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Transcriptional suppression of cytokine-induced iNOS gene expression by IL-13 through IRF-1/ISRE signaling

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Abstract

IL-13 has been reported as one of the major down-regulators of iNOS expression in various tissues and cells. The molecular mechanism of iNOS suppression by IL-13 remains unclear, especially at the transcriptional stage. In this study, we found that IL-13 inhibited the expression of iNOS mRNA, protein, and NO product in a concentration-dependent manner for cytokine-stimulated rat hepatocytes. The most effective dose for IL-13 inhibitory effect is ~5 ng/ml. IL-13 also decreased the rat iNOS transcriptional activity by promoter analysis, but had no effect on iNOS mRNA stability. By using TranSignal Protein/DNA Combo Array, we identified cytokine-stimulated IRF-1/ISRE binding that was decreased by the addition of IL-13. Gel shift assay confirmed that IL-13 reduced the IRF-1/ISRE binding at nucleotides −913 to −923 of the rat iNOS promoter. Western blot revealed that IL-13 diminished the relative amount of IRF-1 protein translocated to the nucleus. Our data demonstrate that IL-13 down-regulates the cytokine-induced iNOS transcription by decreasing iNOS specific IRF-1/ISRE binding activity.

Keywords: iNOS; NO; IL-13; IRF-1; ISRE; Protein–DNA array; Nitric oxide; Cytokine

Inducible nitric oxide synthase (iNOS) is an important gene that is expressed in a number of tissues in response to various inflammatory cytokines [1,2]. Nitric oxide (NO) produced by the iNOS gene was initially identified from murine macrophages [3,4]. We further reported that iNOS could be highly expressed in hepatocytes in response to certain cytokines [5,6]. The maximal nitrite oxide (NO) synthesis and high iNOS activity are induced by the simulation with LPS and cytokine mixture (CM) including TNFα, IL-1β, and IFNγ. The molecular regulatory mechanisms of iNOS expression are mainly through gene transcriptional control and post-translational regulation [1,7].

The transcriptional regulation of iNOS gene expression has been shown to be tightly controlled by positive and negative transcription factors that bind to specific cis-acting DNA motifs [2,8–11]. We have shown that TNFα or IL-1β can activate iNOS transcription through NF-κB signaling pathway while IFNγ can turn on iNOS transcription through IRF-1 or Stat-1 signaling pathway [2,8,12–14]. Further we have identified that NRF transcription factor can medicate the silencing of hiNOS transcription [9]. Interestingly, IL-13, an anti-inflammatory cytokine, was found to down-regulate iNOS expression in various cells [15–19]. The detailed mechanisms for IL-13-mediated inhibition of iNOS expression remain unknown [20]. Recently, IL-13 was shown to control iNOS translation by arginine availability in cytokine-stimulated macrophages [21]. However, the exact mechanism of iNOS transcription suppression by IL-13 has not been identified.

In our present study, we show that IL-13 inhibits cytokine-induced iNOS transcription, and applied novel protein/DNA array to investigate the specific transcriptional pathway responsible for IL-13-mediated down-regulation of iNOS gene expression in rat hepatocytes. We have found that the transcription factor IRF-1 plays as a key

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role for IL-13-mediated negative regulation of iNOS gene transcription.

Materials and methods

Nuclear extraction. The cytokine-stimulated or non-stimulated rat hepatocytes are washed and scraped into phosphate-buffered solution and centrifuged at 4500 rpm for 8 min in a microfuge. The pelletted cells are suspended in buffer A [10 mM Tris (pH 7.5)/1.5 mM $MgCl_2$/10 mM KCl/0.5% Nonidet P-40] at ~10× the packed cell volume and lysed by gentle pipetting. Nuclei were recovered by microcentrifugation at 7000 rpm for 8 min. Nuclear proteins are extracted at 4 °C by gentle resuspension of the nuclei (at ~2× the packed nuclear volume) of buffer containing 20 mM Tris (pH 7.5), 10% glycerol, 1.5 mM $MgC1_2$, and 420 mM NaCl, 0.2 mM EDTA, followed by 30 min on ice incubation with frequently vortexing. The nuclear protein suspension is cleared by microcentrifugation at 13,000 rpm for 15 min. The supernatants are collected and frozen at −80 °C or directly used in protein/DNA array or EMSA. All buffers contain the following additions: 1–2 μg/ml each of aprotinin, chymostatin, leupeptin, pepstatin, 0.2 mM PMSF, 0.5 mM DTT, and 0.1 mM Na-vanadate. All steps are carried out on ice or at 4 °C. Protein concentrations are measured by the BCA protein assay, using BSA as a standard.

Protein/DNA array analysis. The protein/DNA array is performed using TranSignal™ Protein/DNA Combo Arrays (Panomics Inc., Redwood City, CA), which includes 345 major transcription factors. In brief, protein/DNA hybridization is carried out according to the manufacturer's instructions. Twenty-microgram nuclear extract is mixed with probe mix and the mixture was incubated at 15 °C for 30 min. The entire content of the mixture is loaded on a 2% agarose gel and electrophoresed at 120 V in 0.5% TBE for 20 min. The gel area from above the blue dye to the loading well, represents the migration distance of any protein/DNA complexes. The gel area is excised containing the protein/DNA complex and the protein/DNA complex is extracted using the extraction buffer and finally incubated with 6 μl of gel extraction beads and incubated at room temperature for 10 min. The mixture is centrifuged at 10,000 rpm for 30 s to pellet out the beads. The beads are washed and the supernatant is removed. The bound probes are eluted by resuspending the pellet in 50 μl of dH_2O and incubated at room temperature for 10 min with vortexing for 2–3 times during incubation. The recovered DNA probes are denatured at 95 °C for 3 min before being hybridized to the array membrane at 42 °C overnight. The membrane is washed twice in 2× SSC/0.5% SDS at 42 °C for 20 min and then twice in 0.1× SSC/0.5% SDS at 42 °C for 20 min.

