Interferon (IFN)-α Activation of Human Blood Mononuclear Cells In Vitro and In Vivo for Nitric Oxide Synthase (NOS) Type 2 mRNA and Protein Expression: Possible Relationship of Induced NOS2 to the Anti–Hepatitis C Effects of IFN-α In Vivo

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Summary

Although researchers have noted high level activation of rodent mononuclear phagocytes for nitric oxide (NO) synthase type 2 (S2) expression and NO production with a variety of agents such as interferon (IFN) γ and endotoxin, it has been difficult to demonstrate activation of human mononuclear phagocytes. The purpose of this study was to determine if IFN-α serves as an activator in vitro and in vivo in humans. Treatment of normal monocytes or mononuclear cells in vitro with IFN-α caused a dose-dependent increase in monocyte NOS2 activity and NO production, and increased expression of NOS2 protein and mRNA expression. To determine if in vivo administration of IFN-α also modulated NOS2, we studied blood cells from patients with hepatitis C before and after IFN-α therapy. Untreated patients with chronic hepatitis C virus infection had levels of NOS activity and NOS2 antigen in freshly isolated mononuclear cells similar to those of healthy subjects, and they expressed minimal or no NOS2 mRNA. However, IFN-α treatment of patients with hepatitis C infection was associated with a significant elevation in mononuclear cell NOS activity, NOS2 antigen content, and NOS2 mRNA content. IFN-α-treated patients had significant decreases in levels of serum alanine aminotransferase and plasma hepatitis C mRNA. The degree of IFN-α–enhanced mononuclear cell NOS2 antigen content correlated significantly with the degree of reduction in serum alanine aminotransferase levels. Thus, IFN-α treatment of cells in vitro or administration of IFN-α to hepatitis C patients in vivo increases expression of mononuclear cell NOS2 mRNA expression, NOS activity, NOS2 antigen expression, and NO production. Since NO has been reported to have antiviral activity for a variety of viruses, we speculate that induced NO production may be related to the antiviral action(s) of IFN-α in hepatitis C infection.

Nitric oxide (NO) is a simple chemical mediator produced endogenously from l-arginine by the action of NO synthase (NOS), a family of related enzymes encoded by separate genes (1, 2). The inducible (or immune) form of the enzyme, NOS2, is found prominently in mononuclear phagocytes and hepatocytes, and is capable of high level NO production. Its expression is regulated primarily by transcription, and it is modulated by various cytokines and microbial products (1). NO has potent antimicrobial activities in vitro and in vivo against a wide array of organisms including certain viruses (3–5).

IFN-γ, endotoxin, and TNF treatment in vitro can potentially increase NOS2 expression and NO production by rodent macrophages, but it has been difficult to show comparable activation in vitro of normal human mononuclear phagocytes for high level expression (6–8). However, cells from patients with illnesses such as malaria (9), rheumatoid arthritis (7, 10), and tuberculosis (11) have mononuclear phagocytes that clearly express NOS2 and produce NO. In rodent macrophages, exogenous IFN-α cannot activate macrophages for NO production (12), but macrophage-synthesized IFN-α can augment NO production in an autocrine fashion (13). IFN-α had not previously been inves-
tigated thoroughly in human mononuclear phagocytes for this function.

The purpose of this study was to determine whether IFN-α could activate human monocytes in vitro and in vivo for NOS2 expression and NO production. We demonstrate here that IFN-α treatment of normal mononuclear cells in vitro induces increased expression of monocyte NOS activity, NOS2 antigen, and mRNA content, and production of NO, and that treatment of patients with chronic hepatitis C virus infection with IFN-α in vivo causes increased mononuclear cell NOS activity, NOS2 antigen content, and mrRNA content.

Materials and Methods

Subjects. Control normal subjects were recruited locally. Patients with chronic hepatitis C (diagnosed by second generation recombinant immunoblot assay and/or hepatitis C virus RNA measurement and liver biopsy) were recruited consecutively from the hepatology outpatient clinics at Duke University Medical Center (DUMC). The study protocol was approved by the DUMC Institutional Review Board. Informed consent was obtained from each subject before participation. Patients and controls were excluded if they had a coexisting chronic inflammatory condition, active allergy or infection, malignancy, or if they were receiving ribavirin, nitroglycerin, or other nitrate-containing medication. Pregnant women were excluded because of reports of elevated NO production in pregnancy. All subjects and patients were abstinent of alcohol. Patients had no identifiable cause(s) for chronic hepatitis other than hepatitis C; all had confirmed hepatitis C infection and hepatitis as determined by blood tests.

