科技部補助專題研究計畫成果報告
期末報告

黃芩素抑制一氧化氮生成之抗發炎作用於腎絲球腎炎之分子機轉探討及表觀遺傳學研究

計 畫 類 別 ： 個別型計畫
計 畫 編 號 ： MOST 104-2320-B-040-007-
執 行 期 間 ： 104年08月01日至105年07月31日
執 行 單 位 ： 中山醫學大學生物醫學科學學系（所）

計 畫 主 持 人 ： 林庭慧

計畫參與人員： 學士級-專任助理人員：李文汐

中 華 民 國 105 年 10 月 31 日

中 文 摘 要 ： 長期發炎反應會導致許多慢性疾病，環境中許多表觀遺傳之因子如何影響發炎反應，是一重要議題。由誘發型一氧化氮合成酶(iNOS)產生之過量的一氧化氮在腎絲球腎炎扮演重要角色。本研究結果顯示，於腎絲球系膜細胞中，來自環境中病原體釋放出之毒素，如內毒素LPS及由受感染細胞產生之細胞素，如干擾素IFN-γ為表觀遺傳之因子，可以影響受感染細胞之甲基化反應，包括DNA甲基化和蛋白質甲基化。於腎絲球系膜細胞中，於內毒素及干擾素同時刺激下，可產生之一氧化氮以及iNOS蛋白質的表現。 而表觀遺傳學中之甲基化反應，與iNOS基因表達的調控有關。本研究計劃之結果顯示如下。結果一：內毒素及干擾素，影響腎絲球系膜細胞中，甲基化反應相關酵素之表現。結果二：內毒素及干擾素，影響腎絲球系膜細胞中，DNA甲基化及蛋白質甲基化。結果三：S-腺苷高半胱氨酸水解酶抑制劑(AdOx)，為一廣泛使用之甲基轉移酶抑制劑，可抑制此結果與2004年由Yu 及Kone在相同腎絲球系膜細胞中，使用DNA甲基轉移酶抑制劑，卻可加強由第一介白素刺激產生之一氧化氮以及iNOS蛋白質的表現，大大相左。
結果四：AdOx抑制一氧化氮生成以及iNOS蛋白質的表現是可抑制iNOS mRNA之表現但不影響iNOS mRNA穩定性；AdOx並未影響影響HuR蛋白質在細胞核及細胞質之分佈。結果五：AdOx抑制轉錄因子NFκB之入核及活化，但對Akt, ERK, STAT 1α 之訊號路徑並無影響。本研究計劃之重要性，除可提供腎絲球腎炎之基礎研究及臨床治療策略外，亦可應用在其他發炎反應相關疾病，如癌症、糖尿病、類風濕性關節炎等。

中 文 關 鍵 詞 ： 關鍵詞: S-腺苷高半胱氨酸水解酶抑制、 腎絲球腎炎、一氧化氮、一氧化氮合成酶、腎臟絲球體系膜細胞。

英 文 摘 要 ： Abstract
Prolonged inflammation reactions lead to chronic diseases. How epigenetic factors from environments affect inflammatory reactions is an important issue to be investigated. Overproduction of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) induction has been implicated in glomerulonephritis (GN). Our results showed both lipopolysaccharide (LPS) released from pathogens and interferon-γ (IFN-γ) produced from infected cells are epigenetic factors modulating methylation reaction in glomerular mesangial cells. Combination of LPS and IFN-γ induce NO production and iNOS protein expression in glomerular mesangial MES-13 cells. Methylation reactions in epigenetic study including DNA methylation and protein methylation play essential roles in regulation of iNOS expression. Our data provide evidences in glomerular mesangial MES-13 cells as followed: Result 1: LPS and IFN-γ modulate the protein expression central to the cellular methylation reactions. Result 2: LPS and IFN-γ trigger global DNA methylation and protein arginine/lysine methylation. Result 3: AdOx, a broad-spectrum

methyltransferase inhibitor, inhibited LPS/IFNγ-stimulated NO production and iNOS protein expression in glomerular mesangial MES-13 cells which is contrast to previous results from Yu and Kone (2004) who have demonstrated that 5-aza-2'-deoxycytidine (5-Aza-CdR), a potent and specific inhibitor of DNA methylation, enhanced IL-1-mediated NO production and iNOS expression in mesangial cells. Therefore, more complex mechanisms may be involved for the role of methylation in regulating iNOS-stimulated NO synthesis in mesangial cells. We provided further results as followed. Result 4: AdOx inhibited iNOS mRNA expression but showed no effect on iNOSmRNA stability and HuR translocation in MES-13 cells. Result 5: AdOx blocked NFκB translocation but showed no effect on Akt, ERK, STAT 1α signaling pathway. Our data indicate protein methylation plays a role in renal inflammatory state, especiallythrough regulation of iNOS expression.The results of the proposed studies will enhance our understanding about the role of methylation in renal inflammatory state, The significance of this research proposal not only provides therapeutic strategy in glomerulonephritis but also applies to other inflammatory disorders, such as cancer, diabetes and rheumatoid arthritis.

英文關鍵詞：Keyword: adenosine dialdehyde; glomerulonephtitis; nitric oxide; inducible nitric oxide synthase; glomerular mesangial cells

附件一

科技部補助專題研究計畫成果報告

（□期中進度報告/■期末報告）

（計畫名稱）黃芩素抑制一氧化氮生成之抗發炎作用於腎絲球腎炎之分子機轉探討及表觀遺傳學研究

計畫類別：■個別型計畫　□整合型計畫

計畫編號：MOST　104－ 2320　－B　－ 040　－007

執行期間：　104 年　8 月　1 日至　105 年　7 月 31　日

執行機構及系所：中山醫學大學生物醫學科學學系（所）

計畫主持人：林庭慧

共同主持人：

計畫參與人員：李文汐

本計畫除繳交成果報告外，另含下列出國報告，共 ___ 份：

□執行國際合作與移地研究心得報告

□出席國際學術會議心得報告

□出國參訪及考察心得報告

期末報告處理方式：

1. 公開方式：

□非列管計畫亦不具下列情形，立即公開查詢

■涉及專利或其他智慧財產權，□一年■二年後可公開查詢

2.「本研究」是否已有嚴重損及公共利益之發現：■否 □是

3.「本報告」是否建議提供政府單位施政參考 ■否 □是，

中　華　民　國 105　年　10　月 31　日

Abbreviations used:

Ado, adenosine; AdOx, Adenosine dialdehyde; ADMA, asymmetric dimethylarginine; ASYM24, anti-dimethyl-arginine,asymmetric antibody;5-Aza-CdR, 5-aza-2'-deoxycytidine; DAPI, 4′-6-diamidino-2-phenylindole;DNMT1, DNA methyltransferase 1;Erα, estrogen receptor α; GN, glomerulonephritis; iNOS, inducible nitric oxide synthase; IL-1β, interleukin-1β; IFN-γ,interferon-γ; IRF-1, interferon regulatory factor 1; KMTs, Lysine methyltransferases; L-Arg, L-arginine; 5-mC, anti-5-methylcytosine antibody; MMA, mono-methylarginine; MTases, methyltransferases; NF-κB, nuclear factor –κB; NO, nitric oxide; NOS, nitric oxide synthase; PRMT, protein arginine methyltransferase; SAM, S-adenosylmethionine; STAT-1, signal transducer and activator of transcription-1; SAH, S-adenosylhomocysteine; SAH hydrolase, S-Adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine; SDMA, symmetric dimethylarginine; SYM10, anti-dimethyl-arginine,symmetric antibody; TNF-α, tumor necrosis factor-α.

ABSTRACT

Prolonged inflammation reactions lead to chronic diseases. How epigenetic factors from environments affect inflammatory reactions is an important issue to be investigated. Overproduction of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) induction has been implicated in glomerulonephritis (GN). Our results showed both lipopolysaccharide (LPS) released from pathogens and interferon-γ (IFN-γ) produced from infected cells are epigenetic factors modulating methylation reaction in glomerular mesangial cells. Combination of LPS and IFN-γ induce NO production and iNOS protein expression in glomerular mesangial MES-13 cells. Methylation reactions in epigenetic study including DNA methylation and protein methylation play essential roles in regulation of iNOS expression. Our data provide evidences in glomerular mesangial MES-13 cells as followed: **Result 1:** LPS and IFN-γ modulate the protein expression central to the cellular methylation reactions. **Result 2:** LPS and IFN-γ trigger global DNA methylation and protein arginine/lysine methylation. **Result 3:** AdOx, a broad-spectrum methyltransferase inhibitor, inhibited LPS/IFNγ-stimulated NO production and iNOS protein expression in glomerular mesangial MES-13 cells which is contrast to previous results from Yu and Kone (2004) who have demonstrated that 5-aza-2'-deoxycytidine (5-Aza-CdR), a potent and specific inhibitor of DNA methylation, enhanced IL-1-mediated NO production and iNOS expression in mesangial cells. Therefore, more complex mechanisms may be involved for the role of methylation in regulating iNOS-stimulated NO synthesis in mesangial cells. We provided further results as followed. **Result 4:** AdOx inhibited iNOS mRNA expression but showed no effect on iNOS mRNA stability and HuR translocation in MES-13 cells. **Result 5:** AdOx blocked NFκB translocation but showed no effect on Akt, ERK, STAT 1α signaling pathway. Our data indicate protein methylation plays a role in renal inflammatory state, especially through regulation of iNOS expression. The results of the proposed studies will enhance our understanding about the role of methylation in renal inflammatory state. The significance of this research proposal not only provides therapeutic strategy in GN but may also apply to other inflammatory disorders, such as cancer, diabetes and rheumatoid arthritis.

Keyword:

methylation; adenosine dialdehyde; nitric oxide; inducible nitric oxide synthase; NFκB; glomerulonephtitis

1. INTRODUCTION

Prolonged inflammatory reactions lead to chronic diseases. How epigenetic factors affect inflammatory reactions is an important issue. Epigenetic modifications include DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs(1).Among these events, chemical modification of DNA and protein by methylation exert major impact on gene expression. **(1-4)**. DNA methylation usually occurs on cytosines, and is related to gene silencing. Protein methylations occur on a number of amino acid residues including lysine, arginine located on **c**hromatin-associated histone proteins and non-histone proteins **(5).**

Methylation reactions are catalyzed by specific methyltransferases (MTases) which have been classified into three distinct classes. Class I sharing common β-sheet structure is the largest groups of MTases that methylate DNA, RNA and proteins. Class II containing the SET domain is known as lysine MTases (KMTs). Class III is isoprenylcysteine carboxy MTases which are associated with cell membrane(6). All structurally relevant MTases catalyze S-adenosylmethionine (SAM) dependent methylation by transferring the methyl group from SAM to yield S-adenosylhomocysteine (SAH) and a methylated target molecule. Protein methylation is involved in many cellular processes including gene transcriptional regulation, RNA processing, DNA repair and signal transduction (7,8). MTases are emerging as targets for drug discovery since MTases are implicated in normal physiology and human diseases(9).

