NO on integrin regulation and its importance in migratory cancer behavior.

Department:	Pharmacology and Physiology	Student's Signature	....
Field of Study:	Pharmacology and Toxicology	Advisor's Signature	....
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LIST OF ABBREVIATIONS

AKT	=	protein kinase B
ANOVA	=	analysis of variance
Bad	=	BCL2-associated death promoter
Bak	=	BCL2 homologous antagonist/killer
Bax	=	BCL2-associated X
Bcl-2	=	B-cell lymphoma 2
Bcl-XL	=	B-cell lymphoma-extra large
Bid	=	BH3 interacting domain death agonist
Bim	=	BcL-2-like protein 11
Cdc42	=	cell division control protein 42
CO_2	=	carbon dioxide
DMSO	=	dimethyl sulfoxide
ECM	=	extracellular matrix
EMT	=	epithelial-mesenchymal transition
FAK	=	focal adhesion kinase
GDP	=	guanosine diphosphate
GTP	=	guanosine triphosphate
h	=	hour, hours
MAPK	=	mitogen activated protein kinase
min	=	minute (S)
ml	=	milliliter
mM	=	millimolar
MMP	=	matrix metalloproteinase

MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	=	nitric oxide
NSCLC	=	non-small cell lung cancer
PBS	=	phosphate buffer saline
PI	=	propidium iodide
PKG	=	protein kinase G
RPMI	=	Roswell Park Memorial Institute's medium
S.D.	=	standard deviation
Src	=	proto-oncogene tyrosine-protein kinase
U	=	unit
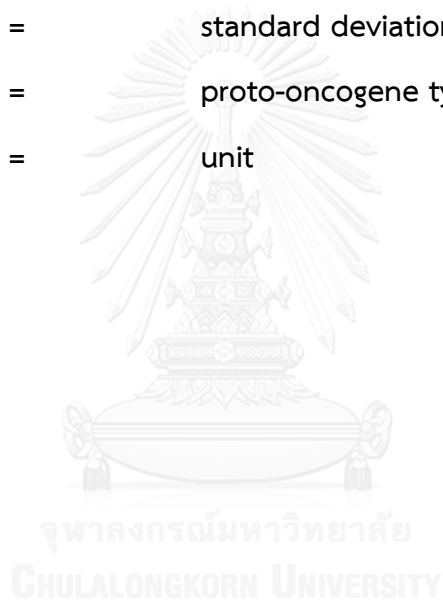
CHAPTER I

INTRODUCTION

Background and rationale

The microenvironment of cancer has a dramatic impact on cancer metastasis and aggressive behavior. As metastasis is one of the crucial factors determining survival of cancer patients, attempts have been made to identify key molecular mediators that control metastasis. It has become evident that the endogenous gas nitric oxide (NO) which is frequently found to be up-regulated in cancer-related area [1], acts as a key transcellular messenger in regulation of the cardiovascular and neuronal systems [2, 3], and plays a role in progression of several pathological conditions including cancer [4, 5]. In terms of cancer, macrophage-derived NO was shown to function as an anti-cancer substance, by directly interacts with DNA and protein resulted in cancer cell toxicity [6]. However, cancer-derived nitric oxide has been shown to have tumor-potentiating functions. In lung cancer, NO was shown to facilitate cell survival after detachment [7].

Furthermore, various studies have pointed out that the excessive and uncontrolled nitric oxide production positively correlated with cancer metastasis in many cancers [4, 8-11]. Regarding cancer metastasis, an essential early step of cancer cell dissemination is activated by the turnover of cell-extracellular matrix (ECM) adhesion resulting in cancer cell migration. Dynamically, new interactions between cell and matrix are made in the front and the old adhesions are disassembled in the rear of cells that motile in response to a gradient of stimulating factors or ECM proteins [12]. The key proteins functioning in cell adhesion are integrins.

Integrins are a diverse family of transmembrane glycoprotein receptors which mediated dynamic interactions between ECM and actin cytoskeleton during cell motility. Integrin interaction with ECM components provides several cellular signals controlling cell survival and motility [13, 14]. In terms of motility, integrin activates the FAK/Src pathways lead to the increase activity of Rho family of small GTPase including Rho, Rac and cell division control protein 42 (Cdc42) [12] resulting in cytoskeletal dynamic shift and formation of lamellipodia and filopodia respectively [15]. Accumulated evidence in recent years suggest that enhanced migration in cancer cells are accompanied by switches in cellular integrin pattern with some integrins decreased and others increased [16, 17]. Importantly, the increase of integrins αv, $\beta 5$, $\alpha 1$, and $\beta 3$ has been demonstrated to enhance migration and metastasis competence in cancer [18-21].

So far, the effect of NO in regulation of integrin expression pattern in lung cancer cells and its contribution to cell motility is largely unknown. Therefore this study aims to investigate the role of NO in regulation of integrins expression and cell migration in human lung cell lines. The expected knowledge gain from this study could be important in the understanding of migration behavior in metastatic of lung cancers.

Research Questions

1. Does NO enhance lung cancer cells migration via up-regulation of αv, $\beta 5$, $\alpha 1$, and $\beta 3$ integrins expression?

2. Does NO mediate integrin expression through AKT dependent mechanism?

Objectives

1. To investigate the effect of NO on integrin expression and migration behavior in H460, H292 and H23 human lung cancer cell lines.

2. To find out specific integrin that influence on H460, H292 and H23 cells migration.

3. To investigate the involvement of AKT protein induced by NO in regulation of αv, $\beta 5$, $\alpha 1$, and $\beta 3$ integrin expression.

Hypothesis

NO enhances human lung cancer cell migration through up-regulation of migration related integrin (αv, $\beta 5$, $\alpha 1$, and $\beta 3$) via AKT dependent mechanism.

Expected benefits

The results of this study may clarify role of NO in regulation of lung cancer cells migration and integrin expression, which can help in development of novel anti-cancer strategy and better understanding in lung cancer biology.

CHAPTER II

LITRATURE REVIEW

1.   Lung cancer

Lung cancer is the most common malignancies worldwide, generated from normal epithelial cells continuously genetic damage leading to uncontrolled proliferation resulting in abnormal cells growth in the airway of lungs. The incidence as well as mortality rate of patients with lung cancer has increase annually. There are two major types of lung cancer, non-small cell lung cancer (NSCLC; 85% of all lung cancer cases) and small cell lung cancer (SCLC; about 15%) [22]. Moreover, histological classification can be divided NSCLC into three major subtypes including squamous cell carcinoma, adenocarcinoma and large-cell carcinoma. The importance of classification is revealed to treatment strategy and prediction of cancer outcome.
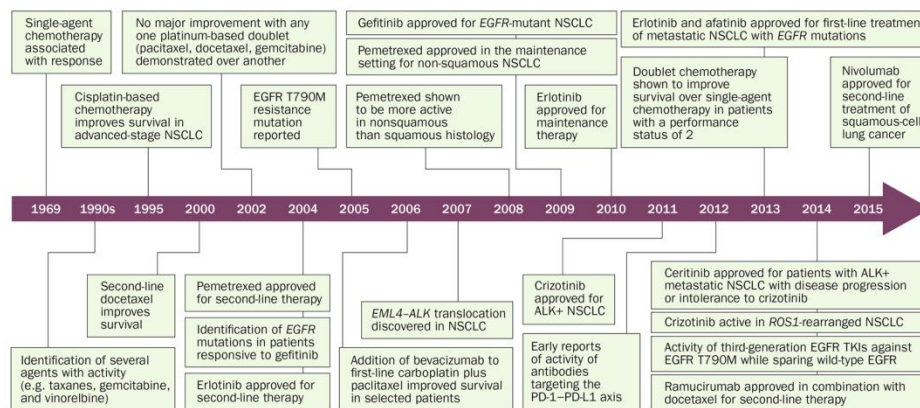
Figure 2.1 Drug developments in lung cancer therapy

At the present day, surgery, radiation, chemotherapy and targeted therapy are used in lung cancer treatment. Despite among multiple approaches and new strategies has been developed continuously in cancer treatment (Figure 2.1), the survival rate of lung cancer patients still very low. The incidence of cancer mortality has indicated that the major cause of lung cancer death is due to metastasis [23].

2. Metastasis of lung cancer

Low survival rate and poor clinical outcome of lung cancer is due to highly metastatic potential of cancer cells. Metastasis is the spread of cancer cells from the origin site to new areas of the body, comprising of five major steps (Figure 2.2) including;

(i)   Migration and Invasion

(ii)   Intravasation

(iii)   Survival in the circulation

(iv)   Extravasation

(v)   Metastatic colonization at the secondary site.

Among the multiple steps, cell migration has implicated as essential early step which referred to a major hallmark of metastasis [24, 25].
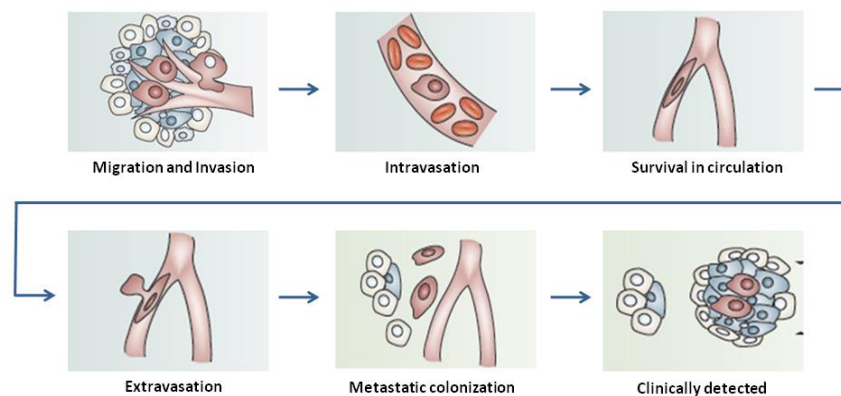
Figure 2.2 The step of cancer metastasis [24]

2.1   Cell migration

Regarding cancer metastasis, an essential early step of cancer cell dissemination from primary tumor site is activated by the turnover of cell-extracellular matrix (ECM) adhesion resulting in cancer cell migration.

Cell migration is the process by which cells move from one location to another by adopting different motility modes in such single cell or multicellular

streaming. The distinct modes of cell migration contribute to diverse type of cellular movement including collective cell migration and single cell migration.

Dynamically, new interactions between cell and basement matrix are made in front of protrusion areas, and the old adhesions are disassembled in rear body of cells that drive cell move forward in response to a gradient of stimulating factors or ECM proteins.
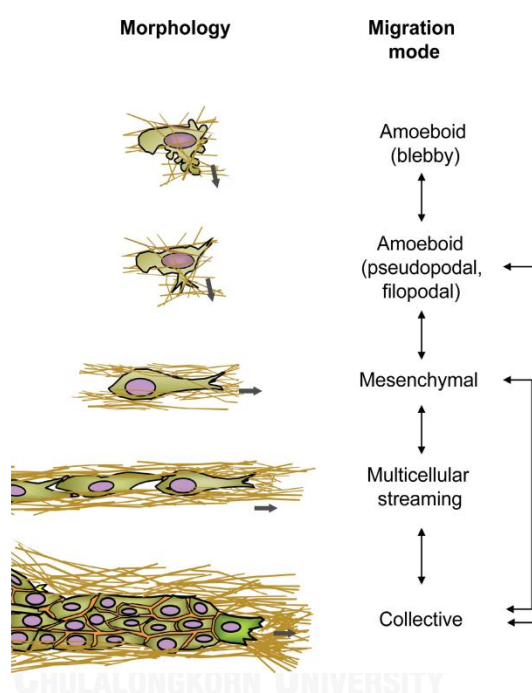
Figure 2.3 Cell morphology and mode of migration [26]

Collective cell migration is the movement while intercellular interaction is retained resulting in co-ordinate movement. In contrast, single cell migration is described as individual movements which can divide into two subtypes consist of mesenchymal migration and amoeboid migration.

Even though, mesenchymal migration and amoeboid migration are shared many feature together, they also behave the difference in characteristic during cellular movement. Mesenchymal migration is the movement which mediated through integrin-extracellular matrix (ECM) contact or integrin-dependent migration

whereas amoeboid migration exhibits relative weak integrin contact and can even be integrin-independent migration [27, 28].

2.2   Migration in lung cancer cells

The metastasis process of lung cancer cell is usually initiated with micrometastasis which involved in mesenchymal migration. This single cell migration type is characterized by the polarization to form leading-direction edge. After polarization, cells extend actin filament on protrusion areas to form new adhesion contact, follow by rearward retraction to achieve complete cellular movement.

These multiple step are associated with cell surface receptor known as "integrin" which play a key function in adhesion of ECM and transduction of signals during the migration [29].
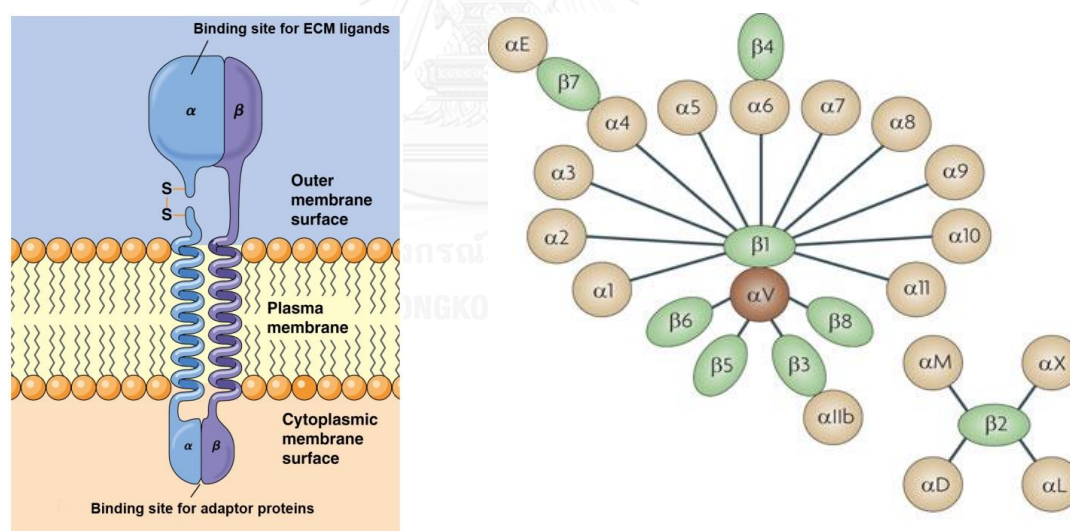
Figure 2.4 Integrin structure and family [30]

3   Integrin

3.1   Integrin structure and family

Integrins are a diverse family of transmembrane glycoprotein receptors which mediated dynamic interactions between ECM and actin cytoskeleton during cellular movement. Integrin family can form at least 24 distinct heterodimer of 18 α-subunits and 8 β-subunits. The heterodimer of integrins generally consist of non-covalent bond link between α- and β-subunit, with each subunit has a large extracellular domain (extracellular region), a single membrane-spanning domain and a short cytoplasmic domain (intracellular region) [31].

3.2   Integrin in biological function

Integrin receptors are the key proteins that regulate diverse cellular functions. The major function of integrin is not only attachment the cell cytoskeleton to ECM but also transduction of biological signaling to cell. These signals are necessary for cell migration, invasion, survival, proliferation and differentiation.

In the migration aspect, Integrin binds to ECM proteins and provide the traction force for cell motility. Initially, integrin ligation activates focal adhesion kinase (FAK) which can stimulate Rho GTPase family including Rho, Rac and Cdc42 [32]. Furthermore, Integrins are also associated with regulating the activities of protease enzymes called matrix metalloproteinase (MMP) that degrade the basement membrane. Numerous studies indicate that MMP activating is involved in $\alpha v \beta 3$ integrin result in cancer cell migration and invasion [33, 34].

Moreover, cell to matrix adhesion is a key factor for cell survival and proliferation. The role of integrins in cell survival is suppressing the apoptosis in attached cells. At least 4 heterodimers of integrin ($\alpha 5 \beta 1$, $\alpha v \beta 3$, $\alpha 1 \beta 1$ and $\alpha 6 \beta 1$) have reported to play an important role in cell survival. Activation signaling from

these heterodimers is able to stimulate survival pathway such as PI3K/AKT and mitogen activated protein kinase (MAPK). These pathways prevent apoptosis cell death by inhibiting pro-apoptotic protein (Bid and Bim) and activating anti-apoptotic protein (Bcl-2, Bcl-XL, and Mcl-1).

