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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 TransSignal 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

Introduction

Inducible nitric oxide synthase (iNOS) is an important gene that is expressed in a number of tissues in response to various inflammatory cytokines [1 and 2]. Nitric Oxide (NO) produced by the iNOS gene was initially identified from murine macrophages [3 and 4]. We further reported that iNOS could be highly expressed in hepatocytes in response to certain cytokines [5 and 6]. The maximal Nitrite oxide (NO) synthesis and high iNOS activity are induced by the simulation with LPS and cytokine mixture (CM) including TNF α , IL-1 β , and IFN γ . The molecular regulatory mechanisms of iNOS expression are mainly through gene transcriptional control and post-translational regulation [1 and 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 [8, 9,10,11, and 12]. We have shown that TNF α or IL-1 β can activate iNOS transcription through NF- κ B signaling pathway while IFN γ can turn on iNOS transcription through IRF-1 or Stat-1

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signaling pathway [8,9,13,14, and 15]. Further we have identified that NRF transcription factor can mediate the silencing of hiNOS transcription [10]. Interestingly, IL-13, an anti-inflammatory cytokine, was found to down-regulate iNOS expression in various cells [16,17, 18,19, and 20]. The detailed mechanisms for IL-13-mediated inhibition of iNOS expression remain unknown [21]. Recently, IL-13 was shown to control iNOS translation by arginine availability in cytokine-stimulated macrophages [22]. 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 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 4,500 rpm for 8 min in a microfuge. The pelleted cells are suspended in buffer A [10 mM Tris (pH 7.5)/1.5 mM MgCl₂/10 mM KCl/0.5% Nonidet P-40] at ~10 × the packed cell volume and lysed by gentle pipetting. Nuclei were recovered by microcentrifugation at 7,000 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 MgCl₂, 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 sec 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 dH₂O and incubated at room temperature for 10 minutes 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 -³²P] dATP (DuPont/NEN) and T4 polynucleotide kinase (Invitrogen) and purified in TEN

by using G-25 resin columns (Amersham). Typically, 5 μ l (10-20 μ g) of nuclear proteins is incubated with $\sim$ 100,000 cpm of 32 P-labeled oligonucleotides ($\sim$ 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 μ g 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 $\times$ TBE running buffer (450 mM Tris borate and 1 μ M EDTA, pH 8.0). After electrophoresis, gels are dried and subjected to autoradiography. Antibody supershift experiments included the addition of 2 μ l 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 [23]. DNA transfections of cells are carried out in six-well plates (Corning), using Lipofectin (Invitrogen). Briefly, cells were exposed to serum-free medium containing 1 μ g of DNA and 20 μ g of liposomes for 6 h, washed, and replenished with medium supplemented with 5% calf serum. To control for transfection efficiency between groups, 0.5 μ g 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 hours, cells are lysed with reporter lysis buffer (Promega) or buffer containing 1% Triton X-100, 5 mM dithiothreitol, 50% glycerol, 10 mM EDTA, and 125 mM Tris-phosphate (pH 7.8). Luciferase activity is assayed with 20 μ l of lysate in a Berthold (Nashua, NH) AutoLumat LB953 luminometer using a commercially available kit (Promega). B-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 [9].

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 TNF α , IL-1 β , and IFN γ . 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 hr, CM stimulated iNOS protein was decreased by IL-13 in a dose-dependent manner. The most effective concentration for IL-13 was $\sim$ 5.0 ng/ml. A similar result was also observed with Northern blot (Fig 1B). Nitrite oxide (NO) synthesis, determined by Griess Assay, was significantly increased 50-fold over control 24 hrs after CM-stimulation (data not shown). IL-13 treatment inhibited the NO production by 50% (data not shown). These data confirm that IL-13 down-regulates cytokine-induced iNOS mRNA and protein expression, and ultimately NO synthesis in rat hepatocytes.

Down-regulation of iNOS transcriptional activity by IL-13

To define the mechanism by which IL-13 down-regulates iNOS expression, we performed iNOS promoter activity assay, and iNOS mRNA stability experiments using Actinomycin-D. Rat hepatocytes cells were transiently transfected with 1.5 kb wild-type rat iNOS promoter luciferase construct. Promoter activities were measured as relative luciferase activities (RLA) in the lysed cells and normalized with β -gal co-transfection. As shown in Fig. 2A, the cytokine mixture of TNF- α + IL-1 β + IFN- γ induced a 6.2-fold increase in luciferase activity compared with resting hepatocytes. IL-13 pretreatment significantly reduced the CM-induced promoter activity by 50%. However, IL-13 pretreatment did not affect basal iNOS promoter activity. These results indicate the inhibition of iNOS by IL-13 occurs by repressing iNOS gene transcription. To investigate whether IL-13 also affected iNOS mRNA stability in rat hepatocytes, cells were pretreated with IL-13 for 16 hr and further stimulated for 6 hours in culture with CM. Actinomycin-D was then added and iNOS mRNA degradation was quantified at various time points to calculate its half-life. The results showed the half-life for rat iNOS mRNA was ~2 hours and was not influenced by IL-13 treatment. Regression analysis showed there was no statistically significant difference for iNOS half-life for the two conditions. Taken together, these results suggest that transcriptional repression is the most likely mechanism responsible for IL-13 mediated inhibition of iNOS expression in rat hepatocytes.

