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Effect of the adenovirus E1A gene on nitric oxide production in alveolar epithelial cells

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ABSTRACT

This study determined the effect of the adenovirus E1A gene on nitric oxide (NO) production in alveolar epithelial (A549) cells. E1A-positive A549 cells (E1A transfectants), E1A-negative A549 cells (control transfectants) and untransfected A549 cells were placed in 96-well tissue culture plates. After stimulation with lipopolysaccharide (LPS) or cytokine mixture (CM), the biochemical reaction products of NO (nitrite and nitrate) in the culture medium were measured by chemiluminescence. The inducible (iNOS) and the endothelial (eNOS) isoforms of nitric oxide synthase (NOS) protein expression were examined by Western blotting. iNOS mRNA expression was examined by Northern blotting and RT-PCR. CM-induced NO production by E1A-positive A549 cells was significantly lower than that of E1A-negative cells ($p < 0.0001$). LPS stimulation failed to enhance NO production in both cell types. CM induced iNOS protein expression in E1A-negative A549 cells, but not in E1A-positive cells. eNOS protein expression was constitutive and was not affected by CM stimulation, LPS stimulation or E1A. CM induced iNOS mRNA expression in E1A-negative A549 cells, but not in E1A-positive cells. In conclusion, the adenovirus E1A gene suppressed NO production through transcriptional control of the iNOS gene in A549 cells. This inhibition of NO production may enable the virus to persist in human tissue, since NO is an antiviral effector of the innate immune system.

Keywords Adenovirus E1A gene, bronchial epithelial cells, inducible nitric oxide synthase, nitric oxide production, pulmonary epithelial cells

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INTRODUCTION

Adenoviruses are a group of DNA viruses that infect the epithelial cells of the respiratory, gastrointestinal and genitourinary tracts. Infection is usually self-limiting, but latent virus infection is implicated as a risk-factor for obstructive lung diseases [1,2]. Adenovirus has been detected at high frequency in children with steroid-resistant asthma [2]. Adenovirus E1A protein and mRNA are detected frequently in the lungs of patients with chronic obstructive pulmonary disease (COPD) [1,3]. Studies of cultured human alveolar epithelial cells have also shown that transfection with this virus gene upregulates the expression of intercellular adhesion molecule-1 (ICAM-1) and interleukin-8 when these cells are challenged with endotoxin or noxious particles [4–7]. These studies suggest that latent adenovirus infections, and the persistence of the E1A gene in the alveolar epithelial cells, may have a role in the pathogenesis of obstructive airway disease by amplifying the inflammation [8].

Nitric oxide (NO) is an antiviral effector of the innate immune system, and inhibits replication of a wide variety of DNA and RNA viruses in cell cultures and animals. Cao *et al.* [9] showed that adenovirus E1A suppressed inducible NO synthase (iNOS) expression in the mouse macrophage cell line RAW264.7. Viral inhibition of NO may affect the host innate immune system and enable viruses to survive in the lung. Therefore, the aim of this study was to determine whether

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NO production by human alveolar epithelial cells is affected by the adenovirus E1A gene. The effect of E1A on iNOS and endothelial NO synthase (eNOS) expression in these cells was also studied.

MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS) from *Escherichia coli* strain 0111:B4 (Sigma, St Louis, MO, USA) was dissolved in sterile distilled water. The final concentration of LPS was 10 μg/L. Recombinant human tumour necrosis factor-α, interferon-γ (IFN-γ) and interleukin-1β were purchased from Roche Diagnostics (Basel, Switzerland) and were used to produce a cytokine mixture (CM: tumour necrosis factor-α 10 ng/mL; interleukin-1β 500 U/mL; IFN-γ 100 U/mL).

Cell culture

The cells used in this study have been described previously [4,5,10]. In brief, A549 cells (ATCC, Rockville, MD, USA) were transfected with the E1A gene of adenovirus 5. The E1A transfectants E4 and E11 investigated in this study were A549 cell clones that were transfected stably with a plasmid carrying the adenovirus 5 E1A gene, driven by its own promoter, while control transfectants C4 and C8 were clones transfected with a control plasmid lacking the E1A gene. Analysis by Western and Northern blots showed that all E1A transfectants produced relatively high levels of E1A mRNA and proteins [4]. Wild-type and transfected A549 cells were grown in Eagle's minimum essential medium supplemented with fetal bovine serum 10% v/v (Hyclone, Logan, UT, USA). Transfectants were maintained under constant selection with active G418 (Gibco BRL, Rockville, MD, USA) 280 mg/L.

