Molecular Pharmacology Fast Forward. Published on December 13, 2007 as doi:10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

MOL#041079

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

Juliann G. Kiang, Sandeep Krishnan, Xinyue Lu, and Yansong Li

Armed Forces Radiobiology Research Institute and Department of Radiation Biology, of Pharmacology, and of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20889; Department of Internal Medicine, Washington Hospital Center, Washington DC; Department of Cellular Injury, Walter Reed Army Institute of Research, Silver Spring, Maryland 20910

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

Running title:

iNOS induces apoptosis

Author correspondence information:

Dr. Juliann G. Kiang
Armed Forces Radiobiology Research Institute
Bldg. 46, Room 2423
8901 Wisconsin Avenue
Bethesda, MD 20889-5603
301-295-1076 (O)
301-295-1731 (Fax)
kiang@afrri.usuhs.mil

Number of text pages: 27

Number of figures: 10

Number of references: 40

Number of words: Abstract = 147

Introduction = 607

Discussion = 1468

Nonstandard abbreviations:

HX: hypoxia

CON: control

iNOS: inducible nitric oxide synthase

cNOS: constitutive nitric oxide synthase

LNNA: Nω-nitro-L-arginine

L-NIL: L-N^6-(1-iminoethyl)-lysine

HSP: heat shock protein

HIF: hypoxia-inducible factor

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

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 LTB4 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.

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

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 appear 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 inducible nitric oxide synthase (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., 2007) that inhibit iNOS and therefore the overproduction of NO have also been shown to limit hypoxia-induced tissue damage. Treatment with Nω-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 and 1999b). Stojadinivic et al. (1995) observed that hypoxia resulting from ischemia increases generation of PGE2 and LTB4

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

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, inducible heat shock protein 70 kDa (HSP-70i), hypoxia inducible factor-1α (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 (Kiang et al., 2003). LNNA treatment blocks iNOS, Bcl-2, and HSP-70 mRNA, but increases p53 mRNA (Kiang et al., 2003). We showed in mice that hemorrhage increases iNOS 7 h prior to HSP-70 and HIF-1α (Kiang et al., 2004) and 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 regulate peripheral responses. In experimental allergic encephalomyelitis (EAE), NO limits inflammatory demyelination by eliminating autoreactive and bystander T cells through apoptotic cell death (Zettl et al., 1997). Interestingly, constitutive upregulation of iNOS in animals that lack glucocorticoid receptor is responsible for immunosuppression and resistance to the development of EAE (Marchetti et al., 2002). In the thymus, NO generated in association with TCR stimulation functions to induce deletion of double-positive thymocytes, especially when their TCR 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.

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Cell Culture. Human Jurkat T cells (American Type Cell Collection, Rockville, MD) were grown in 75 cm^2 tissue culture flasks (Costar, Cambridge, MA) containing RPMI 1640 medium supplemented with 0.03% glutamine, 4.5 g/l glucose, 25 mM HEPES, 10% fetal bovine serum, penicillin (50 µg/ml), and streptomycin (50 U/ml) (Gibco/BRL, Gaithersburg, MD). Cells were incubated in a 5% CO_2 atmosphere at 37^{o}C and fed every 3-4 days.

Chemicals. Chemicals used in this study were albumin, NaCN, Nω-nitro-L-arginine (CAS Registry No.: 2140-70-4), and L-N^6-(1-iminoethyl)-lysine [(CAS Registry No.: 159190-45-1), Sigma Chemical Co., 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 and therefore appear 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 bp coding sequence with Hind III and Xho I restriction sites at each terminus of human iNOS gene was sub-cloned from this full length of human iNOS cDNA. For PCR, we used the forward and reverse primers, 5'-CT

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

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 PCR amplification product, the expression cDNA sequence was used for insertion into the vector. The vector used in this study was pcDNA3.1 (Invitrogen Co., San Diego, CA). The expression cDNA of iNOS gene was inserted between the Hind III and Xho I 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 X 10^7) in 0.5 ml fresh cultured medium were mixed gently with 10 μg 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, Hercules, CA) and electroporation conducted at 250V, 925 microfarads for 15 sec. The electroporated cells were then combined with 2 ml fresh culture medium and placed in one well of a 6-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 (non-silencing 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

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

siRNA silencing potential, siRNAs were heated for 1 min at 90°C, followed by 60 min at 37°C before the siRNA transfection. Prior to transfection, cells were grown in fresh medium without antibiotics for 24 h. Cells were then transferred to 6-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 returned to 37 ^{o}C for 16 h. After incubation, cells were removed from the culture flask and pelleted by centrifugation at 750 x g for 10 min. The pelleted cells were lysed in Tris buffer (pH=6.8) containing 1% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol. Aliquots containing 20 µg of protein were resolved on SDS-polyacrylamide slab gels (Novex precast 10 % gel, San Diego, CA). After electrophoresis, proteins were blotted onto a nitrocellulose membrane (type NC, 0.45 µm, Schleicher and Schuell), 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, CA), or HIF-1α (BD Signal Transduction, KY) at a concentration of 1 µg/ml in PBS - 5% BSA. The blot was then washed 3 times (10 min each)

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

in Tris buffered saline (TBS) - 0.1% Tween 20 before incubating the blot for 60 min at room temperature with a 1000X dilution of species-specific IgG peroxidase conjugate (Santa Cruz, CA) in PBS - 1% gelatin. The blot was washed 6 times (5 min each) in TBS - 0.1% Tween 20 before detection of peroxidase activity using the Enhanced Chemiluminenscence Plus (Amersham Life Science Products, Arlington Height, IL). Actin levels were not altered by NaCN-induced hypoxia (Kiang et al., 2003); we therefore 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 immunoassay kit (Cayman Chemical, Ann Arbor, MI). 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).

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

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% FBS) 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 human IgG (Jackson Immunoresearch, West Grove, PA) to block non-specific binding sites, and then with 1 μg PE-conjugated anti-CD95 (Pharmingen, San Diego, CA) or isotype mouse IgG_1 (κ mAb control, Becton-Dickinson) for 20 min on ice. Cells were washed twice with staining buffer, resuspended in 100 μl staining buffer and 100 μl of 3% paraformaldehyde, and kept in the dark. Antibody-labeled cells were analyzed using a FACSscan flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and the data were analyzed using CellQuest software.

Caspase-3. Caspase-3 cellular activity was measured using a commercial kit (Biomol, Plymouth Meeting, PA). The data are presented in pmol pNA/min/μg protein).

Apoptosis. Apoptosis was measured using the Magic Red Caspase Activity Detection Kit (ICN Biomedicals, Livermore, CA). After specific treatments, 300 μl of cell suspension (0.3×10^6) was transferred into a 12x75mm polypropylene tube. Ten μl of the 31X working dilution DEVD-MR solution was added directly to each 300 μl cell suspension. The mixture was incubated for 1 h at 37 ^oC in air containing 5 % CO_2 with swilling the tubes every 20 min. One hr after incubation, cells were counterstained by adding 1.6 μl Hoechst stain to the 300 μl suspension labeled with DEVD-MR for 15 min. Twenty microliters of the cell suspension was placed onto a microscope slide and covered with a coverslip. Slides were analyzed with a

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

laser scanning confocal fluorescence microscope (1X70, Olympus, Lake Success, NY) with Lasersharp2000 software (Bio-Rad, Richmond, CA).