Blood mononuclear cell preparation. 30 ml of blood was drawn into lithium heparin. Mononuclear cells were prepared using Ficoll/Hypaque as previously noted (7) and stored at −70°C until use. For some in vitro experiments, monocytes were prepared by sequential Ficoll/Hypaque-Percol adherence technique (6). Freshly isolated monocytes were cultured at 3 × 10⁶ cells/6-mm diameter microtiter plate well in 1 ml of DMEM with 10% heated (56°C for 30 min) normal, pooled human serum.

NOS enzyme activity and antigen analyses. Cellular extracts were prepared and analyzed for NOS activity (³⁵⁸⁸C-l-arginine conversion to L-citrulline) and antigen content by immunoblot as previously described (7). In some enzyme assays, 2 mM N°-monomethyl-l-arginine was included to determine if the conversion to l-citrulline was NOS-mediated. Immunoblots were done using either a monoclonal anti-NOS2 antibody from Transduction Laboratories (anti-macNOS; Lexington, KY) or from Research and Diagnostic Antibodies (18B-88; Richmond, CA). We used untreated human colon cancer cell line cells (DLD-1; reference 2) as negative controls. L-DOPA-1 cells treated with human recombinant IFN-γ (100 U/ml), and TNF (100 U/ml), IL-1 (0.5 ng/ml), and IL-6 (200 U/ml) for 3 d were used as positive controls for demonstrating NOS2 antigen. 25–50 μg protein extract of the cells was used in the individual lanes. A positive immunoblot for NOS2 was one in which a clear band was visible at 130–131 kD.

Reverse transcriptase-PCR analyses. Total RNA was isolated by the method of Chomczynski and Sacchi (15), resuspended in diethyl pyrocarbonate-treated water, and reprecipitated overnight with cold isopropanol at −20°C. 1 μg of RNA was reverse transcribed with random hexamers and murine leukemia virus reverse transcriptase (RT; Perkin Elmer, Branchburg, NJ) for 15 min at 42°C. Reactions were stopped by heating for 5 min at 99°C. The final product was then amplified with 2.5 U of AmpliTaq® DNA polymerase (Perkin Elmer) and 0.15 μM of sense and antisense primers in PCR buffer containing 100 mM Tris-HCl, 50 mM KCl, 25 mM MgCl₂, and 10 μM deoxyribonucleotide triphosphate. Mixtures were overlaid with mineral oil and amplified for 40 cycles. The primers for NOS2 were 5′-CCT GAG CTC TTC TTC GAA ATC C-3′ (sense) and 5′-AGG ATG TTT TAG CCG CCT GC TGG AC-3′ (antisense). The expected product is 229 bp. As a control, we used the following primers for the glyceraldehyde-3-phosphate dehydrogenase: 5′-CTA CTA CTG GCG CTT CCC ACC AGG CTG T-3′ (sense) and 5′-GCC ATG AGG TCC ACC ACC CTG T-3′ (antisense). The expected product is 390 bp. Final PCR products were separated on a 1% Tris-borate/EDTA agarose gel and visualized by ethidium bromide staining.

Measurement of serum IFN-α levels. Serum IFN-α levels were done using a previously reported cytokine-specific sandwich ELISA (Endogen, Cambridge, MA); levels were calculated using standard curves generated with recombinant IFN-α. The lower limit of the detection by the assay was 5 pg/ml.

Statistical analysis. Continuous variables were compared using the Student’s t test or ANOVA, as appropriate. Categorical variables were compared using the Fisher’s exact test. The Bonferroni correction was used to control the type I error rate when multiple comparisons were performed. Since the NOS activity data were not normally distributed, we used logarithmic transformation to allow for parametric analysis (ANOVA testing) of the results. The Kruskal-Wallis test was performed on nontransformed data to confirm these results. To test for an increasing dose–response relationship between IFN-α dose in vitro and expression of NOS activity and production of nitrite/nitrate, the Page test for ordered alternatives was used. We used the Statistical Analysis System (SAS Institute, Inc., Cary, NC). P values are two-sided using α = 0.05 as the reference standard for determining significance.