Measurements of nitrite and nitrate

To measure NO production by A549 cells, the biochemical reaction products of NO (nitrite and nitrate) were measured in the culture medium. Cells were placed in 96-well tissue culture plates at 4×10^4 cells/well on the day before stimulation with LPS or CM. The culture medium was collected after incubation for 24 h with or without stimulation. The nitrate (NO_3^-) in culture medium was reduced to nitrite (NO_2^-) with *Aspergillus* nitrate reductase (DOJINDO, Kumamoto, Japan). The resulting nitrite content of the incubation medium was measured by a chemiluminescence technique involving a glass purge–reflux vessel connected to an NO analyser (NOA 280; Sievers Instruments, Boulder, CO, USA). Aliquots (10 μL) of the culture medium that had been treated with the converting enzyme were injected into the purge–reflux vessel, where nitrite was reduced to NO by potassium iodide 1% v/v in glacial acetic acid. NO was then detected by the NO analyser.

Western blot analysis

Cells were lysed with the Mammalian Cell Lysis Kit (Sigma). The protein concentration was measured by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Protein (30 μg) from A549 cells was separated by electrophoresis through SDS-PAGE 10% w/v gels and transferred to nitrocellulose (Hybond-ECL; Amersham, Arlington Heights, IL, USA). Membranes were blocked with Tris-buffered saline containing skimmed milk 5% w/v and Tween-20 0.1% w/v, and then incubated with the primary antibodies, anti-iNOS (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-eNOS (1:100; Santa Cruz Biotechnology), anti-ICAM-1 (1:200; Santa Cruz Biotechnology), anti-β-actin (1:1000; Sigma), anti-adenovirus 5 E1A (1:2000; Sigma) in Tris-buffered saline containing skimmed milk 5% w/v and Tween-20 0.1% w/v at room temperature for 1 h. The blots were washed extensively and then incubated for 1 h with anti-rabbit IgG conjugated to horseradish peroxidase (1:25 000; Amersham) or anti-mouse IgG conjugated to horseradish peroxidase (1:50 000; Amersham). Binding of secondary antibody was detected using an enhanced chemiluminescence detection system (ECL plus; Amersham).

iNOS mRNA expression

iNOS mRNA expression was examined by Northern blotting and RT-PCR. Northern blotting was performed as described previously [11]. A549 cells, grown until they were confluent in 10-cm dishes, were left in media or exposed to LPS 10 μg/L or CM for 6 h, after which total RNA was extracted with Isogen (Wako, Osaka, Japan). This RNA, at 20 μg/lane, was separated on a formaldehyde–agarose 1% w/v gel, transferred to a Hybond-N membrane (Amersham) and fixed to the membrane under vacuum at 80°C. Next, the membrane was prehybridised, and then hybridised for 20 h with the DNA probes, which were labelled with [α-^{32}P]-dCTP (Amersham) using random primers. RT-PCR was performed as described previously [12]. Total RNA (1 μg) was reverse transcribed to cDNA using a Takara RNA-PCR kit (Takara, Kyoto, Japan) according to the manufacturer's recommendations. Briefly, total RNA, random hexadeoxyribonucleotides and avian myeloblastosis virus reverse transcriptase were used for cDNA synthesis. The cDNA was amplified by PCR, using specific primers for iNOS (5′-CAAGTGGAAGTTCACCAACAGC-3′ and 5′-GATATCTTCGTGATAGCGCTTCTGGC-3′ [13,14]) and a control mRNA, GAPDH (5′-ATTCCATGGCACCGTCAAGGCT-3′ and 5′-TCAGGTCCACTGACACGT-3′). Amplification was performed with cycles of 95°C for 1 min, 55°C for 30 s and 72°C for 1 min in a thermal cycler (Perkin-Elmer/Applied BioSystems, Foster City, CA, USA). In preliminary experiments, the appropriate PCR cycle number was selected from cycle numbers that showed a linear relationship to the signal intensity from the PCR product on ethidium bromide-stained agarose gels. For semiquantitative evaluation of iNOS and GAPDH mRNAs, 30 and 26 cycles were chosen, respectively. The PCR products were analysed on an agarose 1% w/v gel, and the intensity of the ethidium bromide fluorescence was evaluated by NIH Image v. 1.62 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data were expressed as a group mean ± SEM, with *n* referring to the number of wells studied in each group. Group comparisons were made through an analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons. Values of $p < 0.05$ were considered significant.

