Interleukin-1β-induced iNOS expression in human lung carcinoma A549 cells: Involvement of STAT and MAPK pathways

Kameswaran Ravichandran¹, Alpna Tyagi¹, Gagan Deep¹,², Chapla Agarwal¹,² & Rajesh Agarwal¹,²*
¹Department of Pharmaceutical Sciences, School of Pharmacy, "University of Colorado Cancer Center,
University of Colorado Denver, Aurora, CO 80045, USA

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For understanding of signaling molecules important in lung cancer growth and progression, IL-1β effect was analyzed on iNOS expression and key signaling molecules in human lung carcinoma A549 cells and established the role of specific signaling molecules by using specific chemical inhibitors. IL-1β exposure (10 ng/ml) induced strong iNOS expression in serum starved A549 cells. Detailed molecular analyses showed that IL-1β increased expression of phosphorylated STAT1 (Tyr701 and Ser727) and STAT3 (Tyr705 and Ser727) both in total cell lysates and nuclear lysates. Further, IL-1β exposure strongly activated MAPKs (ERK1/2, JNK1/2 and p38) and Akt as well as increased nuclear levels of NF-κB and HIF-1α in A549 cells. Use of specific chemical inhibitors for JAK1 kinase (piceatannol), JAK2 kinase (AG-490), MEK1/2 (PD98059) and JNK1/2 (SP600125) revealed that IL-1β-induced iNOS expression involved signaling pathways in addition to JAK-STAT and ERK1/2-JNK1/2 activation. Overall, these results suggested that instead of specific pharmacological inhibitors, use of chemopreventive agents with broad spectrum efficacy to inhibit IL-1β-induced signaling cascades and iNOS expression would be a better strategy towards lung cancer prevention and/or treatment.

Keywords: Human lung carcinoma, iNOS, Interleukin-1β, MAPK, STAT

Lung cancer is a major global health problem and represents the leading cause of cancer related incidences and deaths worldwide. In the year 2010, the number of men and women died by lung cancer in the United States was almost equal to total deaths caused by other three cancers combined [prostate/breast, colon and rectum and pancreas]. Majority of lung cancers are diagnosed at late stages, with less than 20% of lung cancers being diagnosed when the disease is still localized, and accounts for one of the main reasons for high mortality among lung cancer patients. These statistics demands more efforts to find a cure against this deadly human malignancy. Recent studies suggest that most effective strategy against lung cancer can be its prevention and/or intervention by aiming at relevant molecular targets. Numerous studies have established that iNOS (nitric oxide synthase) is one such molecular target which can be investigated for preventive and therapeutic strategies against lung cancer. iNOS is the primary NOS responsible for increased NO (nitric oxide) production and is known to be over expressed in various cancers including lung cancer. The resultant increased production of NO contributes to multistage carcinogenesis by inducing DNA damage, lipid peroxidation and inflammation. Liu et al. have reported increased level of exhaled NO and up-regulation of iNOS expression in patients with primary lung cancer. Further, inhibition of iNOS expression through genetic ablation or chemical inhibitors has been reported to decrease lung tumorigenesis in pre-clinical models. Despite these advances a clear understanding of iNOS regulation in lung cancer cells is still lacking.

Aggressiveness of lung cancer cells have been attributed mainly to the increased production of variety of cytokines and growth factors in the tumor microenvironment. These cytokines and growth factors are known to interact with membrane associated receptors in lung cancer cells and activate

