Effect of nitric oxide synthase on multiple drug resistance is related to Wnt signaling in non-small cell lung cancer

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Abstract. Multiple drug resistance (MDR) is considered a major challenge in the clinical treatment of non-small cell lung cancer (NSCLC). Both nitric oxide synthase (iNOS) and Wnt signaling pathway participate in the regulation of drug resistance, but the interaction between them remains unclear. In the present study, we detected the activation of Wnt/β-catenin signaling in iNOS-induced drug-resistant lung cancer cells, and compared the effect of canonical and noncanonical Wnt pathway on the level of iNOS. Moreover, we investigated the expression of Wnt/β-catenin signaling downstream factors and its main inhibitors. The results indicated iNOS-induced drug resistance was possibly mediated by glutathione S-transferase-π (GST-π) and topoisomerase IIα (TOPO IIα), but not P-glycoprotein (P-gp), and this process was closely associated with the activation of canonical Wnt/β-catenin signaling, but less with noncanonical pathways. The mechanism of iNOS promoting Wnt/β-catenin pathway was mainly dependent on the inverse regulation of Dickkopf-1 (DKK-1) and secreted frizzled-related protein-1 (SFRP-1). Clarifying the relationship between iNOS and Wnt signaling may provide insight into a better understanding of the mechanism of drug resistance development in NSCLC.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, accounting for 26% of all female and 28% of all male cancer deaths in 2013 (1). In China, the crude mortality rates in 2008 were 47.51 per 100,000 men and 22.69 per 100,000 women (2). Of all lung cancer occurrences, ~85% are non-small cell lung cancer (NSCLC) (3), which is a lethal malignancy with a 5-year survival rate of only ~15% (4,5). Standard treatment for patients with NSCLC typically includes radiotherapy, platinum-based chemotherapy and non-platinum agent (6,7). However, the prognosis of lung cancer remains poor, owing mainly to the acquired or inherent drug resistance of cancer cells.

Drug resistance is a highly common phenomenon in the clinical chemotherapy of leukemia or other solid tumors, and these cancer cells may also become cross-resistant to various chemotherapeutics, leading to multiple drug resistance (MDR). Previous research found several mechanisms for MDR, such as overexpression of transporter superfamily members, mutation or alteration in drug target genes, activation of mitogen-activated protein kinase (MAPK) cascade and phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway (8,9).

Nitric oxide (NO) has been shown to play important roles in the innate immune response, neovascularization, cancer metastasis and cell death (10-12). Recently, long-term exposure to NO was found to render lung cancer cells resistant to cisplatin, doxorubicin and etoposide in a dose- and time-dependent manner by increasing the level of caveolin-1 (CAV-1), anti-apoptotic B-cell lymphoma-2 (Bcl-2) and activated protein kinase B (AKT) (13). In the present study, MDR-related factors glutathione S-transferase-π (GST-π) and topoisomerase IIα (TOPO IIα) but not P-glycoprotein (P-gp) were found to be regulated by induced nitric oxide synthase (iNOS) in A549/CDDP, and this process was directly mediated by the Wnt signaling pathway. Moreover, we found iNOS was mainly influenced by canonical Wnt/β-catenin signaling but not noncanonical Wnt pathways. Furthermore, we detected the expression of Wnt/β-catenin downstream factors and inhibitors. The results indicated blocking iNOS could inactivate Wnt/β-catenin signaling, and this function might be mediated by Dickkopf-1 (DKK-1) and secreted frizzled-related protein-1 (SFRP-1). Our findings may help elucidate the relationship between iNOS and Wnt signaling in the process of drug resistance in NSCLC.