Lysine methyltransferases (KMTs), catalyze mono-, di-, or tri-methylation by transferring one, two, or three methyl groups, respectively, from SAM to the lysine residue of proteins. KMTs were initially found to target histones as substrates and have been recognized as a fundamental mechanism for epigenetic regulation of chromatin. Subsequently, numerous KMTs that methylate nonhistone proteins on lysine residue have been identified. These nonhistone substrates include p53, DNA methyltransferase 1 (DNMT1), estrogen receptor alpha (ERα), androgen receptor (AR), p300/CBP-associated factor (PCAF), nuclear factor kappaB (NFκB) Thus, lysine methylation serves a general mechanism that regulates gene transcription through methylation of transcription-related proteins which are implicated in oncogenesis and inflammatory disorders. (2)

Protein arginine methyltransferases (PRMTs), s subset of the class I MTases, catalyze methylation of nitrogen of specific arginine residues in proteins. Both histone

and non-histone proteins are substrates of PRMTs. PRMTs are involved in human diseases including cancer, cardiovascular disorder, pulmonary diseases and virus-related infection(3,10,11) They have been considered as promising targets for the treatment of human disorders (3,10,11). In mammalian cells, PRMTs have been classified into type I (PRMT1, 3, 4, 6, and 8) and type II (PRMT5, 7 and FBXO11). Both types of PRMTs catalyze the formation of mono-methylarginine (MMA) from L-arginine (L-Arg). In a second step, type I PRMTs produce asymmetric dimethylarginine (ADMA), while type II PRMTs form symmetric dimethylarginine (SDMA)(7,8). After proteolytic degradation of proteins containing methylated arginine residues, free MMA, SDMA, or ADMA can be released from cells. MMA and ADMA, but not SDMA, have been identified as potent endogenous competitive inhibitors of nitric oxide synthases (NOSs).(12).

NOSs catalyze the synthesis of nitric oxide (NO) contain three isoforms: inducible (i)NOS, neuronal (n)NOS, and endothelial (e)NOS. Constitutively expressed NOSs including nNOS and eNOS produce a small amount of NO while the inducible form, iNOS, yields a high concentration of NO. Increased NO production by induction of iNOS is harmful to host cells during inflammatory conditions, such as GN. (13,14). Regulation of NO synthesis by iNOS is under precise control with multiple mechanisms involved, predominately at transcriptional level. Endotoxin LPS and inflammatory mediators, such as IFN-γ, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were shown to induce iNOS gene expression through activation of several transcription factors. Two major transcription factors, the signal transducer and activator of transcription (STAT)-1α and NF-κB were reported to modulate *iNOS* gene expression in cell- and species-specific manners (15). By binding to the 5'-flanking regions of the *iNOS* gene, activated STAT-1α and NF-κB work synergistically to elicit iNOS gene expression.

The role of DNA methylation in iNOS induction has been reported in mesangial cells. Cytosines methylation of the iNOS promoter inhibits iNOS expression through blocking NF-κB p50 binding to this element (16). Potential methylation sites in iNOS proteins have been predicted by HPLC/MS/MS analysis (17). Whether methylation is involved in iNOS –stimulated NO production through targeting non-histone transcription factors, such as NF-κB and STAT-1α, have never been elucidated.

Herein, we present data showing that LPS/IFN-γ modulate global methylation reactions in MES-13 glomerular mesangial cells. Adenosine dialdehyde (AdOx), a broad-spectrum MTase, inhibited LPS/IFNγ-stimulated NO production and iNOS

protein expression in MES-13 cells. AdOx inhibited iNOS mRNA expression but showed no effect on iNOS mRNA stability and HuR translocation. Further results demonstrated AdOx inhibited LPS/IFN-γ-stimulated NO production in MES-13 cells via blocking NF-κB pathways but showing no effect on Akt, ERK, STAT 1α signaling pathway.

2. MATERIALS AND METHODS

2.1. Materials

Fetal bovine serum was from Hyclone (Logan, UT). DMEM medium and Ham's F12 medium and medium supplements were obtained from Gibco BRL (Gaithersburg, MD). LPS and AdOX were obtained from Sigma Chemical Company (St. Louis, MO). IFN-γ was purchased from PeproTech EC Ltd. (London, UK). The specific antibodies for iNOS, HuR and GAPDH were products from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylated STAT-1α, STAT-1α, phosphorylated Akt, Akt phosphorylated ERK, ERK antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Oligonucleotide primer sequences of iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for reverse-transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) were selected by using Primer Select (MD Bio, Inc). The specific antibodies for DNMT, MATIIα, SAHH, SET7/9, NF-κB and GAPDH were products from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody for anti-5'methylcytosine antibodies (5-mC), anti-methylated lysine (dimethyl) antibody were purchased from Abcam (Cambridge, MA). The antibody for protein arginine methyltransferase (PRMT) 1, 4, 5 were obtained from Upstate Biotechnology (Thermo Fisher Scientific Inc. (Waltham, MA). The antibody for PRMT6 were obtained from Novus Biologicals (Littleton, CO). The antibody for anti-dimethyl-Arginine, asymmetric (ASYM24) and anti-dimethyl-Arginine, symmetric (SYM10) were purchase form Merck Millipore (Billerica, MA). Secondary antibody for fluorescent microscopy and flow cytometry were purchased from Thermo Fisher Scientific Inc. (Waltham, MA).

2.2. Cell Culture

The MES-13 cell line (glomerular mesangial cells from an SV40 transgenic mouse) was obtained from American Type Culture Collection (CRL-1927; Manassas, VA, USA) and maintained in culture medium with a 3:1 mixture of DMEM medium and Ham's medium, supplemented with 14 mM HEPES, 2 mM glutamine, antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin) and 5 % fetal bovine serum at 37^0 C. The incubation chamber was equilibrated with 5% CO2- 95% air.

2.3.Nitrite Assay

Nitrite assay was performed to measure NO production in MES-13 cells after different treatment. NO is rapidly converted into nitrite as the end product. Thus, the

nitrite accumulation in culture supernatant was used as indirect measures of the amount of NO produced. The Griess assay was used and the nitrite level was measured in triplicate. Briefly, an aliquot of 100 µl of the culture supernatant of MES-13 cells was mixed with 100 µl of Griess reagent (one part 0.1% N -(1-naphtyl) ethylene-diamine dihydrochloride in water and one part 1% sulfanilamide in 5% H_3PO_4; both purchased from Sigma Chemicals). The mixture was incubated for ten minutes at room temperature in the dark. The absorbance at 540 nm was measured and the nitrite concentration was calculated by comparison to standard curves of sodium nitrite in culture medium.

2.4. MTT Assay

Colorimetric MTT assay was used to measure the cell viability. In viable cells, the mitochondria are able to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrrazolium bromide (MTT) to formazan. After different drug treatment, the culture medium of MES-13 cells was aspirated and 0.5 mg/ml MTT was incubated with the cells for 4 hr at 37 ^{0}C followed by solubilization in isopropanol. The purple formazan product was measured by the absorbance at 570 nm on a VersaMaxTM Tunable Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA). The absorbance read from cells treated with 1 µg/ml LPS and10 ng/ml IFN-γ (LPS/IFN-γ) is used to indicate 100% of cell viability.

2.5. Preparation of Whole Cell Extract and Western Blot Analysis

To detect the protein levels of iNOS after exposure to different stimuli, MES-13 cells were washed with 1x PBS, scraped out, and incubated with lysis buffer. The lysis buffer contained 1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail tablet (Roche Applied Science, Mannheim, Germany). Cells suspended in lysis buffer were sonicated. The homogenate was centrifuged at 12,000 rpm for 30 min at 4^{0}C, and the cell supernatant was collected. The protein concentration was measured using a Bio-Rad protein assay kit. Cell lysate was combined with 5 x sample buffer containing 100 mM Tris–HCl (pH 6.8), 20% glycerol, 7% SDS, 5% mercaptoethanol, and 0.1% bromophenol blue. The sample was boiled for 5 min and centrifuged to remove the debris. Equal amounts of protein samples (80 µg) were subjected to SDS–PAGE using 10% polyacrylamide gels. Following electrophoresis, the gel was transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with 5% skim-milk in Tris-buffered saline (TBS) containing10 mM Tris (pH 8.0) and 150 mM NaCl, then incubated with primary antibody at 4 C overnight. TBS containing 0.02% Tween 20 (TBST) was used to wash out the nonspecific binding material on the PVDF membrane. Finally, the membrane

was incubated with secondary antibody for 1 h at room temperature. After washing with TBST, the immunoreactive bands were visualized with a light-emitting kit (ECL, Amersham, UK). The protein amount was quantified by measuring the area of the iNOS band using densitometric analysis with AlphaEaseFC.

2.6. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from MES-13 cells using the Tri reagent RNA isolation reagent (Molecular Research Center, Inc, Cincinnati, OH, USA). Total RNA was reverse transcribed to cDNA using Superscript II reverse transcription RT-PCR kit (Life Technologies, Gaithersburg, MD) followed by amplification with PCR. The oligonucleotide primers for the RT-PCR were as followed:
5'-CAGTTCTGCGCCTTTGCTCAT-3' (forward) and
5'-GGTGGTGCGGCTGGACTTT-3'(reverse) for iNOS;
5'- CATCATCTCCGCCCCTTCT-'3 (forward) and
5'-CTCGTGGTTCACACCCATCA-'3 (reverse) for GAPDH. After an initial denaturation at 94 °C for 3 min, 35 cycles of amplification (94°C for 30 sec, 58.3°C for 30 sec , and 72 °C for 1 min) were performed followed by a 7 min extension at 72^0C for iNOS; an initial denaturation at 94 °C for 3 min, 40 cycles of amplification (94°C for 30 sec, 60°C for 30 sec, and 72 °C for 1 min) were performed followed by a 7 min extension at 72^0C for GAPDH. The amplified PCR products were analyzed on a 1 % agarose gel. The PCR product of GAPDH was used as an internal control for quantitation.

2.7. Real-Time RT-PCR

Total cellular RNA was extracted from MES-13 cells using Tri reagent RNA isolation reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). The cDNAs were synthesized from 3.5 μg of RNA of each sample using a SuperScriptTM II reverse transcription system kit according to the manufacturer's protocol. The primers used were as followed: 5'-CCGATTTAGAGTCTTGGTGAAAGTG -3' (forward) and 5'-TGACCCGTGAAGCCATGA-3' (reverse) for iNOS;
5'-CCGATACAAAGCAGGAGAAAAAG -3' (forward) and
5'-CTCGTGGTTCACACCCATCA-3' (reverse) for GAPDH. The qRT-PCR was carried out in a 25 μl final volume containing: 3 μg cDNA sample, 500 nM primer pairs and 12.5 μl SYBR Green PCR Master Mix, and performed by an initial

denaturation at 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 sec and 60°C for 60 sec in an ABI PRISM 7000 system sequence detector (Applied Biosystems). Each RNA sample was measured in duplication. The specificity of amplified PCR products was evaluated by a comparative Ct method. The threshold cycle value (Ct value), which is inversely proportional to the initial template copy number, is calculated from cycle number at which the PCR product crosses a threshold of detection. The iNOS mRNA expression were normalized against GAPDH and gene expression changes induced by various treatments were determined by the $2^{-\Delta\Delta CT}$ method.