Statistical Analysis. All data are expressed as the mean ± s.e.m. One-way ANOVA, two-way ANOVA, studentized-range test, Bonferroni's inequality, and Student's t-test were used for comparison of groups with 5% as a significant level.

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

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^{o}C, protein levels for iNOS, HSP-70i, and HIF-1α (a key regulator of the cellular response to hypoxia) increased (Fig. 1A, C, 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. 1A, C, 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 fashion (Fig. 1B, D, F), though 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 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 prior to a 1-h exposure to 10 mM NaCN and then 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

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

(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 (see Fig. 1), we wanted to confirm that NaCN treatment resulted in increased NO production and test if the iNOS inhibitors LNNA or L-NIL affected its production. Treatment with LNNA or L-NIL before hypoxia inhibited NO production compared to 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 while 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 peroxynitrite ($ONOO^-$), and peroxynitrite can in turn break down to free radicals such as OH^- and NO_2^+, which then react with tissue elements (Szabo and Thiemermann, 1994; Yasmin et al., 1997). Because we found hypoxia induces a significant elevation of NO production, we determined if free radical damage was occurring in the cell by

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

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).

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 12-120 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). On the other hand, caspase-3 activity, which plays a pivotal role in the process of apoptosis (Lakhani et al., 2006; Jiang and Wang, 2004; Kiang

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

and Tsen, 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 Apaf-1 (Lakhani et al., 2006; Jiang and Wang, 2004; Kiang and Tsen, 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 data show that hypoxia induces increases in iNOS, HSP-70i, HIF-1α, NO, lipid peroxidation, 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.

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

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). [DEM1]

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[DEM2] results reinforce the view that NaCN-induced chemical hypoxia triggers apoptosis mediated by the iNOS pathway.

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

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; while treatment with LNNA (an irreversible inhibitor of cNOS and a reversible inhibitor of iNOS) completely inhibits the hypoxia-induced increase in NO production, 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 upregulate cNOS protein (see Results section). 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 PI3-K/Akt pathway (Dimmerler 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 (Garcia-Cardena et la., 1996).

It has been reported that blocking cNOS activation with the antibiotics geldanamycin or radicicol (inhibitors of HSP-90) suppresses iNOS expression (Vo et al., 2005; Kiang et al., 2004). cNOS-derived NO regulates the activation of NF-κB pathway (Kroncke, 2003) that

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

subsequently binds to the promoter region of iNOS gene to initiate iNOS gene expression (Pittet et al., 2001; 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 (Guzel et al, 2000), and plasma of human perinatal fetus (Schmit 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 (Rb) (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 al., 2003) and iNOS

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

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 peroxynitrite ($ONOO^-$), 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 (Szabo 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 polymorphonuclear leukocyte (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 et al., 2002), chronic fetal anemia cardiac hypertrophy (Martin et al., 1998), injection of CoCl2 (Sharp et al., 2002), 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 appear, for example, 6-12 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 lab has reported elsewhere (Kiang et al., 2007) that the hypoxia-induced increases in caspase-3 activity and apoptosis do not involve the PI-3K/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 SNAP.

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 (Brown, 2007; Hierholzer et al., 1998; Lakhani et al., 2006).The cytosolic cytochrome c then associates with caspase-9 and Apaf-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-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 suggest that down-regulation of iNOS can prevent or minimize

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

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.

REFERENCES

Brown GC (2007) Nitric oxide and mitochondria. *Front Biosci* **12**:1024-1033.

Buonocore G, Zani S, Perrone S, Caciotti B and Bracci R (1998) Intraerythrocyte nonprotein-bound iron and plasma malondialdehyde in the hypoxic newborn. *Free Radic Biol Med* **25**:766-770.

Chabrier PE, Auguet M, Spinnewyn B, Auvin S, Cornet S, Demerle-Pallaedy C, Guilmard-Favre C, Marin JG, Pignol B, Gillard-Roubert V, Roussillot-Charnet C, Schultz J, Viossat I, Bigg D and Moncada S (1999) BN 80933, a dual inhibitor of neuronal nitric oxide synthase and lipid peroxidation: a promising neuroprotective strategy. *Proc Natl Acad Sci USA* **96**:10824-10829a.

Chabrier PE, Demerle-Pallardy C and Auguet M (1999) Nitric oxide synthases: targets for therapeutic strategies in neurological diseases. *Cell Mol Life Sci* **55**:1029-1035b.

Chen X, Li YJ, Deng HW, Yang BC, Li DY and Shen N (1987) Protective effects of ginsenosides on anoxia/reoxygenation of cultured rat myocytes and on reperfusion injuries against lipid peroxidation. *Biomed Biochim Acta* **46**:S646-S649.

Dimmeler S, Fleming I, Fisslthaler B, Hermann C, and Zeiher AM (1999) Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399**:601-605.

Drexler H (1999) Nitric oxide synthases in the failing human heart: a double-edged sword? *Circulation* **99**:2972-2975.

Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujin Y, Walsh K, Franke TF, Papapetropoulos A, and Sessa WC (1999) Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399**:597-601.

Garcia-Cardena G, Fan R, Stern DF, Liu J and Sessa WC (1996) Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *J Biol Chem* **271**:27237-27240.

Guzel NA, Sayan H and Erbas D (2000) Effects of moderate altitude on exhaled nitric oxide, erythrocytes lipid peroxidation and superoxide dismutase levels. *Jpn J Physiol* **50**:187-190.

Han ZY, Chen M, Lu JR, Wen P, Song XL and Wu QY (1995) Hypoxia induced increase of MDA and echinocytes from erythrocytes in rabbit's blood with special reference to inhibition of the increase by MPEG-SOD. *Sheng Li Xue Bao* **47**:565-572.

Hierholzer C, Hatbrecht B, Menezes JM, Kane J, MacMicking J, Nathan CE, Peitzman AB, Billiar TR and Tweardy DJ (1998) Essential role of induced nitric oxide in the initiation of the inflammatory response after hemorrhagic shock. *J Exp Med* **187**:917-929.

Hogg N, Kalyanaraman B, Joseph J, Struck A and Parthasarathy S (1993) Inhibition of low-density lipoprotein oxidation by nitric oxide. Potential role in antherogenesis. *FEBS Lett* **334**:170-174.

Jiang X and Wang X (2004) Cytochrome-c-mediated apoptosis. *Annu Rev Biochem* **73**:87-106.

[0]Kiang JG, Bowman PD, Lu X, Li Y, Wu BW, Loh HH, Tsen KT and Tsokos GC (2007) Geldanamycin inhibits hemorrhage-induced increases in caspase-3 activity: role of inducible nitric oxide synthase. *J Appl Physiol* 103:1045-1055.

