Transcriptional Activation of the Human Inducible Nitric Oxide Synthase (iNOS) Promoter by Kruppel-like Factor 6

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Running Title: Regulation of the human iNOS promoter by KLF6.

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ABSTRACT

Nitric oxide is a ubiquitous free radical that plays a key role in a broad spectrum of signaling pathways in physiological and pathophysiological processes. We have explored the transcriptional regulation of inducible nitric oxide synthase (iNOS) by the Kruppel-like factor 6 (KLF6), an Sp-1 like zinc finger transcription factor. Study of serial truncation constructs of the iNOS promoter revealed that the proximal 0.63 kb region can support a 3 to 6 fold reporter activity similar to that of the full length 16 kb promoter. Within the 0.63 kb region we identified two CACCC sites (-164 to -168 and -261 to -265) which bound KLF6, both in electrophoretic mobility shift and chromatin immunoprecipitation assays. Mutation of both these sites abrogated the KLF6-induced enhancement of the 0.63 kb iNOS promoter activity. The binding of KLF6 with the iNOS promoter was significantly increased in Jurkat cells, primary T lymphocytes and Cos-7 cells subjected to sodium cyanide (NaCN) induced hypoxia, heat shock, serum starvation and phorbol myristic acid/A23187 ionophore stimulation. Furthermore, in KLF6 transfected and NaCN treated Cos-7 cells, there was a 3- to 4-fold increase in the expression of the endogenous iNOS mRNA and protein that correlated with increased production of nitric oxide. These findings indicate that KLF6 is a potential transactivator of the human iNOS promoter in diverse pathophysiological conditions.
Key Words: Transcriptional regulation, cell injury, hypoxia, KLF6/CPBP/Zf9; Nitric oxide synthase, iNOS, Chromatin immunoprecipitation analysis.
INTRODUCTION

Nitric oxide synthases (NOS) are key proteins that produce nitric oxide (NO) and thereby regulate many important biological processes. NO is generated during the oxidation of L-arginine to L-citrulline by at least three different isoforms of NOS. Endothelial and neuronal NOS are constitutively expressed and their activity is Ca\(^{2+}\) and calmodulin dependent, whereas the third isoform is transcriptionally inducible (iNOS) and its activity is independent of Ca\(^{2+}\) and calmodulin, and can produce very high levels of nitric oxide over a sustained period of time (1;2). It has been shown that iNOS is transcriptionally upregulated in pathophysiologic conditions such as hypoxia, ischemia-reperfusion injury, trauma and by reactive oxygen species (ROS)(3;4).

NO is a key central molecule in cellular biochemical processes as it is freely diffusible and traverses cell membranes to reach different targets, alters signaling networks by redox-sensitive modifications and transcriptionally regulates multiple gene families (5-10). NO production following iNOS up-regulation is associated with increased wound healing and repair in tissue injury (11;12). NO is also known to activate multiple gene and cell signaling pathways through processes such as nitrosation and cGMP production (3;9). Furthermore, numerous studies have shown that NO has antitumor effects and forced expression of iNOS causes regression of tumors (13-16).

Kruppel-like factor (KLF6) is a ubiquitously expressed member of the Kruppel like family of transcription factors that have characteristic Cys2/His2 zinc finger motifs and bind very similar “GC-box” or “CACCC element” sites on DNA (17;18). KLF6 is an immediate early gene that regulates the expression of multiple genes and is involved in
tissue differentiation (19-23). KLF6 is rapidly induced in cells after acute injury and directly activates collagen α1 and TGFβ along with TGFβ receptor I and II genes thereby mediating wound healing mechanisms of fibrogenesis and extracellular matrix formation (18;24). Recently, KLF6 has been shown to function as a tumor suppressor gene that was mutated in prostate cancer (25).

The iNOS promoter defines a number of NF-κB and AP-1 sites dispersed throughout the 16 kb region. A number of iNOS inducers including cell injury, heat shock and various cytokines have been found to exert their effect by activating either NF-κB or AP-1 (1;26-28). Since KLF6 and iNOS are involved in common processes such as cell injury and wound repair, embryogenesis, tissue differentiation and suppression of tumorigenesis and because the iNOS promoter defines multiple CACCC sites (KLF6 binding motifs), we hypothesized that KLF6 binds to the iNOS gene and regulates its expression.

In the present study we have identified a novel transcriptional regulator of iNOS. Specifically, we demonstrate that KLF6 binds to CACCC sites within the proximal 0.63 kb and regulates the expression of the iNOS promoter in various cell types. We also show that in cells, exposed to stress conditions, the enhanced expression of KLF6 causes a direct increase in expression of the endogenous iNOS gene and NO.
MATERIALS AND METHODS

Cell Culture

Jurkat cells were cultured in RPMI medium (Life Technologies, Carlsbad, CA) supplemented with 10 % FBS. Cos-7 cells were maintained in DMEM (Life technologies) supplemented with 10 % FBS. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy adult volunteers. Primary T lymphocytes were obtained from PBMCs using a pan T-cell isolation kit according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). The protocol of study was approved by the Health Use Committee of the Walter Reed Army Institute of Research, Silver Spring, MD.