On the other hand, loss of attachment to ECM, cells undergo especially apoptosis in termed "anoikis" [35]. In cell proliferation, cell cycle progression is involved in integrin-mediated adhesion, by regulating of cyclin D1 which is a necessary protein for progression through the G1 phase of the cell cycle. Integrin also activates FAK which plays a role in phosphorylation of ERK and induction of cyclin D1 [36].

3.3   Regulation of integrin

Almost integrins are expressed and remain in a low-affinity binding state until achieve signaling to activate and transforms them into a high-affinity form. There are two directions of integrin activation signaling including "inside out signaling" and "outside in signaling", which have different in biological consequences.

Cytoplasmic domain of integrin plays a central role in integrin activation. During inside out signaling, talin is a major cytoskeleton protein that co-localizes and binds to β-cytoplasmic domain. This binding result in turn activate of integrin leading to change in conformation and increase the affinity to ECM. The affinity strength between integrin and ECM should be sufficiently strong for cell adhesion and migration assembly. On the other hand, outside-in signaling is a transmission of signals from ECM to intracellular proteins. The binding of α-subunit and ECM ligands such as fibronectin, fibrinogen and collagen results in conformation changes to high affinity. Then, integrin is clustered and leading to transmit information into cell [37, 38].
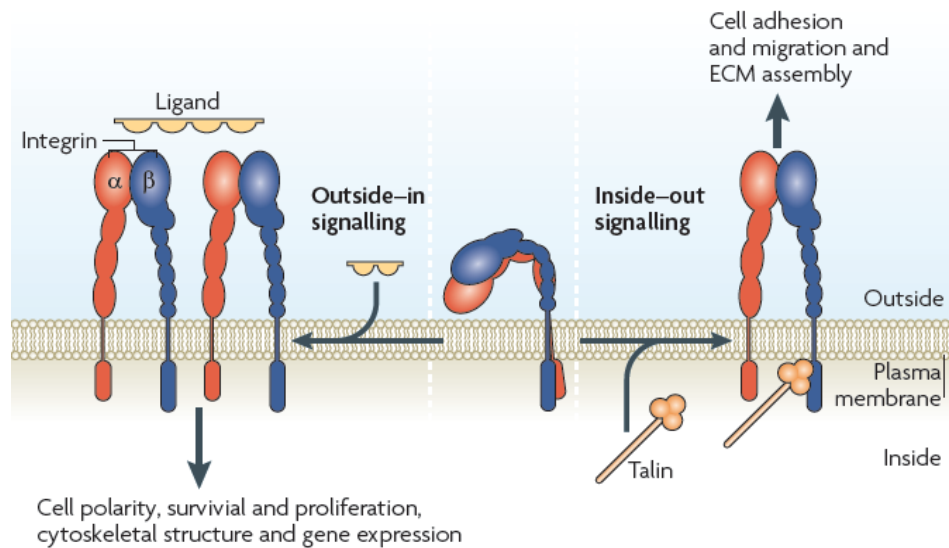
Figure 2.5 Activation of integrin [37]

The combination of inside-out and outside-in signaling control several cellular process such as cell polarity, cytoskeleton structure, gene expression, cell proliferation and cell survival [37].

The biological mechanism that control over-activity of integrin is the trafficking of integrin receptor. This mechanism is a process comprising of intracellular internalization and recycling pathways. There are three different types of endocytosis include Raft/caveolar endocytosis (RCE), clathrin-mediated endocytosis (CME) and pinocytic or macropinocytic mechanisms.

Integrin is internalized via RCE and CME pathways and then is proceeded to the early/late endosome. Briefly, integrin is internalized from the plasma membrane into internal membrane. After that, vesicles fuse with the early endosome and then integrin is recycled back to the cell surface or degraded in the late endosome or lysosome [39].

3.4   Integrin signaling during migration

In the role of migration, the affinity of individual integrin has different in strength of ECM-binding. Thereby, each combination of integrin displays a unique in migration capacity. The diversity of migration is regarded to integrin expression. For example, adhesion binding of fibronectin with $\alpha5\beta1$ are more dynamic than $\alpha v\beta3$ which are associated with more persistent of migration in cancer cell [12].

Activation of integrin consequently stimulates several intracellular signaling pathways. These downstream pathways typically involve in activation of focal adhesion kinase (FAK) protein, by recruit and engage of FAK protein to cytoplasmic domain of β-subunit. Activation of FAK protein subsequently results in phosphorylation and turn activates Rho family of GTPase including Rho, Rac and Cdc42 which mediated actin polymerization during migration [15, 40].

Focal adhesion kinase (FAK)

FAK is a focal adhesion-associated protein, encoded by PTK2 gene (protein tyrosine kinase). FAK act as essential early protein that co-localize with integrin on cytoplasmic domain and facilitate migration through transmit integrin signaling. FAK is recruited and activated by signaling from integrin. The major phosphorylation site of FAK is identified as tyrosine 397 (Tyr^{397}).

In the migration aspect, after FAK protein was phosphorylated at active site, FAK form a complex with Src family kinase proteins, which generate multiple downstream signaling through phosphorylation of other adaptor proteins to regulate cell migration [15, 40].

The signaling of integrin through FAK protein has been shown to promote cell migration in various studies [40, 41]. For example, the over expression or activation of FAK signaling protein was found to be associated with migratory behavior of cancer cells.

Moreover an increasing of FAK expression levels have also been correlated with invasive and metastatic potential in several human tumors [42, 43].
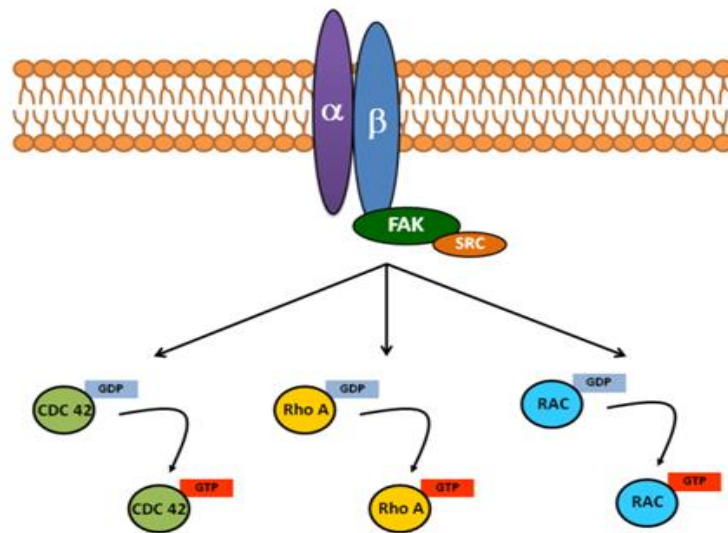
Figure 2.6 Role of integrin on cell migration

Rho GTPase family

Rho GTPase is a family of small signaling G proteins comprised of Rho, Rac and Cdc42. Rho GTPase acts as effector proteins of integrin which mediate many aspects of intracellular actin dynamics. Individual Rho GTPase has played the different role in rearrangement of actin cytoskeleton during the migration [15].

The activation of Rho (Rho-GTP) results in the formation of actin stress fiber and the assembly of focal adhesion, while activation of Cdc42 (Cdc42-GTP) is associated with the formation of filopodia, a slender cytoplasmic projection that extend beyond the leading edge. Activation of Rac (Rac-GTP) promotes membrane ruffling called lamellipodium, a characteristic phenotype of leading edge region that are dynamically alternating between adherent and non-adherent states [15, 44].
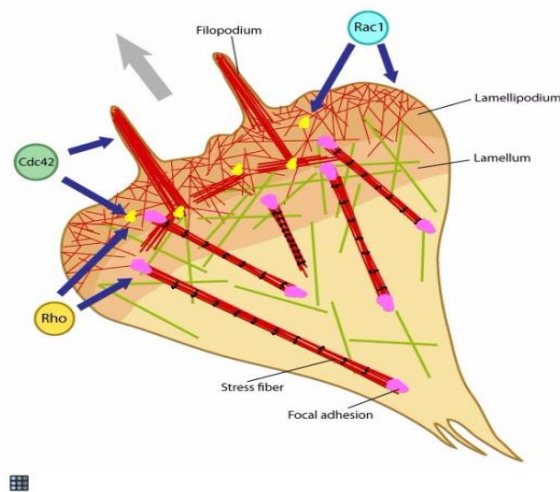
Figure 2.7 Rho GTPase family function [12]

3.5   Integrin and cancer aggressive

Over the last decade, there are many studies examine the association between integrin expression and cancer aggressiveness. Almost experiments exhibited, an increasing of integrin expression is always correlated with cancer poor prognosis. For example, over expression of $\alpha6$ integrin is reported to be associated with metastatic phenotype in human breast cancer [45], whereas integrin $\alpha v\beta3$ is required in bone metastasis of prostate cancer [46].

Table 2.1 Cancer-related integrin expressions [31]

Tumor type	Integrin expression	Associated phenotypes
Melanoma	$\alpha v\beta3$ and $\alpha5\beta1$	Vertical growth phase and lymph node metastasis
Breast	$\alpha6\beta4$ and $\alpha v\beta3$	Increased tumor size and grade, and decreased survival ($\alpha6\beta4$). Increased bone metastasis ($\alpha v\beta3$)
Prostates	$\alpha v\beta3$	Increased bone metastasis
Pancreatic	$\alpha v\beta3$	Lymph node metastasis
Ovarian	$\alpha4\beta1$ and $\alpha v\beta3$	Increased peritoneal metastasis ($\alpha4\beta1$) and tumor proliferation ($\alpha v\beta3$)
Cervical	$\alpha v\beta3$ and $\alpha v\beta6$	Decreased patient survival
Glioblastoma	$\alpha v\beta3$ and $\alpha v\beta5$	Both are expressed at the tumor–normal tissue margin and have a possible role in invasion
NSCLC	$\alpha5\beta1$	Decreased survival in patients with lymph node-negative tumors
Colon	$\alpha v\beta6$	Reduced patient survival

Interestingly, the increasing of some certain integrin expression especially αv, α5, β1, and β3 are correlated with high aggressive and poor prognosis phenotype in human lung cancers. Each subunit was evidenced in promotion of cancer cell migration [18-21]. Therefore, expression of these subunits was investigated in this study.

4   Nitric oxide

4.1   Biological source of nitric oxide

Nitric oxide (NO) is a highly reactive free radical, which plays multifunction in physiological and pathological processes. NO is initially identified as a signaling molecule synthesized from the conversion of L-arginine into L-citrulline by a family of enzyme called nitric oxide synthase (NOS) in the presence of NADPH, oxygen and co-factors. NOS family consist of three isoforms including; neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). Because of nNOS and eNOS isoforms constantly generate NO upon physiological process, therefore nNOS and eNOS are referred as constitutive NOS (cNOS). In contrast, iNOS isoform is regulated and induced under pathological condition [47].

NADPH   **NADP⁺**

L-arginine + O2 ⟶ **L-citrulline + NO**

NOS
FMN/FAD/CaM/Heme/BH₄
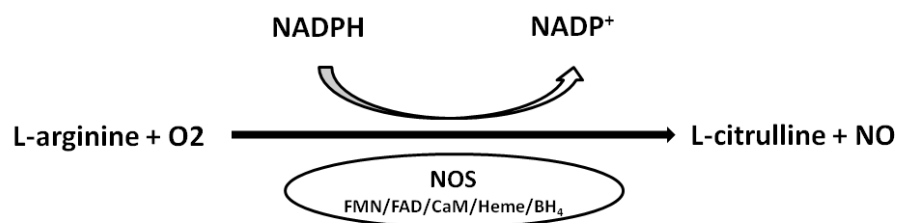
Figure 2.8 Synthesis of nitric oxide

4.2   Biological function of nitric oxide

Role of NO has been first described in the regulation of blood pressure and protection from cardiovascular diseases. By the time, NO have been demonstrated that include its role in immune system, nervous system, inflammation and cancer [48].

NO manifests its biological action mainly through cGMP signaling pathway and S-nitrosylation reaction. cGMP is a cyclic nucleotide which acts as a secondary messenger in several cellular processes such as vasodilation, regulation of mitochondrial function and biogenesis. NO is necessary to activate soluble guanylyl cyclase (sGC) which converse of guanosine 5′-triphosphate (GTP) to cyclic guanosine 3′,5′-monophosphate (cGMP) [49, 50]. Moreover, cGMP also activates cGMP-dependent protein kinase (PKG) and then trigger to activate AKT which plays a role in activation of cell migration [5].

S-nitrosylation is another major mechanism that NO mediates biological function. S-nitrosylation is a kind of post-translational proteins modification which produces covalent bond between nitrogen monoxide and thiol side chain of cysteine residue. The result of S-nitrosylation is changing in protein conformation lead to alteration of protein function [5, 49].
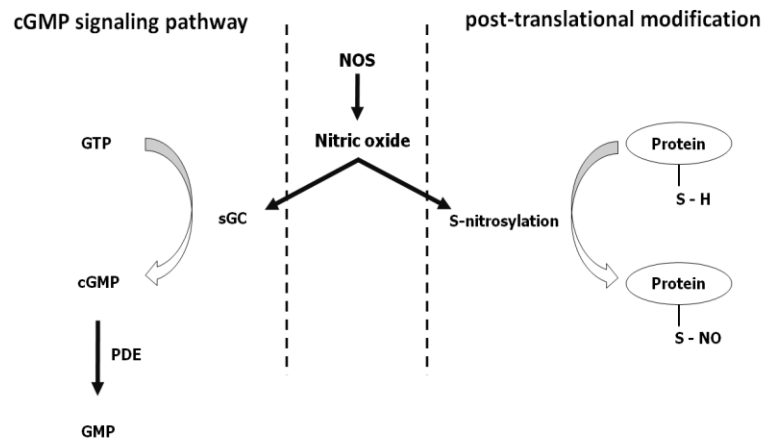
Figure 2.9 Biological action of nitric oxide [5]

4.3   Nitric oxide and cancer

Under normal physiological conditions, NO serves as a key signaling molecule in various functions such as smooth muscle relaxation, blood flow regulation, iron homeostasis, and platelet reactivity. On the other hand, excessive and uncontrolled NO production, an elevation of NO levels have been implicated as causal and contributing to pathological events such as infection, chronic inflammation and cancerous condition [51].

Clinical data has been shown that, an increasing of NO and its derivative is frequently detected in tumor tissues. Moreover the higher level of NO in exhaled breath of lung cancer patients is always correlated with advancing stage and poor clinical outcome [1]. There are strong evidences reported that, the increasing of NO levels which always found in cancer environment is concerned with up-regulation of iNOS in tumor sites [52, 53].

In addition, iNOS is widely presence in cancer cells and distinct neighboring cells such as tumor-associated endothelium, stromal fibroblast and immune cells. Moreover, not only cancer and supporting cells but also inflammatory cytokine such as interferon-γ, interleukin-1β and tumor necrosis factor-α can induce iNOS activation

resulting in increasing of NO levels in tumor site approximately 1,000-fold to micromoles unit [5].
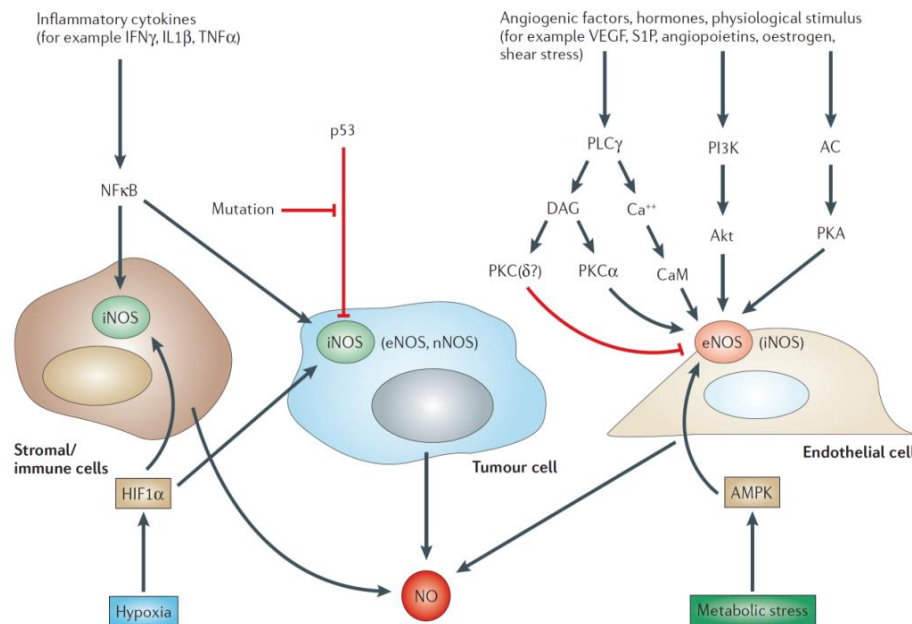
Figure 2.9 Production of nitric oxide in cancer cells [5]

According to clinical finding, in *vivo* study has demonstrated that, NO plays an essential role in promotion of cancer progression by induce cell migration, matrix degradation and angiogenesis [54]. Many interested-report indicated that, an elevation of NO in cancer environment can be increase tumor metastasis through various mechanism such as SRC protein, a key mediator molecules in regulation of migration and invasion can be activated by S-nitrosylation reaction at cysteine 498 (Cys[498]) resulting in enhance metastatic phenotype of cancer cells [55], NO also promotes cell motility by modulating the actin dynamic change through activated Rho GTPases family protein [56]. Moreover NO has been reported in promoting cell migration through activated PI3K/AKT pathway.