Identification of transcription factors/binding motifs for IL-13 suppression mechanism using TranSignal protein/DNA array

A recently developed array technology-TranSignal™ Protein/DNA array (Panomics Inc; Redwood City, CA) was applied for the high-throughput functional analysis of transcription factor (TF) binding. This array-based assay can profile the activities of multiple transcription factors in a single experiment. We utilized this system to identify specific TFs whose DNA-binding activities are regulated by cytokine stimulation and reversed by IL-13 treatment. Expression of the relevant TFs can be determined by the analysis of the relative density for the hybridized spots.

The TranSignal array was utilized that contains 345 transcription factors associated with inflammatory conditions. We compared the binding of TFs present in nuclear extracts of three groups: control hepatocytes (without cytokine treatment), CM (TNF α 500u/ml, IL-1 β 200u/ml, and IFN γ 100u/ml), and CM + IL-13 stimulation. We identified 37 transcription factor binding sites (TFBSs) that were at least 2-fold increased by CM stimulation when compared with control group (Table 1). There were 28 TFBS that showed at least a 2-fold inhibition for CM + IL-13 compared to CM alone. Noteworthy was the TF binding site GAS/ISRE site, which has been identified in the iNOS promoter to functionally bind IRF-1 in response to IFN γ stimulated iNOS transcription [13]. The bindings to GAS/ISRE was increased by cytokine stimulation, and inhibited by IL-13 treatment. These data suggest that IRF-1 transcription factor binding to the cis-acting GAS/ISRE DNA motif accounts (at least in part) for the IL-13 mediated inhibitory effect on iNOS transcription. EMSA and western blot were then applied to verify this finding.

Transcriptional suppression of IL-13 on iNOS expression through IRF-1/ISRE binding

Sequence analysis of rodent iNOS promoter reveals several cytokine responsive DNA elements that can potentially bind to NF- κ B, Stat-1, and IRF-1 proteins. A functional GAS/ISRE (interferon response sequence element) at -913 to -923 was previously identified that binds IRF-1 protein by site-directed mutagenesis and gel shift assay [13]. In order to validate whether IL-13 altered IRF-1 protein-DNA binding, gel shift assay was carried using CM-stimulated rat hepatocytes with or without IL-13 treatment. A specific DNA primer was designed from rat iNOS promoter between -926 and -909 that contained the GAS/ISRE site. As shown in Fig. 3A, resting hepatocytes showed low level expression of a protein-DNA complex (lane 2). This

band was induced by CM treatment (lane 3), but was significantly decreased with the IL-13 pretreatment (lane 4). IL-13 treatment alone did not induce any DNA binding complex (lane 5). To confirm the specificity of the DNA probe, 100-fold excess of cold competition DNA abrogated the protein/DNA complex (lane 1). The DNA complex was completely shifted with IRF-1 antibody (lane 6), confirming that the protein-DNA complex contains IRF-1. The above results indicate that IL-13 suppressed CM-induced iNOS transcription by specifically decreasing IRF-1/ISRE binding at -913 to -923 GAS/ISRE site in the rat iNOS promoter.

To further show that effect of IL-13 on IRF-1 nuclear translocation, we performed Western blot on nuclear extracts from CM-stimulated rat hepatocytes in the presence or absence of IL-13. As depicted in Fig. 3B, IRF-1 protein was enriched in nuclear extracts after CM treatment. Noticeably, treatment with IL-13 significantly decreased the translocated IRF-1 protein. These data are consistent with the decreased IRF-1 DNA binding to the GAS/ISRE in response to IL-13, and indicate that IL-13 elicits an inhibitory effect on CM-stimulated iNOS transcription by decreasing the nuclear translocation of IRF-1.