Plasmid Constructs

The human iNOS luciferase reporter constructs 0.63, 1.3, 3.8, 5.8, 7.2 and 16.0 kb upstream have been previously described (29). The two CACCC sites in the 0.63 kb iNOS promoter were mutated to AAAAA using the Quikchange™ XL site directed mutagenesis kit (Stratagene, La Jolla, CA). The mutations were confirmed by sequencing. The promoterless luciferase gene vectors pXP1 and pXP2 were used as control vectors. The Thymidine kinase-iNOS promoter luciferase constructs have been described previously (29). The KLF6 expression vector, pXCPBP and the control vector pX (pBluescript) have been described previously (30). The p50 and p65 NF-κB vectors were a kind gift from Dr. Barbara Rellahan (Center for Biologics Evaluation and Research, FDA, Bethesda MD)

Transient Transfections and Luciferase Activity Assays
Plasmid DNA transfections of Jurkat T cells and Cos-7 cells were carried out in 24 well plates (Corning Inc., Corning, NY) using LipofectAMINE™ 2000 Reagent (Life Technologies) as per the manufacturer’s protocol. The day before transfection, 6 x 10⁴ Cos-7 cells or 0.6 x 10⁶ Jurkat T cells were plated in 0.5 ml medium per well. For each well, lipofectamine reagent (2-3 µl) was mixed with plasmid DNA (1.5 µg) in serum free OPTI-MEM medium to allow DNA-lipofectamine reagent complexes to form. The complexes were added to respective wells and mixed by gently rocking the plate back and forth. The cells were incubated in a CO₂ incubator at 37°C for 48 h and then lysed with 60 µl reporter lysis buffer (Promega, Madison, WI). Luciferase activity was assayed with 20 µl of lysate and 80 µl of luciferase assay reagent (Promega) in a TD20/20 luminometer (Promega). Transfection efficiency was determined in all samples by cotransfection with 0.5 µg of a plasmid encoding the cytomegalovirus promoter driven β-galactosidase gene and the luciferase activity was normalized to the β-galactosidase activity.

*Electrophoretic Mobility Shift Assay (EMSA)*

Cos-7 cells (2 x 10⁶) were transfected with the KLF6 expression vector overnight. Briefly, the transfected cells were detached using trypsin-EDTA, washed with phosphate buffered saline and nuclear extracts were prepared as described previously (31). The sequences of the oligonucleotides used for EMSA are as follows: Probe 2 - 52 GAT CAG GTC ACC CAC AGG CCC 32 and its complementary sequence 52 GGG
CCT GTG GGT GAC CTG ATC 32. Probe 4 - 52 AGC AGC CAC CCT GCT GAT
GAA C 32 and its complementary sequence 52 GTT CAT CAG CAG GGT GGC TGC T
32. The oligonucleotides were synthesized by Sigma-Genosys (The Woodlands, TX).
Complementary single stranded oligonucleotides were annealed, end-labeled with $[^\gamma-32p]$ ATP (DuPont/NEN) and T4 polynucleotide kinase (Boehringer Mannheim, Germany), then purified by Centri-Sep columns (Princeton Separations, Adelphia, NJ) and used as probes in EMSA. In all experiments, unlabeled oligonucleotides were used as cold competitors. In each experiment, 5-10 µg of nuclear extracts were incubated for 20 minutes at room temperature with the labeled oligonucleotide (2-3 ng) in 20 µl of buffer containing 20 mM HEPES (pH 7.4), 1 mM MgCl$_2$, 10 µM ZnSO$_4$, 20 mM KCl, 15% Ficoll and 2 µg poly dI-dC. For supershift experiments, 3 µg each of control anti-body anti-KLF4 (clone T-16, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-KLF6 antibody (clone R-173, Santa Cruz Biotechnology) were added and incubated for 45 minutes at room temperature prior to the addition of the radiolabeled probe. The protein-DNA complex was separated on a 4% nondenaturing polyacrylamide gel in 0.5X Tris Borate-EDTA buffer. The data were analyzed using the Phosphorimager system (Molecular Dynamics, Sunnyvale, CA) and Quantity One software (Bio-Rad, Hercules, CA).