Materials and methods

Cell lines and reagents. The human cisplatin-tolerant NSCLC cell line A549/CDDP was obtained from the American Type

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Culture Collection (ATCC). Cells were cultured at 37˚C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), containing 10% FBS (Clontech) and penicillin streptomycin solution (Hyclone). Human TNF-α, IL-1β and IFN-γ obtained from R&D Systems were used to induce the production of NO as previously described (14). iNOS selective inhibitor S-methylisothiourea sulfate (SMT) was obtained from Beyotime (China). Recombinant human DKK-1 was from PeproTech, used to generally block Wnt pathways. XAV939 and SP600125 (both from Selleck) and Xec (Merck) were chosen to inhibit Wnt/β-catenin, Wnt/JNK and Wnt/Ca^{2+} pathways respectively. Protein levels were normalized to β-actin.

Analysis of mRNA levels by RT-PCR. Total cellular RNA was isolated with TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using Sprint RT complete products kit (Clontech). The gene-specific primers for RT-PCR are listed in Table I.

Western blot analysis. A549/CDDP cells were plated in 6-well plates ($3x10^6$ cells/well). Following inflammatory cytokine mixture stimulation for 4 h, inhibitors of iNOS and Wnt pathways were added to the medium. After 8 h treatment of these antagonists, cells were harvested and homogenized with lysis buffer. Total protein was separated by denaturing 10% SDS-polyacrylamide gel electrophoresis. Detection was performed with Odyssey system (Gene). The primary antibodies for iNOS, P-gp, TOPO IIα, GST-π, Wnt-3a/5a/8a/11, Fzd-8, β-catenin, Axin, APC, phospho-GSK-3β (Ser9), GSK3β, Wif-1, DKK-1, SFRP-1 and β-actin were all obtained from Santa Cruz Biotechnology. The animal-matched horseradish peroxidase-conjugated secondary antibody was purchased from Santa Cruz Biotechnology.

ELISA. NO is rapidly oxidized to nitrite and nitrate which are used to quantitate NO production. BioVision's Nitric Oxide Colorimetric Assay Kit provided an accurate, convenient measure of total nitrate/nitrite in a simple two-step process. The amount of the azo chromophore accurately reflected NO amount in samples.

Statistical analysis. Data are presented as the mean ± SD. Experiments were carried out in duplicate or triplicate, and were all conducted a minimum of three times. Data were analyzed by the Student's t-test or ANOVA where appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inhibition of Wnt signaling decreases the NO-induced drug resistance in A549/CDDP. In inflammation conditions, the iNOS gene is often activated, resulting in the production of NO. Thus, the pro-inflammatory cytokines TNF-α, IL-1β and IFN-γ were used to trigger the expression of iNOS in our experiment (14-17). To investigate the Wnt signaling pathway, DKK-1 was added into the medium to inhibit the Wnt pathway. The expressions of P-gp, TOPO IIα and GST-π were chosen to reflect the extent of the drug resistance, and the level of NO in the culture media was evaluated by ELISA.

Table I. Primer sequences used in RT-PCR.

Gene	Primer
iNOS	F: 5'-ACAAGCTGGCCTCGC TCTGGAAAGA-3'
	R: 5'-TCCATGCAGACAACCTTGGGGTTGAAG-3'
P-gp	F: 5'-ACTTCCACATCTGCTTCGTCAGTG-3'
	R: 5'-ATTCAGCCACAGGAGGTAGAGAGC-3'
GST-π	F: 5'-TGGGCATCTGAAGCCTTTTG-3'
	R: 5'-GATCTGGTCACCCACGATGAA-3'
TOPO IIα	F: 5'-AAGGTTTGGGCACCAGCAC-3'
	R: 5'-CTCGCTTGTCATTCCGTTTG-3'
Wnt-3a	F: 5'-TCCACGCCATTGCCTCAG-3'
	R: 5'-GACCACCAGCATGTCTTCACC-3'
Wnt-5a	F: 5'-ACAACCTGGCTGATGTGGC-3'
	R: 5'-CGTCTGCACGGTCTTGAACT-3'
Wnt-8a	F: 5'-CCTATCTGACCTACACGACTAGTGT-3'
	R: 5'-CGTTCCCAAGCAAACTGG-3'
Wnt-11	F: 5'-AAGGACTCGGAACTCGTCTATC-3'
	R: 5'-GCAGCACCAGTGGTACTTACAG-3'
Wif-1	F: 5'-ACCTGGATTCTATGGAGTGAACTGT-3'
	R: 5'-GTATGAGGCTGGCTTCGTACCT-3'
SFRP-1	F: 5'-GCTTCCAGTCGGACATCG-3'
	R: 5'-AGCATCTCGGGCCAGTAG-3'
DKK-1	F: 5'-TTCCAACGCTATCAAGAACCT-3'
	R: 5'-CCAAGGTGCTATGATCATTACC-3'
β-actin	F: 5'-ATGGATGATGATATCGCCGCGCT-3'
	R: 5'-GACTCGATGCCCAGGAAGGA-3'