Kiang JG, Bowman PD, Wu BW, Hampton N, Kiang AG, Zhao B, Juang Y-T, Atkins JL and Tsokos GC (2004) Geldanamycin treatment inhibits hemorrhage-induced increases in KLF6 and iNOS expression in unresuscitated mouse organs: role of inducible HSP-70. *J Appl Physiol* **97**:564-569.

Kiang JG, Kiang SC, Zhuang J-T and Tsokos, GC (2002) Nω-nitro-L-arginine inhibits inducible HSP-70 via Ca^{2+}, PKC, and PKA in human intestinal epithelial T84 cells. *Am J Physiol* **282**:G415-G423.

Kiang JG, McClain DE, Warke V, Krishnan S and Tsokos GC (2003) Constitutive NO synthase regulates the Na^{+}/Ca^{2+} exchanger in human T cells: role of $[Ca^{2+}]_i$ and tyrosine phosphorylation. *J Cell Biochem* **89**:1030-1043.

Kiang JG and Smallridge RC (1994) Sodium cyanide increases cytosolic free calcium: Evidence of activation of the reversed mode of Na^{+}/Ca^{2+} exchanger and Ca^{2+} mobilization from inositol trisphosphate-insensitive pools. *Toxicol Appl Pharmacol* **127**:173-181.

Kiang JG and Tsen KT (2006) Biology of hypoxia. *Chin J Physiol* **49**: 223-233.

Kiang JG, Wang XD, Ding XZ, Gist I, and Smallridge RC (1996) Heat shock inhibits the hypoxia-induced effects on iodide uptake and signal transduction and enhances cell survival in rat thyroid FRTL-5 cells. *Thyroid* **6**:475-483.

Kroncke KD (2003) Nitrosative stress and transcription. *Biol Chem* **384**:1365-1377.

Kurhaliuk NM (2001) Effect of nitric oxide synthase inhibitor L-NNA on the activities of antioxidant enzymes and lipid peroxidation in blood and tissues of rats with different resistance to hypoxia. *Fiziol Zh* **47**:52-59.

Lakhani SA, Masud A, Kuida K, Porter GA Jr, Booth CJ, Mehal WZ, Inayat I and

Flavell RA (2006) Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science* **311**:847-51.

Lu H, Forbes RA and Verma A (2002) Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. *J Biol Chem* **277**:23111-23115.

Marchetti B, Morale MC, Brouwer J, Tirolo C, Testa N, Caniglia S, Barden N, Amor S, Smith PA and Dijkstra CD (2002) Exposure to a dysfunctional glucocorticoid receptor from early embryonic life programs the resistance to experimental autoimmune encephalomyelitis via nitric oxide-induced immunosuppression. [0]*J Immunol* **168**:5848-5859.

Martin C, Yu AY, Jiang BH, Davis L, Kimberly D, Hohimer AR and Semenza GL (1998) Cardiac hypertrophy in chronically anemic fetal sheep: Increased vascularization is associated with increased myocardial expression of vascular endothelial growth factor and hypoxia-inducible factor 1. *Am J Obstet Gynecol* **178**:527-534.

Pittet JF, Lu LN, Geiser T, Lee H, Matthay A, and Welch WJ (2001) Stress preconditioning attenuates oxidative injury to the alveolar epithelium of the lung following haemorrhage in rats. *J Physiol* **538**:583-597.

Rubbo H, Parthasarathy S, Barnes S, Kirk M, Kalyanaraman B and Freeman BA (1995) Nitric oxide inhibition of lipoxygenase-dependent liposome and low-density lipoprotein oxidation: termination of radical chain propagation reactions and formation of nitrogen-containing oxidized lipid derivatives. *Arch Biochem Biophys* **324**:15-25.

Sarada SK, Dipti P, Anju B, Pauline T, Kain AK, Sairam M, Sharma SK, Ilavazhagan G, Kumar D and Selvamurthy W (2002) Antioxidant effect of beta-carotene on hypoxia induced oxidative stress in male albino rats. *J Ethnopharmacol* **79**:149-153.

Schmidt H, Grune T, Muller R, Siems WG and Wauer RR (1996) Increased levels of lipid peroxidation products malondialdehyde and 4-hydroxynonenal after perinatal hypoxia. *Pediatr Res* **40**:15-20.

Semenza GL (2002) HIF-1 and tumor progression, pathophysiology and therapeutics. *Trends Mol Med* **8**:S62-S67.

Sharp FR, Bergeron M and Bernaudin M (2002) Hypoxia-inducible factor in brain. *Adv Exp Med Biol* **502**:273-291.

Stock MK, Silvernail KK and Metcalfe J (1990) Prenatal oxidative stress: I. Malondialdehyde in hypoxic and hyperoxic chick embryos. *Free Radic Biol Med* **8**:313-318.

Stojadinovic A, Kiang JG, Smallridge RC, Galloway RL and Shea-Donahue T (1995) Heat shock protein 72 kD induction protects rat intestinal mucosa from ischemia/reperfusion injury. *Gastroenterol* **109**:505-515.

Szabo C and Thiemermann C (1994) Invited opinion: role of nitric oxide in hemorrhagic, traumatic, and anaphylactic shock and thermal injury. *Shock* **2**:145-155.

[0]Tai XG, Toyo-Oka K, Yamamoto N, Yashiro Y, Mu J, Hamaoka T and Fujiwara H (1997) CD9-mediated costimulation of TCR-triggered naive T cells leads to activation followed by apoptosis. *J Immunol* **158**:4696-4703.

Vo PA, Lad B, Tomlinson JAP, Francis S and Ahluwalia A (2005) Autoregulatory role of endothelium-derived nitric oxide (NO) on lipopolysaccharide-induced vascular inducible NO synthase expression and function. *J Biol Chem* **280**:7236-7243.

Ward DT, Lawson SA, Gallagher CM, Conner WC and Shea-Donohue T (2000) Sustained nitric oxide production via L-arginine administration ameliorates effects of intestinal ischemia-reperfusion. *J Surg Res* **89**:13-19.

Yasmin W, Strynadka KD and Schulz R (1997) Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. *Cardiovasc Res* **33**:422-432.

Zettl UK, Mix E, Zielasek J, Stangel M, Hartung HP and Gold R (1997) Apoptosis of myelin-reactive T cells induced by reactive oxygen and nitrogen intermediates in vitro. [0]C*ell Immunol* **178**:1-8.

Footnotes

This work was supported by AFRRI RAB2CF and DOD RAMII STO C .

Disclaimer

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army, Department of Defense, Armed Forces Radiobiology Research Institute, or Uniformed Services University of The Health Sciences.

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 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 (panels A and B), HSP-70i (panels C and D), and HIF-1α (panels E and F). Protein was quantitated densitometrically and normalized with actin. *P<0.05 vs. control groups, determined by one-way ANOVA.