*Chromatin Hybridization Immunoprecipitation Assay (ChIP)*

Cos-7 cells (10 x 10$^6$) were transfected with KLF6 expression vector or were non-transfected (control). Both, the transfected and control cells were divided into two
subgroups, one was untreated and the other treated with 20 mM sodium cyanide (NaCN) for 3 h and both subgroups were used for the ChIP assay. Jurkat and T lymphocytes (10 x 10^6) were also treated similar to Cos-7 cells, however only the Cos-7 cells were subject to KLF6 transfection. Moreover all the cells were also treated with PMA/A23187 or heat shock or serum starved and subjected to ChIP analysis. The ChIP assay was performed as per the manufacturer’s recommendation (Upstate Biotechnology, Lake Placid, NY) and previously published protocols (32-34). Briefly, KLF6 was cross-linked to DNA by adding formaldehyde to a final concentration of 1%. The chromatin samples for studying phosphorylated KLF6 were incubated with anti-phosphoserine (clone PSR-45) (Sigma) and anti-phosphotyrosine (clone 4G10) antibodies (Santa Cruz Biotechnology) overnight at 4°C and immunoprecipitated with salmon sperm DNA-BSA-Sepharose beads, followed by treatment with 10 mM phenylphosphate for 15 min. The supernatants were used for further steps. All the samples (for phosphorylated as well as non-phosphorylated KLF6) were then incubated overnight at 4°C with anti-KLF6 antibody (Santa Cruz Biotechnology) followed by incubation with Sepharose beads. The immunocomplexes were treated with DNAse and RNAse free proteinase K, and DNA was purified by a DNA purification kit (Qiagen, Santa Clara, CA). PCR was performed with primers flanking the proximal as well as distal KLF6 binding sites in the proximal 0.63 kb iNOS promoter (5′ CAG AGA GCT CCC TGC TGA GGA AA 3′ and 5′ GAG AGT TGT TTT TGC ATA AAG GTC TC 3′) (Fig. 5). Amplified fragments (321 bp) were analyzed on a 2% agarose gel by Syber Green (FMC Bioproducts, Rockland, ME) staining. The no-antibody immunoprecipitation samples served as negative control.
Real-time quantitative PCR

Total RNA was isolated from 5 x 10^6 control cells using RNeasy mini kit (Qiagen, Santa Clarita, CA), treated with DNAse I and reverse transcribed using AMV reverse transcriptase and oligo-dT primer (Promega). The PCR primers synthesized by Sigma-Genosys were as follows: KLF6 - forward 52 AGA GCG AGC CCT GCT ATG TTT CAG 32 and reverse 52 CGC TGG TGT GCT TTC AAG TGG GAG 32; GAPDH-forward 52 CAA CTA CAT GGT TTA CAT GTT CC 32 and reverse 52 GGA CTG TGG TCA TGA GTC CT 32; iNOS forward 52 ACC TAC CAC ACC CGA GAT GGC CAG 32 and reverse 52 AGG ATG TCC TGA ACA TAG ACC TTG GG 32. Quantitative PCR was performed by monitoring in real-time the increase in fluorescence of the SYBR Green dye on a SmartCycler™ (Cepheid, Sunnyvale, CA), according to the manufacturer’s instructions. Relative expression level of the target gene in KLF6-transfected cells and NaCN-treated cells was plotted as fold-change compared to control-vector transfected and non NaCN-treated cells respectively. GAPDH gene expression was used for normalization. Each real-time quantitative PCR assay was performed twice using triplicate samples.

SDS-PAGE and Immunoblotting

KLF6 transfected and control Cos-7 cells (0.5 x 10^6 cells) were lysed in cold 1% NP-40 (Sigma Chemical Co., St. Louis, MO) lysis buffer with protease inhibitors as described previously (35), and protein was assayed using a BioRad protein assay kit (Bio-Rad, Hercules, CA). Seventy micrograms of the protein was resolved by 4-12% Bis-Tris
NuPAGE and transferred to PVDF membrane. The blot was probed with anti-β-Actin control antibody (clone 54) (BD Biosciences, San Diego, CA), anti-KLF6 antibody (Santa Cruz Biotechnology) and the anti-iNOS antibody (BD Biosciences). The membranes were washed and incubated with corresponding HRP-conjugated secondary antibody (BioRad). Protein bands were detected by enhanced ECL chemiluminescence reagents (Amersham-Pharmacia, Arlington Heights, IL). The Western blot bands were quantitated by densitometry by using the software GelPro.

**Estimation of Nitrite + Nitrate**

Briefly, 50 µl of cell culture medium (DMEM) was treated with nitrate reductase (N-7265, Sigma) in the presence of NADPH (N-7505, Sigma) to convert nitrate to nitrite (36;37). Upon the addition of 2,3-diaminonaphthalene (Sigma), which reacts with nitrite under acidic conditions to form a fluorescent product (1(H)-napthotriazole), fluorescence intensity was measured with a fluorescence microplate reader with excitation at 365 nm and emission at 450 nm and nitrite quantitated by comparison to a standard curve of NaNO₂.

