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Inhibition of Inducible Nitric-Oxide Synthase Protects Human T Cells from Hypoxia-Induced Apoptosis

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ABSTRACT

Sodium cyanide-induced chemical hypoxia triggers a series of biochemical alterations leading to apoptosis in many cell types, including T cells. It is known that chemical hypoxia promotes inducible nitric-oxide synthase (iNOS) gene transcription by activating its transcription factors. To determine whether iNOS and NO production are responsible for chemical hypoxia-induced apoptosis, we exposed human Jurkat T cells to sodium cyanide in the presence or absence of iNOS inhibitors. We found that iNOS expression is necessary for hypoxia-induced lipid peroxidation and leukotriene B_4 generation. The inhibition of iNOS limited T-cell apoptosis by decreasing the activity of caspase-3 without affecting the expression of Fas/Apo-1/CD95 on the surface membrane of T cells. These data suggest iNOS-mediated NO produced endogenously in the T cell alters overall T-cell function and results in apoptosis. Proper control of iNOS expressed in the T cell may represent a useful approach to immunomodulation.

The low oxygen cellular environment characteristic of maladies such as stroke, heart attack, anemia, ischemia, and hemorrhage has been shown to lead to cell injury both in vivo and in vitro. Hypoxia promotes NO production that leads to polymorphonuclear neutrophil (PMN) infiltration of tissues and leukotriene B_4 (LTB4) generation (Stojadinovic et al., 1995). It has been reported that hypoxia stimulates the inflammatory response by up-regulating the early response gene, inducible nitric-oxide synthase (iNOS), which leads to the rapid overproduction of NO. Excessive amounts of NO seem to cause damage when it combines with superoxide to form peroxynitrite ($ONOO^-$), a powerful oxidant that can be cleaved into highly reactive free radicals such as OH^- and NO_2^+ (Yasmin et al., 1997). It is the overproduction of NO, not NO per se, that leads to cellular damage. In fact, NO at appropriate levels is critical for normal tissue homeostasis. For example, maintenance of vascular integrity requires NO (Drexler, 1999), but abnormally high levels lead to vascular leakage (Ward et al., 2000).

Deletion of the iNOS gene in mice or treatment of mice with L-N^6-(1-iminoethyl)-lysine (L-NIL), a selective inhibitor of iNOs, prevents hypoxia-induced injury (Hierholzer et al., 1998). Other agents such as 5-androstenediol and geldanamycin (Kiang et al., 2007a,b) that inhibit iNOS and therefore the overproduction of NO have also been shown to limit hypoxia-induced tissue damage. Treatment with N^ω-nitro-L-arginine (LNNA), an irreversible inhibitor of constitutive nitric oxide synthase (cNOS) and reversible iNOS inhibitor, results in significant reduction of local tissue damage, PMN infiltration, and LTB4 generation (Chabrier et al., 1999a,b). Stojadinovic et al. (1995) observed that hypoxia resulting from ischemia increases generation of PGE2 and LTB4, and PGE2 and LTB4 are proinflammatory mediators that can lead to multiple organ dysfunction and failure (Kiang and Tsen, 2006). Nonetheless, the relation between iNOS and LTB4 as well as PGE2 was not clear.

Our laboratory has previously investigated various stress response genes involved in hypoxia, including iNOS, 70-kDa inducible heat shock protein (HSP-70i), hypoxia-inducible factor (HIF)-1α, Bcl-2, and p53. In human intestinal epithelial T84 cells and human Jurkat T cells, NaCN-induced hypoxia increases iNOS and HSP-70 mRNA; whereas p53 is induced only in T84 cells and Bcl-2 only in Jurkat T cells

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ABBREVIATIONS: PMN, polymorphonuclear neutrophil; LT, leukotriene; iNOS, inducible nitric-oxide synthase; $ONOO^-$, peroxynitrite; L-NIL, L-N^6-(1-iminoethyl)-lysine; LNNA, N^ω-nitro-L-arginine; cNOS, constitutive nitric oxide synthase; PG, prostaglandin; HSP, heat shock protein; HSP-70i, 70-kDa inducible heat shock protein; HIF, hypoxia-inducible factor; siRNA, small interfering RNA; PBS, phosphate-buffered saline; i, inducible; MDA, malondialdehyde; ANOVA, analysis of variance; CON, control; HX, hypoxia; lipo, Lipofectamine reagent.

(Kiang et al., 2003). LNNA treatment blocks iNOS, Bcl-2, and HSP-70 mRNA, but it increases p53 mRNA (Kiang et al., 2003). We showed in mice that hemorrhage increases iNOS 7 h before HSP-70 and HIF-1α (Kiang et al., 2004), and we hypothesized that the degree of severity of hypoxia correlates with the amount of iNOS mRNA expression.

NO has been suggested to mediate apoptosis of T cells and to regulate peripheral responses. In experimental allergic encephalomyelitis, NO limits inflammatory demyelination by eliminating autoreactive and bystander T cells through apoptotic cell death (Zettl et al., 1997). Interestingly, constitutive up-regulation of iNOS in animals that lack glucocorticoid receptor is responsible for immunosuppression and resistance to the development of experimental allergic encephalomyelitis (Marchetti et al., 2002). In the thymus, NO generated in association with T-cell receptor stimulation functions to induce deletion of double-positive thymocytes, especially when their T-cell receptor is stimulated (Tai et al., 1997).

Although hypoxia promotes apoptosis in various cell types, the mechanisms involved are not known. Since hypoxia increases the expression of iNOS, we sought to determine whether hypoxia mediates apoptosis by increasing the iNOS-mediated production of NO and lipid peroxidation. We also asked whether levels of PGE2, LTB4, HSP-70, and HIF-1α are regulated by iNOS in hypoxic cells. Using sodium cyanide to induce chemical hypoxia in human Jurkat T cells, we show that the increased iNOS is responsible for altered NO, LTB4, lipid peroxidation, caspase-3 activation, and apoptosis.