Figure 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) prior to 1-h treatment with 10 mM NaCN and 16-h recovery. Cell lysates were immunoblotted with antibodies against iNOS (panel A) and HSP-70i (panel B). Protein was quantitated densitometrically and normalized with actin. *P<0.05 vs. control groups, **P<0.05 vs. control and HX, determined by two-way ANOVA. CON: control; HX: hypoxia

Figure 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) prior to 1-h treatment with 10 mM NaCN and 16-h recovery. NO production was measured in cell lysates as described in methods. *P<0.05 vs. control without NaCN, L-NNA, and L-NIL; **P<0.05 vs. control, L-NNA, and L-NIL without NaCN, determined by two-way ANOVA. CON: control; HX: hypoxia

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 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) prior to 1-h treatment with 10 mM NaCN (HX) and 16-h recovery. Panel A: LTB4 levels were measured in cell lysates as described in methods. *P<0.05 vs. control, L-NIL alone, and L-NIL+HX, determined by Chi-square test. Panel B: PGE2 levels were measured in cell lysates as described in methods. *P<0.05 vs. control and L-NIL alone, determined by Chi-square test. CON: control; HX: hypoxia

Figure 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) prior to 1h-treatment with 10 mM NaCN and 16-h recovery. Caspase-3 activity was measured as described in methods. *P<0.05 vs. control without HX, L-NNA, and L-NIL; **P<0.05 vs. control, LNNA without HX and L-NIL, determined by two-way ANOVA. CON: control; HX: hypoxia

Figure 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 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). CON: control

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 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) prior to 1-h treatment with 10 mM NaCN and 23-h recovery. Cells were prepared for microscopy as described in Methods. Data are expressed as percent apoptosis-positive cells (see bar graph inset), *P<0.05 vs. untreated, LNNA, LNNA+NaCN, L-NIL, and L-NIL+NaCN; **P<0.05 vs. untreated, NaCN, LNNA, L-NIL, and L-NIL+NaCN, determined by two-way ANOVA.

Figure 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 vs. CON and iNOS gene-transfected group, **P<0.05 vs. CON and empty vector alone; for MDA, *P<0.05 vs. CON and empty vector alone, determined by one-way ANOVA. CON: control

Figure 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 prior to 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 vs. lipo+vehicle, iNOS siRNA+vehicle, and iNOS siRNA+HX; **P<0.05 vs. lipo+vehicle, lipo+HX, and iNOS siRNA+vehicle; for LTB4, *P<0.05 vs. lipo+vehicle, iNOS siRNA+

Molecular Pharmacology Fast Forward. Published on December 13, 2007 as DOI: 10.1124/mol.107.041079
This article has not been copyedited and formatted. The final version may differ from this version.

vehicle, and iNOS siRNA+HX; for caspase-3, *P<0.05 vs. lipo+vehicle,iNOS siRNA+vehicle, and iNOS siRNA+HX, determined by Chi-square test. HX: hypoxia; lipo: lipofectamine reagent

Figure 10. iNOS inhibition leads to reduction of apoptosis. Jurkat T cells were treated with or without iNOS siRNA for 24 h prior to 1-h treatment with 10 mM NaCN followed by 23-h recovery. Cells were prepared for microscopy as described in Methods. LIPO: lipofectamine reagent