RESULTS

NO production by A549 cells

To determine NO production by A549 cells, nitrite and nitrate were measured in the culture supernatant. In the absence of any stimulation, NO production by A549 cells was not affected by the adenovirus E1A gene (Fig. 1). NO production by A549 cells and E1A-negative cells (control transfectants) was enhanced after stimulation for 24 h with CM, but not after stimulation with LPS. In contrast, NO production by E1A-positive cells was not enhanced after stimulation with either CM or LPS. In the E1A-positive cells (E4), NO production induced by CM was significantly lower than that in the untransfected A549 cells and E1A-negative cells (C8) (untransfected A549 cells, 11.93 ± 0.29 μM; E1A-negative cells, 10.33 ± 0.19 μM; E1A-positive cells, 7.39 ± 0.26 μM; $p < 0.0001$). Similar results were obtained for the second set of E1A-negative and -positive transfected clones (C4 and E11, respectively) (data not shown).

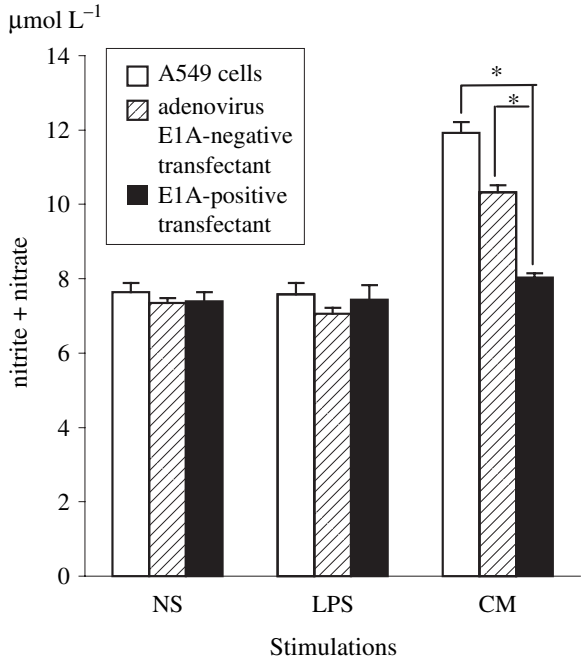

Fig. 1. Nitric oxide (NO) (nitrite and nitrate) concentration in the culture medium of A549 cells, after stimulation with lipopolysaccharide or cytokine mixture for 24 h. Data are expressed as the mean ± SEM of ten experiments. NS, no stimulation; LPS, stimulation with lipopolysaccharide 10 mg/L; CM, stimulation with cytokine mixture (tumour necrosis factor-α 10 ng/mL, interleukin-1β 500 U/mL, and interferon-γ 100 U/mL). *$p < 0.0001$ compared to A549 cells and E1A-negative transfectants.

Western blot analysis of iNOS and eNOS protein expression

To determine which isoform of NOS was suppressed by the E1A gene, iNOS and eNOS proteins were examined by Western blotting. iNOS protein expression was not detected, or was barely detectable, without stimulation, regardless of the presence of E1A (Fig. 2). CM stimulation induced iNOS protein expression in untransfected A549 cells and E1A-negative transfectants (C8); however, CM did not induce iNOS protein expression in E1A-positive transfectants (E4). LPS stimulation did not enhance production of the iNOS protein in either cell type, while LPS induced ICAM-1 protein expression in both cells. LPS-induced ICAM-1 expression was stronger in E1A-positive cells than in E1A-negative cells. eNOS protein expression was constitutive, and was not affected by either E1A or CM stimulation. Similar results were obtained for the second set of E1A-negative and -positive transfected clones (C4 and E11, respectively) (data not shown).