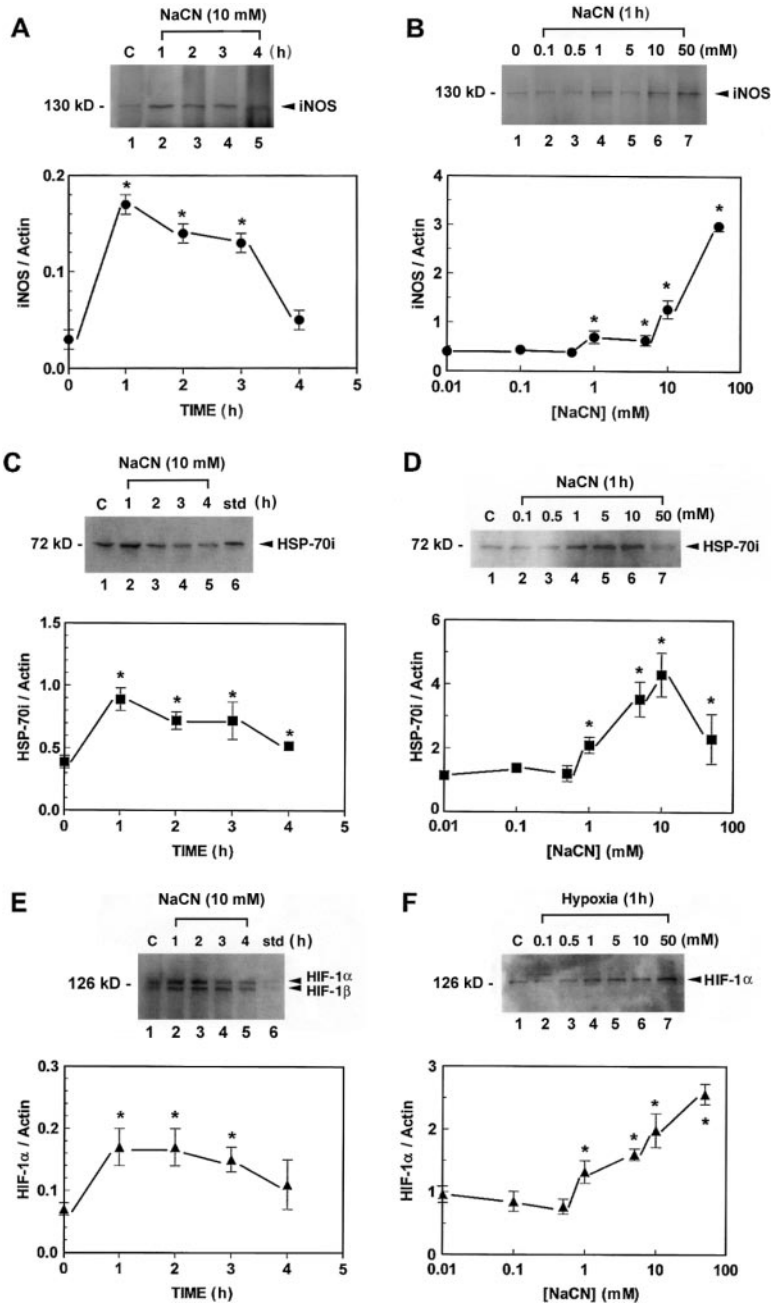

Fig. 1. Chemical hypoxia up-regulates protein expression of iNOS, HSP-70i, and HIF-1α. Human Jurkat T cells were treated with 10 mM NaCN for 1, 2, 3, or 4 h followed by 16-h recovery. In parallel experiment, cells were treated with 0.1, 0.5, 1, 5, 10, or 50 mM NaCN for 1 h followed by 16-h recovery. Cell lysates were immunoblotted with antibodies against iNOS (A and B), HSP-70i (C and D), and HIF-1α (E and F). Protein was quantitated densitometrically and normalized with actin. *, $P < 0.05$ versus control groups, determined by one-way ANOVA and Studentized range test.

Materials and Methods

Cell Culture. Human Jurkat T cells (American Type Cell Collection, Manassas, VA) were grown in 75-cm^2 tissue culture flasks (Costar; Corning Life Sciences, Acton, MA) containing RPMI 1640 medium supplemented with 0.03% glutamine, 4.5 g/l glucose, 25 mM HEPES, 10% fetal bovine serum, 50 μg/ml penicillin, and 50 U/ml streptomycin (Invitrogen, Carlsbad, CA). Cells were incubated in a 5% CO_2 atmosphere at 37°C and fed every 3 to 4 days.

Chemicals. Chemicals used in this study were albumin, NaCN, N^{ω}-nitro-L-arginine (CAS Registry no. 2140-70-4), and L-N^6-(1-iminoethyl)-lysine (CAS Registry no. 159190-45-1); Sigma-Aldrich, St. Louis, MO).

Hypoxia. Hypoxia was induced in cells by treatment with NaCN. Chemically induced hypoxia occurs more rapidly than that induced by lowering oxygen levels in the atmosphere of the cell culture incubator, but both methods cause increases in intracellular calcium concentrations and inositol 1,4,5-trisphosphate levels; therefore, they seem to work through a similar mechanism (Kiang and Smallridge, 1994; Kiang et al., 1996). Optimal conditions for hypoxia induction were determined by treating Jurkat T cells with various concentrations of NaCN (0.01–100 mM) in cell culture medium for various times (1–4 h) before assessment.

iNOS Gene Construct. cDNA of human iNOS was obtained from Dr. N. Tony Eissa (Baylor College of Medicine, Houston, TX). A 3362-base pair coding sequence with HindIII and XhoI restriction sites at each terminus of human iNOS gene was subcloned from this full length of human iNOS cDNA. For polymerase chain reaction, we used the forward and reverse primers, 5′-CT AAG CTT GTC ATG GCC TGT CCT TGG AAA TTT CTG TTC-3′ and 5′-GAC TCG AGC TCA GAG CGC TGA CAT CTC CAG GCT-3′, respectively. After digestion and purification of the polymerase chain reaction amplification product, the expression cDNA sequence was used for insertion into the vector. The vector used in this study was pcDNA3.1 (Invitrogen). The expression cDNA of iNOS gene was inserted between the HindIII and XhoI sites of pcDNA 3.1 vector. The expression construct then was sequenced to confirm its correct sequence and open reading frame.

DNA Transient Transfection. Jurkat T cells (1×10^7) in 0.5 ml of fresh cultured medium were mixed gently with 10 μg of iNOS expression plasmid. An equivalent number of cells was mixed with blank pcDNA 3.1 vector as a control group. The mixtures were then transferred to Gene Pulser cuvettes (Bio-Rad Laboratories, Hercules, CA), and electroporation was conducted at 250 V, 925 microfarads for 15 s. The electroporated cells were then combined with 2 ml of fresh culture medium and placed in one well of a six-well plate for 24 h to allow transfection to occur.

iNOS siRNA Transfection. RNA interference technology was used to decrease iNOS protein levels. Two designed pairs of oligoduplexes targeted against iNOS were purchased from QIAGEN (Valencia, CA). The target sequences of the oligoduplexes are the NOS-S sense strand, 5′-ACAACAGGAACCUACCAGCUTT-3′, and NOS-AS antisense strand, 5′-AGCUGGUAGGUUCCUGUUGUTT-3′, respectively. A nonspecific oligoduplex (nonsilencing control, targeting AAU UCU CCG AAC GUG UCA CGU) at the same final concentrations as the iNOS RNA duplexes was used as a negative control. To maximize siRNA silencing potential, siRNAs were heated for 1 min at 90°C, followed by 60 min at 37°C before the siRNA transfection. Before transfection, cells were grown in fresh medium without antibiotics for 24 h. Cells were then transferred to six-well plates (2×10^6 cells/well). Transient transfection with siRNA duplexes at 50 nM was performed using the Lipofectamine reagent (Invitrogen). The 50 nM concentration used in these experiments was determined to be the highest effective concentration not leading to an elevation of NO in controls. Twenty-four hours after transfection, cells were either exposed to 10 mM NaCN or vehicle (saline) for 1 h to allow hypoxia to occur. Cells were allowed to recover in the incubator for 23 h before harvesting and analysis.