Abbreviations: IL-1β- interleukin-1 beta; iNOS- inducible nitric oxide synthase; NO- nitric oxide; MAPK- mitogen activated protein kinases; JAK- janus activated kinases; STAT- signal transducer and activation of transcription; ERK1/2- extracellular signal regulated kinases 1/2; JNK1/2- c-Jun-N-terminal kinases 1/2; NF-κB- nuclear factor-kappa B; DMSO- dimethyl sulphoxide; EDTA- ethylene diamine tetra acetic acid; HIF-1α- hypoxia inducible factor-1 alpha; PI3K- phosphoinositide 3-kinases
downstream intracellular signaling thereby, contributing to the growth and progression of lung cancer. IL-1β is one such pro-inflammatory cytokine that is secreted by lung tumor cells or its microenvironment cells and is reported to induce a cascade of signaling events including activation of transcription factors and inflammatory genes. The extracellular binding of IL-1β to its receptor IL-1R transduces the signal through activating various signaling pathways including janus activated kinase1/2 (JAK1/2), signal transducer and activation of transcription (STAT), phosphoinositide 3-kinases (PI3K), nuclear factor kappa B (NF-kB), and mitogen activated protein kinase (MAPK) pathways. IL-1β has been reported to enhance iNOS expression in various cells, but a definite role of IL-1β-induced signaling molecules in iNOS up-regulation remains unknown. Therefore, understanding the role of critical signaling molecules in IL-1β-induced iNOS expression may offer novel targets for the chemoprevention/intervention of lung cancer.

In the present study, effect of IL-1β was analyzed on iNOS expression in human lung epithelial carcinoma A549 cells and assessed IL-1β effect on various signaling molecules including STATs, MAPKs, NF-kB, Akt, and hypoxia inducible factor-1alpha (HIF-1α). The present results showed that IL-1β strongly activated iNOS expression and multiple signaling molecules in A549 cells; and that IL-1β induced iNOS expression involved additional pathways together with STAT and MAPK pathways.

Materials and Methods

Cell line and reagents—Human epithelial lung carcinoma A549 cell line was purchased from the American Type Culture Collection (Manassas, VA). RPMI 1640 and other cell culture materials were from Invitrogen Corporation (San Diego, CA). Recombinant human IL-1β was purchased from Chemicon International (Temecula, CA). Piceatannol (50 μM), AG-490 (50 μM), SP600125 (50 μM) and PD98059 (50 μM) were dissolved in DMSO (vehicle). DMSO concentration did not exceed 0.1% (v/v) in any treatment. The inhibitors Piceatannol, AG-490, SP600125 and PD98059 were dissolved in DMSO (vehicle). DMSO concentration did not exceed 0.1% (v/v) in any treatment. In experiments, where A549 cells were treated with specific inhibitor, the inhibitor was added 2 h before IL-1β treatment. After desired treatments medium was aspirated, cells were harvested and total cell lysates were prepared as described previously. Protein concentration in the lysates was determined using Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) following Lowry’s method.

Western immunoblot analysis—As desired, total cell lysates, cytosolic or nuclear extract (40-80 μg protein) were denatured in 2X SDS-PAGE sample buffer, and were subjected to 8 or 12% Tris–glycine gels, and separated proteins were transferred onto nitrocellulose membranes by Western blotting. Membranes were incubated with blocking buffer for 1 h at room temperature, and probed with desired primary antibodies over night at 4°C followed by appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature and protein bands were visualized by enhanced chemiluminescence detection system (GE Healthcare Bioscience, Piscataway, NJ). In each case, blots were subjected to multiple exposures on the X-ray film to ensure that the band density is in the...
linear range, and the bands were scanned with Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA). Each membrane was stripped and re-probed with β-actin antibody to confirm equal protein loading.

**Results and Discussion**

*IL-1β induces iNOS expression in A549 cells*—iNOS is an enzyme involved in production of nitric oxide (NO) via catalyzing the conversion L-arginine to citrulline in the presence of NADPH and oxygen. In response to pro-inflammatory cytokines, lung cancer cells induce iNOS expression and generate nitric oxide, which plays a key role in inflammation, angiogenesis and neoplasia. As mentioned earlier, IL-1β is one such pro-inflammatory cytokine secreted by cancer cells or tumor microenvironment cells that play an important role in the regulation of iNOS expression in lung cancer cells. In the present study, the time-course effect (10-180 min) of IL-1β on iNOS expression was analyzed in lung carcinoma A549 cells. The A549 cells had high basal expression of iNOS (Fig. 1a), which decreased significantly under serum starvation condition in total cell lysates. IL-1β (10 ng/ml) increased significantly the iNOS expression (equal to the expression observed under 10% serum condition) within 10 min of the treatment (Fig. 1A). The increase in iNOS was sustained until 30 minutes after IL-1β treatment, which returned to its basal levels thereafter (Fig. 1A). Then the effect of IL-1β (10 ng/ml) on various signaling molecules was also examined that might be involved in the observed strong increase in iNOS expression.