Fig. 1

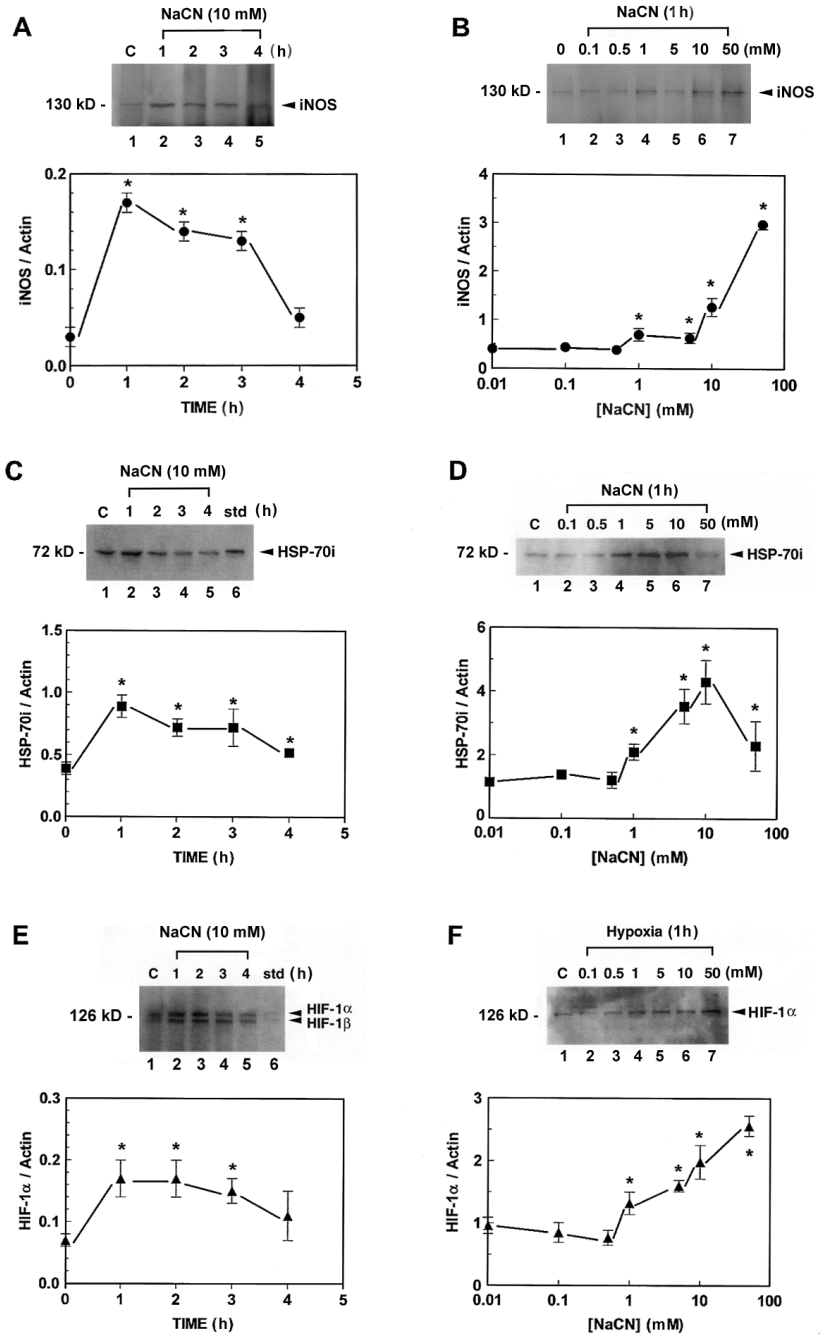


Fig. 2

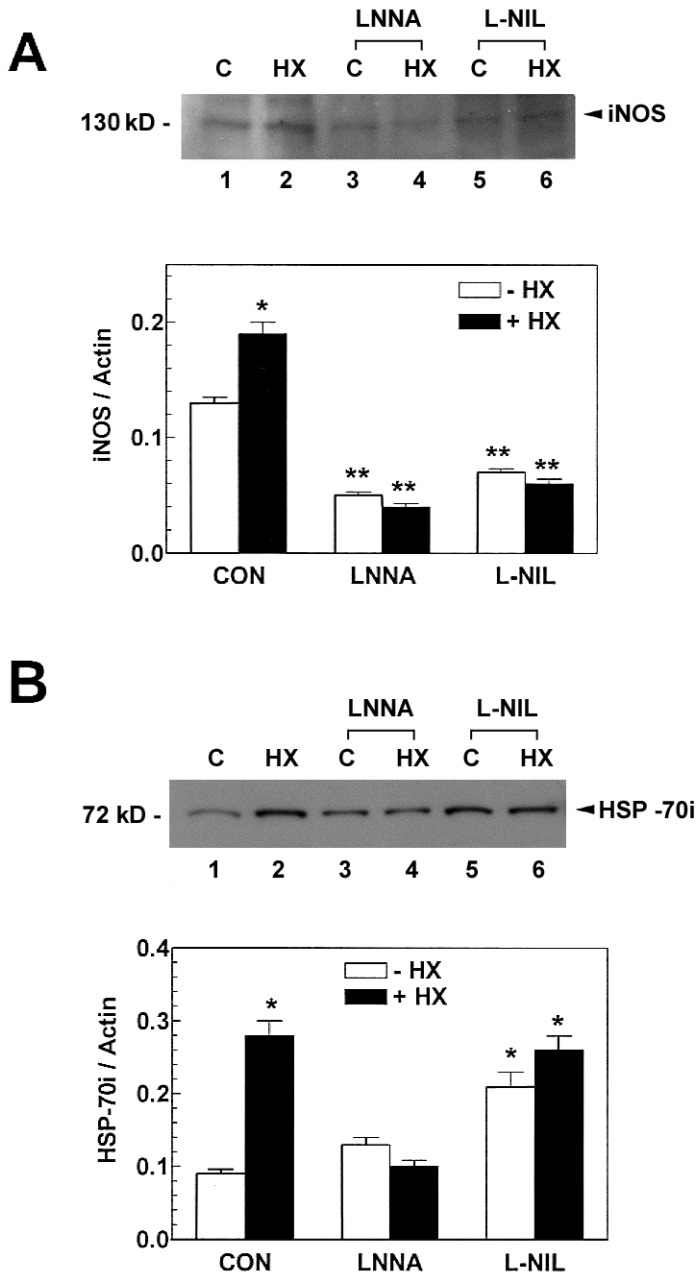
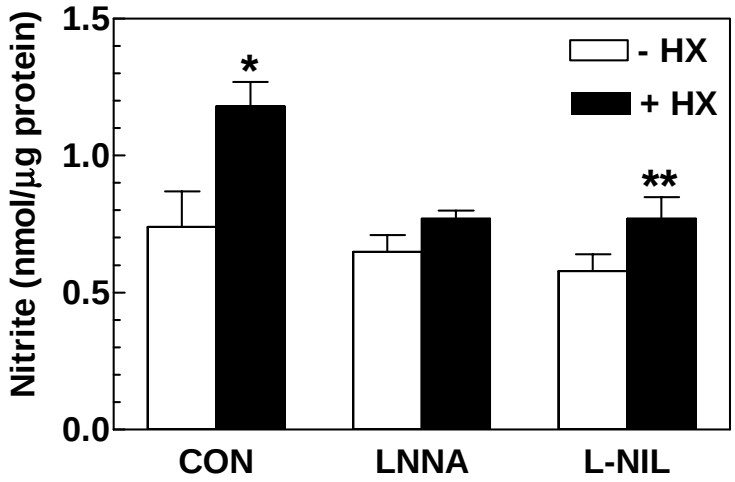


Fig. 3

A. iNOS inhibitors inhibit hypoxia-induced increase in NO production



B. iNOS inhibitors inhibit hypoxia-induced increase in lipid peroxidation

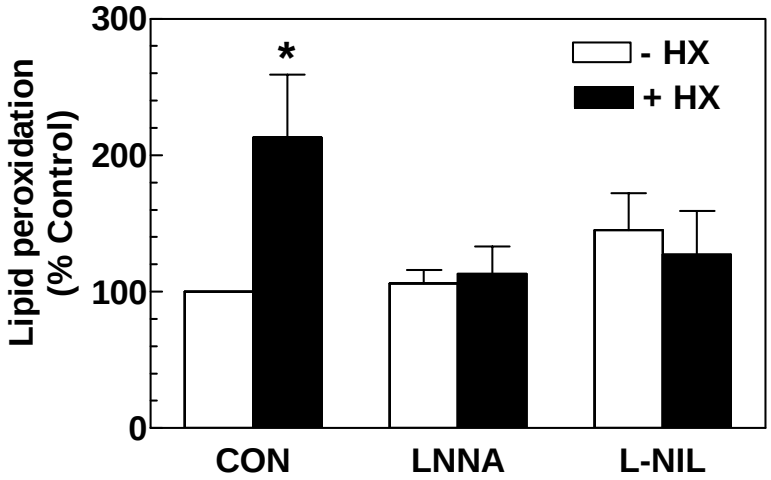
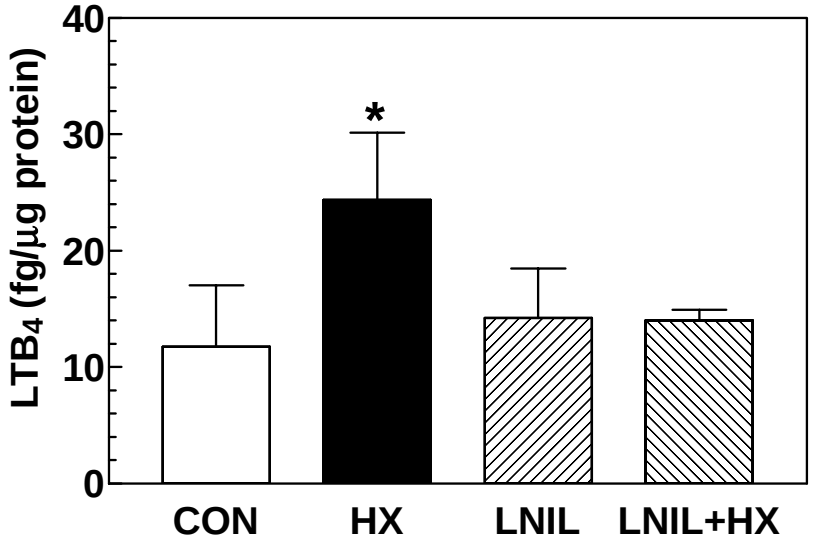