**Quantitation and Statistical Analysis**

Statistical analysis of the data was done with Minitab, Version 14 using student’s t test and p value < 0.05 was considered as significant (Minitab, State College, PA). The Western blot bands were quantitated by GelPro™ software (Media Cybernetics, Silver Spring, MD).
RESULTS

KLF6 induces iNOS promoter activity in Cos-7 cells

A schematic diagram showing putative KLF6 binding motifs in the 52-flanking region of the human iNOS gene is shown in Fig. 1A. In order to investigate the effect of KLF6 on the expression of human iNOS, we performed luciferase reporter assays in Cos-7 cells transfected with the full-length 16 kb iNOS promoter construct and the KLF6 expression vector. As shown in Fig. 1B, transfection of Cos-7 cells with KLF6 induced the luciferase activity 3-fold as compared to the control vector. To compare the induction of iNOS with a well-characterized inducer, we studied the effect of transcription factor NF-κB subunits p50 and p65 on the iNOS promoter. NF-κB has been reported to bind multiple sites on the 7.3 kb iNOS promoter and induce its activity by 4.1 fold in AKN-1 and 3.9 fold in A549 cells following cytokine stimulation (1). Transfection of Cos-7 cells with p65 and p50 with the full-length iNOS promoter construct induced promoter activity by 2 and 4 fold, respectively (Fig. 1B). These results indicate that KLF6 can induce the transcription of the iNOS promoter at levels
comparable to those induced by NF-κB (4;26;29;38;39).

The proximal 0.63 kb iNOS promoter is sufficient for the induction of activity by KLF6

After ascertaining that KLF6 can activate the full-length 16 kb iNOS promoter, we performed transfection experiments to define the region of the iNOS promoter that is required for the induction of transcription by KLF6 (29). Cos-7 and Jurkat cells were transfected with serial deletion constructs of the 16 kb iNOS promoter-luciferase reporter compared to cells transfected with control vector, demonstrated that the induction of iNOS promoter activity remained similar in all constructs compared to the full length 16 kb iNOS promoter (Fig. 2A and B). Previous studies have shown that KLF6 binds to the CACCC motifs of DNA (40). The 0.63 kb iNOS construct has two CACCC motifs and there are a total of ten CACCC binding sites in the first 8 kb of the 16 kb iNOS promoter. Optimum induction (3 fold) of the 0.63 kb iNOS promoter by KLF6 (Fig. 2A and B) suggests that additional upstream KLF6 binding sites do not further enhance the iNOS promoter activity in the presence of CACCC sites in the proximal 0.63 kb. However, when the iNOS core promoter with the CACCC sites (within 0.63 kb) was replaced by thymidine kinase (TK-Luc) core promoter, a 20% increase in the luciferase activity with KLF6 expression vector (compared to transfection with control vector Px) was observed with the 3.8-5.8 kb iNOS and 7.2-16 kb iNOS TK-Luc constructs, whereas there was no luciferase induction with the iNOS promoter region from 5.8-7.0 kb (Fig. 2C). The significance of this finding is presently unknown.

Mutational analysis of the two CACCC sites in the 0.63 kb iNOS promoter

In order to understand the contribution of KLF6 to the activation of the 0.63 kb
iNOS promoter by the two CACCC sites, we created single mutation constructs in which we mutated the proximal or distal CACCC sites individually, or a dual mutation construct with both CACCC sites mutated. Transfection studies with these mutants revealed that the two sites that are separated by 92 bases, had an additive effect in the stimulation of the 0.63 kb iNOS promoter. The distal CACCC site contributed to a 1.6- and 3.2-fold induction of luciferase activity in Cos-7 cells (Fig. 3A) and Jurkat cells (Fig. 3B) respectively. Similarly, the proximal CACCC site contributed to a 1.3- and 2-fold induction in Cos-7 cells (Fig. 3A) Jurkat cells (Fig. 3B), respectively. Mutation of both CACCC sites completely abolished the KLF6-induced iNOS promoter activity suggesting that the CACCC motifs are necessary for the interaction of KLF6 with the 0.63 kb iNOS. These studies demonstrate that the proximal and distal CACCC sites in the 0.63 kb iNOS promoter are necessary for optimal basal promoter activity.

**KLF6 binds to the CACCC sites in the 0.63 kb iNOS promoter**

To demonstrate that KLF6 binds to the human iNOS promoter, we designed two oligonucleotides, each defining the CACCC motif regions at positions -164 to -168 and -261 to -265 in the 0.63 kb iNOS promoter, respectively (41). The primers were end labeled and an EMSA was performed using nuclear extracts from KLF6 transfected Cos-7 cells. Nuclear extracts from these cells bound to both oligonucleotides defined by the 0.63 kb iNOS promoter (Fig. 4A and B). To demonstrate the specificity of the binding, super-shift assays were performed using an antibody specific to KLF6. As shown in lane 4 of both Fig. 4A and B, the shifted band in lane 1 was super-shifted by an anti-KLF6 antibody demonstrating that KLF6 directly interacts with the iNOS promoter in vitro. Absence of a super-shifted band with anti-KLF4 antibody (lane 2 of both Fig. 4A and B) further confirmed the specificity of KLF6 for the CACCC binding sites.

**In vivo binding of KLF6 to the iNOS promoter**
Next, to determine whether KLF6 interacts with iNOS in vivo, we performed ChIP analysis using primary T cells, Jurkat cells and Cos-7 cells. The KLF6-DNA complexes were then immunoprecipitated with anti-KLF6 antibody, followed by reversal of cross-linking and PCR amplification using primers flanking the proximal and distal CACCC binding sites in the 0.63 kb iNOS promoter (Fig. 5A). In transfected Cos-7 cells, the intensity of the PCR product was significantly higher compared to the non-transfected cells (Fig. 5B) (lane 2 versus lane 4) suggesting that increased expression of KLF6 increases its binding to the iNOS promoter.