F, forward; R, reverse.

Following stimulation with the TNF-α/IL-1β/IFN-γ combination, the expression of GST-π was clearly upregulated, while that of TOPO IIα decreased. Although P-gp was also reduced after Wnt pathway blocking, its expression was not significantly altered (Fig. 1A and B). An increasing concentration of NO in the culture media was observed, as shown in Fig. 1C, demonstrating the activation of iNOS. The results indicated that the resistance of A549/CDDP to cisplatin was positively increased by high level of iNOS, and DKK-1 reversed the drug resistance mainly by regulating GST-π and TOPO IIα.

The level of iNOS is positively correlated with the canonical but not the noncanonical Wnt/β-catenin signaling. Although the effect of Wnt signaling in iNOS-induced drug resistance was confirmed in our experiment, the differences between canonical and noncanonical Wnt pathways in regulating the level of iNOS were still unclear. By treatment with Wnt/β-catenin inhibitor XAV939 (18,19), Wnt/Ca^{2+} inhibitor XeC (20,21) and Wnt/JNK inhibitor SP600125 respectively in A549/CDDP, we found a lower iNOS and GST-π, and a higher TOPO IIα in the Wnt/β-catenin-blocking group. However, neither Wnt/JNK nor Wnt/Ca^{2+} pathway were correlated with iNOS, GST-π and