2.8. Fluorescent microscopy

For detection of global DNA methylation, MES-13 Cells were grown on coverslips and fixed in ice-cold methanol, followed by permeablization in 0.2% Triton X-100. Cells were incubated in 1 M HCl for 1 h at 37°C, blocking with 1% BSA and then incubated with anti-5-methylcytosine antibody (5-mC) (Abcam), followed by Alexa Fluor555 secondary Ab. Cells were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific Inc.) and mounted using p-phenylenediamine anti-fade mounting media. Visualization was performed using an Axioplan 2 Zeiss microscope and images were captured with a Carl Zeiss AxioCam system and analyzed by Axiovision Rel 4.8 software (Carl Zeiss). To detect subcellular distribution of NF-κB, incubation of MES-13 cells in 1 M HCl is omitted.

2.9. Flow cytometry

Intracellular staining of global DNA methylation in MES-13 Cells were detected by flow cytometry using 5-mC. Cells were harvested by trypsinization and fixed in 4% paraformaldehye, followed by permeabilization with 0.1% Triton-100/PBS. Staining was then conducted using 5-mC, followed by application of Alexa Fluor 555 secondary Ab. Cells were analyzed using the Beckman Coulter EpicsXL flow cytometry and results were assessed using Coulter EpicsXL-MCL flow cytometer system II software.

2.10. Statistical Analysis.

The values were expressed as the mean ±SD of at least three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by either the Bonferroni's or the Dunnett's method for multi-group comparison tests. A value of $p < 0.05$ was considered as statistical significantly.

3. RESULTs

3.1. LPS/ IFN-γ modulate the protein expression of enzymes central to the cellular methylation reactions in glomerular mesangial MES-13 cells.

Because the role of methylation in inflammation has been studied in many human diseases (18-21) and the enzymes participating in cellular methylation reactions are targets for drug discovery (9), the role of LPS/ IFN-γ on the enzymes associated with cellular methylation reactions in glomerular mesangial cells were firstly investigated. MES-13 cells were cultured for 24 hours with medium only or in the presence of of 1 μg/ml LPS and IFN-γ at various concentrations ranging from 10 ng/ml to 150 ng/ml. The protein levels of enzymes participating in cellular methylation reactions including MAT Ⅱα, SAHH, DNMT3b, PRMT1, 4, 5, 6 and SET7/9 were determined by Western blot analysis. As shown in Fig. 1 (A, B, C and D), LPS/ IFN-γ upregulated protein expression of MAT Ⅱα, DNMT3b, PRMT4 and PRMT 6 in MES-13 cells significantly when compared to untreated MES-13 cells. On the other hand, no significant change of protein expression of SAHH, SET7/9, PRMT 1 and PRMT5 was observed at the same concentrations of LPS/ IFN-γ applied (Fig. 1 B, D and E). These results indicate the impact of LPS/ IFN-γ on global cellular methylation reactions in MES-13 cells**.**

3.2. LPS/ IFN-γ trigger global DNA methylation and protein arginine/lysine methylation in glomerular mesangial MES-13 cells.

To further investigate the effect of LPS/ IFN-γ on cellular methylation reactions in glomerular mesangial cells, global DNA methylation was analyzed. MES-13 cells were cultured for 24 hours with medium only or in the presence of 1 μg/ml LPS and 10 ng/ml IFN-γ. LPS significantly increased global DNA methylation in MES-13 cells as detecting by fluorescent microscopy shown in Fig. 2 (A) and flow cytometry in Fig. 2 (B). In Fig. 2 (A), nuclear staining of global DNA methylation in MES-13 cells was visualized using anti-5'methylcytosine antibodies (5-mC) (middle panels, green signal). The nuclei were stained with DAPI for DNA (left panels, blue signal). The right panel is a merge of the two panels above. Augmentation of global DNA methylation triggered by LPS/ IFN-γ was attenuated when AdOx was applied. Analysis of 5'methylcytosine DNA methylation in MES-13 cells was also conducted by flow cytometry as shown in Fig. 2 (B). Fluorescence intensity of 5'methylcytosine as illustrated by dot plots showed significant increase after LPS/ IFN-γ treatment in MES-13 cells, while treatment with AdOx (10 μM, 20 μM) showed reduction. These results indicate global DNA methylation was increased during inflammation induced

by LPS/ IFN-γ. To investigate the effect of inflammation on protein arginine/lysine methylation, MES-13 cells were cultured for 24 hours with medium only or in the presence of 1 μg/ml LPS and 10 ng/ml IFN-γ., followed by detection with Western blot analysis using anti-dimethyl-arginine, asymmetric antibody (ASYM24), anti-dimethyl-arginine, symmetric antibody (SYM10) and anti- methylated lysine antibody. Alterations in signal for ASYM24 and anti-methylated lysine antibody were observed when MES-13 cells were exposed to 1 μg/ml LPS and 10 ng/ml IFN-γ. Conversely, not much change in signal was observed when SYM10 antibody was applied as shown in Fig. 3 (A, B, C).

3.3. AdOx, a broad-spectrum methyltransferase inhibitor, inhibited LPS/IFNγ-stimulated NO production, iNOS m RNA and protein expression in glomerular mesangial MES-13 cells.

The cell viability of MES-13 cells following exposure to different drugs was evaluated by MTT assay. A 10% decline in cell viability of MES-13 cells was observed after treatment with 1 μg/ml LPS and 10 ng/ml IFN-γ for 24 hr. Co-incubation of 1 μM, 5 μM and 10 μM AdOx with LPS/IFN-γ did not cause a significant decrease in cell viability; more than 90% of MES-13 cells were viable compared to control cells stimulated with LPS/IFN-γ alone (Fig. 4A). On the other hand, exposure of LPS/IFN-γ-stimulated cells to 20 μM AdOx caused a further 25 % reduction in cell viability.

MES-13 cells that were seeded in 6-well plate (1.2×10^5 cells/well) were treated with various concentrations 1 μM, 5 μM and 10 μM of AdOx and then stimulated for 24 hr with LPS/IFN-γ. The cell medium was collected and nitrite concentration within the medium was determined by Griess assay. The nitrite concentration (micromolar) of un-stimulated cells was 1.44 ± 0.30 μM. The nitrite content of the cells that were treated with only LPS, IFN-γ or AdOx had a basal value similar to those of un-stimulated cells (data not shown). Exposure of MES-13 cells with 1 μg/ml LPS+ 10 ng/ml IFN-γ for 24 hr significantly increased the release of nitrite to a concentration of 20.63 ± 1.8 μM. Incubation of 1 μM AdOx in LPS/IFN-γ–induced MES-13 cells did not have any effect on nitrite release (16.3 ± 1.85μM). However, 5 μM ,10 μM and 20 μM AdOx substantially reduced nitrite production in LPS/IFN-γ–induced MES-13 in a dose-dependent manner (10.85 ± 0.74 μM, 6.85 ± 0.83 μM, 2.51±0.85 μM, respectively) (Fig. 4B). Co-incubation of LPS/IFN-γ–induced MES-13 cells with 1 μM, 5 μM and 10 μM AdOx did not further reduce cell viability but significantly reduced nitrite level by 20 %, 48% and 67%

below that of the LPS/IFN-γ–induced cells, proportionately. Additionally, although the cell viability of MES-13 cells was reduced by 25 % upon treatment of 20 μM AdOx, this concentration of AdOx reduced the nitrite level by over 88 % (Fig. 4A and 4B). These results indicated that AdOx, exhibited inhibitory effect on LPS/IFN-γ–induced NO production without affecting cell viability of MES-13 cells. .

The iNOS protein expression in the presence of various concentrations of AdOx was first measured in MES-13 cells. LPS, IFN-γ, or AdOx alone did not trigger iNOS protein expression, which was considerable in cultures that were treated with a combination of 1 μg/ml LPS and 10 ng/ml IFN-γ. As presented in Fig. 4C, the relative amounts of iNOS proteins were quantified as a percentage of the amount of LPS/IFN-γ–induced cells. Adding exogenous AdOx at various concentrations significantly reduced the LPS/IFN-γ–induced iNOS protein expression in a dose-dependent manner. No significant change in the amount of iNOS protein amount was observed upon co-supplementing with 1 μM and 5 μM, of AdOx whereas co-incubation with 5 μM, and 10 μM AdOx significantly reduced iNOS protein expression to 63 %, 37%, respectively. These results suggested that the inhibitory effect of AdOx on NO production in MES-13 cells was accompanied by reduced expression of iNOS protein.

To examine whether the action of AdOx on LPS/IFN-γ-stimulated NO production was correlated with the iNOS gene expression at the transcriptional level, a real-time RT-PCR were performed to determine the iNOS mRNA level in MES-13 cells. MES-13 cells were treated with LPS/IFN-γ in the absence or presence of 10 μM and 20μM AdOx . As revealed by a real-time RT-PCR RT-PCR in Fig. 4, signals of iNOS mRNA diminished with 10 μM and 20μM AdOx. The presence of 10 μM and 20μM AdOx inhibited levels of LPS/IFN-γ-stimulated iNOS mRNA to 78 % and 44% of the control level, respectively (Fig. 4D). These data indicate that the inhibitory effects of AdOx on LPS/IFN-γ-stimulated NO production occurred by transcriptional regulation of iNOS gene expression.

The expression of iNOS is also regulated by post-transcriptional processes. At the post-transcriptional level, an important mechanism of regulation is the modulation of iNOS mRNA stability that is controlled by several RNA binding proteins (RNA-BPs), such as human antigen R (HuR) protein. To test the hypothesis that AdOx could affect iNOS mRNA stability, the levels of iNOS mRNA were quantified in MES-13 cells after different times in the presence of LPS/IFN-γ alone or with10

μM AdOx with addition of actinomycin D after 6 hr incubation with LPS/IFN-γ. The half-life of iNOS mRNA after LPS/IFN-γ treatment showed no significant change by the presence of AdOx (data not shown). Moreover, no change in nucleocytoplasmic HuR shuttling was observed after treatment of AdOx in LPS/IFN-γ stimulated MES-13 cells as detected by immunofluorescent microscopy (data not shown).

3.4 AdOx blocked NFκB translocation but showing no effect on Akt, ERK, STAT 1α signaling pathway.

Both NF-κB and STAT-1α were reported to modulate the iNOS gene expression in cell- and species-specific manners. To further explore the mechanism underlying the inhibitory effect of AdOx on iNOS gene transcription, levels of NF-κB p65 in nuclear extracts were determined by Western blotting (Fig.5A) and immunofluorescent microscopy (Fig. 5B). The nuclear translocation of p65 was observed after the addition of LPS/IFN-γ to MES-13 cells. In addition, the presence of 10 μM AdOx significantly blocked the nuclear translocation of p65 triggered by LPS/IFN-γ. Conversely, as shown in Fig. 5C, AdOx showed no effect on LPS/IFN-γ-modulated STAT-1α phosphorylation and IRF-1, one of the target genes of phosphorylated STAT-1α, which binds to IREs located in the 5′-flanking region of the murine iNOS gene. In addition, AdOx showed no effect on Akt, ERK signaling pathway Fig. 5D.