Western Blots. To investigate synthesis of iNOS, HSP-70i, and HIF-1α proteins after chemical hypoxia, cells were treated with NaCN at various concentrations and times, and then they were returned to 37°C for 16 h. After incubation, cells were removed from the culture flask and pelleted by centrifugation at 750*g* for 10 min. The pelleted cells were lysed in Tris buffer, pH 6.8, containing 1% SDS and 1% 2-mercaptoethanol. Aliquots containing 20 μg of protein were resolved on SDS-polyacrylamide slab gels (precast 10% gel; Novex, San Diego, CA). After electrophoresis, proteins were blotted onto a nitrocellulose membrane (type NC, 0.45 μm; Whatman Schleicher and Schuell, Keene, NH), using a Novex blotting apparatus and the manufacturer's protocol. After blocking the nitrocellulose by incubation in phosphate-buffered saline (PBS) containing 5% nonfat dried milk for 90 min at room temperature, the blot was incubated for 60 min at room temperature with monoclonal antibodies directed against actin, iNOs, HSP-70i (Santa Cruz Biotechnology, Inc., Santa

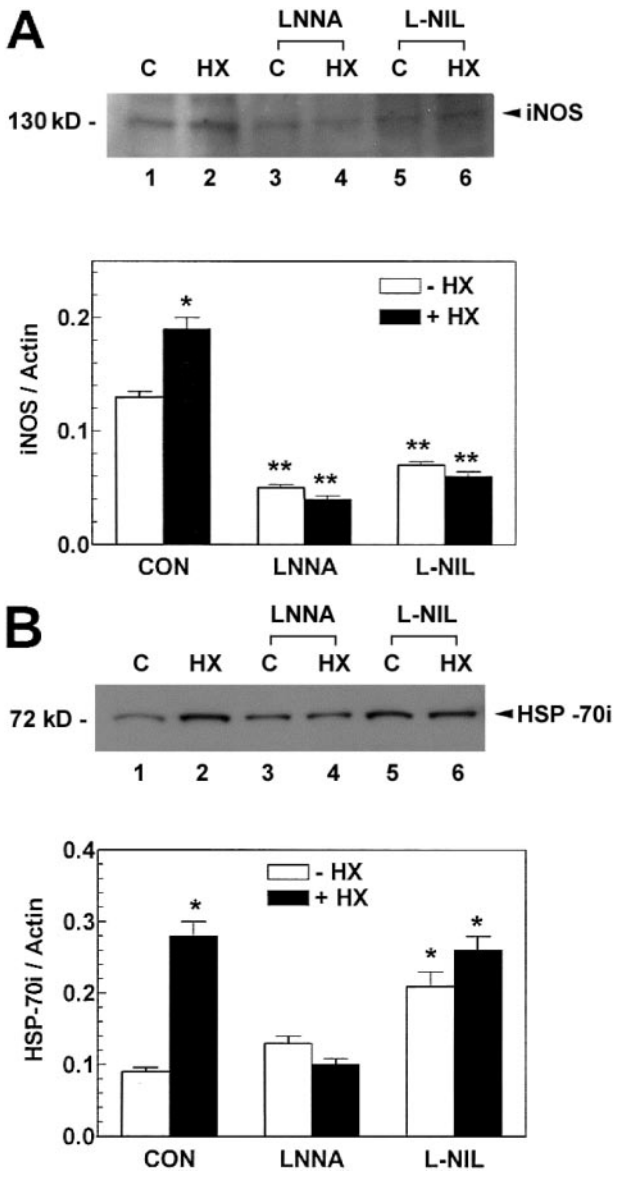

Fig. 2. Effect of iNOS inhibitors on chemical hypoxia-induced increase in iNOS and HSP-70i. Human Jurkat T cells were treated with LNNA (100 μM; 15 min) or L-NIL (100 μM; 15 min) before 1-h treatment with 10 mM NaCN and 16-h recovery. Cell lysates were immunoblotted with antibodies against iNOS (A) and HSP-70i (B). Protein was quantitated densitometrically and normalized with actin. *, $P < 0.05$ versus control groups; **, $P < 0.05$ versus control and HX, determined by two-way ANOVA. CON, control; HX, hypoxia.

Cruz, CA), or HIF-1α (BD Transduction Laboratories, Lexington, KY) at a concentration of 1 μg/ml in PBS and 5% bovine serum albumin. The blot was then washed three times (10 min each) in Tris-buffered saline and 0.1% Tween 20 before incubating the blot for 60 min at room temperature with a 1000 times dilution of species-specific IgG peroxidase conjugate (Santa Cruz Biotechnology, Inc.) in PBS and 1% gelatin. The blot was washed six times (5 min each) in Tris-buffered saline and 0.1% Tween 20 before detection of peroxidase activity using the Enhanced Chemiluminescence Plus (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Actin levels were not altered by NaCN-induced hypoxia (Kiang et al., 2003); therefore, we used actin as a control for protein loading. Protein bands of interest were quantitated densitometrically and normalized to actin.

Nitric Oxide Measurements. NO production was measured under acidic conditions as nitrite, using the Griess Reagent System with sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (Promega, Madison, WI).

LTB4 and PGE2 Measurements. LTB4 levels were determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The assay has <0.01% cross-reactivity with LTC4, LTD4, LTE4, and LTF4. PGE2 levels were determined using an enzyme immuno-

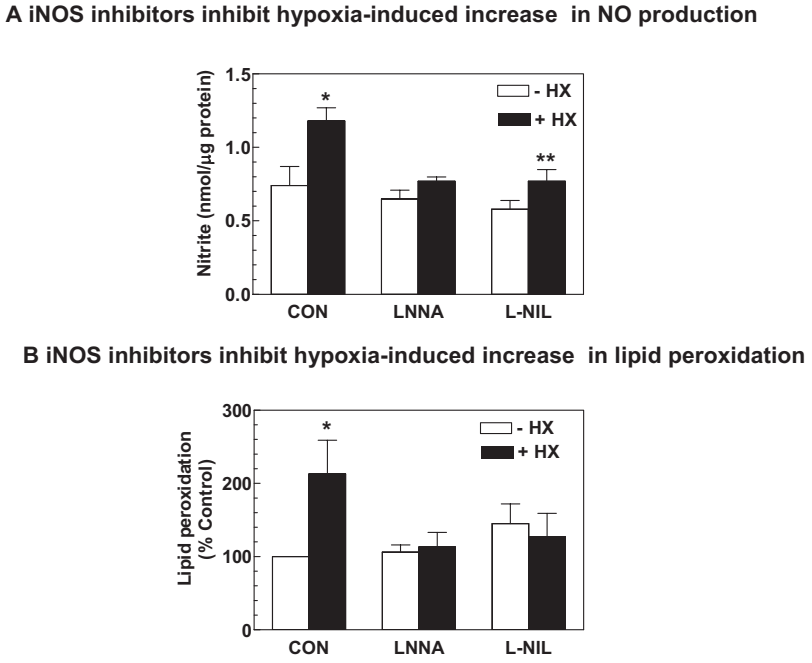

Fig. 3. Inhibition of iNOS blocks chemical hypoxia-induced increase in NO production and lipid peroxidation. Human Jurkat T cells were treated with LNNA (100 μM; 15 min) or L-NIL (100 μM; 15 min) before 1-h treatment with 10 mM NaCN and 16-h recovery. NO production was measured in cell lysates as described under *Materials and Methods*. *, $P < 0.05$ versus control without NaCN, L-NNA, and L-NIL; **, $P < 0.05$ versus control, L-NNA, and L-NIL without NaCN, determined by two-way ANOVA and Studentized range test.