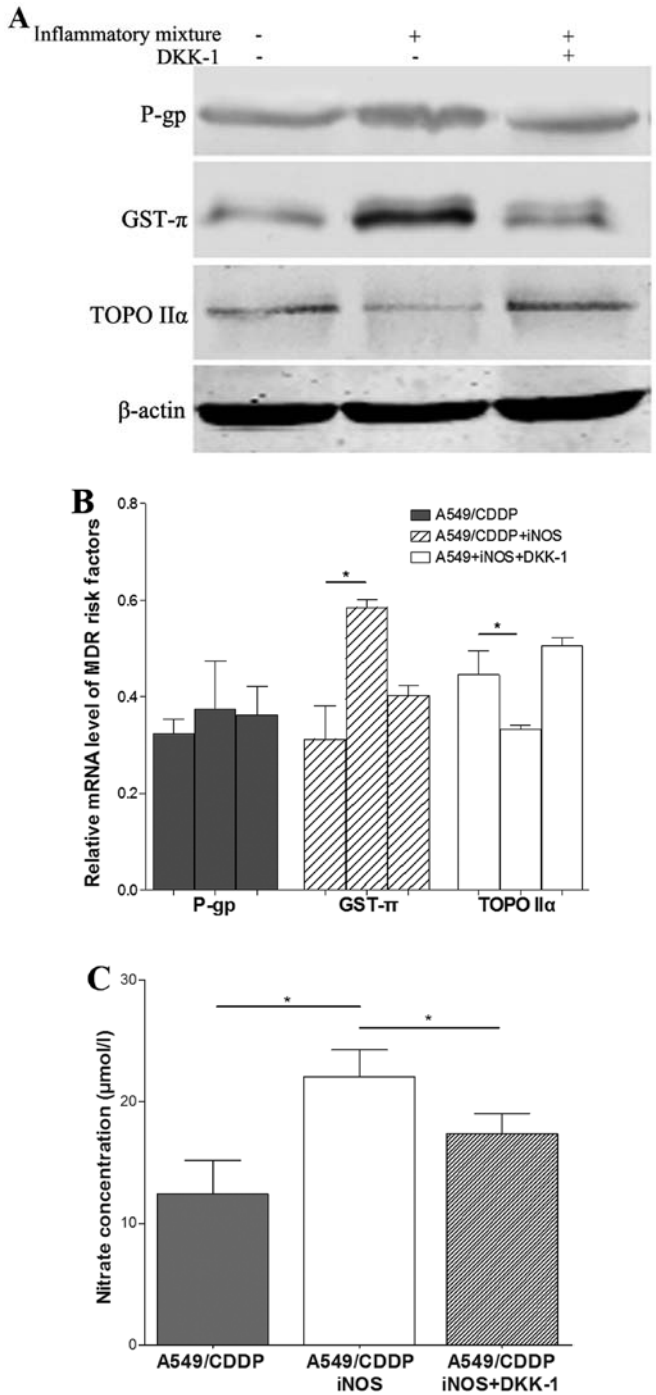

Figure 1. The effect of Wnt signaling on NO-induced drug resistance. (A) A549/CDDP cells were treated with inflammatory mixture containing 1,000 U/ml TNF-α, 100 U/ml IL-1β, and 250 U/ml IFN-γ for 4 h, and 100 ng/ml DKK-1 was added to the medium for 8 h to investigate the level of drug-resistant related factors. (B) Corresponding mRNA levels of GST-π, TOPO IIα and P-gp. (C) The change of nitrate concentration, which was induced by TNF-α/IL-1β/IFN-γ, $^{*}P<0.05$.