DISCUSSION

Epigenetic factors such as infection, hypoxia, stress, nutrition alter the global **e**pigenetic state of an organism known as epigenome. Epigenome is as critical as the genome to control early fetal development and increased disease susceptibility in later life (22). Among different epigenetic events, **c**hemical modification of DNA and protein by methylation exert major impact on gene expression DNA methylation usually occurs on cytosines, and is related to gene silencing. Protein methylations occur on a number of amino acid residues including lysine, arginine which were located on **c**hromatin-associated histone proteins or non-histone proteins(5)**.**Both methylation reactions have emerged as important regulatory mechanisms for epigenetic regulation of gene expression(1).Therefore, the protein expression of enzymes central to the cellular methylation reactions in glomerular mesangial MES-13 cells was investigated. Expression of MATIIα , DNMT3b, PRMT4 and PRMT6 were upregulated in response to LPS/IFN-γ whereas SAHH, PKMT (SET7/9), PRMT1 and PRMT5 were unaffected as shown in Fig.1 (A~E). Global DNA methylation was increased in MES-13 cells exposed to LPS/IFN-γ, as shown in Fig. 2 (A, B). Global protein methylation were alterations in signal for ASYM24 and anti-methylated lysine antibody were observed when MES-13 cells were exposed to LPS/IFN-γ, while not much change in signal was observed when SYM10 antibody was applied as shown in Fig. 3 (A, B). ASYM24 recognizes proteins containing arginines that are asymmetrically dimethylated while SYM10 recognizes proteins that contain multiple symmetrically dimethylated arginines. Thus, LPS/IFN-γ modulates global DNA and protein lysine/arginine methylation by upregulating enzymes associated with cellular methylation reactions. Methylation of histone proteins act as an epigenetic regulator in gene expression at chromatin (5,23). More recently, methylation on non- histone proteins also get attention since this modification may act as another regulator in gene expression.

Among different MTases, protein arginine methyltransferases (PRMTs), s subset of the class I MTases, catalyze methylation of nitrogen of specific arginine residues in proteins. Dyregulation of PRMTs function has been implicated in the pathogenesis of a number of diseases, such as cancer, cardiovascular diseases, viral pathogenesis, multiple sclerosis and spinal muscular atophy (7,23).PRMTs have been recognized as drug targets and specific PRMT inhibitors may be useful in treating tumors or other diseases (7).There are 11 members of the mammalian PRMT family which catalyze the transfer a methyl group from SAM to a arginine. Arginine can be methylated on the terminal guanidine nitrogen in three separate pathways and after proteolytic

degradation of proteins containing methylated arginine residues, mono-methylarginine (MMA), symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA) were generated. Elevated ADMA concentrations have been detected in the plasma and urine of patients with end-stage renal disease.(24). MMA and ADMA, but not SDMA, have been identified as potent endogenous competitive inhibitors of nitric oxide synthases (NOSs)(12). ADMA has been identified as competitive inhibitors of NOSs (25,26). and its impaired effect on endothelium-derived NO may cause cardiovascular complications in hemodialysis patients. ADMA has been recognized as a target for pharmacotherapy (27). Since PRMTs are the major enzymes that generate ADMA, modulating enzymatic activities of PRMTs may provide alterative choice in preventing or treating cardiovascular or renal disease (28). Dysregulation of PRMT activity has been associated with several pathophysiological conditions such as chronic renal failure (11,25,29,30). Our data showed LPS/IFN-γ upregulate PRMT4 and PRMT6 and this indicate dysregulation of PRMT activity during renal inflammatory status.

Excessive production of NO by iNOS, derived from infiltrating macrophages or resident glomerular cells, is associated with inflammatory injury, such as glomerulonephritis, which ultimately lead to and end stage reanl failure (14). The role of DNA methylation in iNOS induction has been reported in mesangial cells. Cytosines methylation of the iNOS promoter inhibits iNOS expression through blocking NF-κB p50 binding to this element (16). Potential methylation sites in iNOS proteins have been predicted by HPLC/MS/MS analysis (17). Whether methylation is involved in iNOS –stimulated NO production through targeting non-histone transcription factors, such as NF-κB and STAT-1α, have never been elucidated. Because both histone and non-histone proteins are substrates of PRMTs, the center hypothesis to be tested in the present study is proinflammatory factors such as LPS and IFN-γ may upregulate PRMTs which in turn targeting non-histone transcription factors, such as NF-κB and STAT-1α, to modulate the expression of iNOS expression. Therefore, the role of protein arginine methylation in moduating iNOS-stimulated NO production was investigated using MES-13 cells. Adenosine dialdehyde (AdOX), a broad-spectrum methyltransferase inhibitor, was use to elucidate whether protein methylation inhibition exert pharmacological effects on NO overproduction in glomerulonephritis. .

AdOX, which is formed by periodate oxidation of adenosine (Ado), was shown to be a potent inhibitor of S-adenosylhomocysteine hydrolase (SAH hydrolase; EC 3.3.1.1). SAH hydrolase catalyzes the metabolism of S-adenosylhomocysteine (SAH) to Ado and homocysteine. Inhibition of SAH hydrolase by AdOX results in the

accumulation of SAH in the cytoplasm, leading to inhibition of cellular methylation reactions via a feedback mechanism. Accumulations in SAH levels inhibited conversion of S-adenosylmethionine (SAM) into SAH and produced a subsequent inhibition of SAM-dependent cellular methylation reactions. Therefore, AdOX decreased the methylation of the methyl-accepting proteins indirectly and was recognized as a broad-spectrum methyltransferase inhibitor (31,32).

In the present study, co-incubation of AdOx with LPS/IFN-γ significantly suppressed LPS/IFNγ-stimulated NO production, iNOS m RNA and protein expression in glomerular mesangial MES-13 cells (Fig.4 A~D). These data indicate AdOX modulates NO synthesis by regulating iNOS gene expression at transcriptional level. Because post- transcriptional regulation of iNOS expression is also important in determining NO synthesis, the effect of AdOx on iNOS mRNA stability has been measured. The half-life of iNOS mRNA after LPS/IFN-γ treatment showed no significant change by the presence of AdOx (data not shown). Therefore, AdOx inhibited LPS/IFNγ-stimulated NO production mainly through transcriptional regulation of iNOS expression in MES-13 cells.

Among different PRMTs, PRMT1 (33), PRMT5 (35) and PRMT6 (36) modulate NF-κB, a chief molecule in inflammation, through diverse mechanisms. These PRMTs modulate NF-κB-dependent gene expression either through forming a complex with transcription factors or methylate NF-κB directly. PRMT1 enhances NF-κB-mediated gene transcription in concert with p300/CREB binding protein, poly (ADP-ribose) polymerase 1 and PRMT4 (CARM1) (33). PRMT4 (CARM1) is a promoter-specific regulator which activates NF-κB-dependent gene expression through forming a complex with p300/CBP binding protein and the p160 family of steroid receptor coactivators (34). PRMT1 and PRMT5 methylate NF-κB directly. Methylation of Rel A at arginine 30 (R30) by PRMT1 represses NF-κB target genes (37) while methylation of Rel A at the same residues by PRMT5 activates NF-κB target genes (35). Therefore, multiple PRMTs are regulators of NF-κB, despite through multiple and controversial mechanisms.

Our data showed LPS/IFN-γ upregulate PRMT4 and PRMT6 (Fig.1 D) and interaction of PRMT with NF-κB may be critical for expression of NF-κB target gene, such as iNOS. As demonstrated in Fig 5 (A and B), the inhibitory effect of AdOx on NF-κB p65 nuclear translocation were observed by Western blotting (Fig.5A) and immunofluorescent microscopy (Fig. 5B). Therefore, AdOx inhibited LPS/IFN-γ-stimulated NO production in MES-13 cells was through blocking nuclear translocation of p65 triggered by LPS/IFN-γ.

Although regulation of NO synthesis by iNOS is under precise control predominately at transcriptional level, (38), post- transcriptional regulation of iNOS expression is also important in determining NO synthesis. Several AU-rich nucleotide sequences located on the 3'-untranslated region (3'-UTR) of iNOS mRNA marked them for rapid breakdown by exosome. iNOS mRNA containing AU-rich regions are highly unstable and several RNA-binding proteins modulate the stability of this mRNA. Of the numerous RNA-binding proteins known to alter mRNA stability, the embryonic lethal abnormal vision (ELAV)-like protein HuR is known to increase the stability of several inducible mRNAs (39). HuR shuttles from nuclear to cytoplasmic compartments, binds to the AU-rich region on the 3'-UTR of iNOS mRNA and increase stability of iNOS gene (40). Whether methylation play a role in HuR shuttling and involved in iNOS mRNA stability has also been investigated. AdOx inhibited iNOS mRNA expression but showed no effect on iNOS mRNA stability and HuR translocation in MES-13 cells. Therefore, the role of methylation in iNOS expression is not through its effect on HuR protein.

In summary, we demonstrated down-regulation of *iNOS* gene expression by AdOx might be caused by its effect on NF-κB protein methylation and subsequent alternation of the nuclear and cytoplasmic shuttling of NF-κB. Our data suggest protein methylation may be implicated in inflammatory disorders though controlling the synthesis of pro-inflammatory mediator, NO. Therapeutic application of methylation inhibitors might be potential in treatment of chronic inflammatory diseases, such as glomerulonephritis.

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FIGURE LEGEND

Fig.1 Lipopolysaccharides upregulated the expression of enzymes associated with cellular methylation reactions in glomerular mesangial cells.
MES-13 cells were cultured for 24 hours with medium only (lane 1: control) or in the presence of of 1 μg/ml LPS and IFN-γ at various concentrations ranging from 10 ng/ml to 150 ng/ml (lane 2 to lane 6). The protein levels of enzymes associated with cellular methylation reactions including MAT Ⅱα, SAHH, DNMT3b, PRMT1, 4, 5, 6 and SET7/9 were determined by Western blot analysis as shown in (A) to (E), respectively. GAPDH was used as an internal control. Relative protein levels were quantified by scanning densitometry and expressed as a percentage of the maximal band intensity of the protein from control cultures. Data represent the mean ± SD of target proteins normalized with GAPDH from at least three separate experiments. Asterisks indicate a significant difference from control (*$p < 0.05$, **$p < 0.01$, *** $p < 0.0001$)

Fig. 2 Lipopolysaccharides increased global DNA methylation in glomerular mesangial cells.
Global DNA methylation in MES-13 cells was detected by fluorescent microscopy and flow cytometry as shown in (A) and (B), respectively. MES-13 cells were treated with medium only (control), 1 μg/ml LPS and 10 ng/ml IFN-γ. alone or in the presence of either AdOx (10 μM and 20 μM) or 5-Aza-2'-dc (10 μM) for 24 hours. In (A), nuclear staining of global DNA methylation in MES-13 cells was visualized using anti-5'methylcytosine antibodies (middle panels, green signal). The nuclei were stained with DAPI for DNA (left panels, blue signal). The right panel is a merge of the two panels above. Analysis of 5-methylcytosine DNA methylation in MES-13 cells was also conducted by flow cytometry as shown in (B). Fluorescence intensity of 5'methylcytosine as illustrated by dot plots showed significant increase after LPS/ IFN-γ treatment in MES-13 cells, while treatment with AdOx or 5-Aza-2'-dc showed reduction. Histogram displayed quantitative analysis of the fluorescence intensity of different treatments. The results were expressed as the mean ± SD for three experiments and asterisks indicated a significant difference from treatment with 1 μg/ml LPS and 10 ng/ml IFN-γ (***$p < 0.0001$ versus LPS-stimulated MES-13 cells).