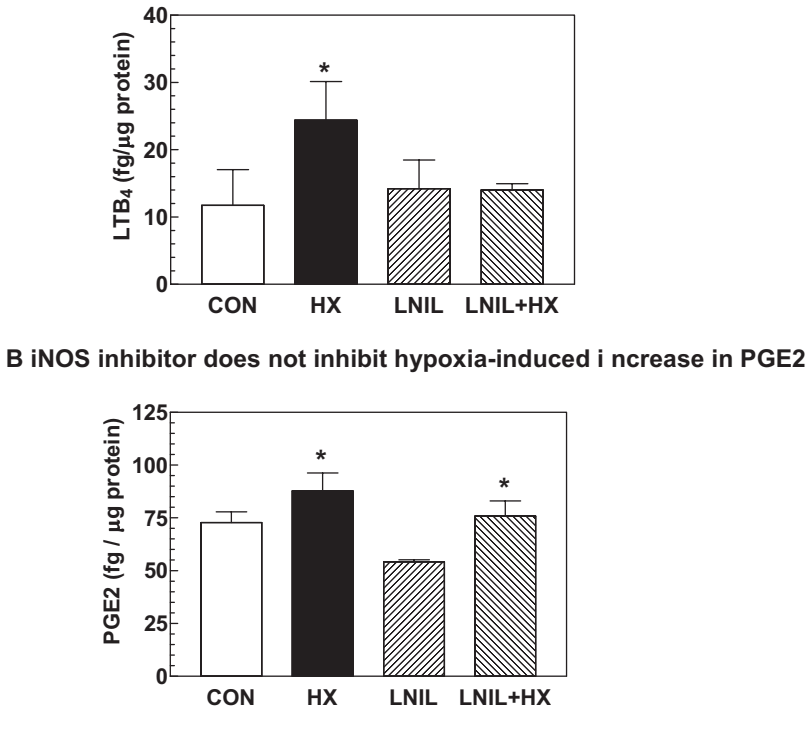

Fig. 4. Inhibition of iNOS blocks chemical hypoxia-induced increase in LTB4 production. Human Jurkat T cells were treated with LNNA (100 μM; 15 min) or L-NIL (100 μM; 15 min) before 1-h treatment with 10 mM NaCN (HX) and 16-h recovery. A, LTB4 levels were measured in cell lysates as described under *Materials and Methods*. *, $P < 0.05$ versus control, L-NIL alone, and L-NIL + HX, determined by χ^2 test. B, PGE2 levels were measured in cell lysates as described under *Materials and Methods*. *, $P < 0.05$ versus control and L-NIL alone, determined by χ^2 test.

assay kit (Cayman Chemical). The assay has <9.2% cross-reactivity with 15-keto PGE2, 5% cross-reactivity with PGE1, and <0.01% cross-reactivity with other metabolites.

Lipid Peroxidation Measurements. Malondialdehyde (MDA), a lipid peroxidation end product, was measured colorimetrically using a commercial lipid peroxidation assay kit (Calbiochem, San Diego, CA).

Flow Cytometry. Expression of surface Fas/Apo-1/CD95 was examined in mildly fixed Jurkat cells by flow cytometry. Briefly, 10^6 cells were washed in staining buffer (PBS + 1% fetal bovine serum) and fixed for 10 min with 0.6% paraformaldehyde on ice before washing again with staining buffer. Fixed cells were incubated for 20 min on ice with 1.5 μg of human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) to block nonspecific binding sites, and then they were incubated with 1 μg of phycoerythrin-conjugated anti-CD95 (BD Biosciences Pharmingen, San Diego, CA) or isotype mouse IgG_1 (κ monoclonal antibody control; BD Biosciences, Franklin Lakes, NJ) for 20 min on ice. Cells were washed twice with staining buffer, resuspended in 100 μl of staining buffer and 100 μl of 3% paraformaldehyde, and kept in the dark. Antibody-labeled cells were analyzed using a FACScan flow cytometer (BD Biosciences), and the data were analyzed using CellQuest software (BD Biosciences).

Caspase-3. Caspase-3 cellular activity was measured using a commercial kit (BIOMOL Research Laboratories, Plymouth Meeting, PA). The data are presented in picomoles of *p*-nitroaniline per minute per microgram of protein.

Apoptosis. Apoptosis was measured using the Magic Red Caspase Activity Detection kit (MP Biomedicals, Irvine, CA). After specific treatments, 300 μl of cell suspension (0.3×10^6) was transferred into a 12- × 75-mm polypropylene tube. Ten microliters of the 31× working dilution DEVD-Magic Red fluorophore solution was added directly to each 300-μl cell suspension. The mixture was incubated for 1 h at 37°C in air containing 5% CO_2, with swilling of the tubes every 20 min. One hour after incubation, cells were counterstained by adding 1.6 μl of Hoechst stain to the 300-μl suspension labeled with DEVD-Magic Red fluorophore for 15 min. Twenty microliters of the cell suspension was placed onto a microscope slide, and the slide was covered with a coverslip. Slides were analyzed with a laser scanning confocal fluorescence microscope (1 × 70; Olympus, Lake Success, NY) with Lasersharp2000 software (Bio-Rad Laboratories, Richmond, CA).

Statistical Analysis. All data are expressed as the mean ± S.E.M. One-way ANOVA, two-way ANOVA, Studentized range test, and χ^2 test were used for comparison of groups, with 5% as a significant level.

Results

Chemical Hypoxia Increases Expression of iNOS, HSP-70i, and HIF-1α Protein. When hypoxia was induced by incubating Jurkat T cells with 10 mM NaCN for various times (1, 2, 3, or 4 h) followed by a 16-h recovery at 37°C, protein levels for iNOS, HSP-70i, and HIF-1α (a key regulator of the cellular response to hypoxia) increased (Fig. 1, A, C, and E). Similar treatment of Jurkat T cells failed to increase the expression of cNOS protein (data not shown). The maximal increase was observed after 1 h of NaCN treatment (Fig. 1, A, C, and E). When cells were treated for 1 h with increasing concentrations of NaCN (0.1–50 mM) and then incubated for 16 h, expression of iNOS, HSP-70i, and HIF-1α proteins increased in a concentration-dependent manner (Fig. 1, B, D, and F), although with somewhat different concentration dependencies. Cells remained viable (trypan blue exclusion assay) for the duration of all treatments except with 50 mM NaCN (data not shown). Based on these results, a 1-h treatment using 10 mM NaCN was considered optimal, and these conditions used for all subsequent experiments.

iNOS Inhibitors Block Hypoxia-Induced Increase in iNOS. We previously observed that the NOS inhibitor LNNA (an irreversible inhibitor of cNOS and reversible inhibitor of iNOS) inhibits HSP-70i protein expression after heat stress, but L-NIL (an irreversible inhibitor of iNOS) does not (Kiang et al., 2002). In this study, we tested the effect of LNNA and L-NIL on the expression of iNOS, iHSP-70i, and HIF-1α protein in hypoxic cells. We treated cells with 100 μM LNNA or L-NIL for 15 min before a 1-h exposure to 10 mM NaCN, and then we allowed the cells to recover for 16 h before measuring protein expression. Figure 2 shows that both LNNA and L-NIL inhibited expression of iNOS protein (Fig. 2A); however, only LNNA blocked the hypoxia-induced expression of HSP-70i protein, whereas L-NIL alone induced significant overexpression of HSP-70i protein, and hypoxia was unable to induce additional increase in HSP-70i (Fig. 2B). HIF-1α protein expression was not affected by either of the iNOS inhibitors (data not shown).

iNOS Inhibitors Block Hypoxia-Induced Increase in NO Production. It has been previously shown that increased iNOS protein expression normally results in increased NO production. Since we had found that NaCN-induced hypoxia increased iNOS mRNA (Kiang et al., 2003) and iNOS protein (Fig. 1), we wanted to confirm that NaCN treatment resulted in increased NO production and to test whether the iNOS inhibitors LNNA or L-NIL affected its production. Treatment with LNNA or L-NIL before hypoxia inhibited NO production compared with NO levels measured in cells treated with hypoxia alone (Fig. 3A), confirming that an inhibition of iNOS blocks the NO production normally stimulated by hypoxia. It is worth noting that LNNA completely inhibited the hypoxia-induced increase in NO production, whereas L-NIL treatment led only to a partial inhibition, suggesting that iNOS and cNOS both contribute to the hypoxia-induced increase in NO.