*IL-1β activates STATs in A549 cells*—STATs are transcription factors activated by various growth factors and cytokines. Upon tyrosine phosphorylation by JAKs, they dimerize and translocate to the nucleus where they modulate the expression of wide range of downstream targets. Members of STAT family are reported to be constitutively activated in number of cancers including lung cancer. The effect of IL-1β on the activation of STAT1/3 in A549 cells has been shown in Fig. 1B. IL-1β (10 ng/ml) treatment induced phosphorylation of STAT1 at both sites (Tyr701 and Ser727) in total cell lysates (Fig. 1B). The phosphorylated STAT1-Tyr701 level remained increased till 180 min post-IL-1β treatment, while the phosphorylated STAT1-Ser727 level started declining after 10 min of IL-1β treatment (Fig. 1B). Similarly, IL-1β treatment increased the phosphorylation of STAT3 at Tyr705 site till the last time-point studied.
(180 min), but IL-1β treatment increased marginally phosphorylation of STAT3 at Ser727 site. Under similar treatment conditions, we did not observe any significant difference in the total STAT1 and STAT3 protein level (Fig. 1B). In each case, membrane was stripped and re-probed with β-actin antibody to confirm equal protein loading.

As mentioned above, nuclear translocation of STATs is necessary for executing their biological roles; therefore, we examined the effect of IL-1β treatment on nuclear levels of STATs. IL-1β treatment resulted in strong increase in nuclear levels of phosphorylated STAT1 (Tyr701 and Ser727) and STAT3 (Tyr705 and Ser727) without affecting the total nuclear level of STAT1 and STAT3 (Fig. 1C). In each case, membrane was stripped and re-probed with nuclear loading control Oct-1 antibody to confirm equal protein loading.

IL-1β activates MAPKs and Akt in A549 cells—MAPKs (ERK1/2, JNK1/2 and p38) belong to a widely conserved family of serine/threonine kinases that are known to regulate many cellular phenomena such as cell proliferation, cell differentiation and cell death, and have been implicated in lung tumorigenesis. Similarly, PI3K family member Akt has been reported to play important role in growth and progression of various cancers including lung cancer. Effect of IL-1β treatment on the activation of MAPKs and Akt in serum starved A549 cells were observed in total cell lysates. Serum starvation decreased the levels of phosphorylated MAPKs (ERK1/2, JNK1/2 and p38) but did not affect the phosphorylated Akt levels (Fig. 2A). Further, IL-1β (10 ng/ml) treatment in serum starved A549 cells strongly increased the phosphorylation of ERK1/2 and JNK1/2, which remain increased till 180 min post-IL-1β treatment (Fig. 2A). A significant increase was observed in phosphorylated p38 levels till 20 min post-IL-1β treatment, but p38 phosphorylation was absent at later time points (30-180 min; Fig. 2A). An increase was also seen in phosphorylation of Akt at Ser473 site post-IL-1β treatment (Fig. 2A). Under similar treatment conditions, we did not observe any significant change in the total protein level of ERK1/2, JNK1/2, p38 and Akt (Fig. 2A). In each case, membrane was stripped and re-probed with β-actin antibody to confirm equal protein loading.