Fig. 3 Modulation of protein methylation by lipopolysaccharides was inhibited by methyltransferases inhibitors in glomerular mesangial cells. The effect of LPS/ IFN-γ on protein arginine methylation in MES-13 cells was analyzed by Western blot using ASYM24, SYM10 antibody (A) while protein lysine methlyation was detected

by anti-methylated lysine antibody (B). MES-13 cells were treated with medium only (control), 1 μg/ml LPS and 10 ng/ml IFN-γ alone.

Fig. 4 AdOx inhibited LPS/IFNγ-stimulated NO production, iNOS m RNA and protein expression in glomerular mesangial MES-13 cells. The cell viability of MES-13 cells following exposure to 1 μg/ml LPS and 10 ng/ml IFN-γ and various concentrations of AdOx (1, 5, 10, 20 μM) were evaluated by MTT assay as shown in (A). The nitrite concentration (micromolar) of each treatment was collected within the medium and determined by Griess assay as shown in (B).The iNOS protein expression in MES-13 cells following exposure to the same treatment as (A) and (B) was analyzed by Western blot as shown in (C). The iNOS mRNA level in MES-13 cells was determined by a real-time RT-PCR to detect the iNOS mRNA level in MES-13 cells with treatment of LPS/IFN-γ in the absence or presence of 10 μM and 20μM AdOx as shown in (D). Relative protein levels were quantified by scanning densitometry and expressed as a percentage of the maximal band intensity of the protein from control cultures. Data represent the mean ± SD of target proteins normalized with GAPDH from at least three separate experiments. Asterisks indicate a significant difference from control ($*p < 0.05$, $**p < 0.01$, $***p < 0.0001$).

Fig. 5 AdOx blocked NFκB translocation but showing no effect on Akt, ERK, STAT 1α signaling pathway. To further explore the mechanism underlying the inhibitory effect of **AdOx** on iNOS gene transcription, levels of NF-κB p65 in nuclear extracts were determined by Western blotting (Fig.5A) and immunofluorescent microscopy (Fig. 5B). AdOx showed no effect on LPS/IFN-γ-modulated STAT-1α phosphorylation and IRF-1 as shown in Fig. 5C. AdOx showed no effect on Akt, ERK signaling pathway Fig. 5D.

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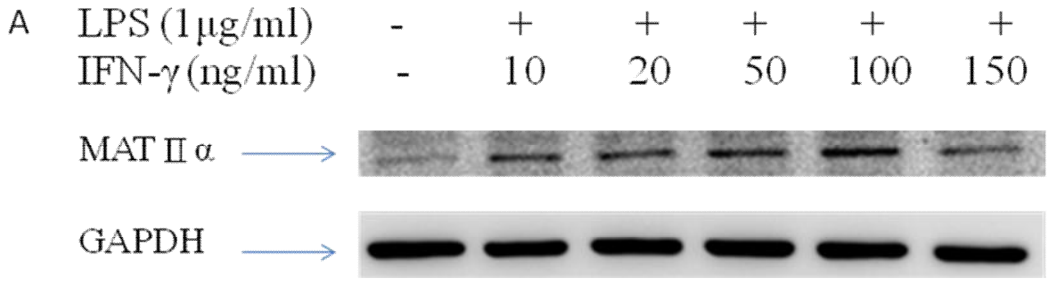
Fig. 1(A)



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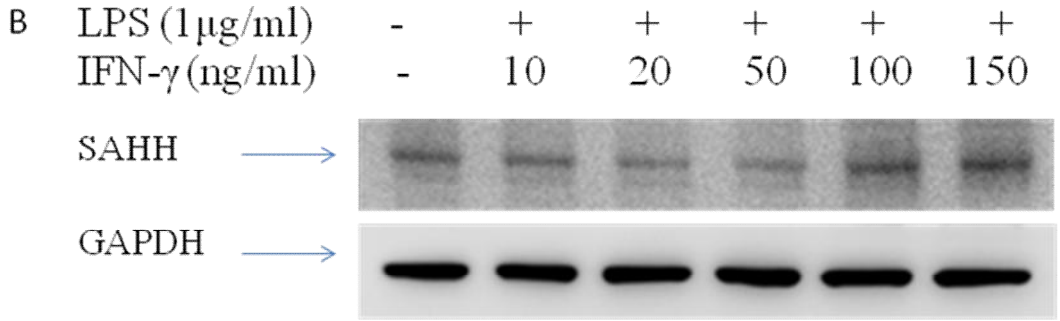

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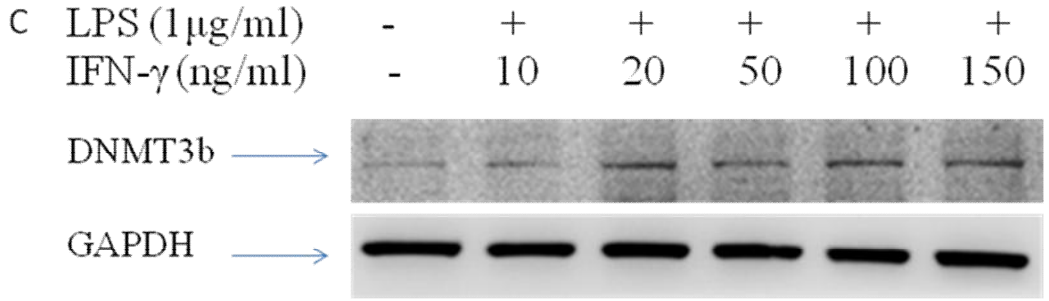

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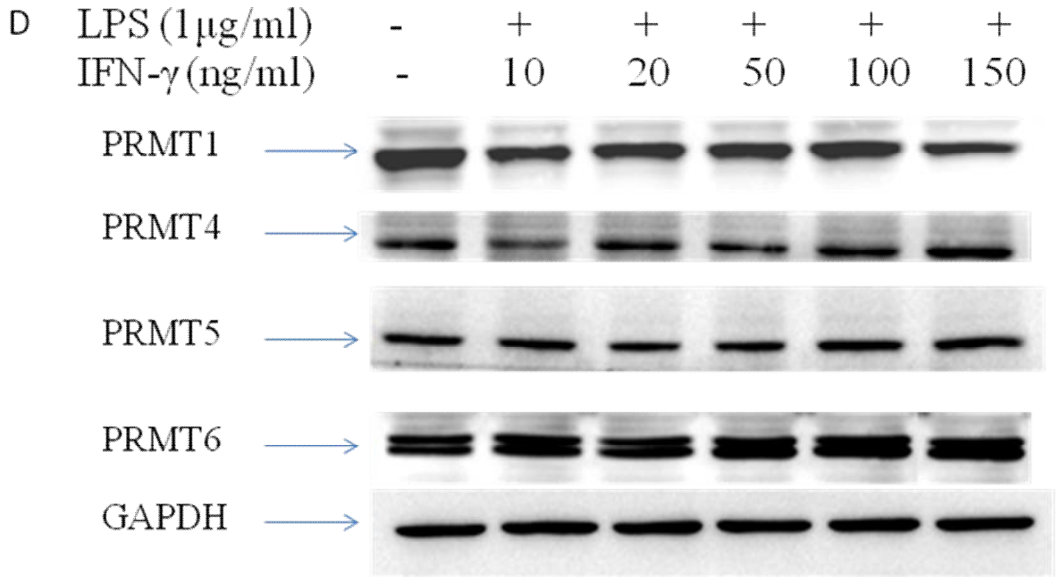


Fig. 1(E)

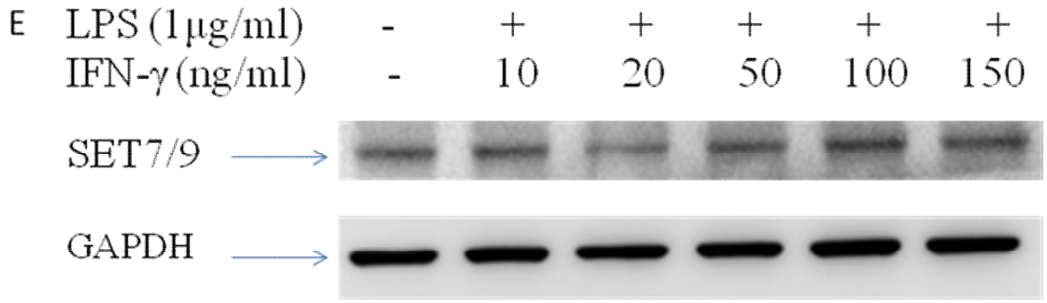

E
LPS (1μg/ml) - + + + + +
IFN-γ (ng/ml) - 10 20 50 100 150
SET7/9
GAPDH

Fig. 2 (A)

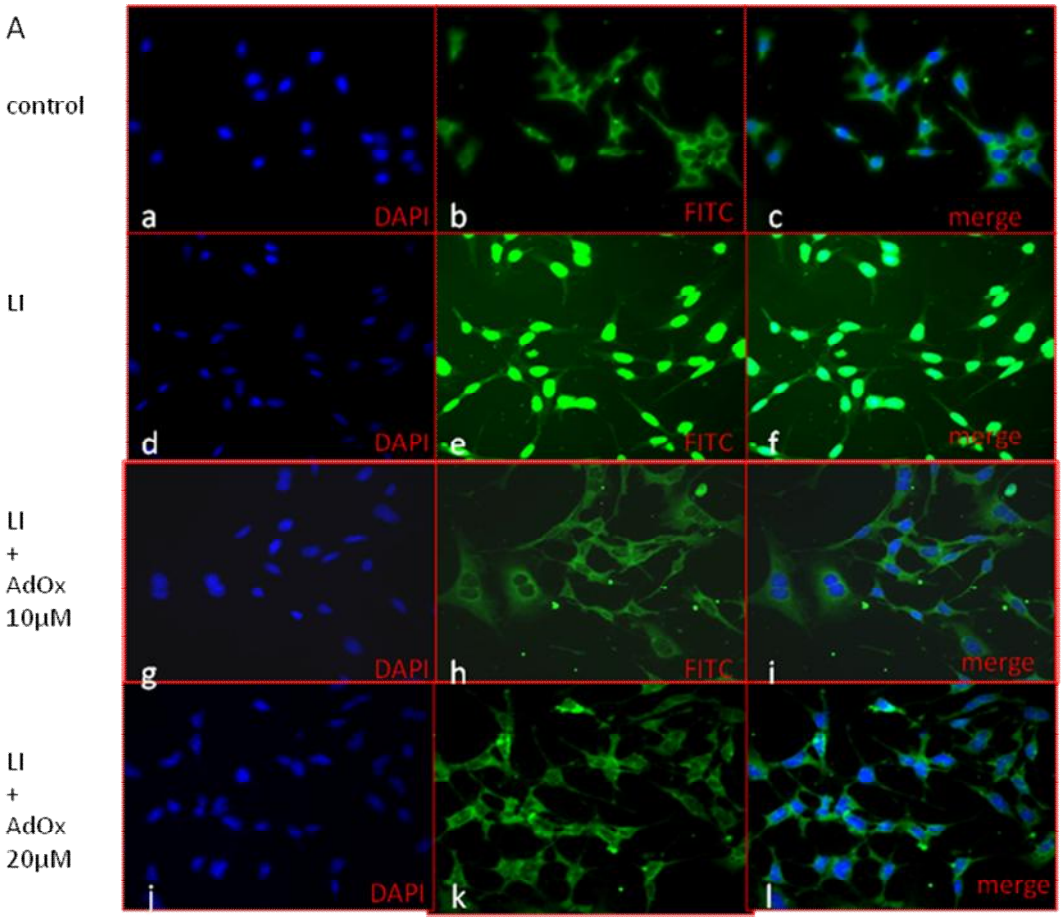
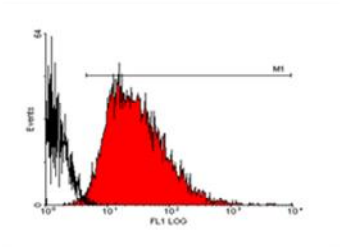
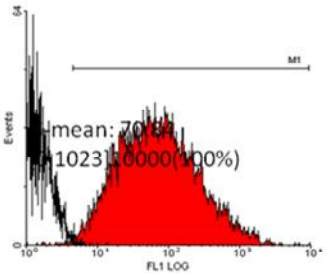