Now a day, NO also activates phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway which regulate multiple aspects of cellular function including migration and invasion. For example, NO enhanced endothelial cell migration and

invasion by activating of AKT signaling protein [57, 58]. Therefore, an activation of AKT may involve in alteration of integrin mediated cell migration.

Although the role of NO on migration has already been described but the effects of NO on integrin expression are remained unclear. Therefore, this study aims to investigate the effect of NO on integrin expression, migration capacity and the involved signaling in human lung cancer cell lines (H460, H292 and H23 cells). The expected benefit gain from this study may provide role of NO in regulation of lung cancer cell migration and integrin expression, which can help in development of novel anti-cancer strategy and better understanding in lung cancer biology.

CHAPTER III

MATERIALS AND METHODS

1   Cells cultures

Human lung cancer cell lines; NCI-H460, NCI-H292 and NCI-H23 (Table 3.1) were obtained from the American Type Culture Collection ATCC$^{®}$ (Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 units/ml of each penicillin and streptomycin (Gibco, MD, USA) at 37 °C with 5% CO2 in a humidified incubator. The medium was changed at an interval of 2-3days. At 70-80% confluence, the cells were rinsed with phosphate buffer saline (PBS) and trypsinized by trypsin-EDTA.

Table 3.1: Human lung cancer cell line description

	NCI-H460 cells	**NCI-H292 cells**	**NCI-H23 cells**
Organism	Homo sapiens	Homo sapiens	Homo sapiens
Tissue	Pleural effusion	Lymph node	Primary tumor
Morphology	Epithelial	Epithelial	Epithelial
Disease	Large cell carcinoma	Mucoepidermoid carcinoma (MEC)	Adenocarcinoma

2   Reagents

NO donor; Dipropylenetriamine (DPTA) NONOate (Figure 3.1) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), dimethyl sulfoxide (DMSO), Hoechst33342, propidium iodide (PI), phalloidin-rhodamine fluorescent dye were purchased from Sigma Chemical, Inc. (St. Louis, MO,USA). BCA protein assay kits were purchased from Pierce Biotechnology (Rockford, USA). Perifosine (Akt inhibitor reagent), FAK inhibitor14 (FAK inhibitor reagent) and antibodies for integrin αv, α5, β1, β3, PKG,

p-Akt (Tyr308), Akt, p-FAK (Tyr397), FAK, β-actin and secondary antibodies were purchased from Cell Signaling (Danvers, MA, USA). Antibodies for Rho-A GTP and Cdc42 were purchased from New-East Bioscience (King of Prussia, PA, USA). Chemi-luminescence substrates were purchased from Supersignal West Pico; Pierce (Rockford, USA).
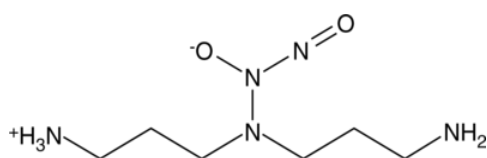
Figure 3.1 Dipropylenetriamine (DPTA) NONOate

3   Instruments

- Fluorescence microplate reader (Anthros, Durham, USA)

- Fluorescence microscope (Olympus IX51 with DP70, Japan)

- Automated cell counter (TC20™ Bio-Rad, Singapore)

- Auto-pipette 0.2-2 µl, 2-20 µl, 20-200 µl and 100-1,000 µl (Corning®, USA)

- Cell culture plate: 6-well and 96-well (Corning®, USA)

- Boyden chamber / transwell plate (BD Bioscience, USA)

- Centrifuge (CF-10 Wise spin®, Korea)

- Conical tube 15 ml and 50 ml (Corning®, USA)

- Duran bottle 100 ml, 250 ml, 500 ml and 1000 ml (Mainz Germany)

- Laminar air flow cabinet (Boss tech, Thailand)

- Carbon dioxide humidified incubator (Thermo scientific, USA)

- pH meter (Mettler-Toledo, Switzerland)

- Vortex mixer (Bohemia, USA)

4   Cytotoxicity analysis

4.1   Cell viability assay

Cell viability was determined by MTT assay. Human lung cancer cell lines (H460, H292 and H23) were seeded at density 1.0×10^4 cells/well in 96-well plate, and then treated with DPTA NONOate at various concentrations (0-250 μM) for 24 h. After exposing to NO donor, cells were treated with MTT (400 μg/mL) and incubated for 4 h at 37°C. Then MTT supernatant solution was removed, and 100 μL of DMSO was added to dissolve formazan product. The optical density (OD) of formazan product was measured by spectrophotometer at 570 nm using microplate reader. The percentage cell viability was calculated as absorbance of NO-treated cells related to non-treated control as follow.

$$\text{Percent cell viability} = \frac{\text{A570 of treatment group}}{\text{A570 of control group}} \times 100$$

4.2   Apoptosis and necrosis evaluation assay

Human lung cancer cell lines (H460, H292, H23 cells) were seeded on 96-well plate at a density 1.0×104 cells/well and incubated overnight, and then cells were treated with DPTA NONOate (0-100 μM) for 24 h. After NO exposure, cells were rinsed with phosphate-buffers saline (PBS) and incubated with 10 μg/mL Hoechst 33342 and 5 μg/mL propidium iodide (PI) for 30 min. Nuclear condensation and DNA fragmentation of apoptotic cells and PI-positive of necrotic cells were visualized and scored under fluorescence microscope.

5   Proliferation analysis

Human lung cancer cell lines (H460, H292 and H23) were seeded at a density 2.0×103 cells/well overnight. After attachment, cells were exposed to NO donor at

various concentrations (0-100 µM) for 24 h and were subjected to proliferation assay at 0, 24 and 48 h.

Proliferative rate was measured through incubation with MTT (400 µg/mL) for 4 h and dissolved crystal formazan product with absolute DMSO. Relative proliferation was calculated by comparing the absorbance of formazan product at 570 nm using microplate reader compare to non-treated control cells.

6   Cell migration analysis

6.1   Wound-healing migration assay

Human lung cancer cells lines (H460, H292 and H23) were seeded on 96-well plate at a density 3×10^4 cells/well and incubated with non-toxic concentration of NO donor for 24 h. After exposing to NO, the monolayer cells (95% confluence) were allowed to migrate by scratched with a P200 micropipette tip to make wound scrape. Detached cells were removed by rinsing once with PBS and replaced with RPMI complete medium.

At the indicated times (0, 12 and 24 h), Phase contrast images of wound space were captured under microscope (20X) and the wound spaces were measured using Olympus DP controller software. Relative cell migration was calculated by dividing the percent change in the space of the NO-treated cells by that one of non-treated control in each experiment as follow.

% change of space	=	$\underline{\text{(Space at time 0)} - \text{(Space at time 12 or 24)}} \times 100$
		(Space at time 0)
Relative migration	=	$\underline{\text{Percent change of treatment group}}$
		Percent change of control group

6.2   Trans-well migration assay

After NO treatment with non-toxic concentration for 24 h, trans-well migration assays were performed using a Boyden chamber plate. H460 cells were trypsinized and seeded at a density 3.0×10^4 cells/well in the upper chamber unit with RPMI serum free condition, while RPMI complete medium containing 10% FBS was added to the lower chamber unit.

After incubation for 24 h at 37 °C, non-penetrated cells on the top chamber were gently removed with cotton swab and migrated cells in the lower chamber unit were fixed with cold absolute methanol for 10 min and stained with 10 µg/ml of Hoechst33342 for 10 min. The migrating cells were visualized and scored under a fluorescence microscope.

Relative migration	=	<u>Number of migrated cell in treatment group</u>
		Number of migrated cell in control group

7   Cell morphological characterization assay

The cell morphology of NCI-H460 was investigated using phalloidin-rhodamine staining assay. After exposing to non-toxic concentration of NO, cells were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min and permeabilized membrane with 0.1% Triton-X 100 in PBS for 5 min, then rinsed cells with PBS and blocked for unspecific binding by incubation with 0.2% BSA in PBS for 30 min. Fixed cells were incubated with 1:100 dilution of phalloidin-rhodamine fluorescent dye in PBS for 30 min, then were rinsed with PBS three times and mounted with 50% glycerol in PBS. The cell morphology images of actin or stress fiber were visualized under fluorescence emitting light.

8 Western blot analysis

Cellular lysates were prepared using incubation with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail for 30 mins on ice.

Cellular lysates were collected and determined for protein content using the BCA protein assay kit. Equal amounts of protein from each sample were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After separation, proteins were transferred onto 0.45 μm nitrocellulose membranes. The membrane blots were blocked with 5% non-fat dry milk in TBST (Tris-buffer saline with 0.1% Tween containing 25 mM Tris-HCl (pH 7.5), 125 mM NaCl, and 0.1% Tween 20) for 1 h and incubated with the appropriate primary antibodies at 4 °C overnight.

After three washes with TBST, membrane blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. Immune blots were detected by enhanced with chemi-luminescence substrate and quantified using analyst/PC densitometry software.

9 Statistical analysis

The data values from three or more independent experiments were presented as the mean ± standard deviation (SD). Multiple comparisons were examined for significant difference of multiple groups, using analysis of variance (ANOVA), followed by individual comparisons with Scheffe's post-hoc test. Statistical significance was considered at $p < 0.05$
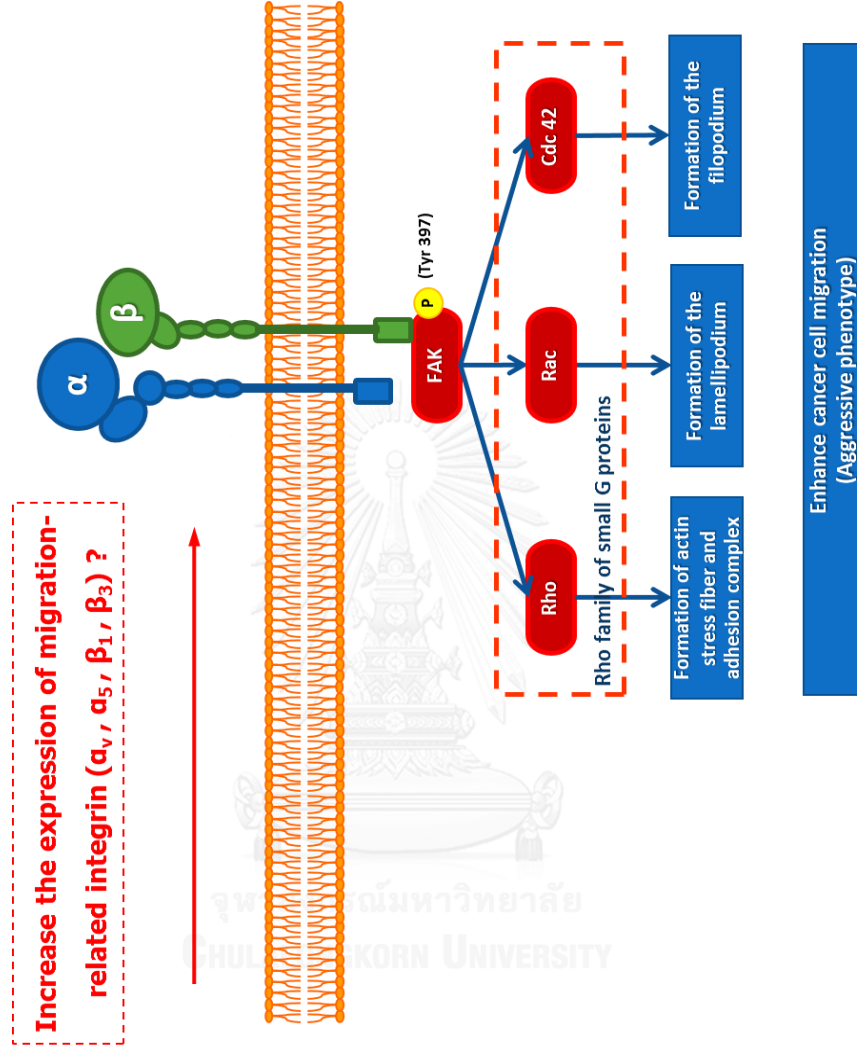
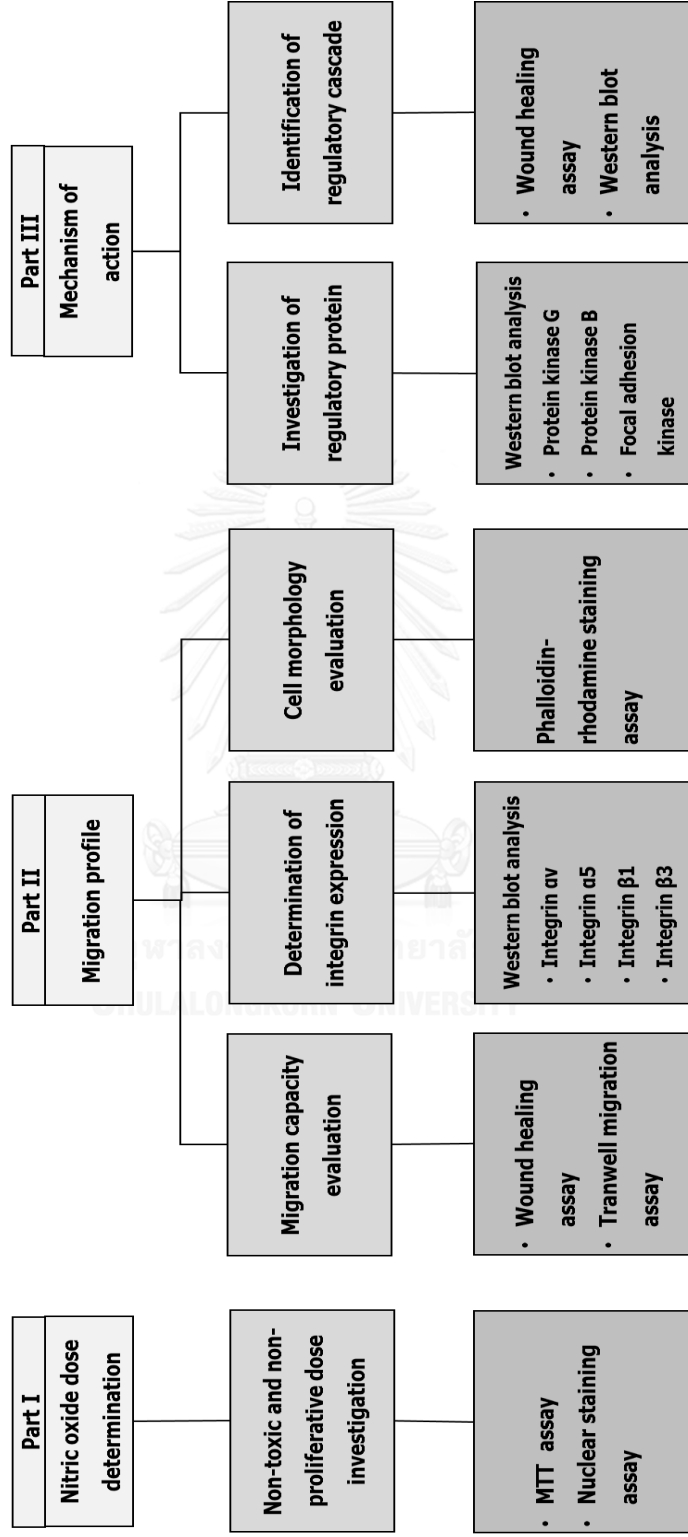Figure 3.2 Conceptual framework

11   Experimental design

Figure 3.3 Experimental design

Part I Cytotoxic effects of nitric oxide on human lung cancer cell lines

Cytotoxicity of NO on human lung cancer cell lines

To determine the optimum concentrations of NO that cause no cytotoxic effect on human lung cancer cell lines (NCI-H460, NCI-H292 and NCI-H23), percent cell viability was evaluated by MTT assay. Initially, cells were treated with DPTA NONOate at wide range concentrations (0-250 μM) for 24 h. After NO treatment, percent of cell viability were determined by MTT colorimetric assay.