iNOS Inhibitors Block Hypoxia-Induced Increase in Lipid Peroxidation. Excess NO production can lead to free radical-mediated tissue injury. NO can combine with superoxide to form $ONOO^-$, and peroxynitrite can in turn break down to free radicals such as OH^- and NO_2^+, which then react with tissue elements (Szabó and Thiemermann, 1994; Yasmin et al., 1997). Because we found hypoxia induces a significant elevation of NO production, we determined

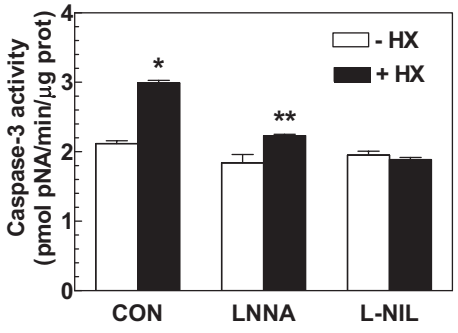

Fig. 5. Inhibition of iNOS blocks chemical hypoxia-induced increase in caspase-3 activity. Human Jurkat T cells were treated with LNNA (100 μM; 15 min) or L-NIL (100 μM; 15 min) before 1-h treatment with 10 mM NaCN and 16-h recovery. Caspase-3 activity was measured as described under *Materials and Methods*. *, $P < 0.05$ versus control without HX, L-NNA, and L-NIL; **, $P < 0.05$ versus control, LNNA without HX and L-NIL, determined by two-way ANOVA and Studentized range test.

whether free radical damage was occurring in the cell by measuring lipid peroxidation levels. Using an assay for MDA, a lipid peroxidation end product commonly used as an indicator of lipid peroxidation, we found that MDA increased in hypoxic cells. Pretreatment with LNNA or L-NIL completely blocked MDA production. LNNA treatment alone (no hypoxia) had no effect on baseline levels of MDA in cells; L-NIL treatment by itself caused a slight rise in baseline MDA levels (Fig. 3B).

Inhibition of iNOS Blocks Hypoxia-Induced Increase in LTB4 Production. Stojadinovic et al. (1995) reported that ischemia and reperfusion increase LTB4 and PGE2 levels in ileum, a process that is not associated with PMN infiltration to ileum. Similarly, our results indicate that LTB4 and PGE2 production increases in hypoxic cells. Treatment with L-NIL inhibited LTB4 (Fig. 4A) but not PGE2 (Fig. 4B), a result consistent with that found with rats subjected to ischemia and reperfusion (Stojadinovic et al., 1995).

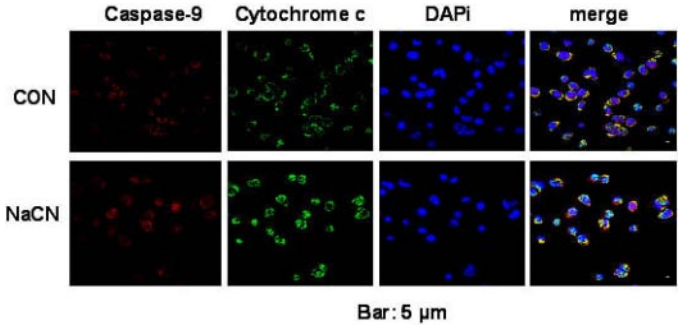

Fig. 6. Hypoxia leads to increase in caspase-9-cytochrome *c* complex. Jurkat T cells were treated with 10 mM NaCN for 1 h, and then they were allowed to recover for 16 h ($n = 3$). Treated cells were stained immunofluorescently with antibodies directed against caspase-9 (red), cytochrome *c* (green), or DAPI (blue). Merged images depict colocalization of caspase-9 and cytochrome *c* (yellow).

iNOS Inhibitors Block Hypoxia-Induced Increase in Caspase-3 Activity. We previously reported that heat stress significantly increases CD95 expression on the cell membrane of Jurkat T cells (Kiang et al., 2003). We hypothesized that hypoxic stress would also increase the expression of Fas/Apo-1/CD95 on the surface membrane of T cells. Accordingly, cells were made hypoxic by exposure to NaCN using our standard protocol. Treated cells were collected immediately and at selected times 12 to 120 h after chemical hypoxia, and Fas/Apo-1/CD95 expression levels and caspase-3 activity were determined. Hypoxia had no effect on Faso/Apo-1/CD95 expression on cell membrane of cells treated with or without LNNA (data not shown). Conversely, caspase-3 activity, which plays a pivotal role in the process of apoptosis (Jiang and Wang, 2004; Kiang and Tsen, 2006; Lakhani et al., 2006; Kiang et al., 2007), was elevated in hypoxic cells, and the increase was significantly inhibited by LNNA or L-NIL (Fig. 5).

Chemical Hypoxia Increases Apoptosome Formation. It is reported that caspase-3 activation is triggered by apoptosomes, a complex of caspase-9, cytochrome *c*, and apoptotic protease activating factor-1 (Jiang and Wang, 2004; Kiang and Tsen, 2006; Lakhani et al., 2006; Kiang et al., 2007). Using immunohistofluorescence, we found significant increases in caspase-9 and cytochrome *c* in hypoxic cells (Fig. 6).

Inhibition of iNOS Blocks Hypoxia-Induced Increase in Apoptosis. The above-mentioned data show that hypoxia induces increases in iNOS, HSP-70i, HIF-1α, NO, lipid per-