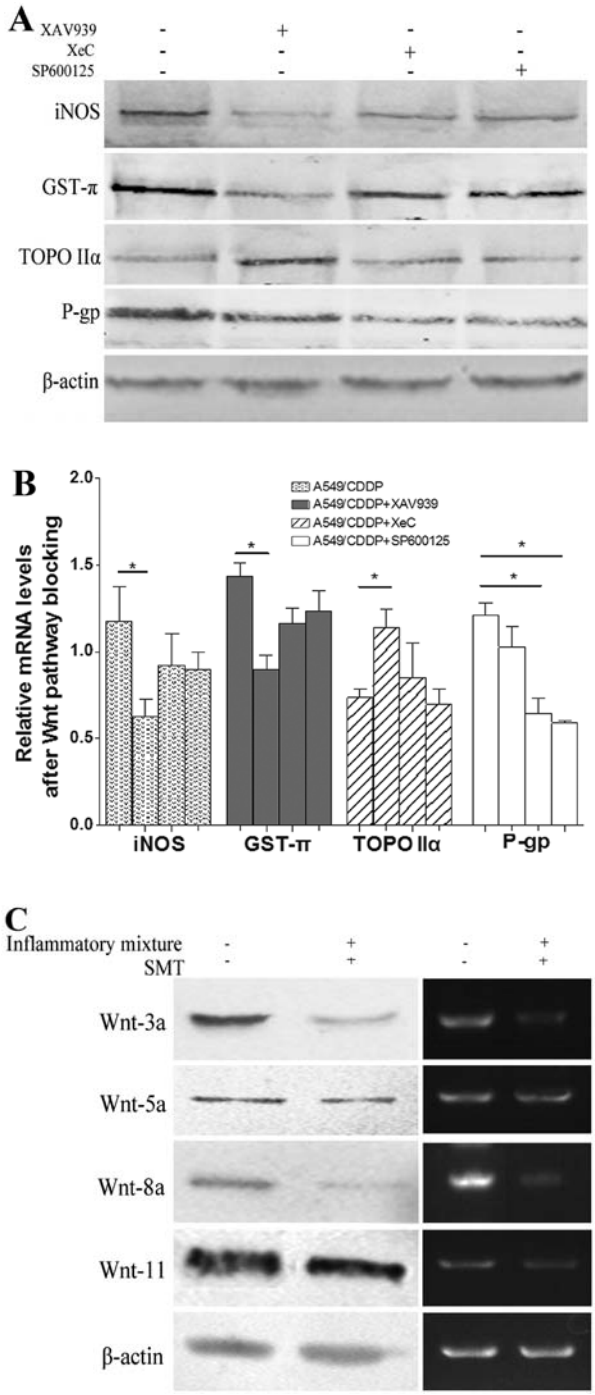

Figure 2. The relationship between iNOS and canonical/noncanonical Wnt pathways. (A) The change of iNOS and drug-resistant factors after treatment with Wnt/β-catenin inhibitor XAV939 (1 μmol/l), Wnt/Ca^{2+} inhibitor XeC (2 μmol/l) and Wnt/JNK inhibitor SP600125 (10 μmol/l). (B) Corresponding mRNA levels of iNOS, GST-π, TOPO IIα and P-gp, $^{*}P<0.05$. (C) The effect of iNOS on canonical Wnt-3a/Wnt-8a and noncanonical Wnt-5a/Wnt-11.

TOPO IIα as shown in Fig. 2A. P-gp was clearly downregulated in noncanonical Wnt pathways, and that might be related to other signals influenced by XeC and SP600125.

Furthermore, we detected the effect of iNOS on canonical and noncanonical Wnt signaling represented secretions (Wnt-3a/Wnt-8a and Wnt-5a/Wnt-11, respectively) (22-24). Consistent with our previous results, inhibition of iNOS led to an obviously decreased expression of Wnt-3a and Wnt-8a which indicated canonical Wnt signaling, but noncanonical Wnt-5a and Wnt-11 were not significantly influenced by iNOS, as shown in Fig. 2B.

Inhibition of iNOS is positively associated with the Wnt/β-catenin signaling pathway and its downstream factors.

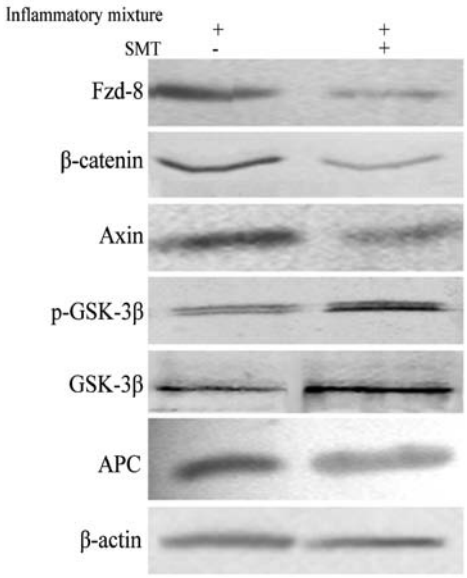

Figure 3. The effect of iNOS on Wnt/β-catenin downstream factors. By preventing the expression of iNOS by its highly selective inhibitor SMT (1 mmol/l) in A549/CDDP, a lower Fzd-8, β-catenin and Axin, and a higher p-GSK-3β and GSK-3β were observed. The expression of APC had no statistical significance after iNOS blocking.

The signaling transduction of canonical Wnt/β-catenin pathway has been well described. Following binding of Wnt to its receptor frizzled (FZD) and lipoprotein receptor-related protein 5/6 (LRP5/6), dishevelled proteins (DSH) become activated, leading to the inactivation of the Axin/adenomatous *polyposis coli* (APC)/glycogen synthase kinase (GSK)3β complex which mediated β-catenin degradation, and resulting in the accumulation of β-catenin. Then, the β-catenin proteins translocated to the nucleus and interacted with transcription factors of the T cell factor (TCF) and lymphoid-enhancing factor (LEF) families, promoting the transcription of many oncogenic factors, such as c-Myc, cyclin D1 and VEGF (25-29). By preventing the expression of iNOS by its highly selective inhibitor SMT in A549/CDDP, we observed a decreasing level of Fzd-8, β-catenin and Axin, and an increased p-GSK-3β and GSK-3β-expression. However, the change in APC showed no statistical significance compared with that in no-SMT control as shown in Fig. 3.