Mode of cell death in response to NO treatment

To confirm the previous MTT results, mode of cell death in response to NO treatment were determined Hoechest 33342/PI nuclear co-staining assay. H460 cells were seed treated with DPTA NONOate (0-100 μM) for 24 h then mode of cell death were determined by apoptosis and necrosis evaluation assay. Non-toxic concentrations were referred to percent viability not less than 80% and will be selected to further experiments.
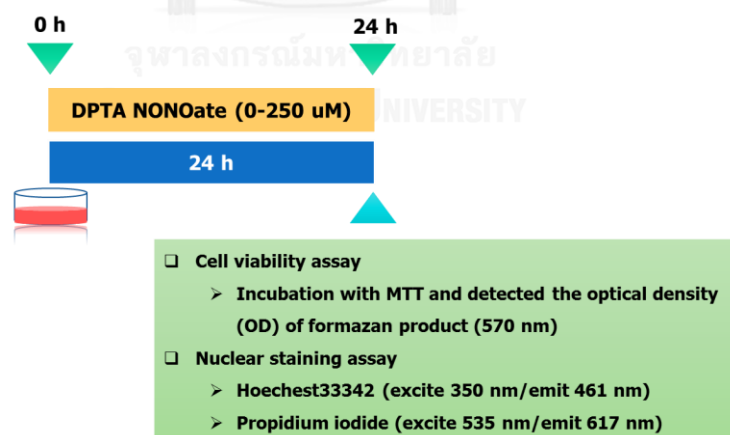
Figure 3.4 Diagram of cytotoxic test

Proliferative effect of NO on human lung cancer cell lines

In order to eradicate the proliferative effect of NO which able to interfere in migration activity, H460, H292 and H23 cells were treated with DPTA NONOate at various concentrations (0-100 µM) for 24 h. After exposed to NO, percent of cell proliferations in all tested-cell were detected by MTT colorimetric assay at indicated time point (0, 24 and 48h).

The appropriate concentration should be non-significant in stimulation and/or inhibition of proliferative effect compare to non-treated control. Non-proliferative doses would be taken to migration assay and other migratory characteristic.
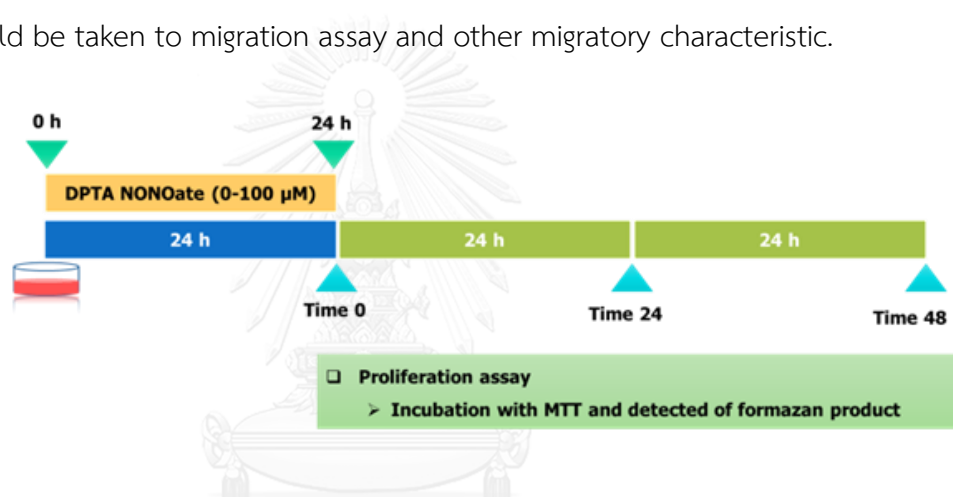

Figure 3.5 Diagram of proliferative test

Part II Migratory characterization in response of NO treatment

The effect of NO on human lung cancer cell migration

An elevation of metastasis rate in lung cancer has been reported in the NO rich environment [54, 57]. Migration capacities were investigated in response to NO treatment. Briefly, a monolayer of cells was cultured at density 3.0×10^4 cells/well, and cells were treated with non-toxic concentrations of DPTA NONOate for 24 h.

Migration capacity was determined by wound healing migration assay and transwell migration assay which represent the collective cell migration and single cell migration respectively. Relative cell migration was calculated by dividing NO-treatment group compare to non-treated control.
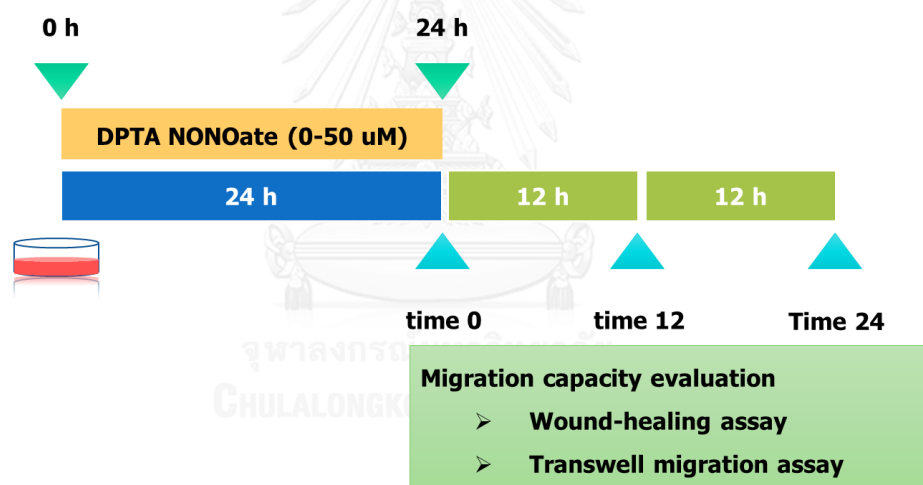
Figure 3.6 Diagram of migration evaluation

The effect of NO on cell morphology and filopodia formation

Regarding cell morphology and filopodia formation has been shown to play an essential role upon cellular movement. The evidence also support that, cellular filopodia which are regulated by a family Rho GTPase proteins generates by actin polymerization and rearrangement of actin stress fiber in the protrusion edge [59].

Therefore, to investigate the effect of NO on filopodia formation, H460 cells were treated with non-toxic concentration of NO for 24 h, and the presence of filopodia was determined by using a phalloidin-rhodamine staining assay. The numbers of filopodia per cell were counted under the fluorescent microscope.
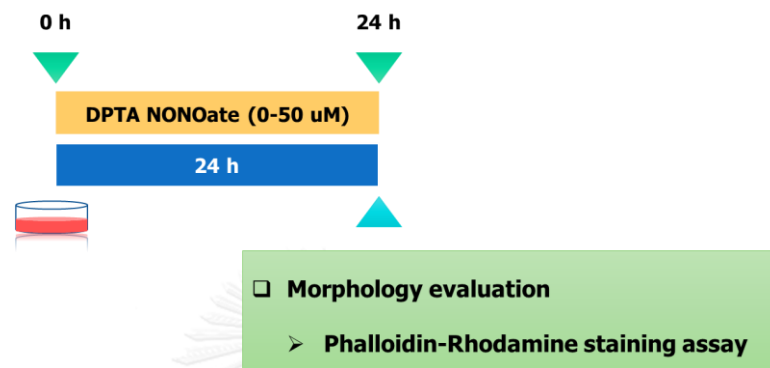

Figure 3.7 Diagram of cell morphology evaluation

The effect of NO on integrin expression

Switching of integrin expression patterns is explained as the central role in migration model. There are previous studies have shown that activation of integrin αv, $\alpha 5$, $\beta 1$ and $\beta 3$ are able to enhance motility in cancer cells [18-21]. This study also hypothesized of these integrins may be involved in NO mediated lung cancer cell migration.

Human lung cancer cell lines (NCI-H460, NCI-H292 and NCI-H23) were seeded at density 1.5×10^5 cells/well in a 6-well plate then cells were treated with non-toxic concentration of NO for 24 h. After NO treatment, the correlation of integrin expression and migration potency were investigated, this study examines the integrin expression levels by western blot analysis. β-actin was used as a loading control of each treatment.
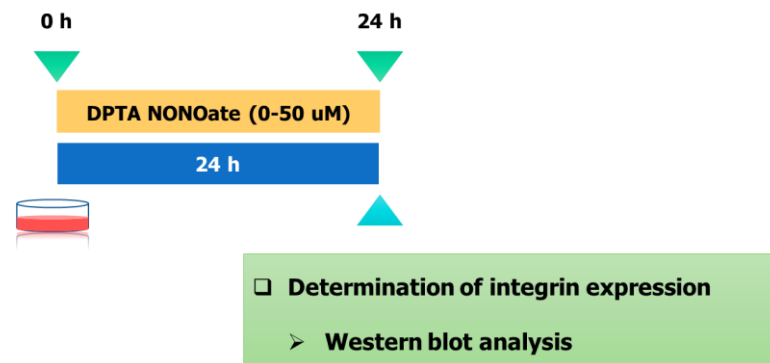
Figure 3.8 Diagram of integrin expression determination

Part III Mechanisms of NO which mediated lung cancer cell migration and alteration of integrin expression

Integrin mediated cell migration has been described through activation of downstream signaling protein such as FAK and a Rho GTPase of small   G protein family. Thus, identification of some specific signaling proteins that conforming to integrin expression could be explained for a regulatory mechanism of integrin expression and migration behavior in NO-treated cells.

The effect of NO on signaling proteins

In order to determine NO regulating cascade, downstream signaling proteins which regulate migration activity as well as integrin were investigated. Western blot analysis was used for detection of involved-signaling protein including PKG, AKT, FAK and small G proteins after NO treatment.

Figure 3.9 Diagram of signaling protein determination
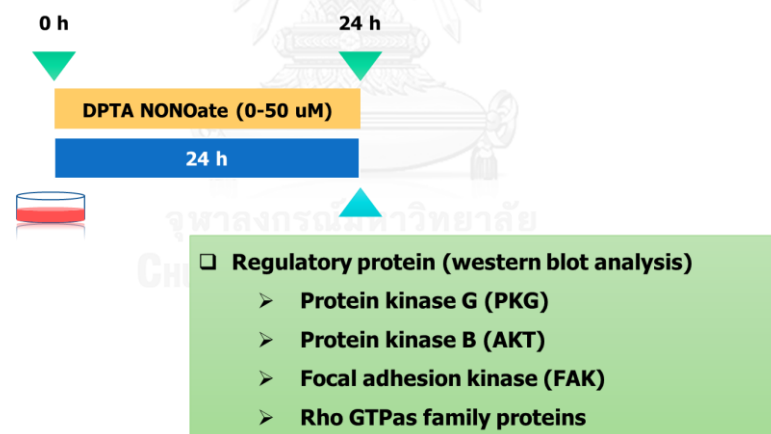
Involvement of AKT in regulation of integrin expression and migration

Since NO has been reported in promotion of cancer cell migration and invasion through activation of AKT signaling pathway [57]. Meanwhile, there is a study reported that AKT regulated integrin which associated to migration [60]. Thus, we also hypothesized that AKT may alter integrin expression.

Therefore, to investigate role of AKT on integrin expression, AKT inhibitor (5 μM Perifosine) were applied in NO treated-cells. Briefly, cells were seeded in 6-well plate at density 1.5×10^{5} cells/well. Then, cells were treated with NO alone or combination with AKT inhibitor. After indicated treatment integrin (αv, α5, β1 and β3) and related proteins including PKG, AKT and FAK were determined by western blotting.

Involvement of FAK in regulation of integrin expression and migration

In order to confirm the role of FAK protein in regulation of migration and integrin expression, FAK inhibitor (10 μM FAK inhibitor14) were applied in NO treated-cells. Initially H460 cells were seeded in 6-well plate at density 1.5×10^{5} cells/well, then cells were treated with NO alone or combination with FAK inhibitor at indicated concentration. After treatment for 24 h, integrin (αv, α5, β1 and β3) and related proteins including PKG, AKT and FAK were determined by western blot analysis.
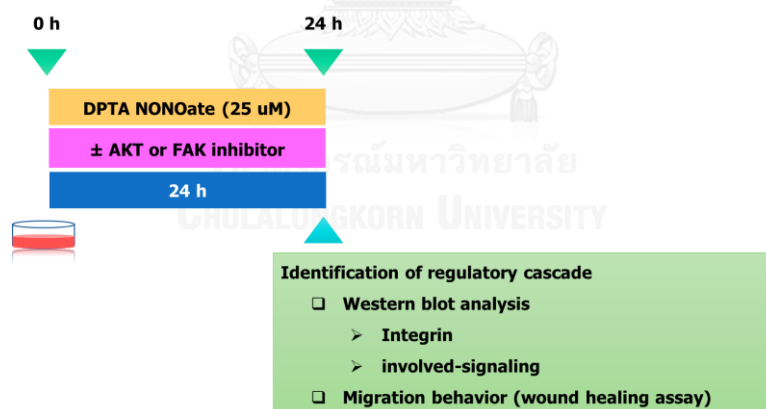

Figure 3.10 Diagram of regulatory cascade identification

CHAPTER IV
RESULTS

Part I Cytotoxic effects of nitric oxide on human lung cancer cell lines

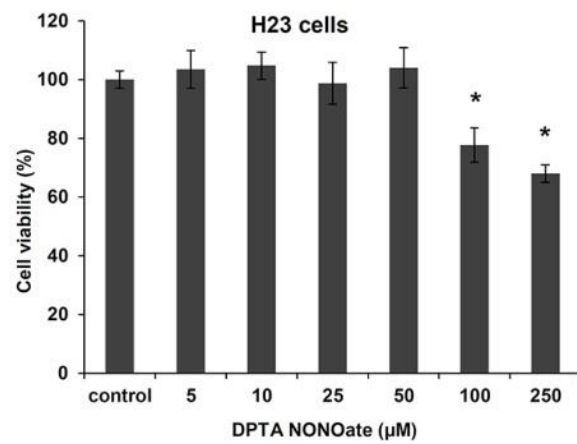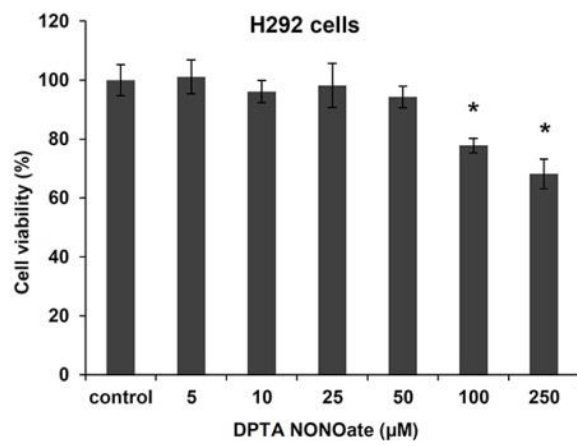### Cytotoxicity of NO on human lung cancer cell lines

In order to study the effect of NO on cell susceptibility to apoptosis and/or necrosis, this study first characterized the effect of NO on cell viability in all-tested cells (NCI-H460, NCI-H292 and NCI-H23). MTT colorimetric assay and apoptosis and necrosis assay were used to evaluate cytotoxic effect and mode of cell death in NO-treated cells.

Cell viability of human lung cancer cells was characterized. Cells were treated various concentrations of NO donor DPTA NONOate (0-250 μM) for 24 h and cell viability was determined by MTT colorimetric assay. Figure 4.1 show that NO donor at the concentrations of 0-50 μM had neither cytotoxic nor proliferative effects on NCI-H460, NCI-H292 and NCI-H233 cells. A significant decrease in cell viability was first detected in NO-treated with NO donor at the concentration 100 μM.

Mode of cell death in response of NO treatment

To confirm non-cytotoxic concentration of NO, apoptotic and necrotic cell deaths in such pre-treatment were determined by Hoechst 33342/PI co-staining assay. The results revealed that, the appearance of cell death was not detected in H460 cell that pre-treated with NO donor 0-50 μM for 24 h, whereas NO donor at 100 μM significantly increased the apoptotic and necrotic cells in comparison to non-treated control.