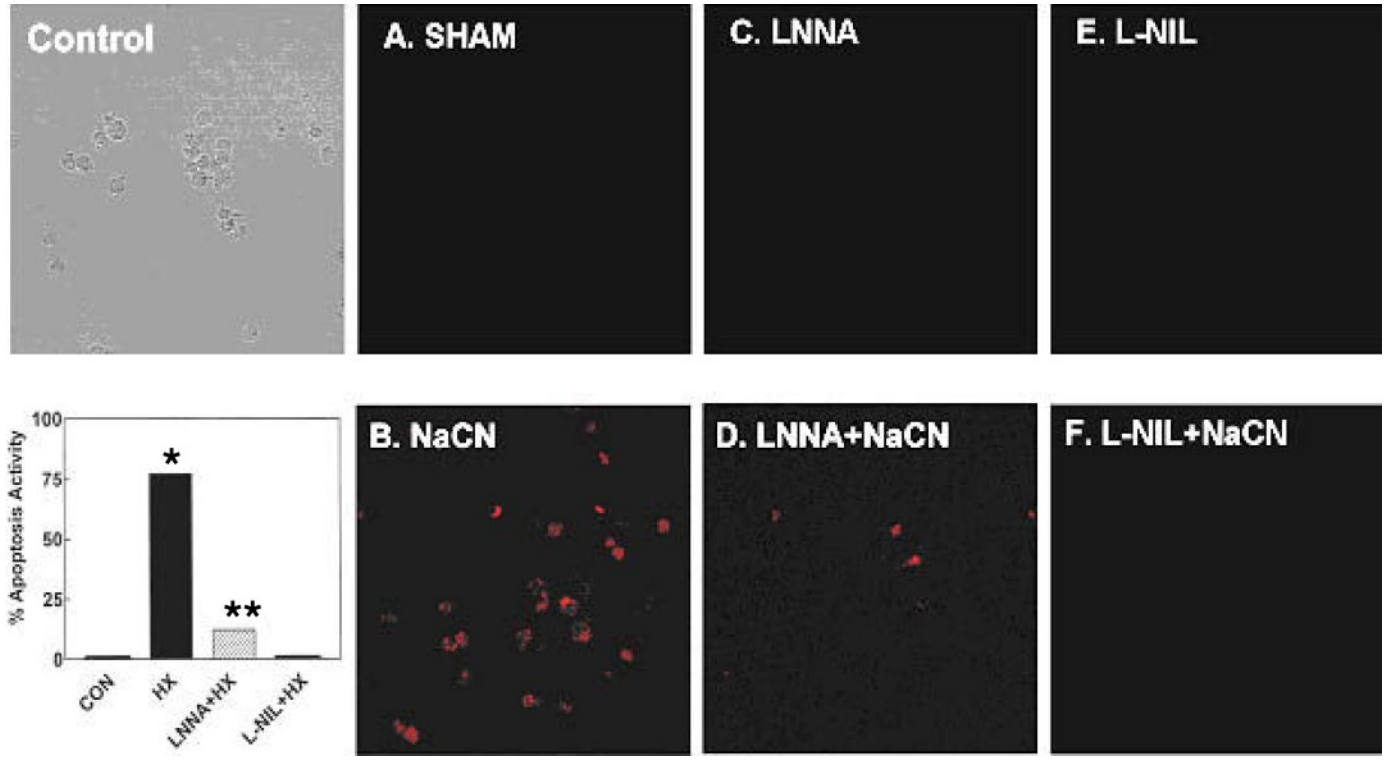

Fig. 7. Inhibition of iNOS blocks chemical hypoxia-induced increase in apoptosis. Human Jurkat T cells were treated with LNNA (100 μM; 15 min) or L-NIL (100 μM; 15 min) before 1-h treatment with 10 mM NaCN and 23-h recovery. Cells were prepared for microscopy as described under *Materials and Methods*. Data are expressed as percent apoptosis-positive cells (see bar graph inset). *, $P < 0.05$ versus untreated, LNNA, LNNA + NaCN, L-NIL, and L-NIL + NaCN; **, $P < 0.05$ versus CON, NaCN, and L-NIL + NaCN, determined by χ^2 test. CON, control; HX, NaCN.

oxidation, LTB4, PGE2, and caspase-3 activity. These increases, except for HSP-70i, HIF-1α, and PGE2 are all indicators of a cell responding to potentially lethal stress. Except for HSP-70i, HIF-1α, and PGE2, the increases were inhibited by inhibitors of iNOS. We asked whether the ability of an iNOS inhibitor to block or inhibit many of these stress indicators was sufficient to prevent cell death. To answer this question, cells were rendered hypoxic in the presence or absence of LNNA or L-NIL, and rates of apoptosis were measured 16 h later using the apoptosis indicator Magic Red. Figure 7 shows that 77% of cells were apoptotic after hypoxia treatment alone. Treatment with LNNA before hypoxia reduced the number of apoptotic cells to 12%, and L-NIL completely blocked apoptotic cell death after hypoxia. These results suggest that NaCN-induced hypoxia triggers apoptosis in cells, and both LNNA and L-NIL prevent it.

iNOS Is Correlated with Apoptosis-Associated Elements. To confirm the relationship between iNOS and apoptosis in hypoxic T cells, Jurkat T cells were transfected with iNOS gene to overexpress iNOS. In these iNOS-overexpressing cells, we observed increases in NO production, lipid peroxidation, LTB4 generation, and caspase-3 activation (Fig. 8). Transfection with vector alone significantly increased NO production (Fig. 8A) and caspase-3 activity (Fig. 8B), probably due to the transfection process.

To further verify the link between iNOS and apoptosis, Jurkat T cells were transfected with iNOS siRNA to silence iNOS expression. In these cells, hypoxia failed to increase NO production, lipid peroxidation, LTB4 generation, and caspase-3 activation, whereas in the cells transfected without oligo, hypoxia induced increases in all of four parameters (Fig. 9).

Rates of apoptosis were also measured in iNOS siRNA-treated T cells. Silencing iNOS expression before hypoxia reduced the number of apoptotic cells (Fig. 10). These results reinforce the view that NaCN-induced chemical hypoxia triggers apoptosis mediated by the iNOS pathway.

Discussion

This report presents evidence that NaCN-induced chemical hypoxia triggers increases iNOS protein expression, NO production, lipid peroxidation, LTB4 and PGE2 production, expression of HSP-70i and HIF-1α proteins, caspase-3 activation, and apoptosis in human Jurkat T cells. We show that iNOS plays a primary role in mediating the chemical hypoxia-induced increases in NO production, lipid peroxidation, LTB4 generation, caspase-3 activation, and apoptosis

We cannot conclude that iNOS alone is responsible for the hypoxia-induced increase in NO production. Our experiments suggest that cNOS-derived NO production apparently still occurs; whereas treatment with LNNA (an irreversible inhibitor of cNOS and a reversible inhibitor of iNOS) completely inhibits the hypoxia-induced increase in NO produc-

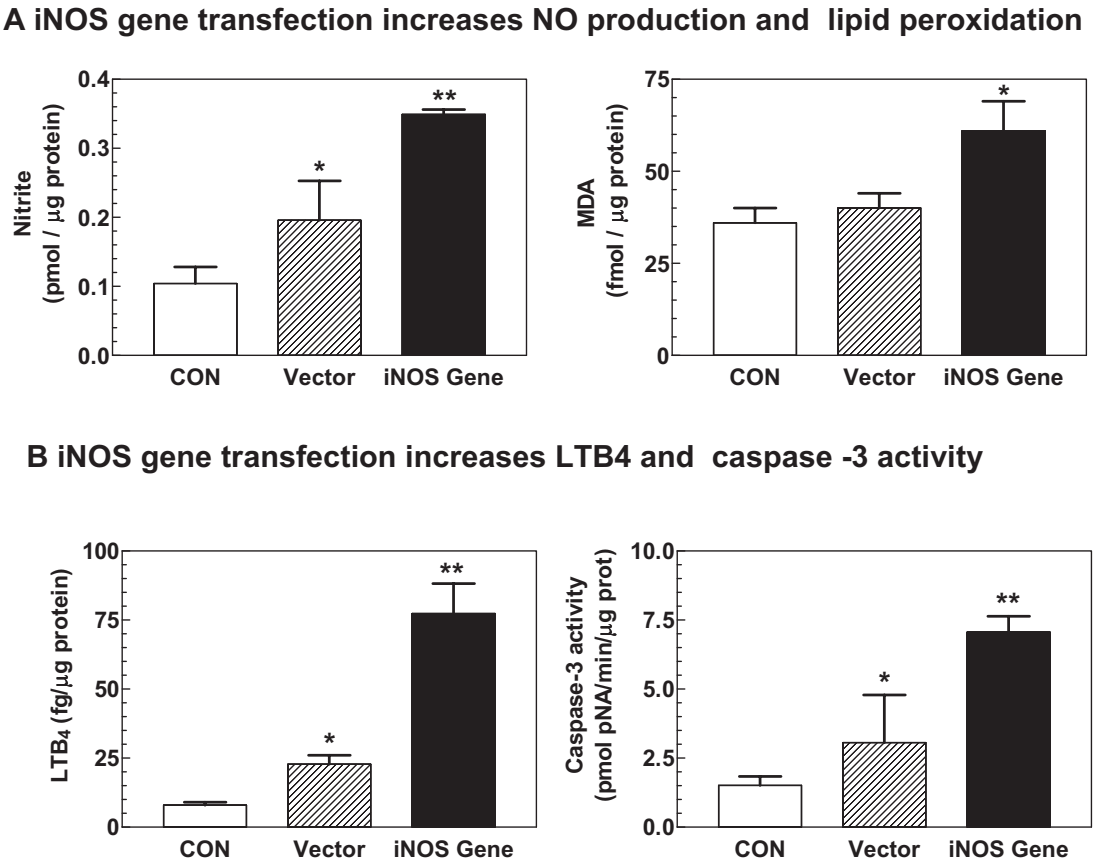

Fig. 8. iNOS overexpression leads to increases in NO production, lipid peroxidation, LTB4 generation, and caspase-3 activity. Jurkat T cells were transfected for 16 h with empty vector alone or iNOS expression vector ($n = 3$). Nitrite (representing NO), MDA (representing lipid peroxidation), LTB4 generation, and caspase-3 activity were measured. For nitrite, LTB4, and caspase-3; *, $P < 0.05$ versus CON and iNOS gene-transfected group; **, $P < 0.05$ versus CON and empty vector alone. For MDA, *, $P < 0.05$ versus CON and empty vector alone, determined by one-way ANOVA and Studentized range test.