iNOS mRNA expression

In the absence of stimulation, iNOS mRNA expression was not detected, regardless of the

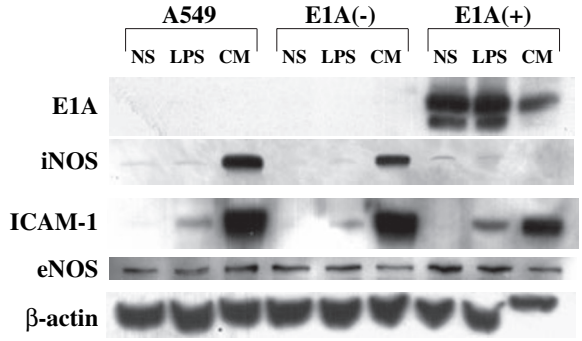

Fig. 2. Western blot analysis of inducible nitric oxide synthase, endothelial nitric oxide synthase, adenovirus E1A and intercellular adhesion molecule-1 (ICAM-1) protein expression. EIA(–), adenovirus E1A-negative transfectant; E1A(+), adenovirus E1A-positive transfectant; NS, no stimulation; LPS, stimulation with lipopolysacharide; CM, stimulation with cytokine mixture (tumour necrosis factor-α 10 ng/mL, interleukin-1β 500 U/mL, and interferon-γ 100 U/mL); iNOS, inducible isoform of nitric oxide synthase; eNOS, endothelial isoform of nitric oxide synthase.

presence of E1A (Fig. 3A). LPS stimulation did not induce iNOS mRNA expression. CM induced iNOS mRNA expression in untransfected A549 cells and the E1A-negative transfectants (C8), but not in the E1A-positive transfectants (E4). To confirm the results from Northern blotting, RT-PCR of iNOS mRNA was performed for two different pairs of transfected A549 cells (Fig. 3B). In the absence of stimulation, iNOS mRNA expression was not detected in A549 cells or in the E1A-negative transfectant C8 and the E1A-positive transfectant E4 of the first pair. Individual stimulation with each cytokine (IFN-γ, tumour necrosis factor-α or interleukin-1β), LPS, or the