(A)

(B)

H460 cells

Hoechst33342

PI

control | 25 | 100
DPTA NONOate (uM)

H292 cells

Hoechst33342

PI

control | 25 | 100
DPTA NONOate (uM)

H23 cells

Hoechst33342

PI

control | 25 | 100
DPTA NONOate (uM)
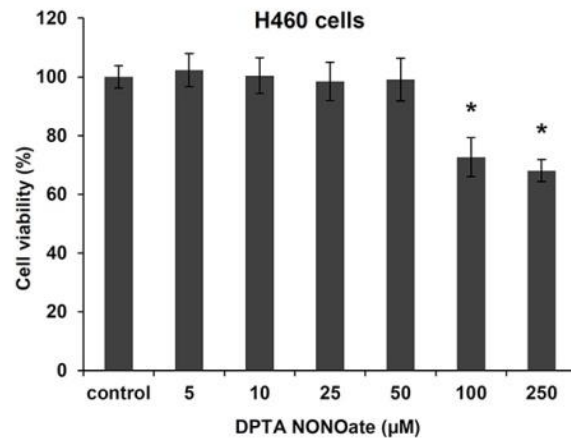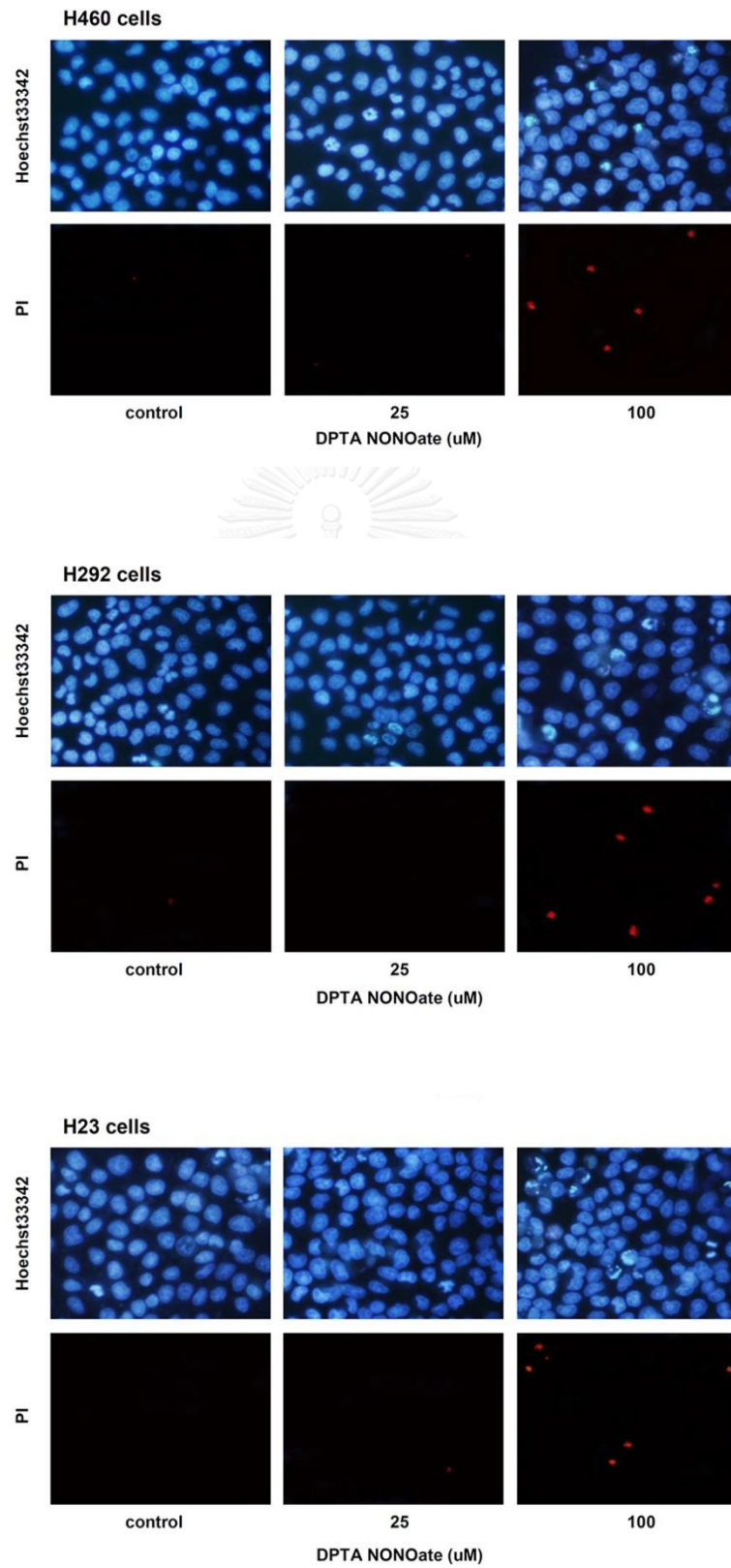
(C)

Figure 4.1 Cytotoxic effect of NO on human lung cancer cell lines (H460, H292 and H23 cells). Cells were treated with DPTA NONOate for 24 h. (A) Cell viability was determined by MTT assay. (B) Nuclear morphology was obtained from Hoechest 33342/PI staining assay. (C) Percentage of apoptotic and necrotic nuclei was analyzed. Data represent mean ± SD (n=3). * p < 0.05 versus non-treated control.

Effects of NO on cell proliferation in human lung cancer cell lines

In order to obtain the appropriated concentrations of NO, proliferative effect which may interfere in migration capacity should be eradicated before characterized the migration activity. The optimum concentration of NO should be non-significant in stimulation and/or inhibition of cell growth compare to non-treated control. NCI-H460, NCI-H292 and NCI-H23 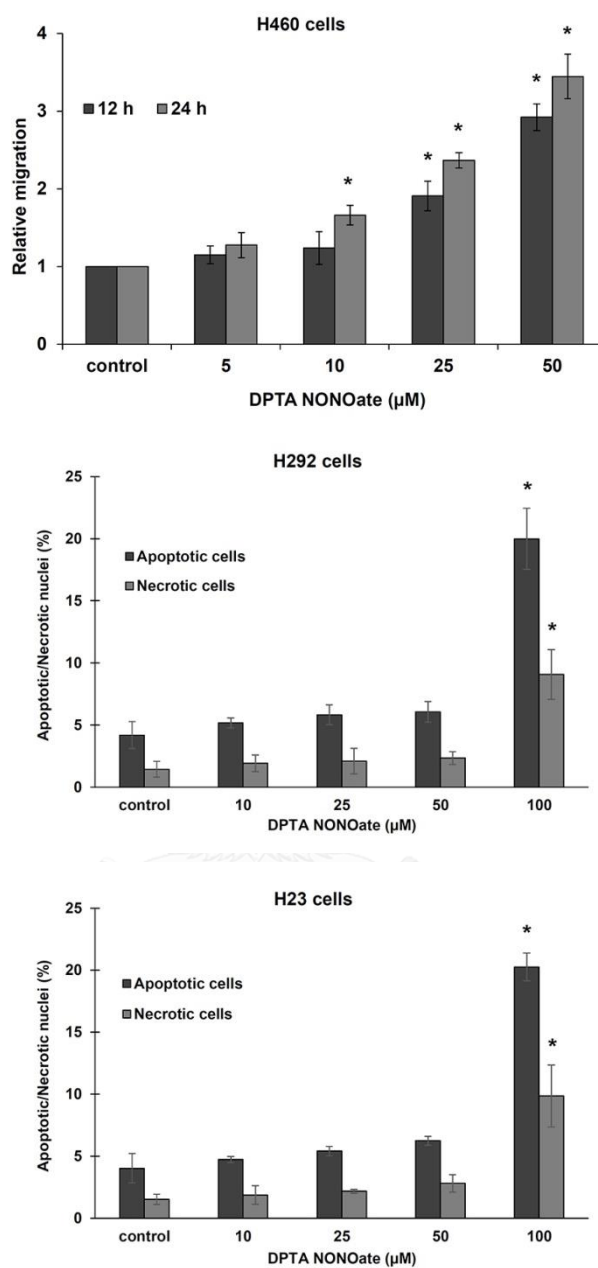cells were pre-treated with DPTA NONOate at various concentrations (0-100 μM) for 24 h. After exposed to NO, relative proliferations of all tested cells were evaluated by MTT colorimetric assay at indicated time point (0, 24, and 48 h).

The results indicated that, at 24 and 48 h after exposed to NO, pre-treatment of all type cell lines with 25 and 50 μM of DPTA NONOate had no significant on cell growth and survival compared non-treated control. However, pre-treatment of cells with higher concentration (100 μM) caused a significant reduction in cell survival as show in Figure 4.2. Furthermore, from previous Hoechest 33342 and PI co-staining results also supported that, the high concentration of NO donor (100 μM) was induced toxicity to cell. An increasing of both apoptosis and necrosis cell death were initially found at 100 μM of DPTA NONOate treatment.
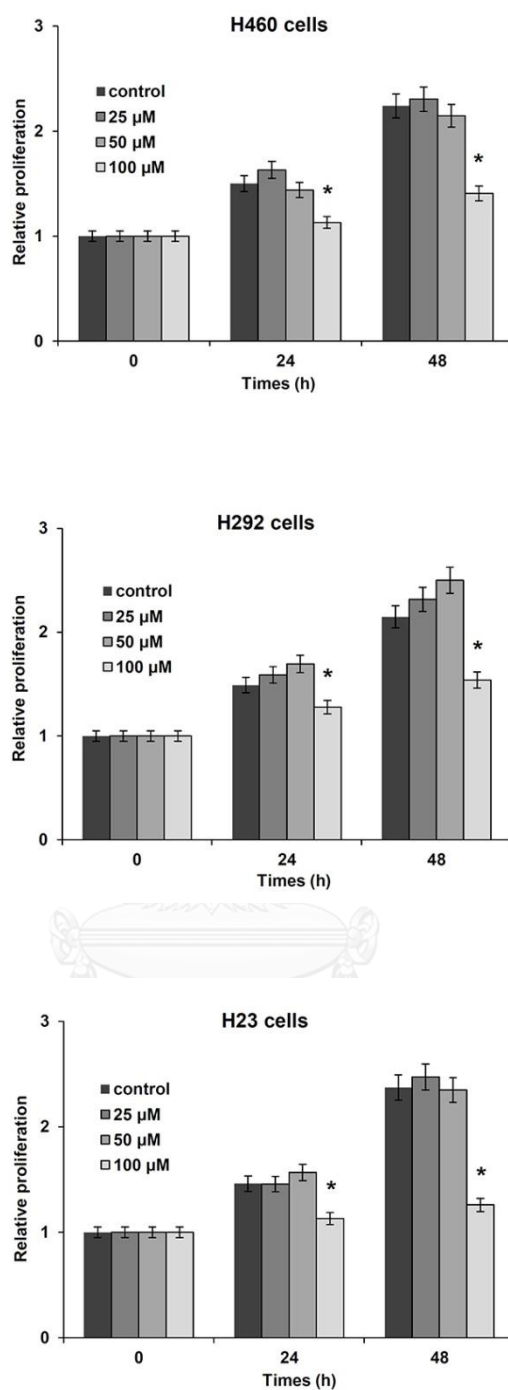
Figure 4.2 Proliferative effect of NO on human lung cancer cell lines (H460, H292 and H23). Cells were treated with DPTA NONOate (0-100 µM) for 24 h. Relative proliferation was determined by MTT assay at indicated time point. * p < 0.05 versus non-treated control.

Part II Migratory characterization in NO treatment

Effect of NO on cell migration in human lung cancer cell lines

Wound healing assays and transwell migration assays were performed to determine the effect of NO on lung cancer cell migration.

Wound healing migration

For wound healing assay, lung cancer cell lines (NCI-H460, NCI-H292 and NCI-H23 cells) were exposed to non-toxic concentrations of NO donor (0-50 μM) for 24 h, cells were subjected to wound healing assay, and allowed to migrate for 0-24 h. Figure 4.3 shown that, NO treatment significantly enhanced the motility of all lung cancer cells in a dose-dependent manner.

Transwell migration

Likewise, the transwell migration results further confirmed that NO increased migration activity in all tested cells. The migrated cells which penetrated through filter membrane to the lower chamber side after treatment with NO for 24 h. Figure 4.4 exhibited, relative cell migration of NCI-H460, NCI-H292 and NCI-H23 cells was found to be significant increase in response to NO in a dose-dependent manner. The significant values could be first observed in 10, 25, and 25 μM DPTA treated H460, H292, and H23 cells respectively.

Taken together, these results indicated that NO enhanced migratory behavior in all human lung cancer cell lines, however, susceptibility of each cell type may be vary due to variation in cell biology.

(A)

H460 cells

0 h

12 h

24 h

control   5   10   25   50

DPTA NONOate (uM)

H292 cells

0 h

12 h

24 h

control   5   10   25   50

DPTA NONOate (uM)

H23 cells

0 h

12 h

24 h

control   5   10   25   50

DPTA NONOate (uM)
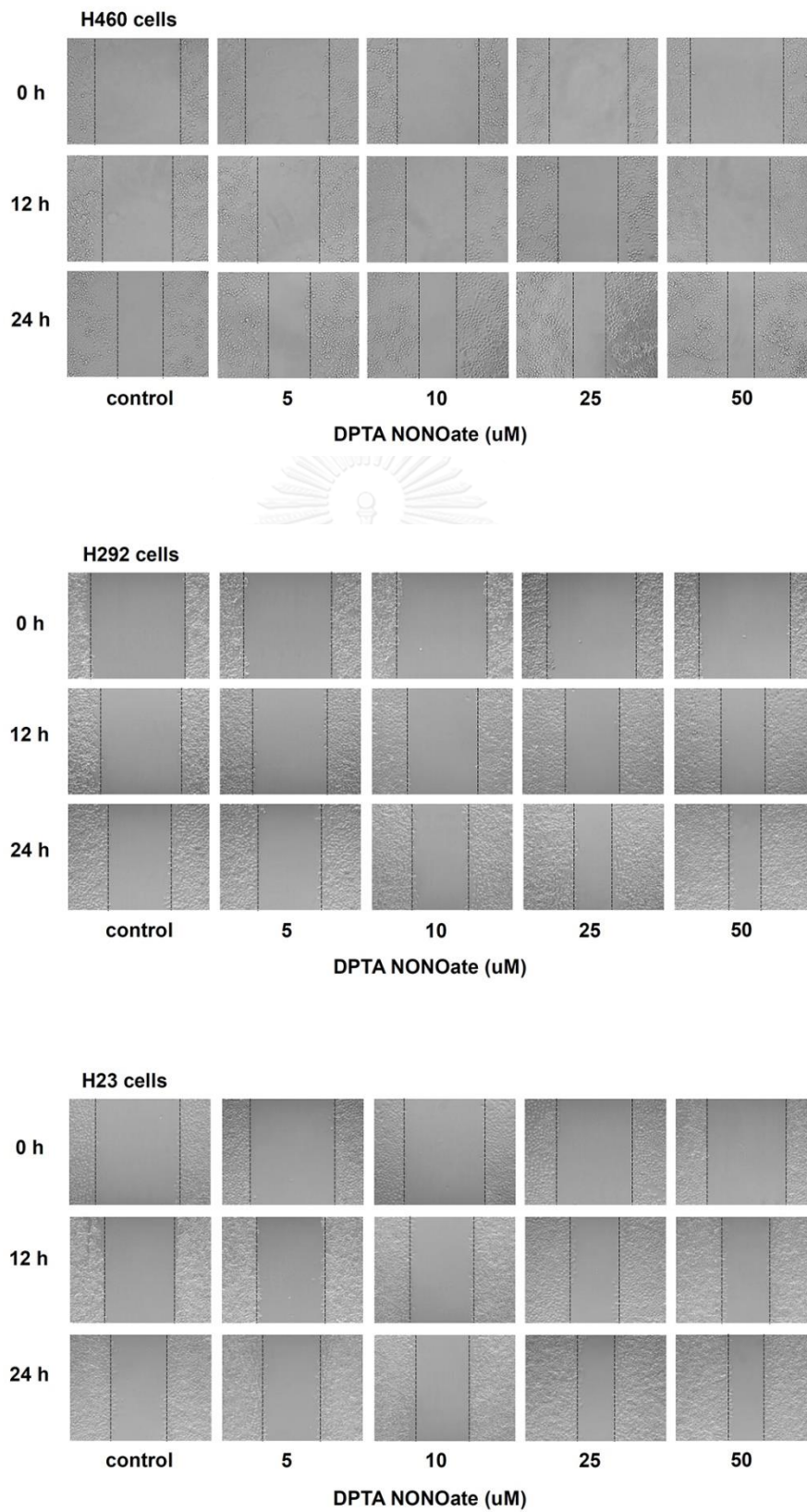
(B)
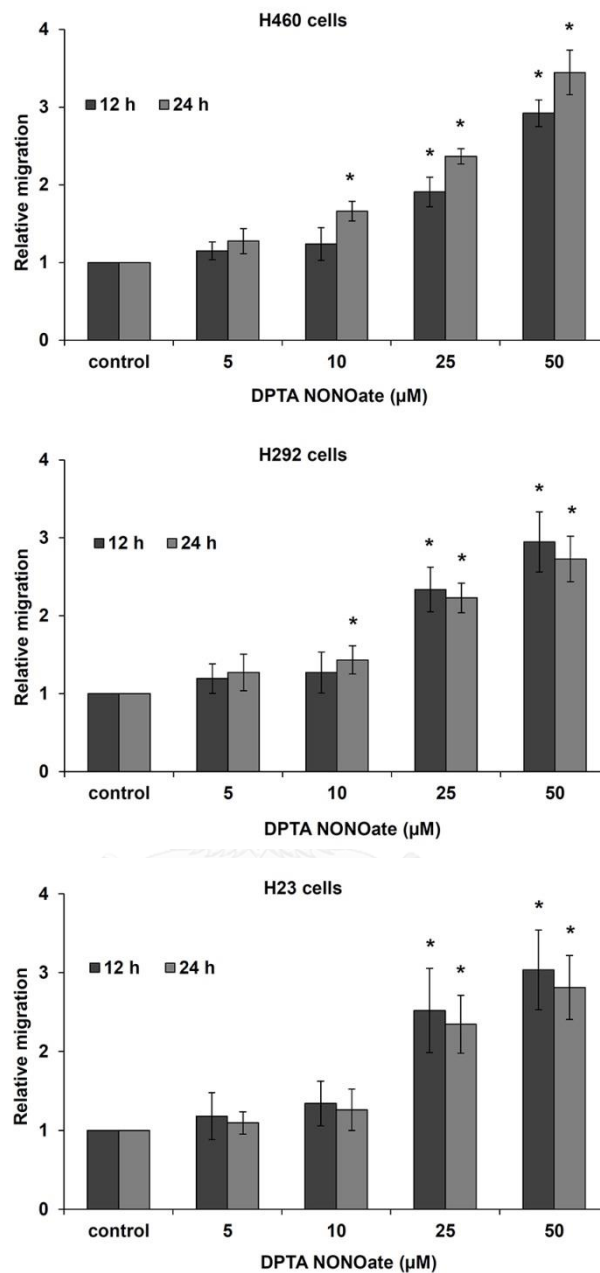
Figure 4.3 NO enhanced monolayer wound healing. Human lung cancer cell lines were pre-treated with NO donor (0-50 uM) for 24 h, then were subjected to wound healing migration assay. (A) Phase-contrast images were captured at 0, 12 and 24 h. (B) Relative migration was determined by comparing wound space to non-treated control. Data represent mean ± SD (n=3). * $p < 0.05$ versus non-treated control.