Results

Freshly isolated monocytes from eight healthy male volunteers showed an increase in NOS activity in response to IFN-α2b treatment in vitro. The increase peaked at 500 U/ml (Fig. 1A). Similarly, production of NO (measured as nitrate and nitrite, the stable catabolites of NO) increased with IFN-α2b treatment (Fig. 1B). Increasing doses of IFN-α2b were associated with significantly increased NOS activity and nitrate/nitrite production (P <0.001). Studies also demonstrated that treatment of monocytes in vitro with IFN-α2a (RIFERON®; Roche Laboratories, Nutley, NJ) augmented NOS activity and NO production (data not shown). In immunoblot studies, normal subject monocytes from four out of six treated in vitro with IFN-α2b had increased expression of NOS2 antigen (Fig. 2A). We did RT-PCR analysis of RNA from blood mononuclear cells to determine if IFN-α induced increased levels of NOS2 mRNA. M ononuclear cells from normal subjects were cultured for 3 d without or with 500 U/ml IFN-α. Untreated cells from zero out of six normal individuals had NOS2 mRNA ex-
expression, whereas all those treated with IFN-α in vitro expressed NOS2 mRNA (six out of six) (Fig. 2 B).

In an attempt to determine if IFN-α treatment in vivo augmented NOS2 expression, we studied patients with hepatitis C before and after IFN-α therapy. Table 1 displays characteristics of the subjects and details of their treatments. As expected, hepatitis C patients receiving IFN-α treatment had higher levels of IFN-α than did normal subjects or hepatitis C patients not receiving IFN-α. There was an overall difference in the blood mononuclear cell NOS activity levels among the three groups (P < 0.004; Fig. 3 A). Untreated patients with chronic hepatitis C and healthy controls had comparable NOS activity (ability to convert L-arginine to L-citrulline) in freshly isolated blood mononuclear cells. This activity was inhibited by > 80% by inclusion of N-monomethyl-L-arginine in the assay (data not shown), indicating that the activity was mediated by NOS. Patients receiving IFN-α2b therapy had significantly higher NOS activity levels than did healthy controls (adjusted P < 0.05) and hepatitis C patients not receiving IFN-α2b therapy (adjusted P < 0.05). Although IFN-α2b treatment caused increases in NOS2 levels, levels of serum alanine aminotransferase (ALT; an indicator of active hepatitis) and plasma hepatitis C RNA decreased with IFN-α2b therapy (Fig. 3, A and B; P = 0.002 and 0.02, respectively, by paired Students’ t test). When we analyzed samples from individuals both before and after receiving IFN-α2b, there was a significant increase in NOS activity after the IFN-α2b treatment (2-8-wk time interval) (P < 0.02, paired t test; Fig. 3 D).

To determine if the increase in NOS activity after IFN-α treatment was accompanied by an increase in NOS2 protein, we analyzed cells for NOS2 antigen content. NOS2 antigen in extracts of blood mononuclear cells was detectable by immunoblot analysis in zero out of seven of the untreated hepatitis C patients and in zero out of five of the healthy control subjects tested. However, eight out of eight samples from hepatitis C patients treated with IFN-α2b had cells with detectable NOS2 antigen. These differences in NOS2 antigen expression among the groups were significant (P < 0.00001, Fisher’s exact test). Similarly, analysis of matched sets of cells from hepatitis C patients before and after IFN-α2b treatment revealed zero out of four with detectable NOS2 before treatment and four out of four with detectable NOS2 after treatment (Fig. 4 A). Using RT-PCR analyses with cells isolated from six normal individuals and examined without any in vitro culture, we could find no NOS2 mRNA (zero out of six) (Fig. 4 B). In two out of three patients with hepatitis C not on IFN-α treatment, we noted relatively low level expression of NOS2 mRNA, while three out of three hepatitis C patients on IFN-α treatment had relatively higher levels of NOS2 mRNA expression. Fig. 4 B shows representative results.