IL-1β increases nuclear levels of NF-kB and HIF-1α in A549 cells—NF-kB, a dimeric transcriptional factor, is known to control expression of numerous genes that are crucial in regulating cell survival, apoptosis, angiogenesis, metastasis etc. and is considered a crucial link between inflammation and oncogenesis. The phosphorylation of p65 subunit of NF-kB is considered necessary for its nuclear translocation as well as for its DNA binding and...
activation of gene transcription. Effect of IL-1β treatment on the activation of NF-κB was observed in serum starved A549 cells. IL-1β treatment increased the levels of phosphorylated p65-Ser536 as well as total level of p50 in the nuclear cell lysates (Fig. 2B). HIF-1α is also an important transcriptional factor that regulates neo-angiogenesis as well as invasive and metastatic behavior of cancer cells. Further, the effect of IL-1β treatment on HIF-1α levels was also examined in A549 cells. Nuclear HIF-1α levels decreased after 24 h of serum starvation, while IL-1β exposure increased significantly the HIF-1α expression that was up-regulated until 60 min post-IL-1β treatment. These studies clearly showed that IL-1β treatment activated multiple signaling cascades that might be involved in increasing the expression of iNOS. Then we used pharmacological inhibitors to understand the roles of these signaling molecules in IL-1β-induced iNOS expression.

Effect of JAK1 and JAK2 inhibitors on IL-1β-induced iNOS expression in A549 cells—As mentioned above, iNOS expression and most of the signaling molecules were activated 20 and 30 minutes post-IL-1β treatment. Accordingly, these two time points were selected to establish the role of signaling pathways in IL-1β-induced iNOS expression. To understand the role of STATs in IL-1β-induced iNOS expression, A549 cells were pre-treated with JAK1 inhibitor piceatannol (50 µM) or JAK2 inhibitor AG-490 (50 µM), which are established inhibitors of STAT signaling. Treatment of A549 cells with piceatannol alone or along with IL-1β decreased significantly the levels of phosphorylated STAT1-Tyr701 and STAT3-Tyr705, but affected marginally the levels of phosphorylated STAT1-Ser727 and STAT3-Ser727 (Fig. 3). Under similar treatment conditions, AG-490 pre-treatment did not affect the IL-1β-induced phosphorylation of STAT1 (Tyr701) and STAT3 (Tyr705), however phosphorylation of STAT1 (Ser727) and STAT3 (Ser727) remained unaffected (Fig. 3). Under similar treatment conditions, AG-490 pre-treatment did not affect the IL-1β-induced levels of phosphorylated ERK1/2 and JNK1/2, but decreased significantly the levels of phosphorylated Akt (Fig. 3). Importantly, AG-490 pre-treatment did not affect the IL-1β-induced expression of iNOS in serum starved A549 cells (Fig. 3). Overall, these results suggested that once JAK1-STAT signaling was inhibited, IL-1β-induced iNOS expression in A549 cells involved additional signaling pathway(s).

Effect of MEK1/2 and JNK1/2 inhibitors on IL-1β-induced iNOS expression in A549 cells—To understand the role of ERK1/2 in IL-1β-induced iNOS expression, A549 cells were pre-treated with MEK1/2 inhibitor PD98059 (50 µM) that decreased significantly the IL-1β-induced expression of iNOS. PD98059
pre-treatment did not significantly affect the IL-1β-induced phosphorylation of STAT1/3, JNK1/2 and Akt phosphorylation (Fig. 4). Importantly, PD98059 pre-treatment did not affect the IL-1β-induced expression of iNOS in serum starved A549 cells (Fig. 4).

To understand the role of JNK1/2 in IL-1β-induced iNOS expression, A549 cells were pre-treated with JNK1/2 inhibitor SP600125 (50 µM) that decreased the IL-1β-induced JNK1/2 phosphorylation. SP600125 pre-treatment affected marginally IL-1β-induced phosphorylation of STAT1/3. Further, SP600125 pre-treatment increased IL-1β-induced phosphorylation of ERK1/2, but decreased IL-1β-induced phosphorylation of Akt (Fig. 4). However, SP600125 pre-treatment did not affect IL-1β-induced expression of iNOS in serum starved A549 cells (Fig. 4). Overall, these results suggested that once MAPKs signaling was inhibited, IL-1β-induced iNOS expression in A549 cells involved additional signaling pathway(s).