A
control
LI
LI + AdOx 10μM
LI + AdOx 20μM
a DAPI
b FITC
c merge
d DAPI
e FITC
f merge
g DAPI
h FITC
i merge
j DAPI
k
l merge

Fig. 2 (B)



G-mean: 29.21
[0,1023]10000(100%)

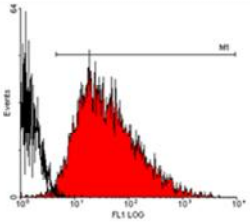


5-methylcytosine wt

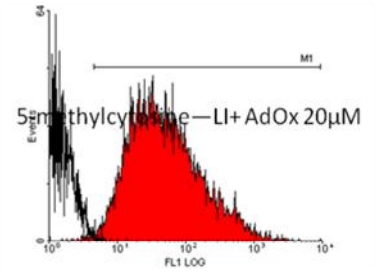
G-mean: 41.01
[0,1023]10000(100%)

5-methylcytosine—LI

G-mean:49.39
[0,1023]10000(100%)



5-methylcytosine—LI+ AdOx 10μM



5-methylcytosine—LI+ AdOx 20μM

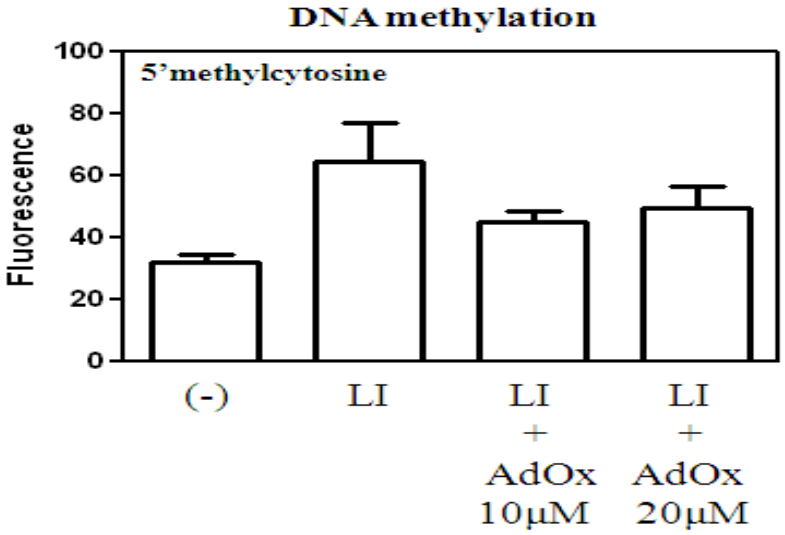


Fig. 3 (A)

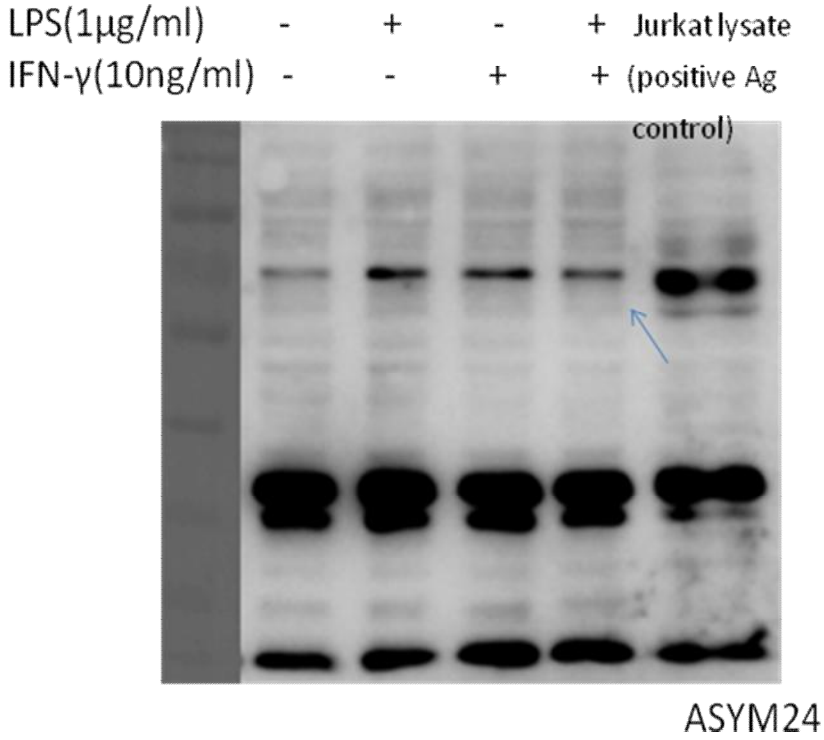
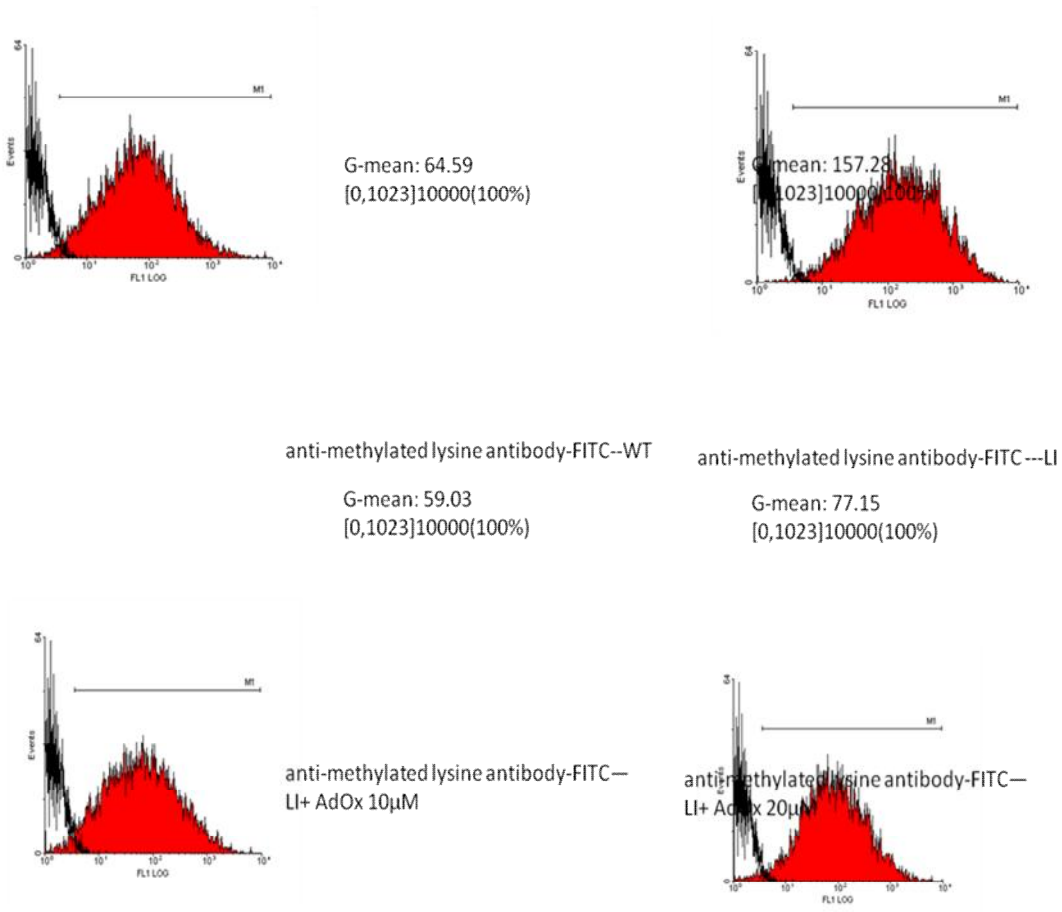

LPS(1μg/ml) - + - + Jurkat lysate
IFN-γ(10ng/ml) - - + + (positive Ag control)
ASYM24

Fig. 3(B)


G-mean: 64.59
[0,1023]10000(100%)
anti-methylated lysine antibody-FITC--WT
G-mean: 59.03
[0,1023]10000(100%)
anti-methylated lysine antibody-FITC---LI
G-mean: 77.15
[0,1023]10000(100%)
anti-methylated lysine antibody-FITC—LI+ AdOx 10μM

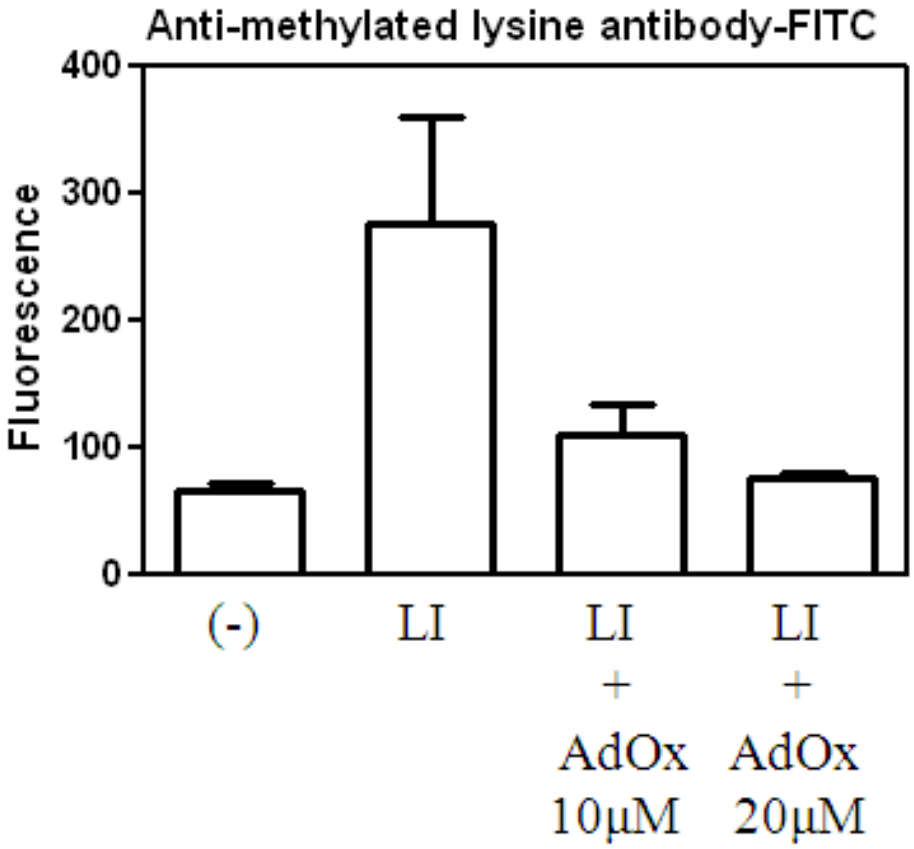

Anti-methylated lysine antibody-FITC
Fluorescence
400
300
200
100
0
(-)
LI
LI + AdOx 10μM
LI + AdOx 20μM