There was a statistically significant correlation between the fold increase in NOS2 antigen immunoblot band density and the degree of reduction of the serum ALT level (fraction of pretreatment ALT level after IFN-α therapy); with increasing NOS2 antigen expression, there was a greater treatment-associated decrease in serum ALT (Fig. 5). The correlation was statistically significant (P < 0.001 by paired Students’ t test).
ANOVA, and <0.05 by Spearman testing). Because of a lack of certain simultaneous determinations, we could analyze data from only five of the eight subjects before and after treatment. While the NOS2 antigen immunoblot band density and the degree of reduction of the serum ALT level correlated, the correlation between NOS activity (pmoles citrulline/mg protein) and ALT reduction was not significant. Nevertheless, these data suggest that the augmentation in NOS2 levels may be causally related to the observed decreases in serum ALT levels and decrease in liver inflammation associated with IFN-α therapy.

**Discussion**

IFNs are a family of proteins with established antiviral and immunomodulatory properties (16). While much is known regarding the possible molecular and cellular modes of action of IFNs, the precise mechanism(s) by which IFN-α

| Normal subjects | n = 9 (5 M, 4 F) | Age 39.4 ± 5.8 yr* |
|-----------------|-----------------|-------------------|
| Total number of hepatitis C patients | n = 26 (17 M, 9 F) | Chronic hepatitis (18) |
| Chronic hepatitis with cirrhosis (8) | n = 18 (14 M, 4 F) | Age 43.8 ± 10.5 yr |
| Hepatitis C patients not on IFN-α treatment (never treated or blood sampled before started on IFN-α treatment) | n = 15 (10 M, 5 F) | Age 40.7 ± 10.5 yr |
| 3 to 10 million units IFN-α three times/wk | 1–40 wk of treatment | 26.4 ± 12.0 h from last injection until blood draw |
| Serum IFN-α levels | Normal subjects: 13.8 ± 0.2 pg/ml |
| | Hepatitis C: 14.5 ± 0.8 pg/ml |
| | Hepatitis C on IFN-α: 34.0 ± 13.9 pg/ml† |

*Mean ± SD.
†P <0.04.
M, male; F, female.
mediates its anti–hepatitis C virus effect in vivo is not known. Our study shows that IFN-α2b treatment of patients with hepatitis C increases their blood mononuclear cell NOS enzyme activity and their NOS2 antigen and mRNA expression. In addition, we demonstrate that treatment of purified monocytes or mononuclear cells from healthy donors with IFN-α2b or IFN-α2a in vitro enhances their N0 production, and NOS enzyme activity, and their NOS2 antigen and mRNA expression. Among isolated mononuclear cells, monocytes are those most likely to express NOS2 and produce NO (6, 7). Our work provides good evidence that IFN-α serves to activate human mononuclear cells (most likely mononuclear phagocytes) for NOS2 expression and NO production both in vitro and in vivo. More than 90% of patients with chronic hepatitis C have circulating immune complexes (17), and immune complexes may enhance NO formation by rodent mononuclear phagocytes (18, 19); thus, immune complexes and IFN-α could have cooperated in vivo to enhance NOS2 expression.

NO has potent antimicrobial activities against a wide array of organisms. These include protozoa, fungi, and bacteria (including Leishmania major, Mycobacterium leprae, Mycobacterium tuberculosis, Toxoplasma gondii, Cryptosporidium parvum, and Schistosoma mansoni; references 3, 4). Also, NO is a critical effector for macrophage-mediated tumor cell cytotoxicity (20). In rodent systems, pharmacological inhibition of NOS or genetic disruption of NOS2 reduces host resistance to infection (3, 4, 21, 22). NO can inhibit infection with DNA viruses (e.g., vaccinia, and Epstein-Barr virus) and RNA viruses (e.g., vesicular stomatitis virus and Coxackie virus; references 5, 23–29).