(A)
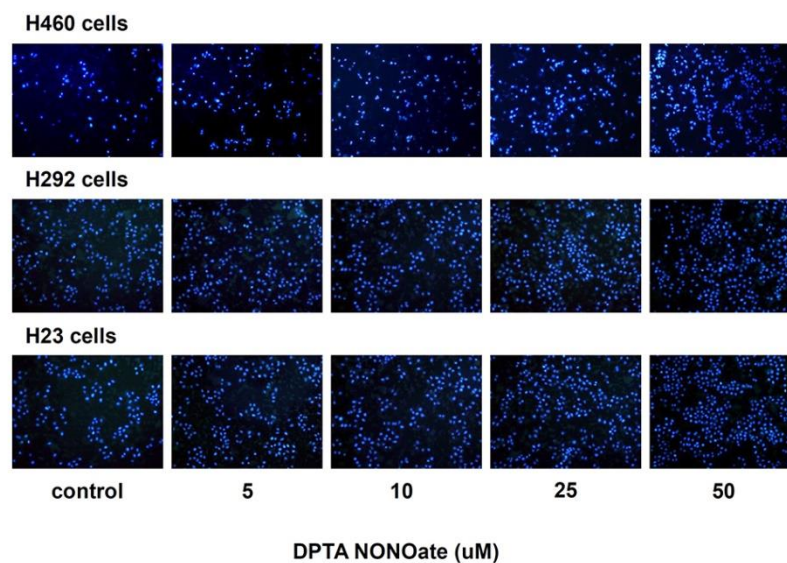
(B)
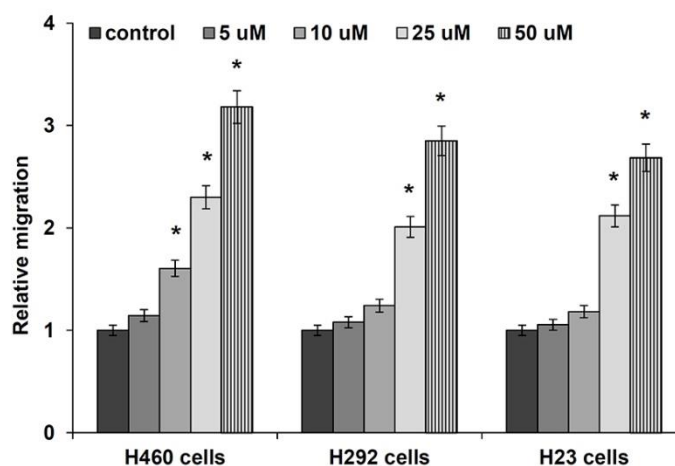
Figure 4.4 NO enhanced human lung cancer cell migration. Cells were pre-treated DPTA NONOate (0-50 uM) for 24 h. After treatment, transwell migration assay was performed using Boyden chamber. (A) Migrated-cells in lower chamber compartment were stained with Hoechst 33342 and visualized under fluorescence microscope. (B) Relative migration was determined by comparing to non-treated control. Data represent mean ± SD (n=3). * p < 0.05 versus non-treated control.

Effect of NO on cell morphology and filopodia formation

Filopodia was shown to play an essential role in cellular movement and used as an indicator for highly motile cells. Importantly, filopodia at the protrusion areas, i.e., the fronts of the cells, have been found to contain integrins and other molecules facilitating anchorage formation [59, 61].

To test whether NO could mediate filopodia formation, H460 cells were treated with NO donor as previously described, and the presence of filopodia was determined using a Phalloidin-Rhodamine staining method. Figure 4.5 indicated that NO-treated cells exhibited significant increase in filopodia number at the protrusion edge of cells.

In addition, this study also confirmed the effect of NO on cell migration by assessing migration regulatory proteins. Human lung cancer cell lines were treated with NO donor and cellular levels of Rho-GTP, Rac-GTP, and Cdc42-GTP were determined by western blot analysis. Results revealed that treatment of the cells with NO caused the significant increase of Rho-GTP and Cdc42-GTP; however, Rac-GTP was found to be slightly decreased (figure 4.6).

(A)
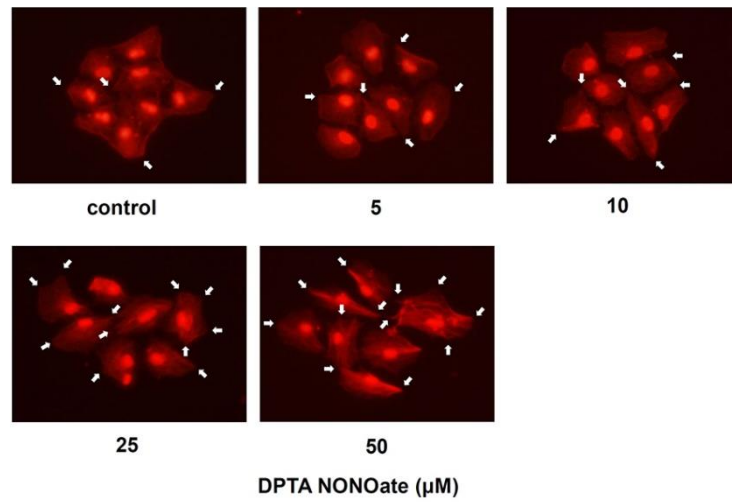
DPTA NONOate (μM)

(B)
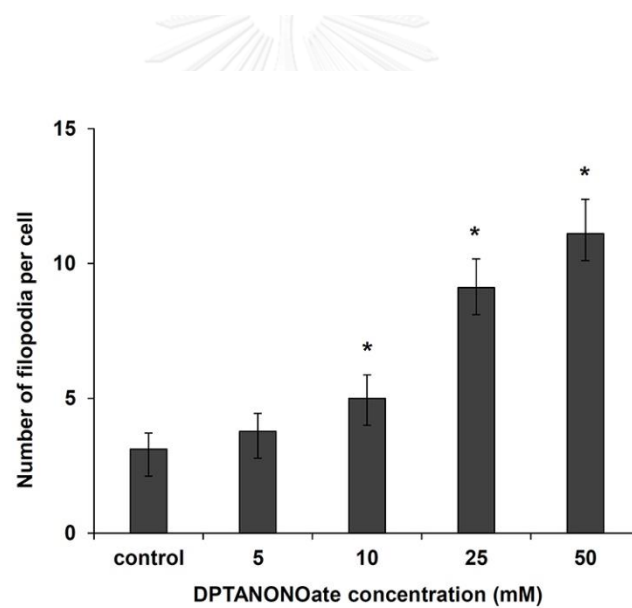
Figure 4.5 NO induced filopodia formation. H460 cells were treated with NO donor (0-50 uM DPTA NONOate) for 24 h. After treatment, cells were fixed with paraformaldehyde and stained with phalloidin-rhodamine fluorescent dye. (A) The actin filaments and stress fibers were visualized under microscope. (B) The number of filopodia per cell was analyzed and compared to non-treated control. Data represent mean ± SD (n=3). * p < 0.05 versus non-treated control.

(A)
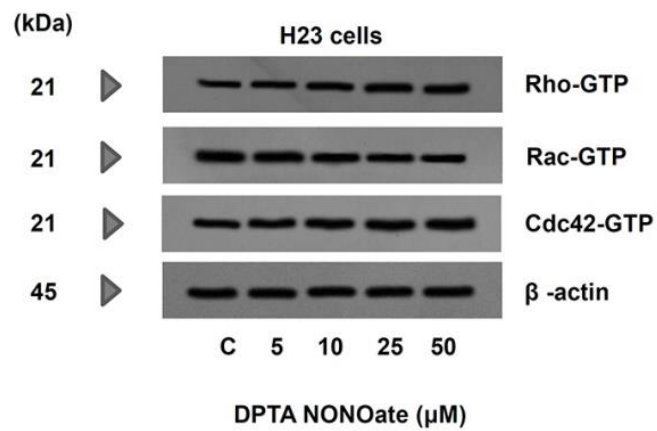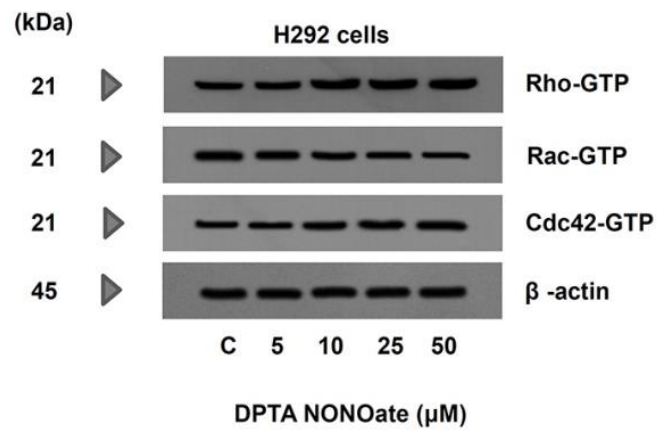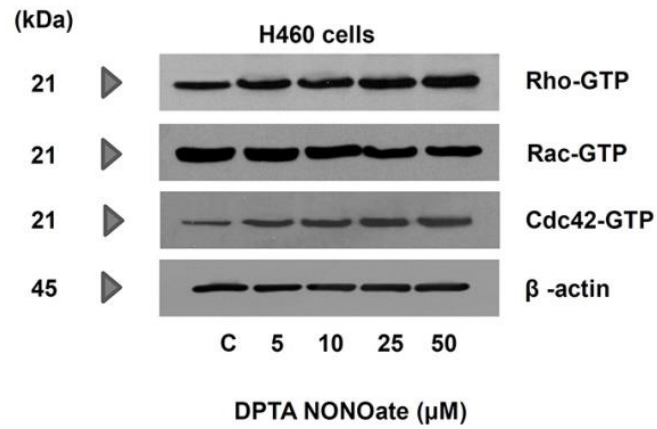
(B)
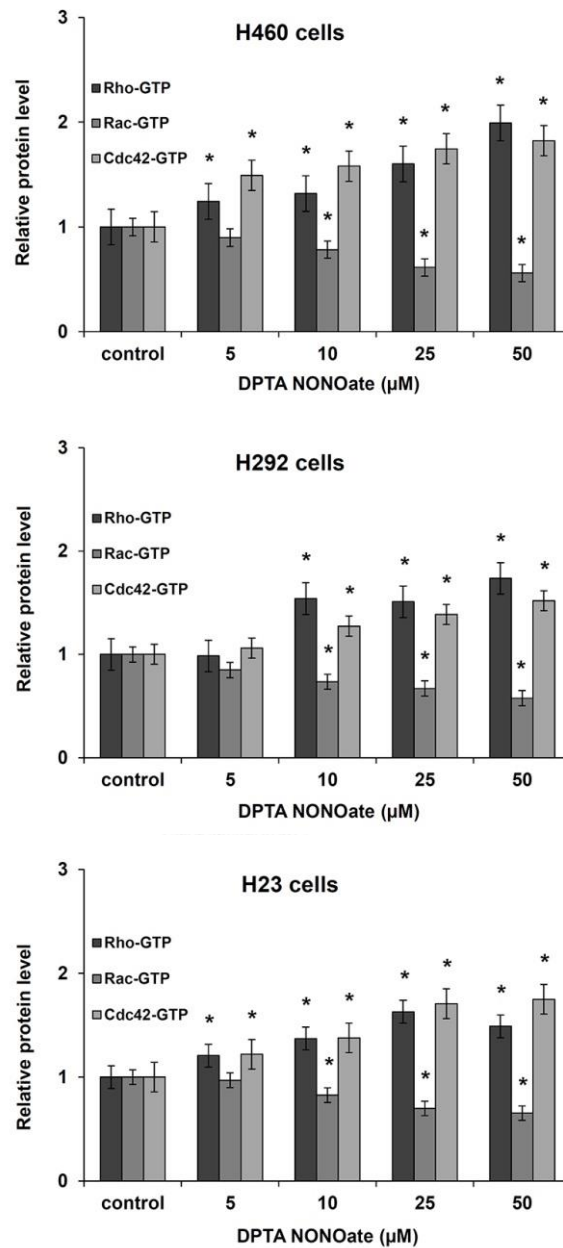
Figure 4.6 The alteration of Rho GTPase proteins during cell migration. Cells were treated with DPTA NONOate (0-50 **μ**M) for 24 h. After treatment, (A) the expression levels of Rho GTPase proteins were determined by western blot analysis. (B) Relative protein levels were quantified by densitometry. Data represent mean ± SD (n=3). * $p < 0.05$ versus non-treated control.

Effect of NO on integrin expression

Switching of integrin was accepted as a determining factor of cell motility [12, 17, 62]. In particular, the increase of specific integrins including αv, α5, β1 and β3 was shown to enhance motility in cancer cells [18-21]. Therefore, we next investigate the effect of NO on integrin expression. Lung cancer H460, H292 and H23 cells were cultured in the presence of NO donor (0-50 μM) for 24 h, and the expression of integrins αv, α5, β1, and β3 was determined by western blotting.

Figure 4.7 shows that, NO treatment caused a dramatic increase in the expression of integrins αv and β1 in all type of human lung cancer cell lines, while integrin β3 was found to be slightly increased in H292 and H23 cells. NO treatment has no significant effect on integrin α5. Having mentioned that the augmented of specific integrins, especially αv, α5, β1 and β3 associated with high migratory activity, this study has provided new information that NO induces migration of lung cancer cells, at least in part, through integrin shift toward increasing of integrins αv and β1.