Fig.4 (A)

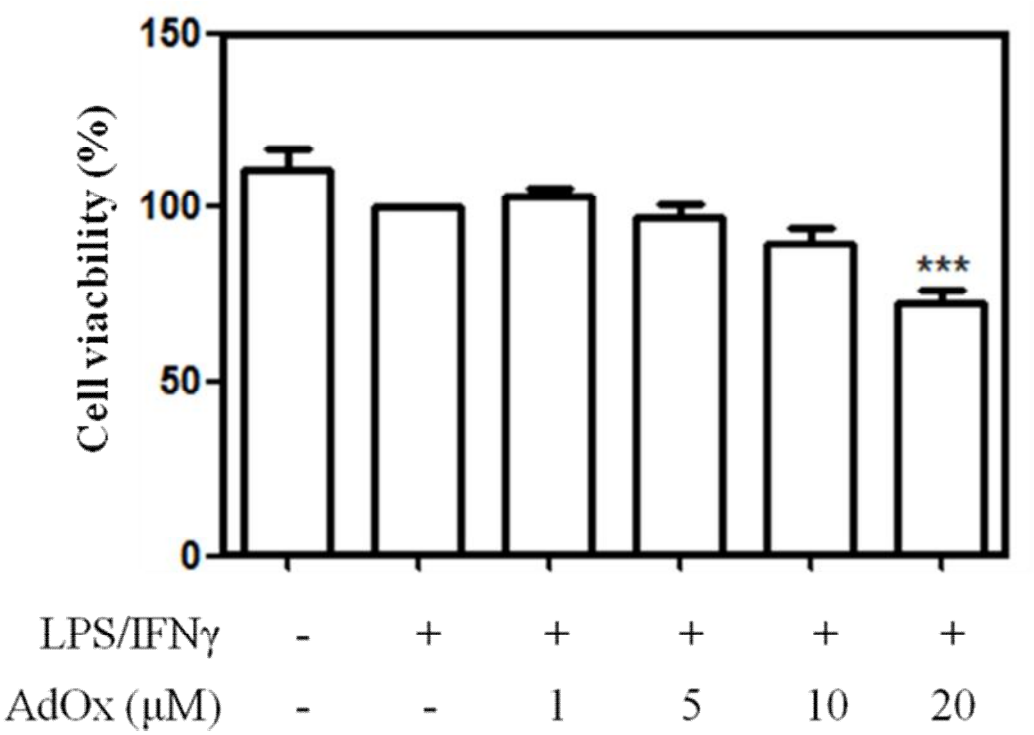

Cell viacbility (%)
150
100
50
0
***
LPS/IFNγ - + + + + +
AdOx (μM) - - 1 5 10 20

Fig.4 (B)

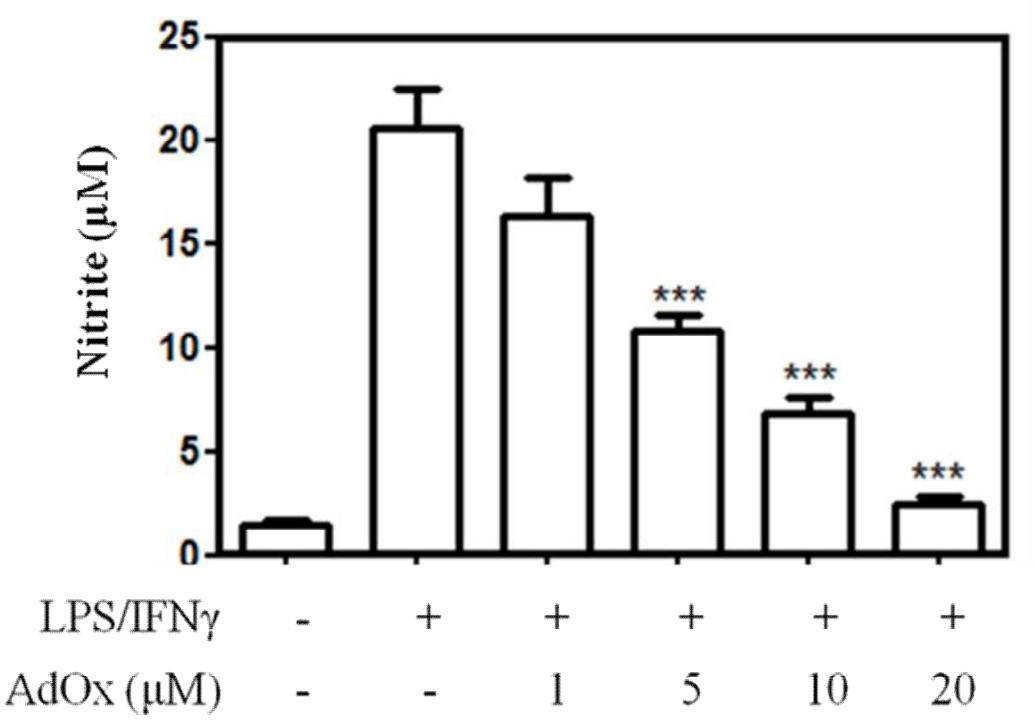

Nitrite (μM)
25
20
15
10
5
0
***
***
***
LPS/IFNγ - + + + + +
AdOx (μM) - - 1 5 10 20

Fig. 4 (C)

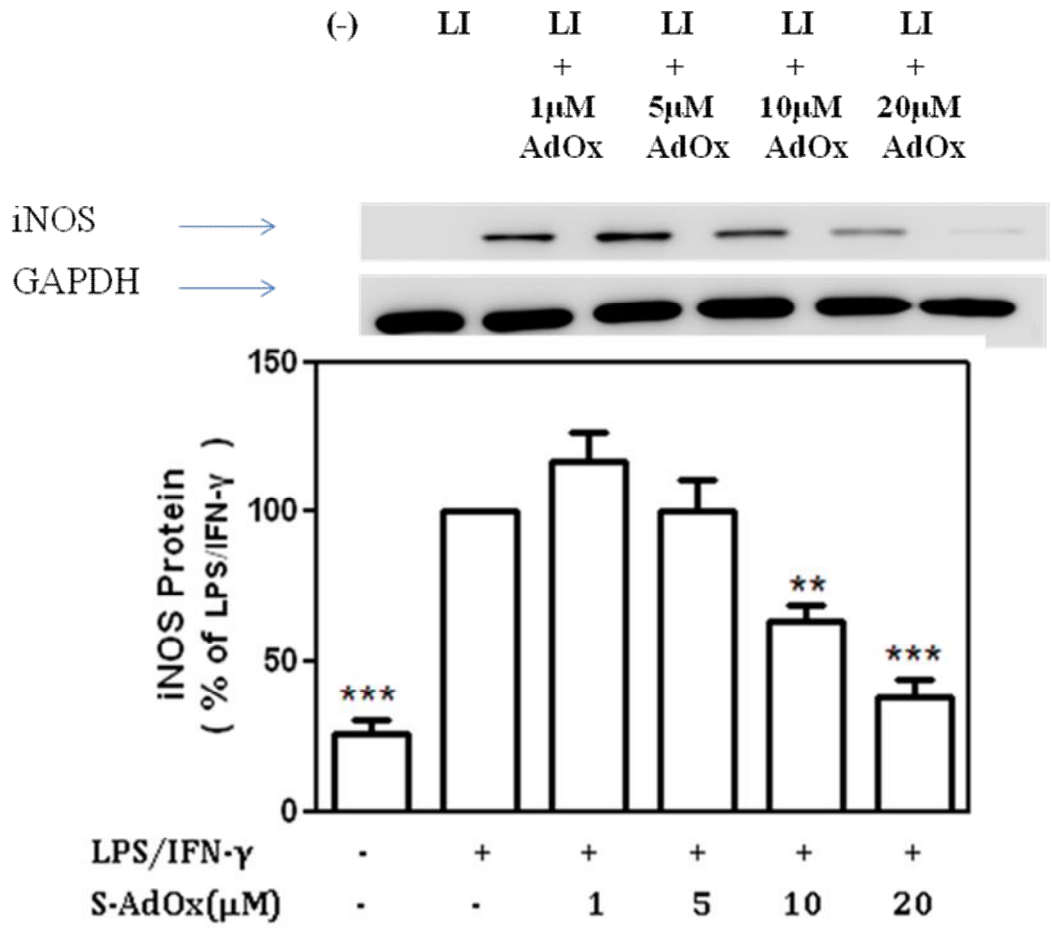

(-)
LI
LI + 1μM AdOx
LI + 5μM AdOx
LI + 10μM AdOx
LI + 20μM AdOx
iNOS
GAPDH
iNOS Protein (% of LPS/IFN-γ)
150
100
50
0
***
**
***
LPS/IFN-γ - + + + + +
S-AdOx(μM) - - 1 5 10 20

Fig. 4 (D)

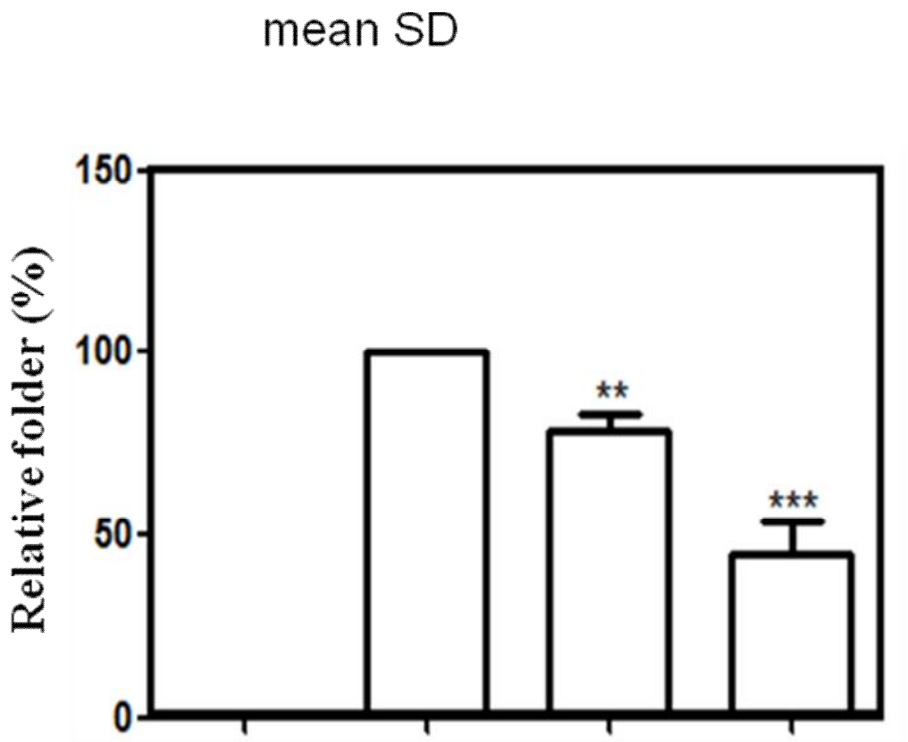

mean SD
Relative folder (%)
150
100
50
0
**
***
LPS/IFNγ - + + +
AdOx (μM) - - 10 20