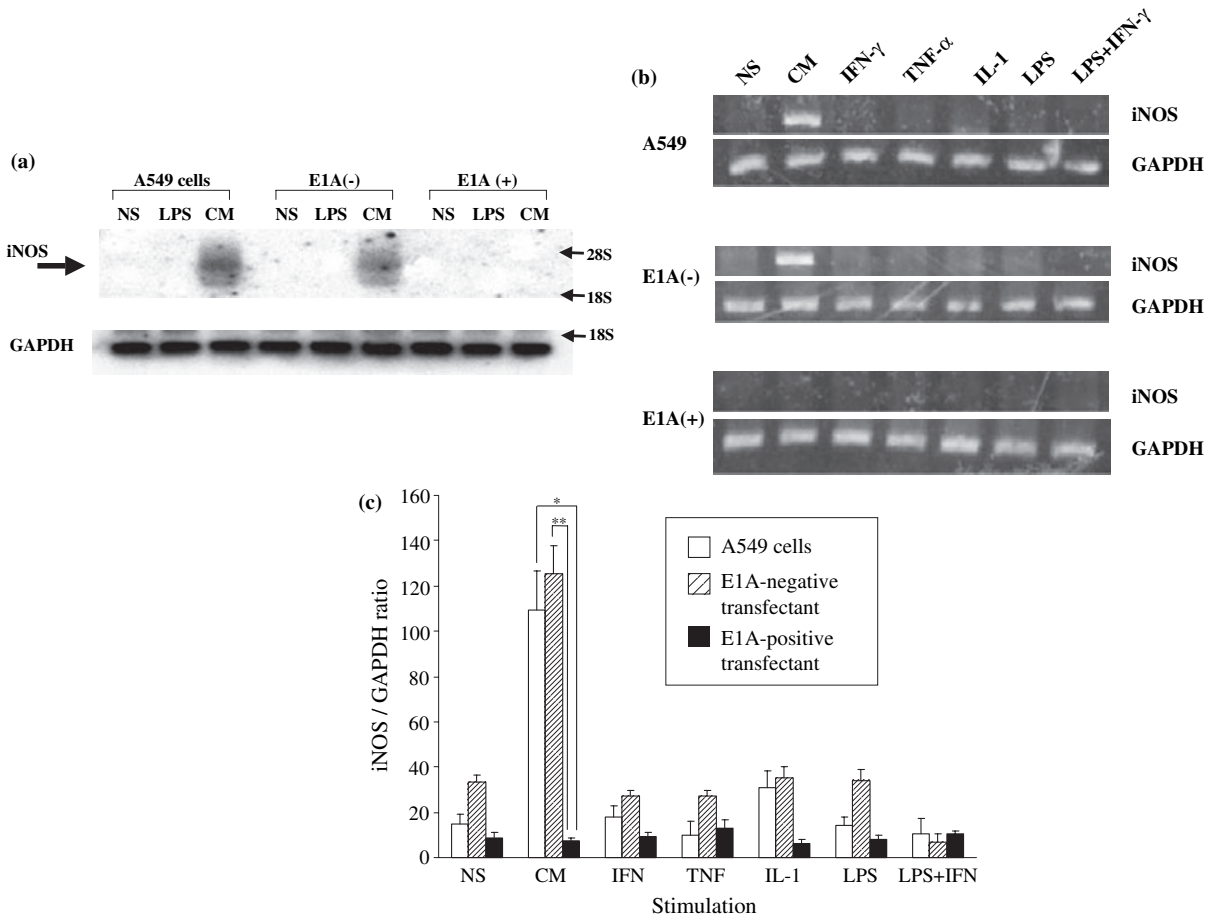

Fig. 3. Inducible nitric oxide synthase mRNA expression in A549 cells. (A) Northern blot analysis. EIA(–), adenovirus E1A-negative transfectant; E1A(+), adenovirus E1A-positive transfectant; NS, no stimulation; LPS, stimulation with lipopolysaccharide; CM, stimulation with cytokine mixture (tumour necrosis factor-α 10 ng/mL, interleukin-1β 500 U/mL, and interferon-γ 100 U/mL); iNOS, inducible isoform of nitric oxide synthase. (B) Inducible nitric oxide synthase and GAPDH RT-PCR; a representative ethidium bromide-stained agarose gel of the RT-PCR products. iNOS, inducible isoform of nitric oxide synthase; EIA(–), adenovirus E1A-negative transfectant; E1A(+), adenovirus E1A-positive transfectant; NS, no stimulation; CM, stimulation with cytokine mixture (tumour necrosis factor-α 10 ng/mL, interleukin-1β 500 U/mL, and interferon-γ 100 U/mL); IFN-γ, stimulation with interferon-γ; TNF-α, stimulation with tumour necrosis factor-α; IL-1, stimulation with interleukin-1; LPS, stimulation with lipopolysaccharide; LPS+IFN-γ, stimulation with lipopolysaccharide and interferon-γ. (C) Densitometric analysis of the RT-PCR products from (A). The density of the inducible nitric oxide synthase (iNOS) RT-PCR product was corrected by that of the corresponding GAPDH band. Data are shown as the mean ± SEM of four experiments. NS, no stimulation; CM, stimulation with cytokine mixture (tumour necrosis factor-α 10 ng/mL, interleukin-1β 500 U/mL, and interferon-γ 100 U/mL); IFN-γ, stimulation with interferon-γ; TNF-α, stimulation with tumour necrosis factor-α; IL-1, stimulation with interleukin-1; LPS, stimulation with lipopolysaccharide; LPS+IFN-γ, stimulation with lipopolysaccharide and interferon-γ. *$p < 0.001$, **$p < 0.0001$ compared to A549 cells and the E1A-negative transfectant.

combination of LPS and IFN-γ, did not induce iNOS mRNA in either set of cells. CM induced iNOS mRNA expression in A549 cells and the E1A-negative transfectant, but not in the E1A-positive transfectant.

Densitometric analysis of the ethidium bromide-stained bands, representing the iNOS and GAPDH mRNA RT-PCR products, confirmed that expression of the iNOS mRNA was significantly lower in the E1A-positive transfectants than in A549 cells and the E1A-negative transfectants (Fig. 3C; $n = 4$, $p < 0.0001$). Similar results were obtained for the second set of E1A-negative and -positive transfected clones (C4 and E11, respectively) (data not shown).