Fig. 4

A. iNOS inhibitor inhibits hypoxia-induced increase in LTB4



B. iNOS inhibitor does not inhibit hypoxia-induced increase in PGE2

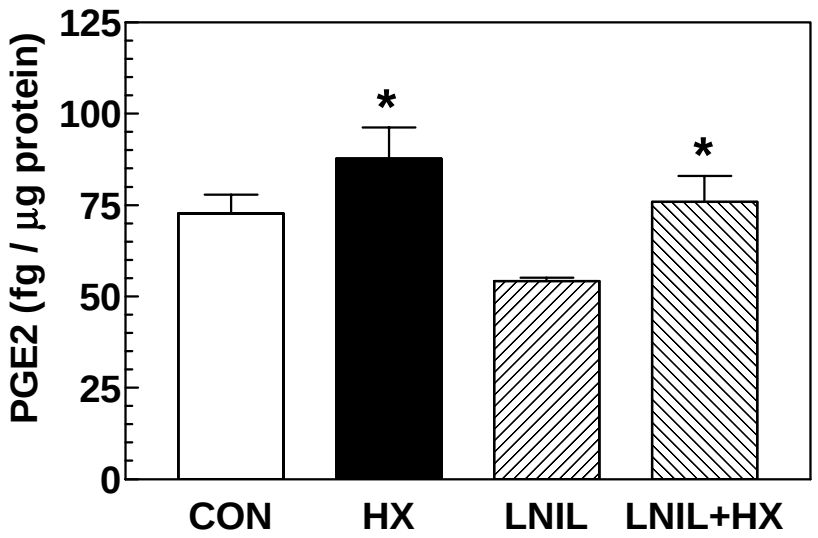


Fig. 5

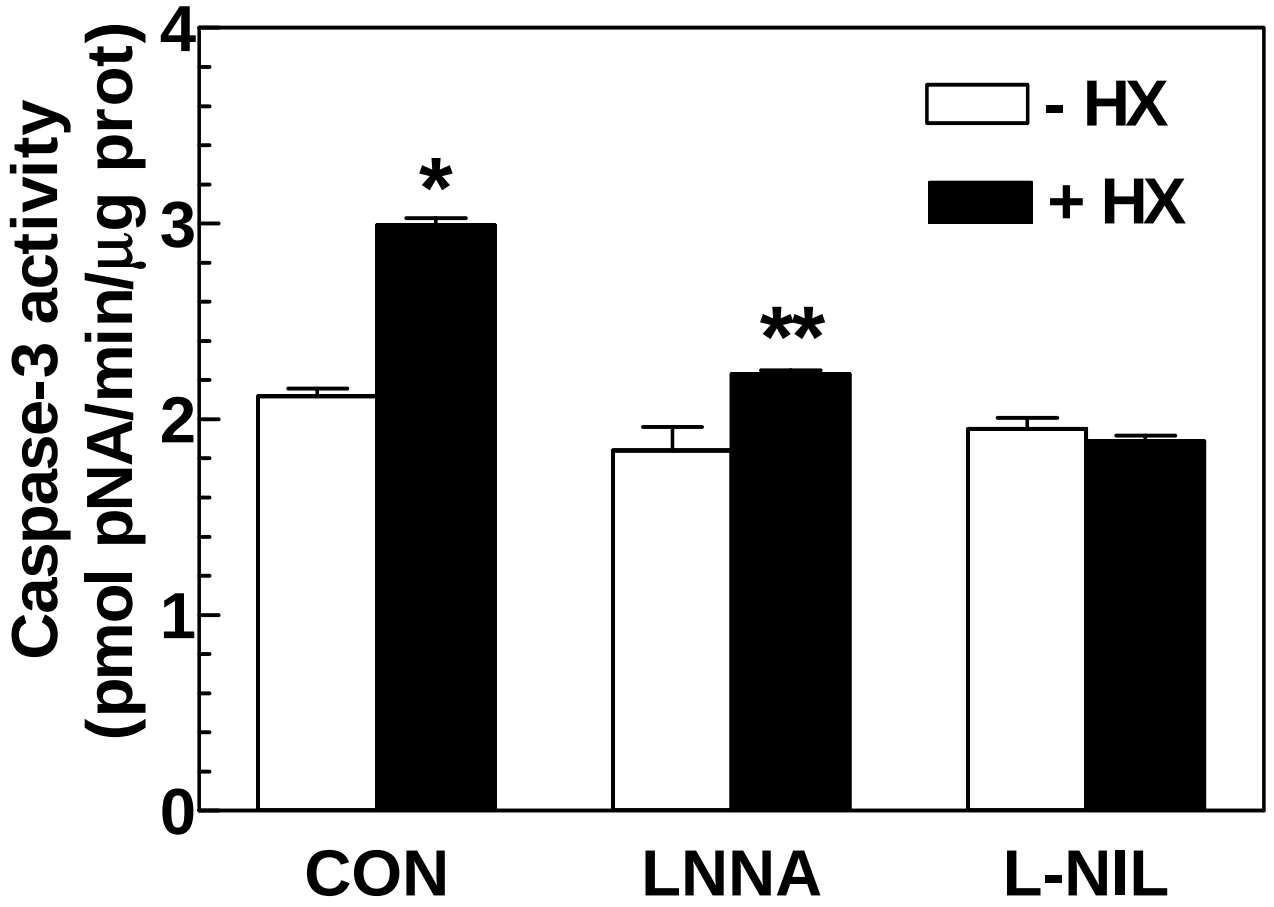


Fig. 6

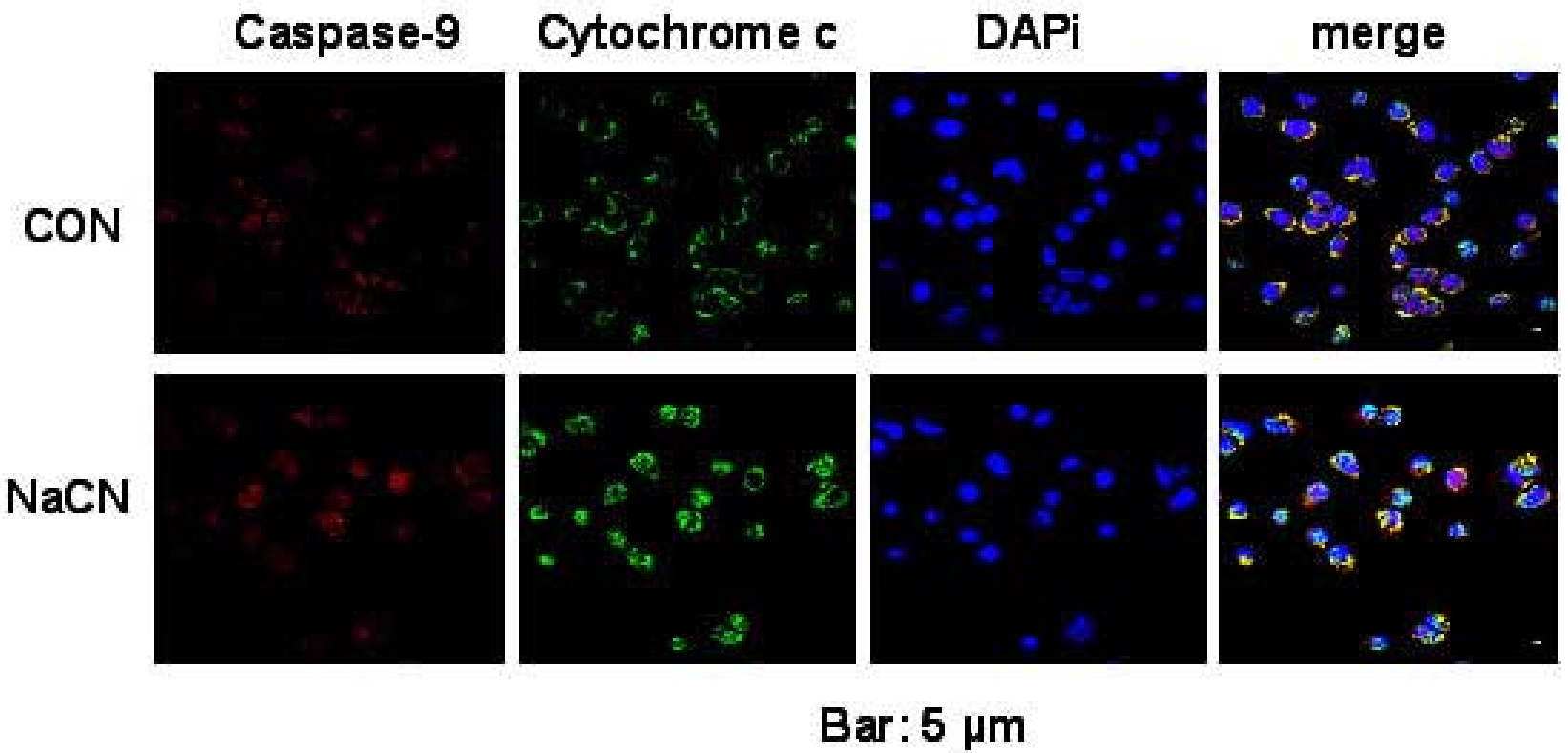


Fig. 7