Fig. 5(A)

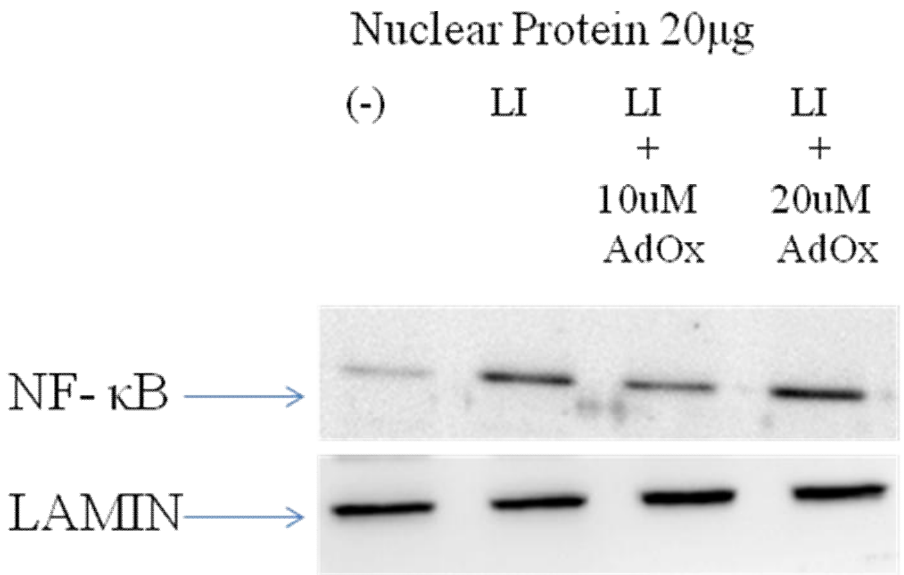

Nuclear Protein 20μg
(-)
LI
LI + 10uM AdOx
LI + 20uM AdOx
NF-κB
LAMIN

Fig. 5(B)

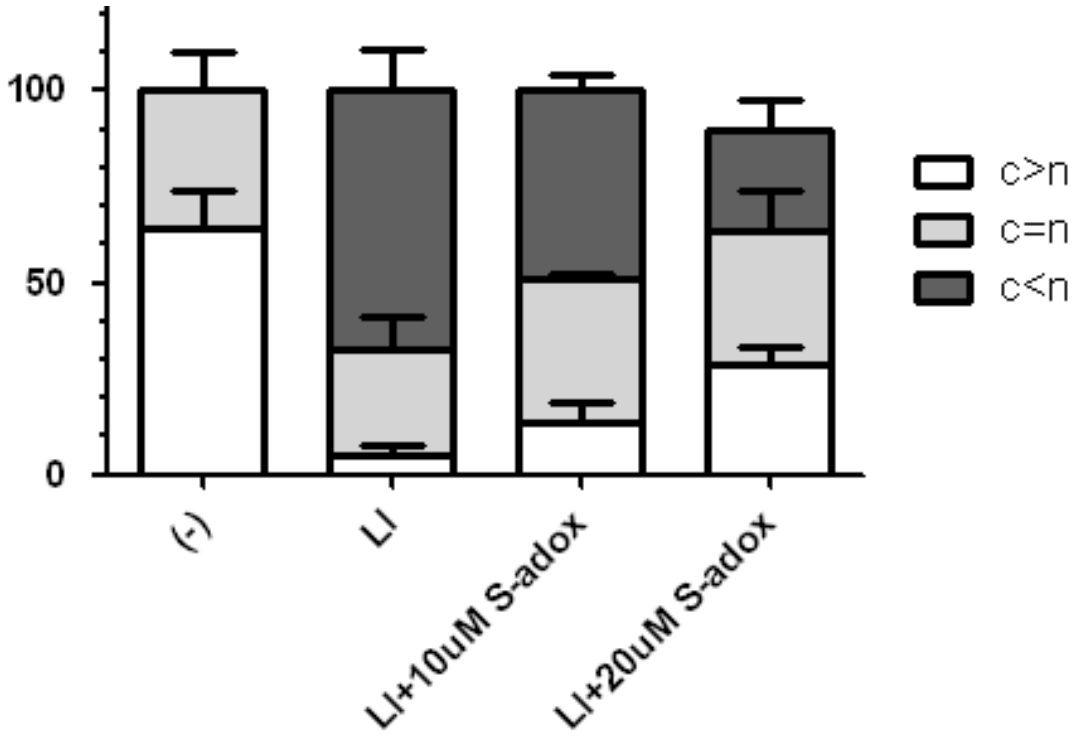

100
50
0
(-)
LI
LI+10uM S-adox
LI+20uM S-adox
c>n
c=n
c<n

Fig. 5 (C)

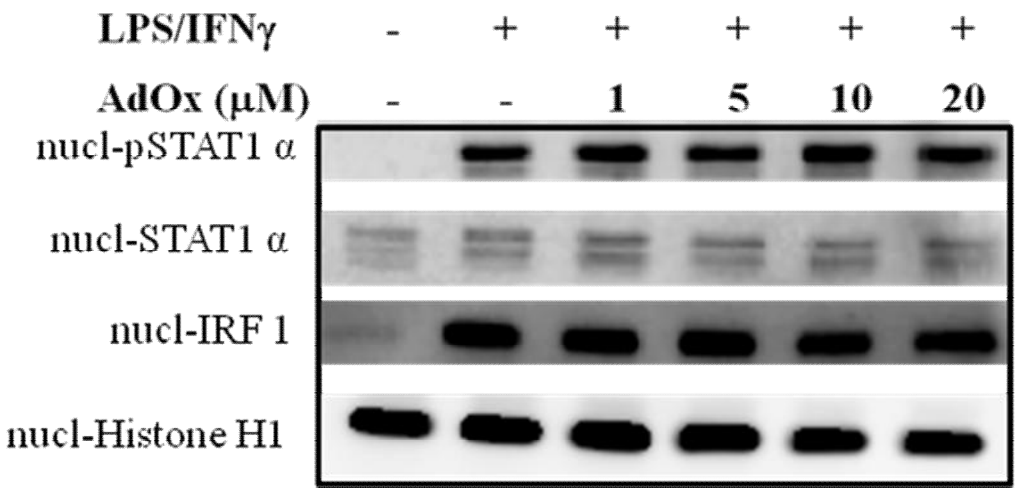
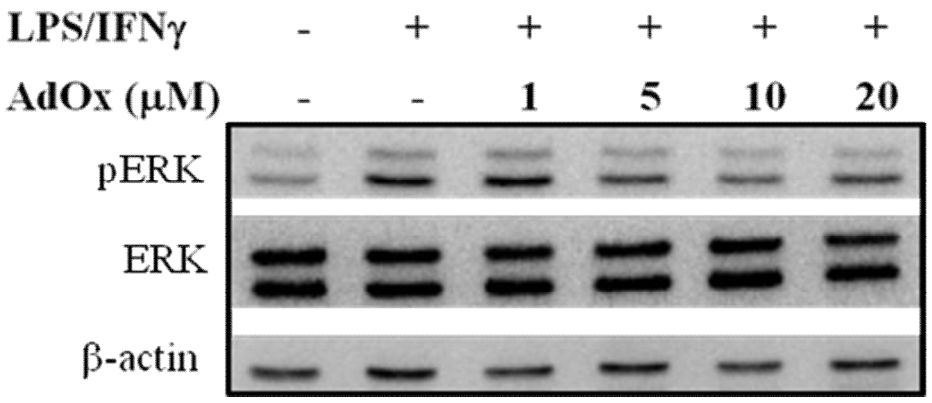

LPS/IFNγ - + + + + +
AdOx (μM) - - 1 5 10 20
nucl-pSTAT1 α
nucl-STAT1 α
nucl-IRF 1
nucl-Histone H1

Fig. 5(D)



科技部補助計畫衍生研發成果推廣資料表

日期:2016/10/30

科技部補助計畫	計畫名稱: 黃芩素抑制一氧化氮生成之抗發炎作用於腎絲球腎炎之分子機轉探討及表觀遺傳學研究
	計畫主持人: 林庭慧
	計畫編號: 104-2320-B-040-007- 學門領域: 營養保健

無研發成果推廣資料

104年度專題研究計畫成果彙整表

計畫主持人：林庭慧	**計畫編號：**104-2320-B-040-007-
計畫名稱：黃芩素抑制一氧化氮生成之抗發炎作用於腎絲球腎炎之分子機轉探討及表觀遺傳學研究	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			1	篇	
		研討會論文			1		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		

	技術移轉	件數	0	件	
		收入	0	千元	
參與計畫人力	本國籍	大專生	0	人次	
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）					

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   □達成目標
   ■未達成目標（請說明，以100字為限）
      □實驗失敗
      □因故實驗中斷
      ■其他原因
   說明：
   原本研究計畫因實驗結果不如預期，因此修改實驗目標。本份成果報告之研究內容與原計畫仍有一定程度相關，即由原本探討論黃芩素抑制一氧化氮生成之表觀遺傳學研究改為甲基化對誘導型一氧化氮合成之調控機轉研究

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：□已發表 □未發表之文稿 ■撰寫中 □無
   專利：□已獲得 □申請中 ■無
   技轉：□已技轉 □洽談中 ■無
   其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   我們的研究結果指出，在腎絲球細胞，黃芩素可減弱LPS / IFN -γ誘導內源性一氧化氮（Nitric Oxide, NO）的產生乃藉由減弱誘導型一氧化氮合酶（iNOS）之表現，其分子機轉乃因黃芩素可降低一個調節免疫反應主要的轉錄因子，NF-κB，之激活。進一步探討黃芩素是否會經由影響表觀遺傳學因子如DNA甲基化來調控細胞內發炎反應因子。結果顯示，黃芩素可影響整體DNA甲基化，但對特定基因起始子甲基化之作用並不明顯。另一方面，黃芩素對腎絲球細胞整體蛋白質甲基化之作用並不明顯。相反的，甲基化反應抑制劑卻可減弱LPS / IFN -γ誘導NO的產生及iNOS之表現。我們證明，甲基化反應與腎臟發炎疾病有關。此份研究報告提供腎臟發炎之可能致病機轉及提供腎臟發炎之治療策略。

4. 主要發現
   本研究具有政策應用參考價值：□否 ■是，建議提供機關衛福部
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：□否 ■是
   說明：（以150字為限）

本研究具有應用參考價值，主要是本研究成果提出以下新穎觀點：環境中來自病原體釋放出之毒素如內毒素LPS及由受感染細胞產生之細胞素如干擾素IFN-γ為表觀遺傳之因子，影響受感染細胞之甲基化反應。甲基化反應抑制劑可作為提供慢性發炎疾病之治療策略。