The level of DKK-1 and SFRP-1 inversely regulate the iNOS and Wnt/β-catenin signaling. As a core modulator, Wnt/β-catenin transduction pathway was regulated by a precise mechanism, containing positive and negative feedback. The general opposite control has been considered to be mediated by Wnt antagonists such as endogenic DKK-1, SFRP-1 and Wif-1. The main inhibitory mechanism is the interference of the combination between Wnt and its receptors (14,30-33). Fig. 4 shows the influence of iNOS on Wif-1, DKK-1 and SFRP-1. In accordance with a previous study, the results indicated DKK-1 was increased after iNOS blocking (14). In addition, the expression of SFRP-1 also showed a negative correlation with iNOS level, but Wif-1 appeared to be less associated with this factor. Thus, we concluded that iNOS could increase drug resistance in NSCLC by inhibiting DKK-1 and SFRP-1.

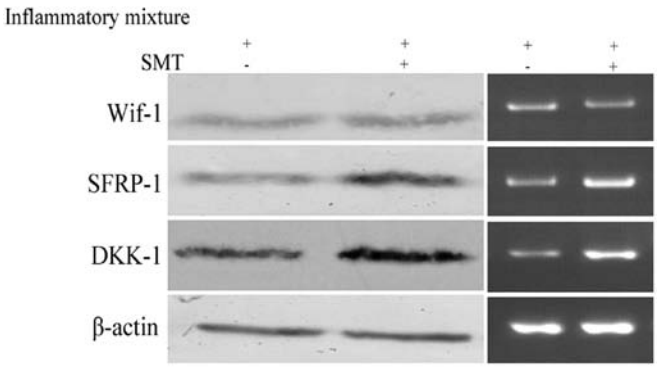

Figure 4. The influence of iNOS on Wnt antagonists. By blocking iNOS, endogenic Wnt inhibitors Wif-1, DKK-1 and SFRP-1 were increased but Wif-1 seemed to be less influenced by iNOS. Thus, the positive regulation of iNOS to Wnt signaling was mainly by decreasing DKK-1 and SFRP-1.

Discussion

Extensive studies have been performed to elucidate the mechanism underlying multiple drug resistance (MDR) in non-small cell lung cancer (NSCLC) in the past ten years. One of the important components of the tumor microenvironment, nitric oxide (NO), has been found to be markedly increased in drug-resistant NSCLC. As a reactive nitrogen species, NO is catalytically synthesized by iNOS, promoting tumor formation, metastasis and differentiation through P53, NF-κB, EGFR and other transduction pathways, including Wnt signaling, which is also considered a core pathway highly activated in drug-resistant lung cancer cells. Previous studies have shown the human iNOS gene is a transcriptional target of Wnt signaling, while iNOS-overexpression increased the levels of downstream effectors of the Wnt pathway such as c-Myc and cyclin D1 (14,34-36).

In this study, we focused on the relationship between iNOS and Wnt signaling in cisplatin-resistant lung cells A549/CDDP. By inhibiting the Wnt pathway by DKK-1, the iNOS-induced drug resistance was confirmed to be reversed. Furthermore, we found Wnt signaling could influence TOPO IIα and GST-π, but affected P-gp less directly. As is known, P-gp-related resistance mainly acts against natural and lipophilic anti-cancer drugs (37,38), thus it may not play a key role in this non-lipophilic drug-induced cell line, leading to a slight change of P-gp levels.