It has been demonstrated in independent studies, that iNOS and KLF6 are upregulated in cell stress (3;4;18;24). In order to examine whether upregulation of iNOS is mediated by KLF6 under conditions of stress, we treated Cos-7 cells, Jurkat cells and primary T lymphocytes with NaCN. NaCN blocks mitochondrial respiration and induces cellular hypoxia (42). As shown in Fig. 5B (lanes 3, 5, 7 and 9) treatment with NaCN strongly increased the binding of KLF6 to the iNOS promoter as evidenced by the increased intensity of the PCR product.

We also subjected Cos-7, Jurkat and primary T cells to heat stress, serum starvation and PMA/A23187 and analyzed the binding of KLF6 to the iNOS promoter by ChIP. As shown in Fig. 5C, compared to the control cells, the KLF6 binding to iNOS promoter was increased in a similar fashion in all cell types subjected to these conditions. These data conclusively show that the association of KLF6 to the iNOS promoter is increased in cells subjected to conditions of stress and stimulation.

Next we asked if the phosphorylation status of KLF6 could play a role in its binding to the iNOS promoter. To ascertain whether serine and tyrosine phosphorylation of KLF6 plays a role in binding to the iNOS promoter, we performed ChIP analysis involving a two-step immunoprecipitation process using anti-phosphoserine or anti-phosphotyrosine followed by treatment with phenyl phosphate (to separate the phosphorylated protein from the bound antibody) and further immunoprecipitation with anti-KLF6 antibodies. The data shows that the KLF6 that binds to iNOS is serine phosphorylated in resting T cells which increases in PMA/A23187 and NaCN treated cells (Fig. 5D). Interestingly, however we observed that tyrosine phosphorylated KLF6 binds to the iNOS promoter only in NaCN treated cells. The absence of any PCR products for the no-antibody and mouse IgG immunoprecipitation controls further confirmed the
specificity of our findings (data not shown). Thus, differential phosphorylation of KLF6 mediates
differential binding to iNOS in various conditions.

**Induction of iNOS mRNA, protein and NO in KLF6 transfected cells**

To establish the functional association between KLF6 expression and expression of iNOS, we measured the iNOS mRNA, protein and NO production in KLF6 transfected Cos-7 cells. We performed real-time PCR to estimate the fold induction of mRNA, based on the fluorescence cycle threshold (Ct) differences between various time points compared to their controls. As shown in Fig. 6A, the production of KLF6 mRNA was induced up to 14-fold 24 h post-transfection and 13-fold 48 h post-transfection, respectively (Fig. 6A). In tandem with the KLF6 mRNA there was a corresponding increase in the iNOS mRNA which showed a steady increase ranging from 3.5-fold (24 h) to 6-fold (48 h) (Fig. 6A). There was no increase in the control (GAPDH) mRNA.

Next, in order to study the kinetics of iNOS protein induction by KLF6 following KLF6 expression vector transfection, we performed Western blot experiments at earlier time points (0-18h) (Fig. 6B) and pursued the kinetics of KLF6 and iNOS expression over 72 h (Fig. 6C). KLF6 protein levels increased starting at 6h post-transfection, whereas iNOS protein levels increased starting at 12h post-transfection, suggesting that KLF6 is able to initiate transcription and produce iNOS protein within 6 h (Fig. 6B). It was previously observed that an increase in KLF6 mRNA in culture activated cells was accompanied by an even greater increase in KLF6 protein (18), moreover the rate of degradation of KLF6 protein was also lower (18). In light of these data, we believe that the induction of KLF6 protein seen beyond 48 h could be attributed to accumulation of KLF6 as a result of decreased protein degradation (Fig. 6C). Furthermore, NO production, as measured by assay for NO metabolites, nitrite and nitrate, indicated a significant increase in cells transiently transfected with KLF6 (Fig. 6D). There was no increase in either KLF6 or iNOS mRNA, protein (data not shown) or NO in control vector transfected cells (Fig. 6D) and cells subjected to lipofectamine transfection agent (data not shown), incubated under similar culture conditions as the KLF6 transfected cells. These data, taken together with the ChIP results (Fig. 5), demonstrate that KLF6 acts as a transactivator of the iNOS gene.
ChIP analysis (Fig. 5A) demonstrated increased binding of KLF6 to the iNOS promoter in cells exposed to NaCN. Therefore, to establish a functional association between the expression of KLF6 and iNOS in pathophysiological conditions, we measured the KLF6 and iNOS mRNA, protein and NO production in NaCN treated Cos-7 cells. The cells were transiently treated with 20 mM NaCN for 4 h and then incubated in fresh medium for various time periods. Following exposure of cells to NaCN, the production of KLF6 mRNA was analyzed by real-time PCR. As shown in Fig. 7A, a significant induction of KLF6 mRNA was seen, with levels reaching 4.5-fold and 8-fold over control by 24 h and 48 h, respectively. Consistent with the KLF6 mRNA results, there was an increase in the KLF6 protein by 24 h and the high amounts were sustained over a 72 h period. The iNOS protein levels were also induced by 12 h and the levels reached a peak by 48 h. (Fig. 7B). Furthermore, NO production, as assayed by its metabolites nitrite and nitrate, also indicated a significant increase in the nitrite + nitrate levels, starting at 6 h with a steady and sustained increase over 72 h (Fig. 7C). Thus, even though we cannot rule out the additional effect of other transcription factors in the induction of iNOS following NaCN treatment, the above data combined with our ChIP findings (Fig. 5B) and increased iNOS mRNA and protein expression data following KLF6 transfection (Fig. 6 A and B), strongly suggest that KLF6 plays a role in iNOS induction following treatment of cells with NaCN.
DISCUSSION