DISCUSSION

The adenovirus E1A gene inhibited NO production by blocking expression of iNOS in human alveolar epithelial cells. These results are consistent with a report showing that E1A suppressed iNOS expression in a mouse macrophage cell line [9]. In the present study, E1A suppressed not only iNOS expression, but also total NO production, in human alveolar epithelial cells. NO is an antiviral effector of the innate immune system [15]. Viral inhibition of NO production by alveolar cells may result in the repression of the innate immune system in the lung, and may enable viruses to persist in the lung. Studies with a guinea-pig model of human adenovirus 5 infection have shown that deposition of the virus in the nasal cavity is followed by virus replication in the lung and persistence of the virus E1A gene, with expression of its protein, in lung epithelial cells long after virus replication stops [16]. Studies suggest that latent adenovirus infection is one of the factors that can amplify lung inflammation [1,3,8]. The adenovirus targets lung epithelium, and its DNA persists in large quantities in the lungs of patients with COPD, while E1A proteins, which act as adenovirus transactivators, have been demonstrated in the epithelium of the conducting airways, bronchial glands and alveolar epithelium [16,17]. Retamales *et al.* [3] reported a 40-fold increase in the number of alveolar epithelial cells expressing adenovirus E1A protein in the lungs of patients with severe COPD, compared to patients without COPD. Previous studies showed enhanced expression of ICAM-1 and interleukin-8 mRNA in A549 cells expressing E1A protein [4,5,7,11]. These data support the hypothesis that adenovirus E1A protein amplifies inflammation in the lung and participates in the destruction of the lung.

The suppressive effect of E1A on NO production may appear to contradict the results of previous studies that showed the inflammatory enhancement effect of E1A [1–6], because NO has been convincingly identified as a pro-inflammatory mediator [18]. However, it should be emphasised that NO also has an anti-inflammatory effect in some conditions [19,20]. NO has a biphasic effect on nuclear factor kappa B binding activity and regulation of the transcription of inflammatory mediators [19]. The effect of NO is likely to depend on the local concentration of NO, and enables it to both upregulate and downregulate the expression of host defence proteins [19]. NO also has anti-apoptotic activity, and thus a protective role in the LPS-induced acute lung injury model [21]. Therefore, suppression of NO by E1A may result in disruption of this complex regulating system of inflammation in the lung.

Previous studies have shown that E1A enhances the expression of ICAM-1 and interleukin-8 induced by LPS in A549 cells [4,5]. However, in the present study, LPS had no effect on NO production or iNOS protein expression in A549 cells, regardless of the presence of E1A, while it induced ICAM-1 expression, with stronger expression in E1A-positive cells. These results also showed that the LPS used in this study was biologically active, although it did not induce NO in A549 cells. CM induced iNOS mRNA and protein, but there was no induction with LPS or any individual cytokine in A549 cells. This result is consistent with previous reports showing that iNOS mRNA was induced by CM, or culture supernatants of monocytes stimulated with LPS, but not by individual cytokines or LPS alone [22].

E1A proteins localise in the nuclei of target cells, where they interact with a variety of cellular transcription factors that regulate the expression of certain cellular genes. In this manner, E1A could affect transcription factors which, in response to CM stimulation, bind to the promoter–enhancer regions of inflammatory genes. Cao *et al.* [9] showed that E1A suppressed iNOS expression by inhibiting the nuclear factor kappa B transcription factor. Nuclear factor kappa B is clearly important in the induction of murine

iNOS, but appears to be less important in the regulation of the human gene [23–25]. Human iNOS regulation appears to require IFN-γ and the activation of the transcription factor signal transducers and activators of transcription (STAT)-1 [24,26]. Adenovirus E1A suppresses the activation of STAT-1 [27,28]. Therefore, the inhibitory effect of E1A on iNOS mRNA expression could be attributed to a block of the STAT-1 pathway required for the IFN-γ response.

In conclusion, the adenovirus E1A gene suppressed NO production through transcriptional control of the iNOS gene in A549 cells. This inhibition of NO production may enable the virus to persist in human tissue, since NO is an antiviral effector of the innate immune system.

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