To further differentiate among three Wnt signaling pathways in the regulation of iNOS, we chose XAV939, XeC and SP600125 to inhibit Wnt/β-catenin, Wnt/Ca^{2+} and Wnt/JNK pathways respectively. The results clearly demonstrated higher TOPO IIα and lower iNOS/GST-π levels in the XAV939 treatment group compared with that in the other two inhibitor groups. The expression of P-gp was only slightly altered in the XAV939 group, but it was downregulated in the XeC and SP600125 groups. That is possibly because SP600125 and XeC could disturb other core signal transductions related to P-gp-expression, except the inhibition of JNK1/2 and Ca^{2+}.

To confirm the effect of iNOS on canonical and noncanonical Wnt signaling, we also investigated the corresponding secretions, Wnt-3a/Wnt-8a and Wnt-5a/Wnt-11, respectively.

Consistent with what we observed, inhibition of iNOS led to an obviously decreased Wnt-3a and Wnt-8a level, which indicated canonical Wnt signaling, but noncanonical Wnt-5a and Wnt-11 levels were less altered. The results indicated the iNOS-induced drug resistance was mainly mediated by canonical Wnt/β-catenin signaling, but not by the other two noncanonical pathways.

After establishing the relationship between iNOS and Wnt/β-catenin signaling in A549/CDDP, we detected the effect of iNOS on downstream factors of this pathway, containing membrane co-receptor Fzd, and β-catenin/APC/GSK-3β/Axin compound. In humans, there are 10 Fzd genes which may be divided into five subgroups: Fzd-1/2/7, Fzd-3/6, Fzd-5/8, Fzd-9/10 and Fzd-4 (39). Among them, Fzd-8 was confirmed to form a complex with Wnt3α *in vitro* (40,41). Thus, we tested the expression of Fzd-8, β-catenin, APC and Axin, and the phosphorylation of GSK-3β was assessed as well. By blocking iNOS by SMT, we observed a decreasing level of Fzd-8, β-catenin and Axin, a higher p-GSK-3β and GSK-3β expression, but a slight change of APC. Thus the effect of iNOS on the Wnt/β-catenin pathway was mainly mediated by Fzd-8 and p-GSK-3β. It is of note that Axin, as a negative modulator in the canonical Wnt pathway, was downregulated after iNOS inhibition, and we speculated it might be because Axin has multi functions influenced by iNOS in tumor proliferation or other processes.

To explain the mechanism of iNOS-induced positive regulation on Wnt/β-catenin signaling, we further investigated three widely accepted antagonists of this pathway, Wif-1, DKK-1 and SFRP-1. Human Wif-1 protein contains a Wnt inhibitory factor (Wif) domain, can bind to seven Wnts (3a, 4, 5a, 7a, 8, 9a and 11) (42,43), directly competing with Wnt for binding to its membrane receptors. DKK-1 works by inhibiting Wnt co-receptors LRP5/6 through binding cell surface Kremen-1 or Kremen-2 and thus promoting the internalization of LRP5/6. As a type of secreted frizzled-related protein, SFRP can suppress the transduction of Wnt pathway signaling by competitively binding with Fzd receptor. Consistent with other reports that DKK1 expression is inversely correlated with iNOS and β-catenin translocation, we also observed the negative correlation between DKK-1 and iNOS. Furthermore, SFRP-1 was indicated to be inversely regulated by iNOS as well, but Wif-1 seemed to be less associated with this factor. In the present study, we presumed that there exists a balance between Fzd and its relative secreted protein SFRP, and blocking iNOS might promote the balance switches from Fzd to SFRP, inducing Wnt/β-catenin pathway inactivation, and finally increasing the sensitivity of A549/CDDP cells to cisplatin.

The relationship between iNOS and Wnt signaling has attracted considerable attention for its multiple functions in tumor; hence, clarifying the detailed mechanism of its regulation may help to better understand the mechanism of drug resistance, and may aid in the development of new targets for reversing drug resistance in NSCLC.

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