Our study provides first evidence for the regulation of the human iNOS promoter by a member of the Kruppel-like family of transcription factors. Using luciferase reporter gene assays, EMSA and ChIP, we demonstrate that KLF6, a member of the Kruppel-like family of transcription factors, directly interacts with the iNOS promoter in resting cells, and with greater intensity following cell stress, injury and stimulation. Furthermore, we show that the upregulated KLF6 increases both iNOS mRNA and protein that correlates functionally with concomitant nitric oxide expression. The presented evidence strongly suggests that KLF6 is a major transcriptional regulator of iNOS in conditions of hypoxia and cell stress.

The human iNOS promoter is 16 kb long and is one of the largest known promoters (1). Its regulation is complex and occurs at multiple levels, orchestrated by multiple transcription factors, in
response to diverse conditions in tissue specific context. Multiple transcription factors such as NF-κB, AP1, Stat 1α and interferon regulatory factor are known to regulate the iNOS promoter (reviewed in detail in (1)). NF-κB is ubiquitously expressed and regulates iNOS in multiple cell types and the regulation of iNOS by NF-κB is well characterized (4;26;29;38;39). Our data demonstrate that the induction of iNOS by KLF6 was comparable to that by the NF-κB subunits, suggesting that KLF6 could be an equally important regulator of iNOS.

Despite the presence of at least ten CACCC sites in the 16 kb iNOS promoter, the KLF6 induced transcriptional activation of the 0.63 kb construct was similar to that of the 16 kb construct in Cos-7 and Jurkat cells (Fig. 2). The CACCC sites in the 0.63 kb construct are placed in close proximity at -164 and -261 as compared to the other CACCC sites, the nearest of which is much farther upstream at -2736. Previous studies have reported similar binding of KLF6 to either tandem sites or sites in close proximity to each other in other promoters and placed in close proximity to the basal promoter elements. KLF6 is known to bind the leukotriene C4 synthase promoter at two tandem CACCC sites located between -135 and -149 close to the basal promoter elements (40). In yet another study, KLF6 was shown to interact with TGF-β1 and both TGF-β receptor I and II promoters at multiple sites. KLF6 strongly transactivates TGFβ-1 by binding to two tandem Sp1 binding sites between -239 and -209 as compared to much lesser interactions with promoter regions including single Sp1 binding sites (24). The transactivation of the TGF-β type II receptor promoter required the presence of closely placed GC rich regions from -152 to -127 and -118 and -85 (24). Thus it is very likely that the two closely placed CACCC sites, in proximity to the basal promoter elements, in the
0.63 kb iNOS promoter are sufficient for induction of the iNOS gene by KLF6. Our data suggest that CACCC sites located between 3.8 and 5.8 kb upstream and between 7.0 and 16.0 kb upstream in the iNOS promoter may play a role in KLF6 mediated regulation of the iNOS gene only in the absence of proximal (0.63kb) CACCC sites.

The fact that KLF is very rich in serines (30) and its transactivation domain contains serines as well as tyrosines (30), suggests that the activity and binding of KLF6 to the iNOS promoter could also be regulated by serine and tyrosine phosphorylation. There are several examples of transcription factors being regulated by their phosphorylation status. For example, the activation and intermolecular interactions of KLF1 (EKLF) are known to be dependent on its serine phosphorylation (43). Similarly, studies have indicated that two distinct phosphorylation events i.e. phosphorylation of tyrosine 701 and serine 727 are necessary for full activation of STAT1 by IFN-γ (44;45). Thus our observation that serine phosphorylated KLF6 binds to the iNOS promoter in resting, stimulated (PMA/A23187) and stressed (NaCN-treated) cells, and serine as well as tyrosine phosphorylated KLF6 binds to the iNOS promoter in NaCN-treated cells, indicates that differential phosphorylation of KLF6 could play a very important role in gene regulation. The precise mechanism of this interaction is currently under investigation.