Based on our results, we think that IFN-α-induced NOS2 expression and NO production may be responsible (at least in part) for the anti–hepatitis C effects of IFN-α in vivo. There was a statistically significant correlation between the degree of increase in NOS2 antigen in blood mononuclear cells and the degree of improvement in hepatitis C as reflected by a decrease in serum ALT. We do not know the precise mechanism of this possible NO-mediated inhibition; NO likely affects both cellular and viral factors that modify the infectivity. With vaccinia virus, NO-mediated interference with ribonucleotide reductase function appears to be important (30, 31). NO may also damage nucleic acids, alter cellular growth and differentiation, and modify a variety of transcription factors (2, 5, 27, 32, 33); all of these effects might alter viral infectivity. There have been no demonstrations of NO antiviral activity for hepatitis C. There is currently no efficient and reliable cell culture system for growth of hepatitis C virus in vitro, and chimpanzees are the only suitable nonhuman hosts for hepatitis C virus growth in vivo.

In a subset of patients with hepatitis C virus infection, treatment with IFN effectively inhibits viral replication and reduces liver injury, but the effect is usually brief, with an overall response rate of 10–25%. Predictors of a poor response to IFN include viral factors such as high serum hepatitis C virus RNA levels, viral genotype 1, and the absence of sequence mutations in the NS5 region of the viral genome; host-specific factors such as age, weight, duration of infection, and immune status also are apparently important factors (34). The responses of patients to IFN-α treatment correlate inversely with plasma iron saturation and liver iron content (the response is worse with more iron). In some cases, depletion of iron by repeated phlebotomy is associated with reduction in serum ALT abnormalities, reduction in plasma hepatitis C virus RNA levels, and improved responsiveness to IFN-α treatment (35–37). We speculate that iron in these patients may blunt IFN-α-induced NOS2 expression (38), or quench NO antiviral effects (26). NO targets iron- and thiol-containing proteins such as hemoglobin, guanylate cyclase, ribonucleotide reductase, aconitase, and mitochondrial electron transport enzymes, and glyceraldehyde phosphate dehydrogenase (2, 20, 31, 39). Excess iron decreases NOS2 mRNA transcription, and reduction of iron (e.g., by treatment with the iron chelator deferoxamine) can increase NOS2 mRNA levels in vitro (38).

Patients receiving IFN-α for a variety of indications...
(e.g., hepatitis B or C and malignancies) may develop "autoimmune" illnesses with inflammation similar to rheumatoid arthritis (RA) and SLE (40–43). Our work and that by others has indicated that NO may be a mediator of inflammation in human autoimmune diseases such as RA and SLE. NO is increased in synovial fluid and serum of patients with RA (44). Synovial tissues from patients with RA contain increased amounts of NOS2 and overproduce NO (10, 45), and RA patients overproduce NO systemically and have blood mononuclear cells with increased NOS2 expression and NO production (7, 46). It is possible that the IFN-α treatment-related inflammatory illnesses are due (at least in part) to an IFN-α-mediated increase in NO production.

This study provides the first evidence that IFN-α can augment NOS2 expression and NO production by human blood mononuclear cells in vitro and in vivo. It is not known whether the magnitude of NOS expression will be predictive of response to IFN-α treatment in patients with hepatitis C virus infection; future studies may determine this. Amaro et al. recently showed that patients with hepatitis C had reduced levels of serum nitrite as compared to control subjects. However, the nitrite levels were higher in hepatitis C patients who responded to IFN-α therapy (47).

In their study, serum nitrate levels were not measured, dietary intake of nitrite and nitrate was not controlled, and levels of NOS activity and NOS2 antigen were not measured (47).

Although in this study we focus on monocytes as the producers of NO, hepatocytes can also express NOS2 and produce large amounts of NO after activation (48). We did not test hepatocytes for the ability to produce NO in response to IFN-α. Kane et al. recently reported that tissue in 60% of liver biopsies from hepatitis C patients (but none of "normal" subjects) expressed NOS2 mRNA by RT-PCR analysis (49). The authors did not report whether the patients were receiving IFN-α treatment. IFN-α-induced NOS2 expression and NO production by hepatocytes would provide an efficient manner of delivering an antiviral effector molecule to the offending pathogen in its primary cellular target. In addition to indirectly stimulating endogenous NO production by treatment with agents such as IFN-α, delivery of NO per se, perhaps selectively to the liver (50), might be effective in inhibiting hepatitis C virus or other viruses in vivo.

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