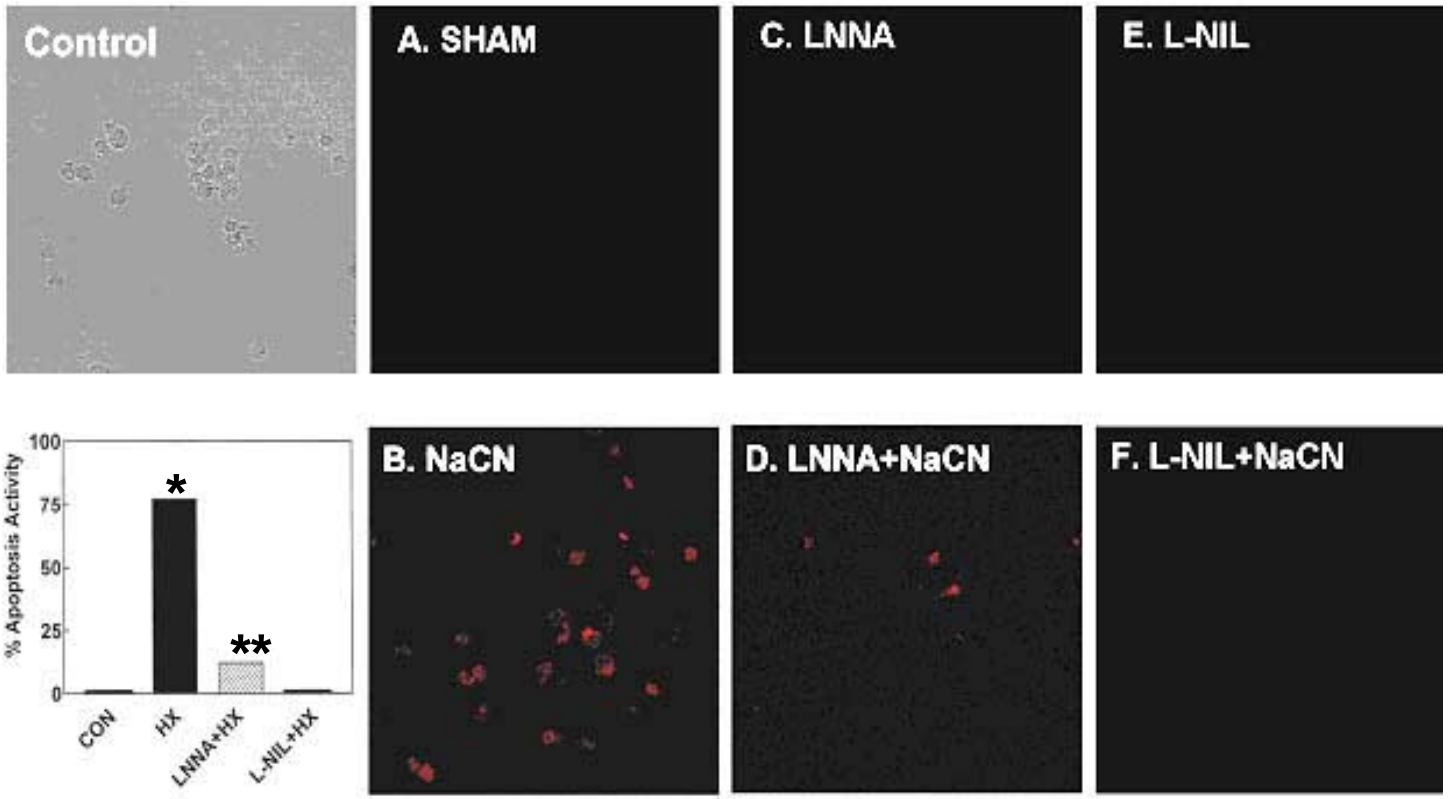


Fig. 8

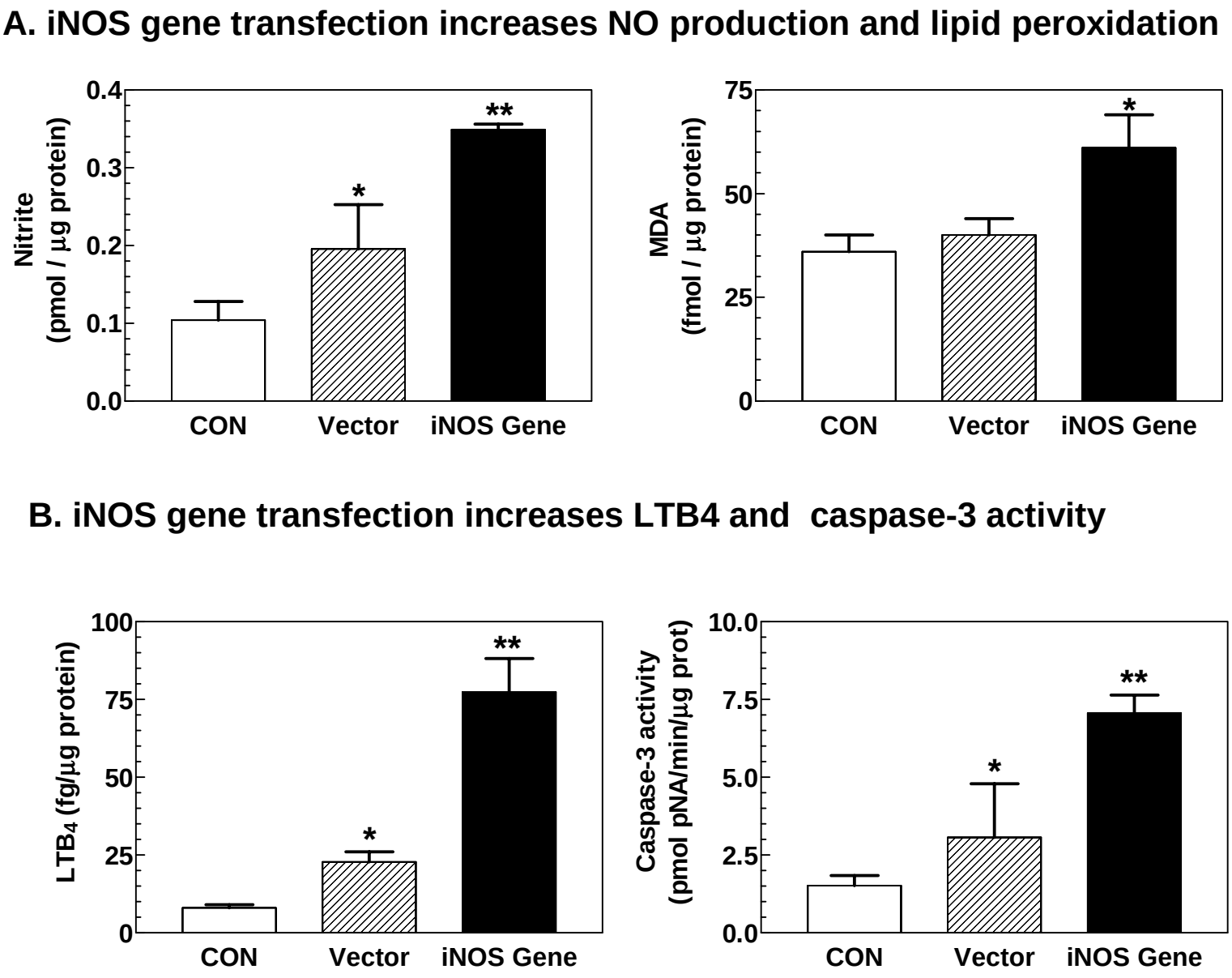


Fig. 9

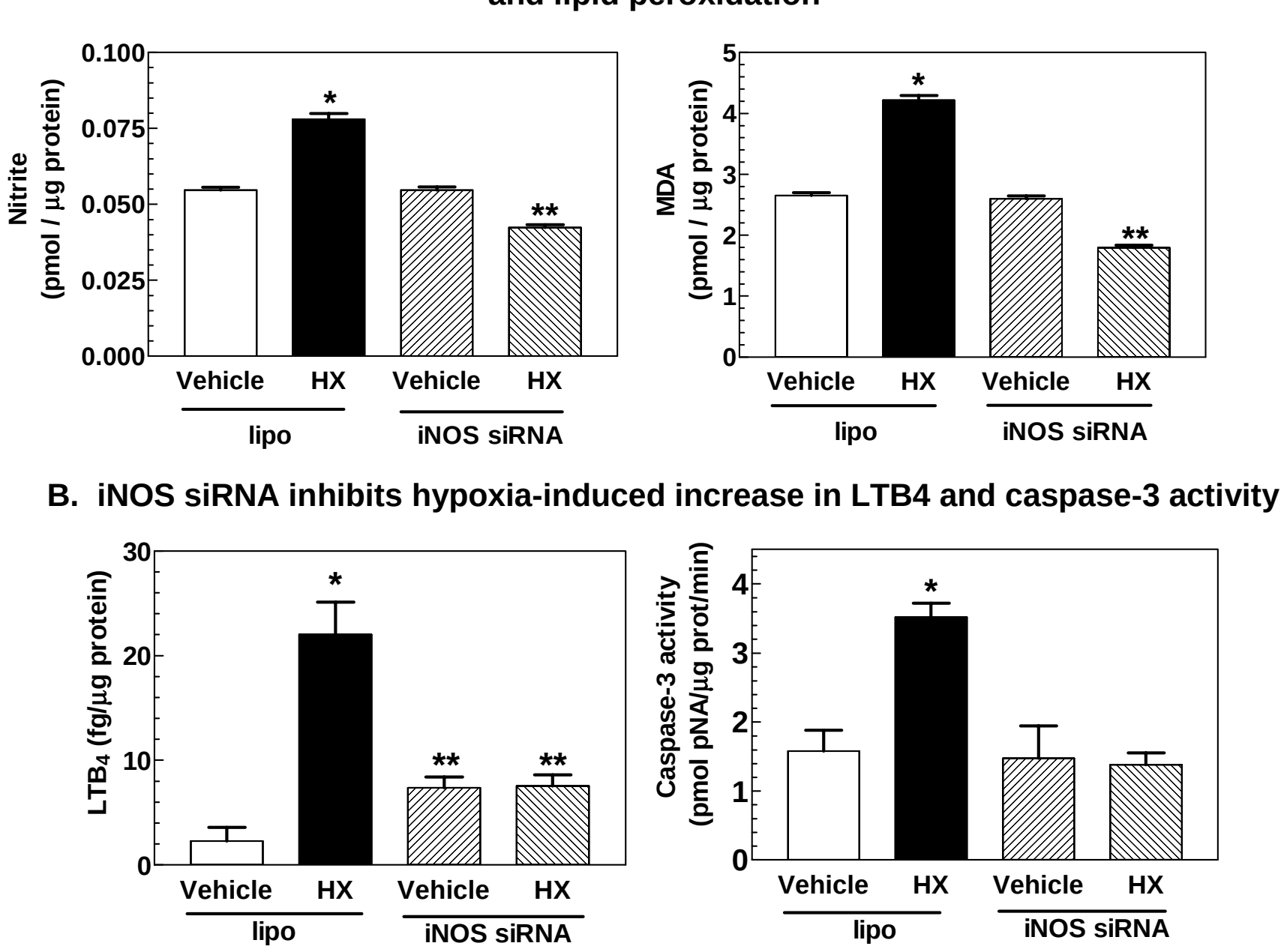


Fig. 10