Induction of iNOS by KLF6 may have important implications in the prevention of apoptosis, tissue-injury repair and cancer. Previous studies have addressed the protective role of NO in apoptosis. NO blocks apoptosis by multiple mechanisms. Firstly, NO inhibits caspases (46) and inhibiting IL1β -converting enzyme- like and cysteine protease protein-32-like proteases (47). Secondly, NO protects the mitochondria, lowers
cytochrome c release and inhibits calcium fluxes (48). Thirdly, NO prevents an increase in Bcl-2 and induces the expression of heat shock proteins such as Hsp-70 that have an anti-apoptotic role. NaCN triggers apoptosis of cells predominantly by inducing cytotoxic hypoxia by inhibiting cytochrome c oxidase, the terminal enzyme of the respiratory chain and by causing activation of voltage-sensitive calcium channels and calcium fluxes (49). In NaCN treated cells, we observed increased binding of KLF6 to the iNOS promoter (Fig. 5B), in addition to increased levels of iNOS protein and concomitant NO production (Fig. 7B and C). In a similar study, it has been shown that NO protected NaCN treated chick embryonic neurons from cyanide-induced apoptosis (42).

NO plays a role in wound healing and tissue repair. In several studies including colon anastomosis, bone fracture and cutaneous wound healing, the reparative role of NO through upregulation of iNOS has been well demonstrated (10;50;51). Similarly, KLF6 plays a direct anti-apoptotic role in conditions of acute injury. KLF6 was found to be responsible for healing of acutely injured hepatic stellate cells (18) and aortic endothelial cells (52). Our ChIP data (Fig. 5B) demonstrate that in heat shock and NaCN treated cells there is a strong binding of KLF6 to the iNOS promoter, thereby suggesting that KLF6 can orchestrate its anti-apoptotic and protective effects by upregulating NO through iNOS (Fig. 6).

Recently, KLF6 was demonstrated to be a tumor suppressor gene that was found mutated in 77% of prostate cancer patients, and it was shown to act in a p53 independent manner through the p21 (Waf1/Cip1) pathway (25). The tumor suppressive role of iNOS is also well documented (13;15;16). NO production by iNOS through its transcriptional upregulation
by KLF6 of could serve as another mechanism for the anti-tumor effect of KLF6.

In conclusion, our findings provide evidence that KLF6 binds to the human iNOS promoter and regulates its expression in conditions of cell stress, injury and stimulation, with possible implications in the treatment of organ injury and cancer.

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REFERENCES


FIGURE LEGENDS

FIG. 1. **KLF6 induces iNOS expression in Cos-7 cells.**

A. Schematic diagram of the 16 kb full length Human iNOS promoter. The positions of KLF6 binding site, CACCC, identified in the 8 kb sequenced region have been indicated with arrow marks. The numbers indicate the position of the beginning nucleotide of the CACCC site according to Spitsin et al. (41).

B. 16 kb iNOS-luciferase constructs was transfected with KLF6, p50 or p65 subunits of NF-κB into Cos-7 cells and the luciferase activity was determined. Data are representative of three independent experiments performed in triplicate.
FIG. 2. The effect of KLF6 on iNOS promoter activity in cells transfected with various deletion constructs of iNOS promoter. Schematic view of the iNOS promoter indicating the restriction enzymes used to generate various deletion constructs with luciferase. A and B. Cos-7 and Jurkat cells were transfected with KLF6 expression vector (pXCPBP, 1.5 µg) and iNOS promoter deletion constructs (1.5 µg) or with the empty KLF6 vector (1.5 µg) and iNOS promoter deletion constructs (1.5 µg) along with (0.5 µg) β-galactosidase expressing vector (as transfection efficiency control). pXP1 and pXP2 are empty vectors of iNOS transfected with KLF6 or the empty KLF6 vector (pX). The luciferase activity was determined after 24-48 h post-transfection. The effect of KLF6 on the respective constructs is indicated as fold induction of luciferase activity over that of the control vector pX. C. Cos-7 cells were transfected with KLF6 expression vector (pXCPBP, 1.5 µg) and thymidine kinase-luciferase-iNOS promoter deletion constructs or with the empty KLF6 vector (1.5 µg) and iNOS promoter deletion constructs (1.5 µg) along with (0.5 µg) β-galactosidase expressing vector (as transfection efficiency control). The luciferase activity was determined after 24-48 h post-transfection. The effect of KLF6 on the respective constructs is indicated as percent increase of luciferase activity over that of the control vector pX. Data are representative of five independent experiments performed in triplicate.

FIG. 3. CACCC motifs in the 0.63 kb iNOS promoter are necessary for activation by KLF6. The two CACCC sites in the 0.63 kb iNOS promoter were mutated to AAAAAA by PCR based mutagenesis. A and B. The wild type and mutant reporter genes (1.5 µg)
were cotransfected with either empty vector (pX, 1.5 µg) or KLF6 expression vector (pXCPBP, 1.5 µg) in the presence of β-galactosidase expressing vector (0.5 µg) in either Cos-7 or Jurkat cells. Luciferase activity was measured 24-48 h post transfection and normalized to β-galactosidase levels. Data are presented as fold induction over that of the empty vector. Data are representative of three similar experiments performed in triplicate. D, distal; P, proximal; N, no mutations; *, mutation.