tion, treatment with L-NIL (an irreversible inhibitor of iNOS) does not (Figs. 3A and 9A). This cNOS-mediated NO production occurs even though hypoxia does not up-regulate cNOS protein (see *Results*). Increased activity of pre-existing cNOS is possible as a result of protein phosphorylations or through protein-protein interactions. For example, phosphorylation of specific serine residues by the phosphatidylinositol 3-kinase/Akt pathway (Dimmeler et al., 1999; Fulton et al., 1999) or phosphorylation of specific tyrosine residues by protein tyrosine kinase (Kiang et al., 2003) has been shown to activate cNOS; and formation of HSP-90-cNOS complexes enhances cNOS activity (García-Cardeña et al., 1996).

It has been reported that blocking cNOS activation with the antibiotics geldanamycin or radicicola (inhibitors of HSP-90) suppresses iNOS expression (Kiang et al., 2004; Vo et al., 2005). cNOS-derived NO regulates the activation of nuclear factor-κB pathway (Kröncke, 2003) that subsequently binds to the promoter region of iNOS gene to initiate iNOS gene expression (Pittet et al., 2002; Kiang et al., 2004). Therefore, it is appropriate to conclude that NO production derived from cNOS activity plays a role in enhancing the activity of hypoxia-induced iNOS.

Our data indicate the induced iNOS is not responsible for the increases in PGE2 (Fig. 4B), and HSP-70i (Fig. 2B) and HIF-1α protein levels. The results suggest that inhibition of iNOS by LNNA and L-NIL is rather specific for the iNOS pathway.

Hypoxia triggered an increase in lipid peroxidation in the Jurkat T cells (Fig. 3B). Hypoxia has been previously shown to increase lipid peroxidation in a variety of other biological systems. After hypoxia, levels of the lipid peroxidation indicator MDA increase in plasma and tissues of male albino rats (Kurhaliuk, 2001; Sarada et al., 2002), lung of adult human skiers (Güzel et al., 2000), and plasma of human perinatal fetus (Schmidt et al., 1996) and newborn infants (Buonocore et al., 1998). Hypoxia also induces an increase in MDA in liver, brain, and heart of chicken embryo (Stock et al., 1990) and rat heart (Chen et al., 1987). Red blood cells and plasma lipoproteins are common targets of free radical-induced oxidative damage in hypoxic human newborn infants (Buonocore et al., 1998). Lipid peroxidation levels increase in both erythrocytes and plasma of acutely hypobaric rabbits (Han et al., 1995), and the increases in that system were prevented by vitamin E treatment (Han et al., 1995). The increase in lipid peroxidation observed after rat myocardial infarction can be blocked by retinoblastoma protein (Chen et al., 1987).

In our experiments, inhibition of iNOS by the iNOS inhibitors LNNA and L-NIL effectively blocked lipid peroxidation in hypoxic Jurkat T cells (Fig. 3B). The inhibition of iNOS is characterized by both a reduction in iNOS mRNA (Kiang et

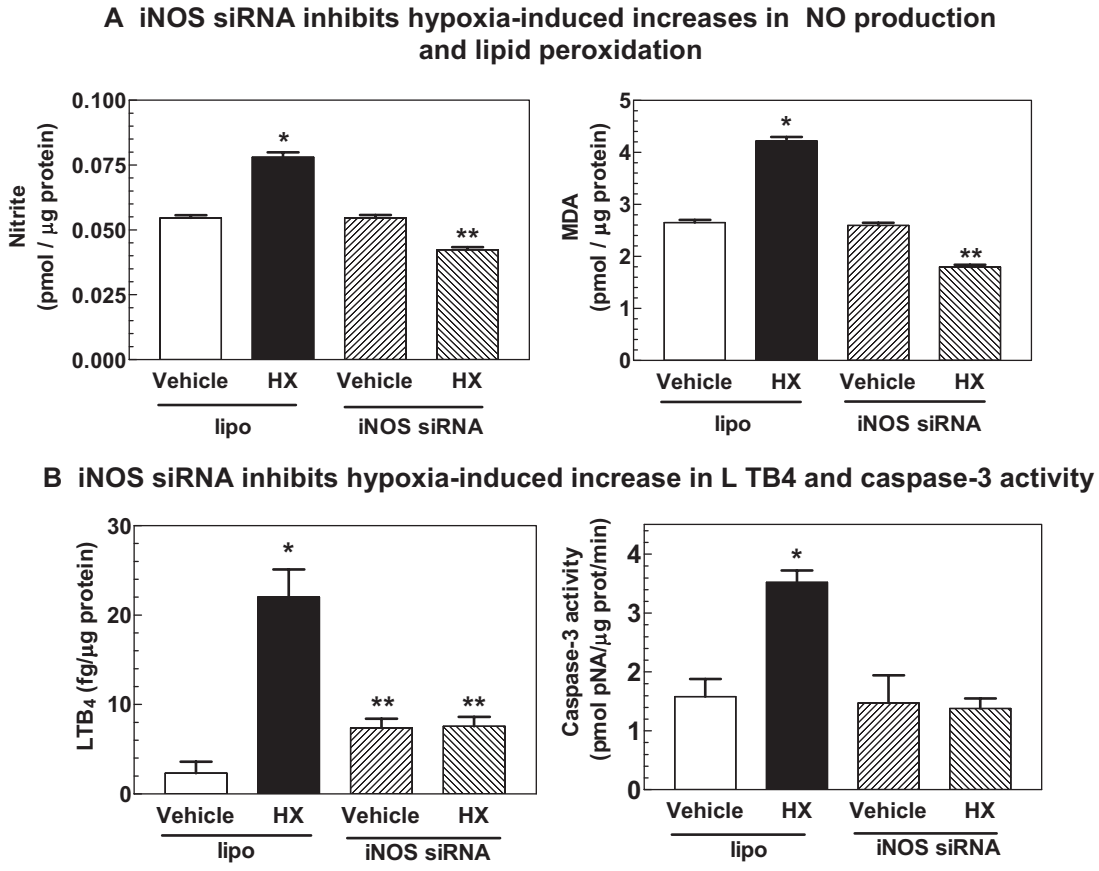

Fig. 9. iNOS inhibition leads to reduction of NO production, lipid peroxidation, LTB4 generation, and caspase-3 activity. Jurkat T cells were treated with or without iNOS siRNA for 24 h before 1-h treatment with 10 mM NaCN followed by 23-h recovery ($n = 3$). Nitrite (representing NO), MDA (representing lipid peroxidation), LTB4 generation, and caspase-3 activity were measured. For nitrite and MDA, $*$, $P < 0.05$ versus lipo + vehicle, iNOS siRNA + vehicle, and iNOS siRNA + HX; $**$, $P < 0.05$ versus lipo + vehicle, lipo + HX, and iNOS siRNA + vehicle; for LTB4, $*$, $P < 0.05$ versus lipo + vehicle, iNOS siRNA+ vehicle, and iNOS siRNA + HX $**$, $P < 0.05$ vs. lipo + vehicle and lipo + HX; and for caspase-3, $*$, $P < 0.05$ versus lipo + vehicle, iNOS siRNA + vehicle, and iNOS siRNA + HX, determined by χ^2 test.

al., 2003) and iNOS protein expression (Fig. 2). Our observation that both of the iNOS inhibitors reduced the levels of NO stimulated by hypoxia in the cells (Fig. 3A) is consistent with an interpretation that NO production probably plays a central role in causing the lipid peroxidation. Excess NO has been shown to cause lipid peroxidation and other oxidative damage indirectly when it combines with superoxide to form $ONOO^-$, which is subsequently cleaved to free radicals such as OH^- and NO_2^+ (Hogg et al., 1993; Rubbo et al., 1995), a process shown to cause local tissue injury (Szabó and Thiemermann, 1994; Yasmin et al., 1997).