(A)

(kDa)

H460 cells

135/140 — Integrin αv
150 — Integrin α5
115/135 — Integrin β1
97/110 — Integrin β3
45 — β -actin

C 5 10 25 50

DPTA NONOate (µM)

(kDa)

H292 cells

135/140 — Integrin αv
150 — Integrin α5
115/135 — Integrin β1
97/110 — Integrin β3
45 — β -actin

C 5 10 25 50

DPTA NONOate (µM)

(kDa)

H23 cells

135/140 — Integrin αv
150 — Integrin α5
115/135 — Integrin β1
97/110 — Integrin β3
45 — β -actin

C 5 10 25 50

DPTA NONOate (µM)

(B)
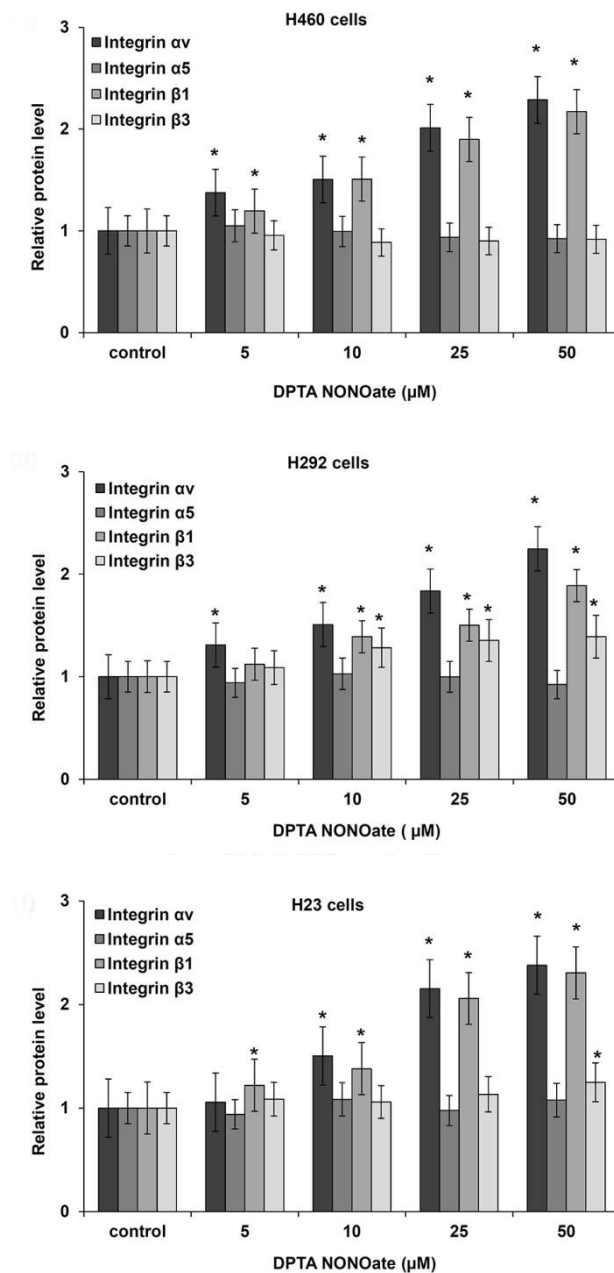
Figure 4.7 Effect of NO on integrin expression. Human lung cancer cell lines were treated with DPTA NONOate (0-50 uM) for 24 h. After treatment, (A) the expression levels of integrin (αv, α5, β1 and β3) were determined by western blot analysis. (B) Relative protein levels were quantified by densitometry. Data represent mean ± SD (n=3). * p < 0.05 versus non-treated control.
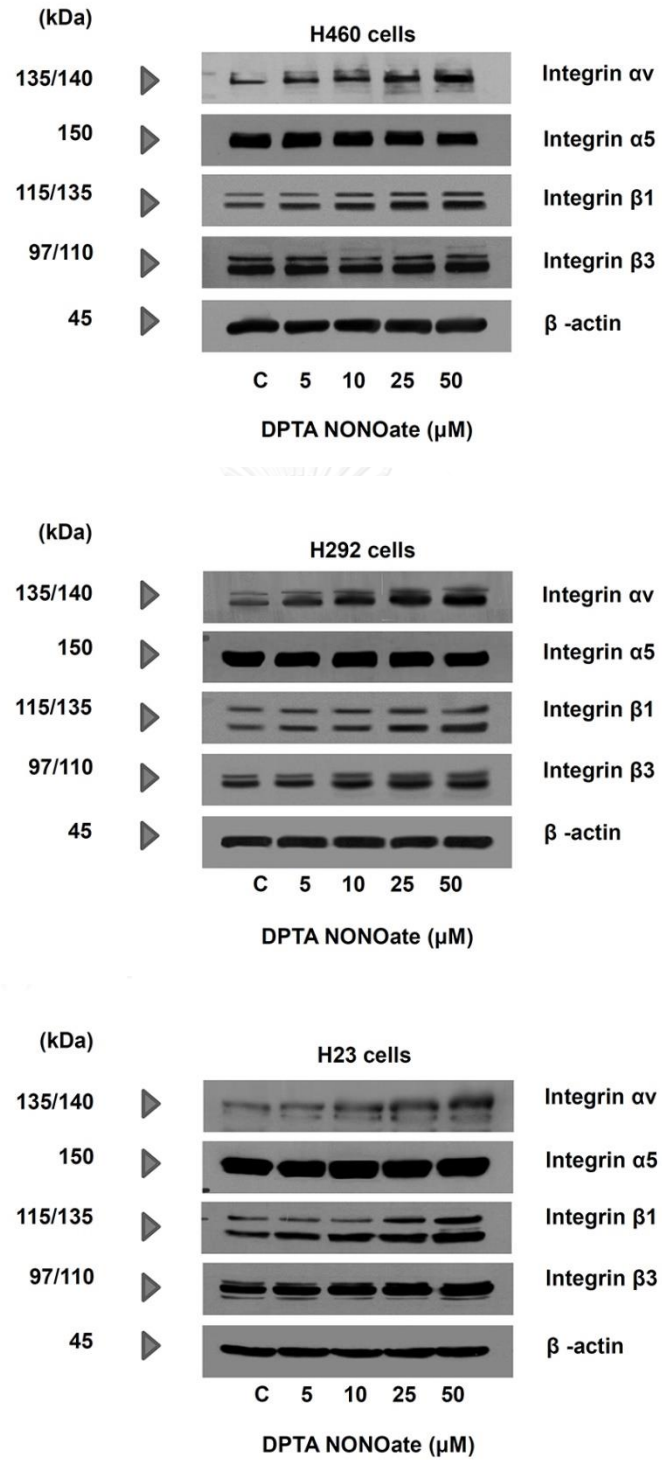
Part III Mechanism of NO which mediated human lung cancer cell migration and integrin expression

The effect of NO on regulatory proteins

Active PKG is known to act as a target of cGMP induced by NO [49]. Also, AKT has been shown to act as a downstream phosphorylation targets of PKG [63]. In order to unravel the mechanism by which NO regulated integrin expressions, the increasing of PKG level and activation of AKT, and FAK in response to NO treatment was assessed in NSCLC-derived H460, H292, and H23 cells.

The results of western blot analysis revealed that, the treatment of human lung cancer cell lines with non-toxic concentration of NO significantly increased active PKG and proportion of p-AKT/AKT and p-FAK/FAK in a dose-dependent manner in all tested-cells (Figure 4.8).

(A)

H460 cells

(kDa)			
78	▷		PKG
60	▷		p-AKT (Thr 308)
60	▷		AKT
60	▷		p-FAK (Tyr 397)
125	▷		FAK
125	▷		β -actin

C   5   10   25   50

DPTA NONOate (µM)

H292 cells

(kDa)			
78	▷		PKG
60	▷		p-AKT (Thr 308)
60	▷		AKT
60	▷		p-FAK (Tyr 397)
125	▷		FAK
125	▷		β -actin

C   5   10   25   50

DPTA NONOate (µM)

H23 cells

(kDa)			
78	▷		PKG
60	▷		p-AKT (Thr 308)
60	▷		AKT
60	▷		p-FAK (Tyr 397)
125	▷		FAK
125	▷		β -actin

C   5   10   25   50
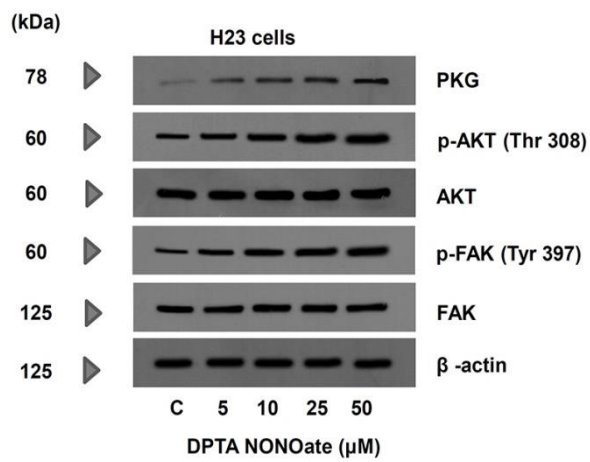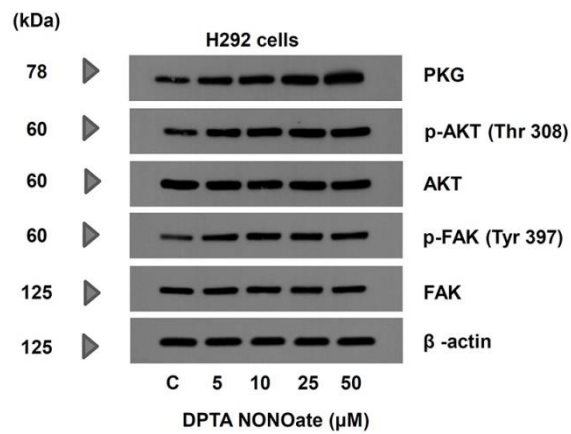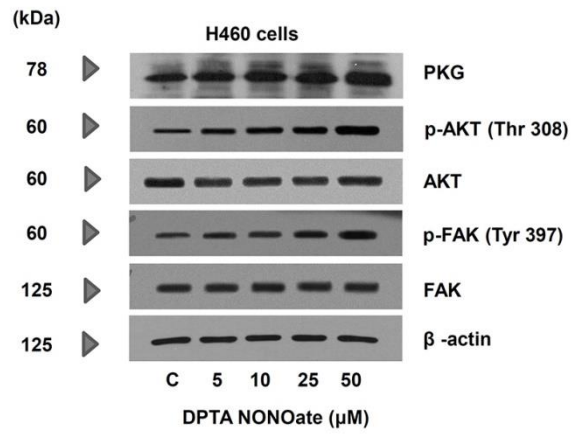
DPTA NONOate (µM)

(B)
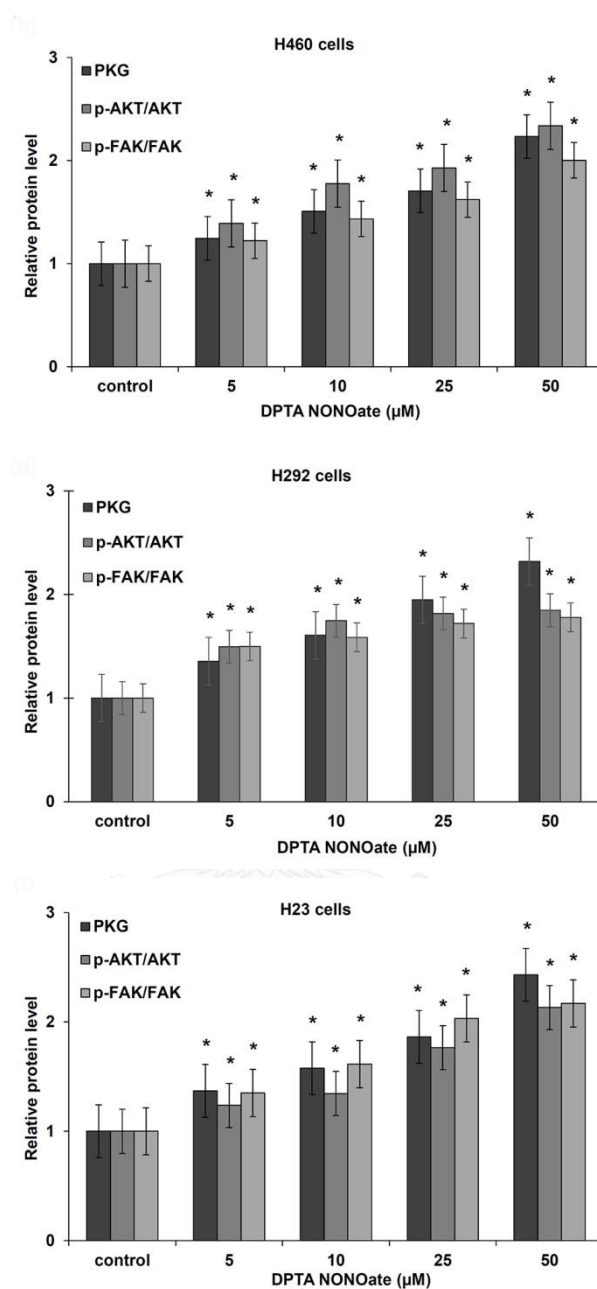
Figure 4.8 NO activated PKG, AKT and FAK signaling proteins. Human lung cancer cell lines were treated with DPTA NONOate (0-50 μM) for 24 h. After treatment, (A) the expression levels of PKG, p-AKT, AKT, p-FAK and FAK were determined by western blot analysis. (B) Relative protein levels were quantified by densitometry. Data represent mean ± SD (n=3). * p < 0.05 versus non-treated control.

Involvement of AKT and FAK protein in regulation of integrin expression and migration

AKT and FAK signaling proteins were shown as a key mediator molecules in regulation of integrins expression [40, 64, 65], thus AKT and FAK inhibitors were next used for evaluating the underlying mechanism. In order to clarify the involved-mechanism, AKT inhibitor (5 μM perifosine) was applied to test whether integrins αv and β1 is upregulated in response to AKT activation.

The results indicated that, treatment of the cells with NO significantly increase both integrin αv and β1. AKT inhibitor inhibited the activation of AKT mediated by NO. Interestingly, AKT inhibitor diminished the effect of NO on integrins αv and β1 induction, suggesting that NO mediated these integrin alteration via AKT-dependent mechanism (Figure 4.9). It is worth noting that treatment of the cells with AKT inhibitor also diminish the effect of NO on FAK activation, implying that the FAK signaling in our condition was mediated through AKT-dependent mechanism. In addition, AKT inhibitor was shown to abolish the migration enhancing effect of NO treatment.

For FAK signaling, while FAK inhibitor (10 μM FAK inhibitor14) significantly suppressed FAK in the cells, it had no significant effect on NO-mediated integrin switch (Figure 4.10). Also, the activation of AKT in response to NO was found to be not affected by FAK inhibitor. Interestingly, FAK inhibitor significantly decreased the migratory response of the cells to NO.

Taken together, our results indicated that NO mediated integrin induction through AKT-dependent mean and FAK is a downstream executer for NO/PKG/AKT cascade in controlling cancer cell migration.