FIG. 4. **KLF6 binds to oligonucleotides containing the two CACCC motifs in 0.63 kb iNOS promoter.** Nuclear extracts were prepared from KLF6 transfected Cos-7 cells and incubated with labeled oligonucleotides from iNOS promoter followed by EMSA analysis as described in materials and methods. A. EMSA was done by using the oligonucleotide defining the proximal CACCC site in Lane 1; a non-specific antibody (anti-KLF4) was added in Lane 2; hundred-fold excess of specific unlabeled competitor in was added in Lane 3 and Lane 4 depicts a super-shifted band with the anti-KLF6 mAb. 2 µg antibody was added to the oligonucleotide and nuclear extract. (f)- free probe B. EMSA experiments were repeated as in panel A with an oligonucleotide defining the distal CACCC site.

FIG. 5. *In vivo* binding of KLF6 to the iNOS promoter in Cos-7, Jurkat and T lymphocytes. Cos-7 cells (10 x 10^6/per sample) were treated as per protocol, fixed by formalin, washed, lysed and sonicated. The DNA-protein complexes were immunoprecipitated with the anti-KLF6 antibody and extracted by protein A agarose
beads. The DNA was purified and amplified with primers flanking the iNOS promoter.

A. The nucleotide sequence of iNOS promoter in the 0.63 kb fragment depicting the primers used for PCR of the ChIP DNA. Primer sequences are underlined and shown by arrows. The two KLF6 binding CACCC sites are shown in bold letters. The nucleotides in upper case represent the iNOS promoter starting from the origin of transcription initiation. B. ChIP analysis of the iNOS promoter in KLF6 transfected Cos-7 cells and non-transfected Cos-7, Jurkat and T cells after treatment with sodium cyanide. Lane 1, the PCR product from the input (positive control) DNA. Lanes 2 and 3, KLF6 transfected Cos-7 cells, Lanes 4 and 5, 6 and 7, 8 and 9 depict non-transfected Cos-7, Jurkat and primary T cells respectively. Cells in lanes 3, 5, 7, and 9 were treated with NaCN. PCR products were resolved on a 2 % agarose gel. C. ChIP analysis of control and treated Jurkat cells, primary T cells and Cos-7 cells. D. ChIP analysis of serine and tyrosine phosphorylated KLF6 in PMA/A23187-, sodium cyanide-treated and control primary T cells. Data are representative of two similar experiments.

FIG. 6. **KLF6 transfection upregulates iNOS mRNA, protein and NO in Cos-7 cells.** A. Cos-7 cells were transfected with KLF6 and incubated for various time periods. Total RNA isolated, cDNA synthesized and KLF6, iNOS and GAPDH (control) mRNA were amplified by real-time quantitative PCR. B and C. KLF6, iNOS, β-Actin protein levels were estimated by western blot. D. NO production was estimated by assay for NO metabolites Nitrite+Nitrate from the same samples. Data are representative of three
similar experiments.

FIG. 7. NaCN exposure upregulates KLF6 and iNOS mRNA, protein and NO in Cos-7 cells. A. Cos-7 cells were exposed to 20mM NaCN for 4 h, washed and incubated for various time periods in normal medium. Total RNA isolated, cDNA synthesized and KLF6, iNOS and GAPDH (control) mRNA were amplified by real-time quantitative PCR. B. KLF6, iNOS, β-Actin protein levels were estimated by western blot. C. NO production was estimated by assay for NO metabolites Nitrite+Nitrate from the same samples. Data are representative of three similar experiments.
Fig. 1

A

CACCC sites

16 kb

-7480 -5927 -5470 -5411 -5249 -4192 -3883 -2740 -265 -168

B

Luciferase activity

(Fold increase)

-7480 -5927 -5470 -5411 -5249 -4192 -3883 -2740 -265 -168

KLF6
p65
p50
Fig. 4

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<th>A</th>
<th>B</th>
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<td>KLF6 Ab</td>
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<td>-  -  + -</td>
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<tr>
<td>Non-specific Ab</td>
<td>+  -  - -</td>
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KLF6 supershift-

Shift band-

Free probe-

1  2  3  4  1  2  3  4
Fig. 7

A

Relative KLF6 mRNA expression (fold induction)

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<td>β-Actin</td>
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B

Time (h) 0 12 24 48 72

KLF6 iNOS β-Actin

C

Nitrite + Nitrate (μM)

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Transcriptional activation of the human inducible nitric oxide synthase (iNOS) promoter by Kruppel-like factor 6
Vishal G. Warke, Madhusoodana P. Nambiar, Sandeep Krishnan, Klaus Tenbrock, David A. Geller, Nicolas P. Koritschoner, James L. Atkins, Donna L. Farber and George C. Tsokos

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