Chemical hypoxia increases LTB4 and PGE2 in Jurkat cells (Fig. 4), which is consistent with findings in ischemic rodent ileum (Stojadinovic et al., 1995). Similar to observations in ischemic rodent ileum (Stojadinovic et al., 1995), iNOS inhibitors blocked LTB4 in our experiments (Fig. 4A), but not PGE2 (Fig. 4B). LTB4, a product of the 5-lipoxygenase pathway, is a potent enhancer of PMN adherence, activation, and extravasation. It also increases the expression of CD11/CD18, which leads to granulocyte adherence.

We previously showed that an inhibition of HSP-70i occurs after inhibiting cNOS but not iNOS in heat-treated cells (Kiang et al., 2002). We demonstrate here a similar response of HSP-70i in hypoxic cells (Fig. 2), suggesting that the pathways up-regulating HSP-70i are the same for both stressors. Induction of HIF-1α by hypoxia was not inhibited significantly by either LNNA or L-NIL (data not shown), indicating that neither cNOS nor iNOS is involved in regulating HIF-1α protein expression after hypoxia. Differences in the response to NaCN concentration that we observed between iNOS and both HSP-70i and HIF-1α also support such a conclusion. HIF-1α is a heterodimeric protein consisting of a constitutively expressed β subunit and an oxygen- and growth factor-regulated α subunit. HIF-1α is normally rapidly degraded in cells when adequate oxygen levels are present, but it is overexpressed in stressful circumstances such as intratumoral hypoxia (Semenza, 2002), chronic fetal anemia cardiac hypertrophy (Martin et al., 1998), injection of $CoCl_2$ (Sharp et al., 2001), or treatment with pyruvate (Lu et al., 2002).

The increases in HSP-70i and HIF-1α, like PGE2, may not play a role in the cellular injury observed after hypoxia. Induction of HSP-70i has been shown to be a self-defense mechanism for the cell (Kiang and Tsen, 2006). Both HSP-70i and HIF-1α are late response proteins that occur, for example, 6 to 12 h after hemorrhage in mouse (Kiang et al., 2004), much later than the increase in iNOS observed. The late responses of HSP-70i and HIF-1α, their apparent independence from iNOS, and the protection they demonstrate in other systems indicate they may play a role in limiting, not producing, hypoxia injury.

Our results show levels of caspase-3 (Fig. 5) and apoptosis (Fig. 7) increase in hypoxic Jurkat T cells. Caspase-3 is an aspartate-specific cysteinyl protease that plays a central role in apoptosis (Lakhani et al., 2006; Kiang et al., 2007). When iNOS is down-regulated by treatment with iNOS inhibitors, the hypoxia-induced increase in caspase-3 activity and apoptosis is inhibited. Stimulated increases in caspase-3 activity in the cell can occur through a variety of mechanisms. Hypoxia does not cause an increased expression of CD95 on cell membrane of Jurkat T cells, suggesting that the extrinsic apoptotic pathway may not be involved. Our laboratory has reported previously (Kiang et al., 2007) that the hypoxia-induced increases in caspase-3 activity and apoptosis do not involve the phosphatidylinositol 3-kinase/Akt pathway. Increases in caspase-3 activity and apoptosis are inhibited by the DVED caspase-3 inhibitors, an inhibition that can be reversed by the NO donor *S*-nitroso-*N*-acetylpenicillamine.

NO production and lipid peroxidation increases after hypoxia are known to lead to nitrosative stress and oxidative stress and the release of cytochrome *c* from the mitochondria to the cytosol (Hierholzer et al., 1998; Lakhani et al., 2006; Brown, 2007).The cytosolic cytochrome *c* then associates with caspase-9 and apoptotic protease activating factor-1 to form apoptosomes (Fig. 6), which are directly responsible for triggering caspase-3 activity (Jiang and Wang, 2004; Kiang and Tsen, 2006; Kiang et al., 2007) and apoptosis.

The view that iNOS activates caspase-3 activity through increases in LTB4, NO, and lipid peroxidation is further supported by our experiments in which we manipulated expression of the iNOS gene. Forced overexpression of iNOS led to increases in NO production, lipid peroxidation, LTB4 generation, and caspase-3 activity (Fig. 8). We also observed that blocking iNOS protein expression by treating cells with iNOS siRNA completely inhibited the increase in NO production, lipid peroxidation, LTB4 generation, and caspase-3 activity (Fig. 9, A and B). Furthermore, silencing the iNOS gene results in reduction of apoptosis (Fig. 10). Together, these data provide convincing evidence of a central role for iNOS in the response to hypoxia. Silencing the iNOS gene using siRNA treatment can potentially be therapeutic for maladies associated with hypoxia.

In summary, we report that chemical hypoxia increases iNOS activity, NO production, lipid peroxidation, LTB4 and PGE2 levels, HIF-1α and HSP-70i protein expression, caspase-3 activity, and apoptosis. Inhibition of iNOS with iNOS inhibitors or siRNA reduces the hypoxia-induced increases in NO production and lipid peroxidation, LTB4 levels, caspase-3 activity, and apoptosis. The results indicate that iNOS plays a key role in the cellular injury caused by hypoxia, and they suggest that down-regulation of iNOS can prevent or minimize hypoxia-induced injury. We propose that modulation of the iNOS activity in T cells may prove valuable in the control of the magnitude of T cell response as needed.

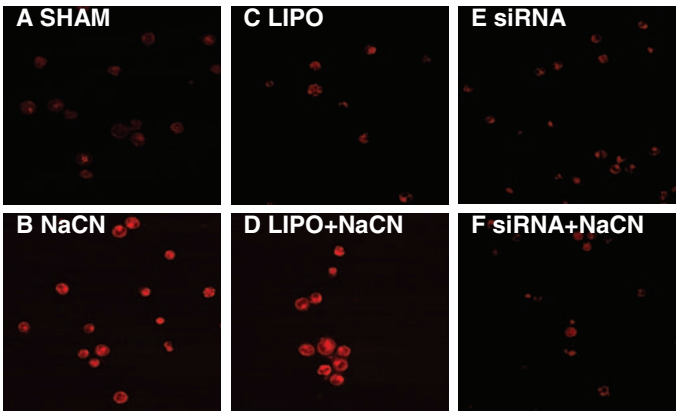

Fig. 10. iNOS inhibition leads to reduction of apoptosis. Jurkat T cells were treated with or without iNOS siRNA for 24 h before 1-h treatment with 10 mM NaCN followed by 23-h recovery. Cells were prepared for microscopy as described under *Materials and Methods*.

Acknowledgments

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