EMSA assay. DNA probes presenting the cis-elements for specific TFs are prepared by end-labeling with [γ-^{32}P]dATP (DuPont/NEN) and T4 polynucleotide kinase (Invitrogen) and purified in TEN by using G-25 resin columns (Amersham). Typically, 5 μl (10–20 μg) of nuclear proteins is incubated with ~100,000 cpm of ^{32}P-labeled oligonucleotides (~0.5 ng) for 2 h at room temperature. The nuclear proteins and various oligonucleotide probes are incubated in a buffer containing 10 mM Tris (pH 7.5), 10% glycerol, and 0.2% Nonidet P-40. Additionally, 2–4 μg of poly(dI-dC) (Amersham) is included as a nonspecific competitor DNA. Protein–DNA complexes were resolved on 4% nondenaturing polyacrylamide gels in 0.4× TBE running buffer (450 mM Tris borate and 1 μM EDTA, pH 8.0). After electrophoresis, gels are dried and subjected to autoradiography. Antibody supershift experiments included the addition of 2 μl of various antibodies, all of which were purchased from Santa Cruz Biotechnology.

Plasmid construction and luciferase activity assay. The rat iNOS promoter–reporter plasmid pRatiNOS(1.7)Luc contains −1.7 kb of upstream 5′-flanking DNA linked to the luciferase reporter gene and has been described [22]. DNA transfections of cells are carried out in six-well plates (Corning), using Lipofecin (Invitrogen). Briefly, cells were exposed to serum-free medium containing 1 μg of DNA and 20 μg of liposomes for 6 h, washed, and replenished with medium supplemented with 5% calf serum. To control for transfection efficiency between groups, 0.5 μg of a plasmid containing a cytomegalovirus promoter-driven β-galactosidase gene (pIEP-Lacz) is added to each well. As a positive control, cells are transfected with PRSV-Luc while transfection of the promoter-less plasmid pXP2 served as a negative control. After treated with cytokines for 6 h, cells are lysed with reporter lysis buffer (Promega) or buffer containing 1% Triton X-100, 5 mM dithiotreitol, 50% glycerol, 10 mM EDTA, and 125 mM Tris–phosphate (pH 7.8). Luciferase activity is assayed with 20 μl of lysate in a Berthold Nashua, (NH) AutoLumat LB953 luminometer using a commercially available kit (Promega). β-Galactosidase activity was determined as recommended (Promega), using a 96-well multiplate reader with SOFTMAX software (Molecular Devices). Luciferase activity is normalized to β-galactosidase activity.

Northern and Western blotting. Northern and Western blot experiments were performed as described according to established protocol [8].

Results and discussion

Suppression of cytokine-stimulated iNOS protein and mRNA expression by IL-13 in rat hepatocytes

Our previous work demonstrated that iNOS expression can be highly induced with the cytokine mixture (CM) of TNFα, IL-1β, and IFNγ. In this study, we further explored the role of IL-13 in the regulation of iNOS gene expression in rat hepatocytes. We first tested the effect of IL-13 on iNOS protein and mRNA expression as well as NO production. We performed the Western and Northern blot experiments with CM-stimulated rat hepatocytes. As depicted in Fig. 1A, pretreatment with IL-13 inhibited cytokine-induced iNOS protein expression in a dose-dependent manner. Western blot showed that iNOS protein was greatly induced by CM stimulation in rat hepatocytes without IL-13 pretreatment. After IL-13 (0.1–20 ng/ml) pretreatment for 16 h, CM-stimulated iNOS protein was decreased by IL-13 in a dose-dependent manner. The most effective concentration for IL-13 was ~5.0 ng/ml. A similar

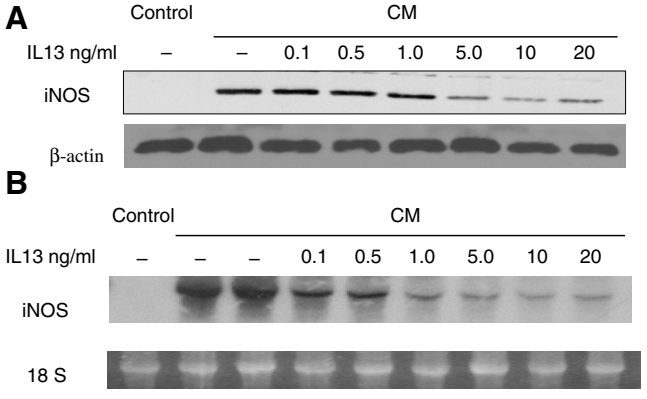

Fig. 1. IL-13 inhibits cytokine-induced iNOS protein and mRNA expression in a dose-dependent manner. (A) Western blot analysis of iNOS protein expression in rat hepatocytes. (B) Northern blot analysis of iNOS mRNA expression in rat hepatocytes. After pretreatment in culture medium alone or with different doses of IL-13 (0.1–20 ng/ml) for 16 h, rat hepatocytes were stimulated with a cytokine mixture containing of 500 U/ml of TNFα, 200 U/ml of IL-1β, and 100 U/ml of IFNγ. Protein and mRNA were extracted from rat hepatocytes after CM treatment for 8 and 6 h, respectively.