IRF-1 was originally identified as a nuclear factor specifically binding to the IFN- β promoter and its cDNA was subsequently cloned from murine L929 fibroblasts [24]. IRF-1 has also been shown to mediate IFN γ -induced iNOS expression by binding to the cis-acting IRF-1 responsive elements in the rodent iNOS promoters [13,25, and 26]. In addition, IRF-1 defective mice failed to induce iNOS expression [27 and 28]. Besides IRF-1 responsive elements in the iNOS promoters, there also exist functional GAS/ISRE sites in the rodent and human iNOS promoters that bind Stat-1 and/or IRF-1 [9,29,30, and 31]. Our current study further defines an important role for IRF-1 in mediating trans-repression of iNOS expression by its diminished binding to the GAS/ISRE site following IL-13 treatment. This highlights that certain transcription factors can exhibit complex functions in regulating inflammatory gene expression depending on the local cytokine milieu.

In summary, we observed that IL-13 inhibited the expression of iNOS protein, mRNA, and NO production in cytokine-stimulated rat hepatocytes. Subsequent promoter assay confirmed that IL-13 also decreased rat iNOS transcriptional activity. We also excluded a potential posttranscriptional effect of IL-13 on iNOS mRNA stability by observing no changes in iNOS mRNA half-life experiments. We further investigated possible TF binding sites responsible for the IL-13 inhibitory mechanism by using TransSignal Protein/DNA Combo Array. From 345 TF binding sites, we identified that GAS/ISRE site was a potential candidate. We further confirmed that the binding activities for a functional IRF-1/ISRE site in the rat iNOS promoter that was inhibited with IL-13 treatment. Further, the relative amounts of IRF-1 protein translocated to the nucleus was also decreased by IL-13 pretreatment. Taken together, our data reveal that IRF-1/ISRE signaling accounts as one mechanism by which IL-13 down-regulates cytokine-induced iNOS transcription in rat hepatocytes.

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Figure 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 to 20 ng/ml) for 16 hr, rat hepatocytes were stimulated with a cytokine mixture containing of 500 units/ml of $\text{TNF}\alpha$, 200 units/ml of IL-1 β , and 100 units/ml of IFN γ . Protein and mRNA were extracted from rat hepatocytes after CM treatment for 8 hr and 6 hrs, respectively.

Figure 2.

Effect of IL-13 on iNOS gene transcription and mRNA stability. A. Suppression of iNOS promoter activity by IL-13 in CM stimulated rat hepatocytes. Cells were transfected with iNOS-luciferase reporter construct. Basal and induced luciferase activity was determined 6 hrs after cytokine stimulation. White bars represent the expression of luciferase reporter gene in untreated cells as control; black bars represent the expression of luciferase reporter gene with cytokine mixture treatment. IL-13 treatment alone had no effect (gray bar). Relative luciferase activity (RLA) was normalized to cotransfected β -galactosidase as an internal standard. Values shown are the means $\pm$ sd of at least three separate experiments performed in triplicate. IL-13 groups indicated the pretreatment of IL-13 (5 ng/ml) for 16 hr. * Indicates $P < 0.05$ vs. IL-13 + CM. B. Effect of IL-13 on iNOS mRNA stability for CM stimulated rat hepatocytes. After pretreatment of IL-13 (5 ng/ml) for 16 hr, rat hepatocytes were stimulated with CM for 6 hours. 5 μ g/ml Actinomycin-D was added to cells and mRNA decay was analyzed at 0, 30, 90, or 150 minutes by Northern blot probing for iNOS mRNA. GAPDH was used as the internal control, and expression levels of iNOS mRNA were normalized to that of GAPDH mRNA.

Figure 3.

Effect of IL-13 on cytokine-stimulated rat hepatocyte IRF-1 DNA binding and nuclear translocation. A. Gel mobility shift assay of nuclear proteins from cytokine-stimulated rat hepatocytes pretreated with IL-13. Nuclear extracts were prepared from rat hepatocytes with or without IL-13 pretreatments and 2 hrs of CM stimulation. 10 μ g of nuclear extract were used in each lane. For supershift, antibody against IRF-1 was pre-incubated with nuclear extracts from 2 hr CM stimulated cells. B. Western Blot analysis of IRF-1 protein from rat hepatocyte nuclear extracts. After pretreatment in culture medium alone or with IL-13 (5 ng/ml) for 16 hr, rat hepatocytes were stimulated with CM for 2hrs, and nuclear proteins were extracted and used in western blot analysis. Histone H1 protein complex served as internal control.

Table 1

Changes of DNA binding activities for multiple transcription factors after cytokine stimulation and IL-13 treatment

Comparison	Increase (≥ 2x)
Control vs CM CM vs CM+IL13	<u>GAS/ISRE</u> , PTF1, TR(DR-4), PYR, ODC, TEF1, MEF-1, RAR(DR-5), USF-1, EKLF(1), WT(1), HFN-3, c-mybBP, ETF, ISGF, Freac-2, RFX123, GRE, T FAST-1, Oct-1, Smad SBE, C-Rel, GATA-1/2

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