(A)
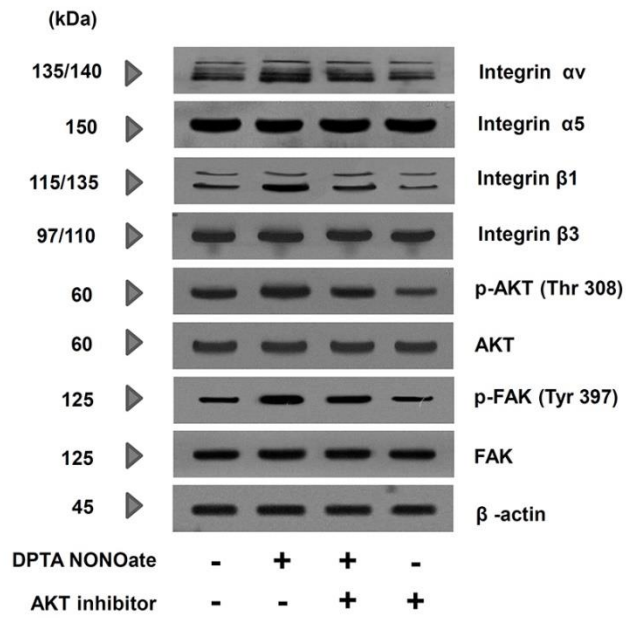
(B)
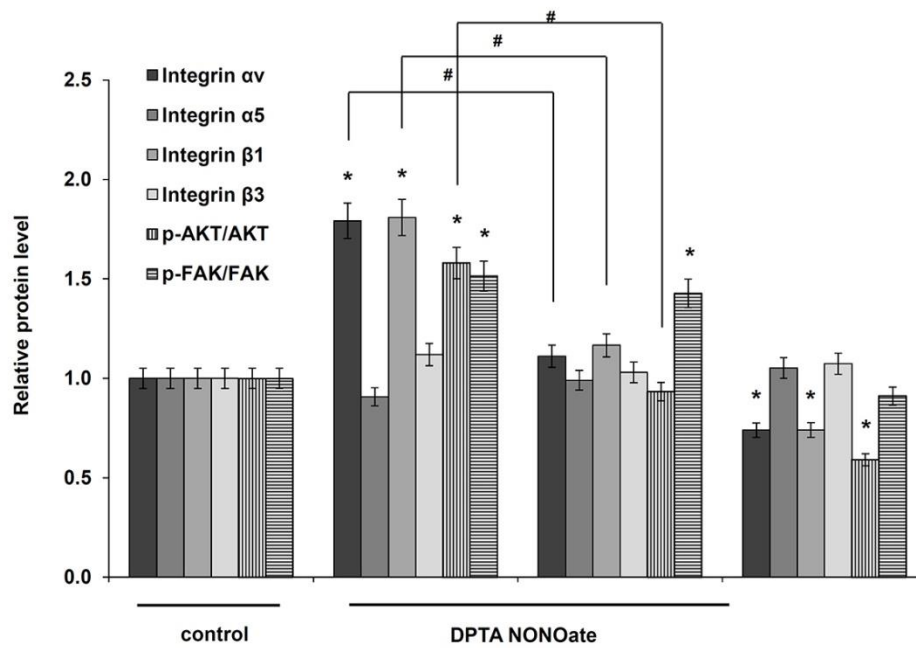
(C)
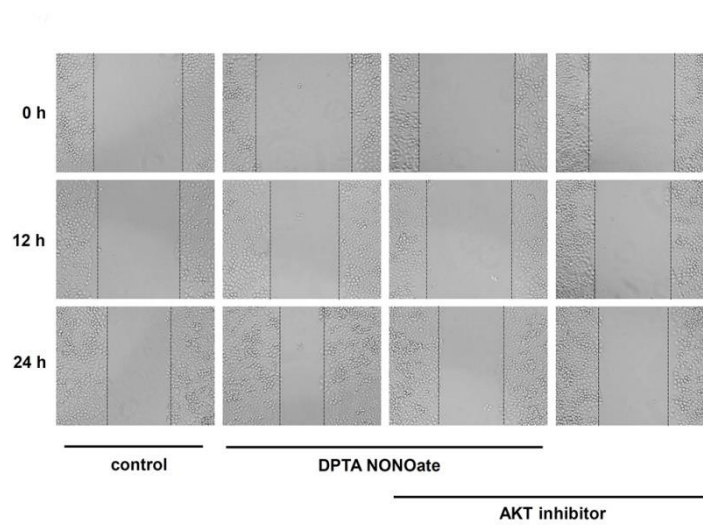
(D)
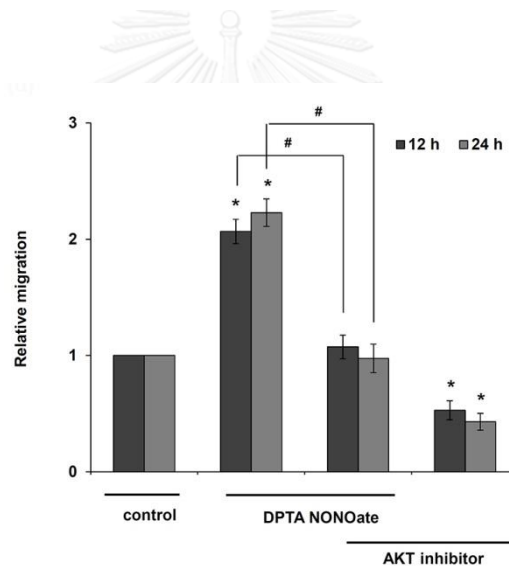
Figure 4.9 NO mediated integrin switch and enhanced cell migration via AKT dependent mechanism. H460 cells were treated with or without DPTA NONOate (25 μM) and AKT inhibitor (5 μM Perifosine) for 24 h. After treatment, (A) the expression levels of integrin and related proteins were determined by western blot. (B) Relative protein levels were quantified by densitometry. (C) Phase contrast images of wound healing were captured at 0, 12 and 24 h. (D) Relative migration was determined by comparing to control. Data represent mean ± SD (n=3). * p < 0.05 versus non-treated control, [#] p < 0.05 versus NO-treated control.

(A)
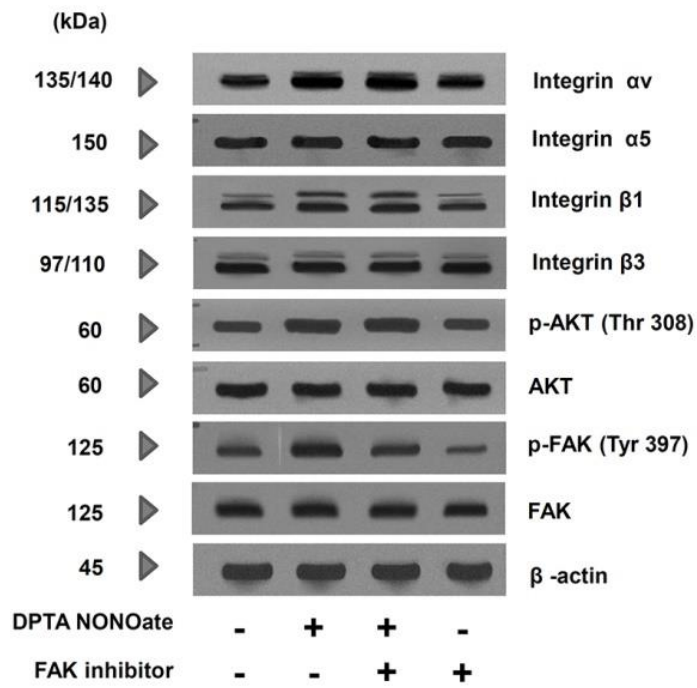
(B)
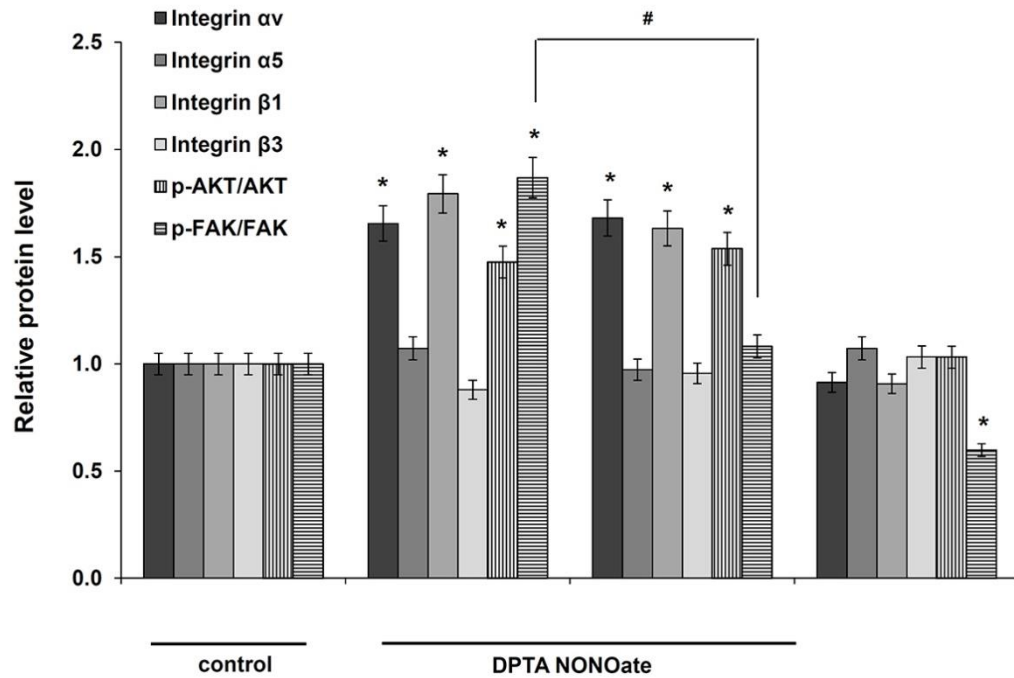
(C)
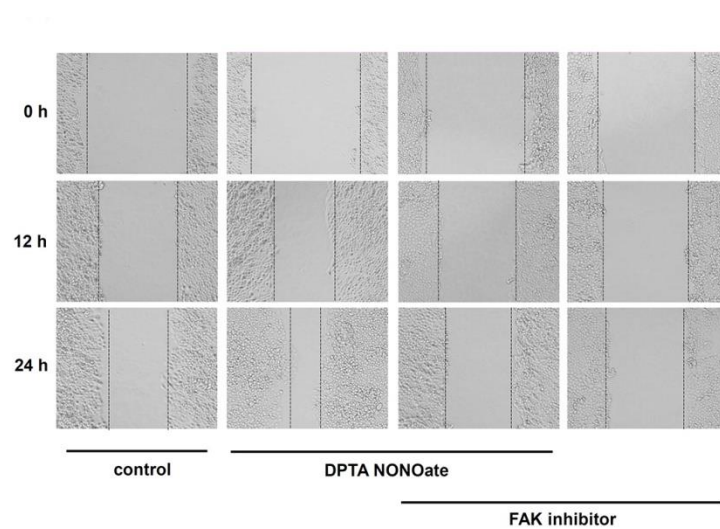
(D)
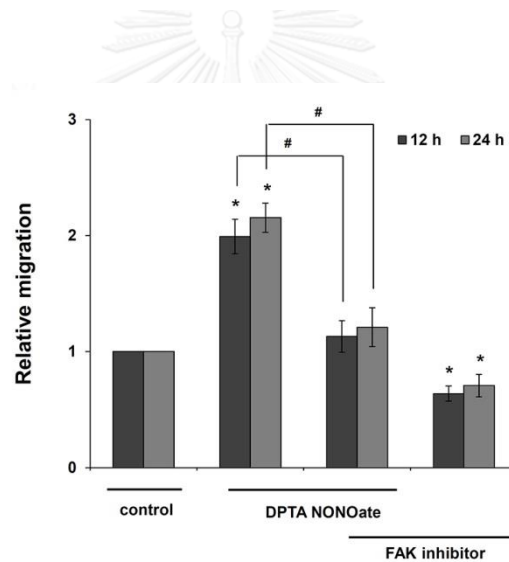
Figure 4.10 Integrin signaling mediated cell migration through FAK protein. H460 cells were treated with or without DPTA NONOate (25 μM) and FAK inhibitor (10 μM FAK inhibitor14) for 24 h. After treatment, (A) the expression levels of integrin αv, α5, β1, β3 and related proteins were determined by western blot. (B) Relative protein levels were quantified by densitometry. (C) Phase contrast images of wound healing were captured at 0, 12 and 24 h. (D) Relative migration was determined by comparing to control. Data represent mean ± SD (n=3). * $p < 0.05$ versus non-treated control, [#] $p < 0.05$ versus NO-treated control.

CHAPTER V

DISCUSSION AND CONCLUSION

It is well accepted that an elevation of NO level in cancer tissue is the dominant cause of cancer metastasis. In this study demonstrated that NO, an important endogenous gas, functions as integrins modulator in human lung cancer cells. The results from PKG, a known downstream molecular target of NO signal suggest that, the increasing of integrins αv and $\beta 1$ augments the migratory activity of H460, H292, and H23 cells via the AKT activation.

NO is synthesized through the function of NOS enzyme, which was found abundantly in the lung cancer tissue [52]. Besides, the immune cells locate around tumor site are responsible for the high level of NO in cancer microenvironment [66]. As lung cancer is a leading cause of cancer-related death [67], identification of biological molecules that potentiate this cancer aggressiveness as well as metastasis is of very interest and may lead to novel strategies that improve the outcome of treatment. Certain studies have indicated that NO is cytotoxic to the cancer cells. NO was shown to directly induced oxidative stress and DNA damage signal that resulted in cell apoptosis [68]. However, more recent studies have shown that NO at physiological or relatively low level is not only non-cytotoxic, but also potentiate cellular functions [69]. In terms of cancer, NO was shown to attenuate response of the lung cancer cells to cisplatin [70, 71] and Fas ligand-induced apoptosis [72]. Besides, NO was shown to mediate anoikis resistance through Caveolin-1 upregulation [7, 73]. As integrin switch or alteration of integrin pattern on the surface of cancer cells have intensively shown to positively influence migration and invasion behaviors of the cells [15, 17].

Integrins are a family of transmembrane glycoprotein receptors transmitting the signals from ECM to intracellular pathways. Their functions regulate a number of

cellular processes including survival, proliferation, adhesion and motility [12, 62]. Certain integrins including integrin αv and β1 were shown to potentiate motility of cells [19, 21, 74]. Consistent with our finding, the increase of such integrins mediated by NO treatment was well correlated with the increase in cell migration.

In light of its importance in both cell migration and proliferation control, AKT is pointed to as a critical target for anticancer therapy. AKT signal was linked to the chemotherapeutic resistance and potentiated cancer metastasis [75]. In lung cancer, PI3K pathway, an up-stream signal of AKT, is frequently deregulated due to genetic instability resulting in increased PI3K and AKT signals [76]. Enhanced activation of AKT has been found in NSCLC cells, and was shown to correlate with chemotherapeutic and radio resistance [77]. In addition, activated AKT in primary NSCLC tumors was suggested as a poor prognostic factor [75].

In addition to those contexts, this study has provided important evidence indicating that the alteration of integrins induced by NO was depended on AKT status. Treatment of the lung cancer cells with NO caused the activation of PKG which further activated AKT. The specific inhibitor of AKT suppressed the NO-mediated integrin change in these lung cancer cells, pointing out the importance of AKT pathway in regulation of integrin expression.

Upon cell motility, FAK in the area of cellular protrusion is activated by phosphorylation at Tyr 397 position. The active p-FAK stimulates the down-stream pathways resulting in actin polymerization and filopodia formation [78]. Several GTPase have been characterized to regulate cytoskeleton dynamics in migration response. Cdc42-GTP promotes filopodia formation and extended forward protrusion. Likewise, Rho-GTP regulates stress fiber accumulation and controls rearward contraction. Rac-GTP regulates actin rearrangement and induces lamellipodia formation [44]. In this study, we observed the involvement of these GTPase signals and found that Cdc42-GTP and Rho-GTP were significantly increased in NO-treated

cells. Consistent with the previous findings, the up regulation of Cdc42-GTP in NO-treated cells correlated with the increase of filopodia formation.

In closing, we show here for the first time the function of NO in inducing integrins αv and β1 in human lung cancer cells and identify a mechanism involving PKG/AKT. As integrin switch has been shown to correlate with chemotherapeutic resistance, enhanced metastasis potentials, and poor prognosis in many cancers, the findings from the present study may help fulfill the knowledge involving role of NO on aggressiveness of lung cancer, at least in part, through integrin regulation.
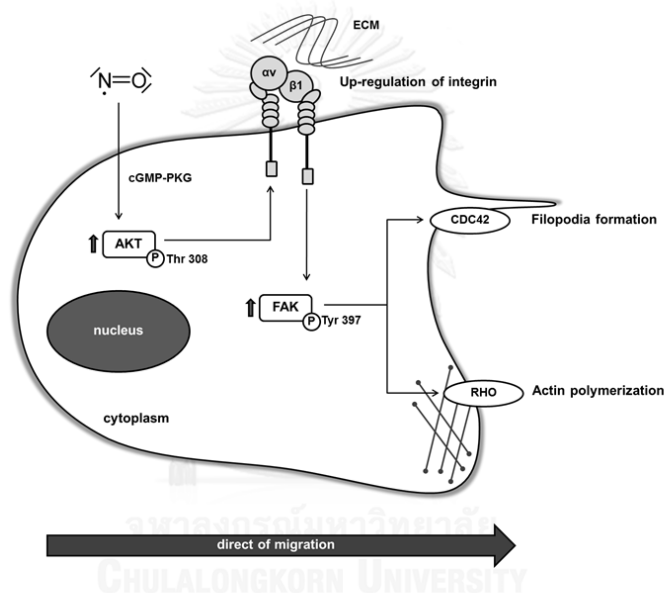
Figure 5.1 Schematic overview of nitric oxide mediates integrin switch via AKT-dependent mechanism. Nitric oxide increase p-AKT which stimulate the up-regulation of integrin αv and β1. The high level of certain integrin recruits and activates FAK to mediate effector molecules such Cdc42-GTP and Rho-GTP, thus promote migratory process.

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APPENDIX

VITA

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