Each year more than one million people die due to lung cancer around the world. Lung cancer cells are highly resistant to conventional cancer therapies and more than 90% of patients diagnosed with lung cancer die due to this disease. Therefore, new approaches are desperately required to lower the mortality associated with lung cancer. In recent years, there has been greater focus on understanding the specific molecular alterations in cancer cells which could be targeted for drug development. For example, based upon the observation that EGFR expression is higher in lung cancer cells compared to normal cells, synthetic small-molecule inhibitors (gefitinib, erlotinib etc) targeting EGFR have been developed for treatment of lung cancer. Therefore, a better understanding of signaling molecules regulating the development or progression of lung cancer would be helpful to devise more focused and effective preventive as well as therapeutic strategies.

Recent studies have shown that iNOS overexpression plays an important role at both early and late stage lung carcinogenesis. iNOS mediated production of NO is reported to facilitate tumor neo-vascularization and to promote tumor invasiveness through regulating the balance between matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs). Further, Kisley et al. have clearly demonstrated that the genetic disruption of iNOS significantly inhibits chemical carcinogen-induced lung tumorigenesis as well as expression of pro-angiogenic factor VEGF expression in lung tumors, suggesting the essential role of iNOS in lung tumorigenesis. Chen et al. study also demonstrated that cigarette smoking promotes iNOS/NO level which could lead to increased proliferation and growth of lung cancer cells. Recent studies have also shown that targeting iNOS expression by specific chemical inhibitors or chemopreventive agents is effective in preventing lung carcinogenesis. In this regard, we have reported earlier that a chemopreventive agent silibinin prevents neo-angiogenesis in urethane-induced lung tumors by inhibiting iNOS expression. These results were confirmed in our recently completed study where using iNOS knockout mice we established that the cancer preventive and anti-angiogenic efficacy of silibinin against lung carcinogenesis is mainly through iNOS inhibition. Based upon the central role of iNOS in lung tumorigenesis, results from the present study revealed the role of key signaling molecules in the regulation of iNOS expression in lung cancer cells.
Extensive epidemiological studies have clearly established tobacco smoking as the prime cause of lung cancer. The pro-inflammatory milieu in the lung due to smoking as well as due to exposure to chemicals or airborne particles is considered critical in lung cancer development. In response to chronic inflammation, alveolar macrophages become activated and release inflammatory mediators such as cytokines and growth factors, which play important role in growth and progression of cancer cells including the regulation of neo-angiogenesis, invasion and metastasis. Recent studies suggest that IL-1β is a key cytokine that plays important role in lung inflammatory diseases including lung cancer. IL-1β is known to signal through various adaptor proteins and kinases that lead to the activation of various downstream targets, thereby promoting tumor cell proliferation, angiogenesis and tumor invasion. Even though, the role of IL-1β in the regulation of iNOS expression is shown earlier, present study provides detailed understanding towards the role of various signaling molecules in IL-1β-induced expression of iNOS in A549 lung cancer cells. Briefly, the major findings from the present study are: (a) IL-1β activates iNOS and multiple signaling cascades involving STATs, MAPKs, Akt, NF-κB and HIF-1α in lung carcinoma A549 cells; (b) Akt seems to be down-stream of STAT or JNK1/2 in IL-1β-activated signaling events in A549 cells; and (c) IL-1β-induced iNOS expression involves additional pathways together with JAK-STAT and ERK1/2/JNK1/2 in A549 cells.

In summary, the results of our present study suggested that targeting STATs or MAPKs signaling alone would not be sufficient towards targeting iNOS expression in lung cancer cells; specifically in tumor microenvironment where the cancer cells are continuously exposed to several cytokines/chemokines which activate additional signaling pathways and transcriptional factors. Accordingly, the efforts should be focused on the agents that inhibit/target various signaling cascades; the chemopreventive agents are one such class of agents. Therefore, instead of specific pharmacological inhibitors, the use of chemopreventive agents with broad spectrum efficacy to inhibit IL-1β-induced signaling cascades and iNOS expression would be a better strategy towards lung cancer prevention and/or treatment.

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