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Constitutive NO Synthase Regulates the Na^+/Ca^{2+} Exchanger in human T cells: Role of $[Ca^{2+}]_i$ and Tyrosine Phosphorylation

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Abstract For many types of cells, heat stress leads to an increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) that has been shown to trigger a wide variety of cellular responses. In T lymphocytes, for example, heat stress stimulates pathways that make them more susceptible to Fas/CD95-mediated apoptosis. Because of our interest in understanding more about the response of lymphocytes to various stressors, we used human peripheral and Jurkat T lymphocytes to investigate the effect of heat stress on calcium homeostasis. We found that peripheral and Jurkat T cells both exhibit cNOs activity but not iNOs activity. Heat stress increased NO production, which was inhibited by LNNA (a cNOs inhibitor) but not L-NIL (an iNOs inhibitor). Heat stress increased $[Ca^{2+}]_i$ in Jurkat T cells by decreasing the K_m of the cell surface membrane Na^+/Ca^{2+} exchanger for extracellular Ca^{2+}. Heating also increased cNOs phosphorylation at tyrosine residues. In cells incubated with LNNA, heat stress promoted an increase in $[Ca^{2+}]_i$ and a decrease in $[Na^+]_i$ greater than in cells heated without LNNA, a larger decrease in K_m of the Na^+/Ca^{2+} exchanger for extracellular Ca^{2+}, and decreased phosphorylation of cNOs. Our results suggest that cNOs plays an important regulatory role after heat stress. Heating appears to increase the phosphorylation of cNOs that is complexed with the Na^+/Ca^{2+} exchanger to decrease its activity. This process is related to increased expression of Fas/CD95 on the cell surface, which might explain the apoptotic diathesis of lymphocytes after heat stress. J. Cell. Biochem. 89: 1030–1043, 2003. Published 2003 Wiley-Liss, Inc.†

Key words: Na^+/Ca^{2+} exchanger; Ca^{2+}; NO synthase; heat stress; lymphocytes; T cells; tyrosine phosphorylation; CD95; Fas

Cells regulate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a variety of ways. Systems located at the cell membrane include ATPase-mediated Ca^{2+} pumps, Na^+/Ca^{2+} antiporter (exchanger), Ca^{2+}-adenosine triphosphatase-regulated pumps, and Ca^{2+} channels (including voltage-gated, second messenger-operated, and receptor-operated channels). Intracellular systems include Ca^{2+} mobilization from intracellular pools and cytosolic Ca^{2+}-binding proteins such as calmodulin and calcineurin [Waldron et al., 1997; Kiang and Tsokos, 1998; Ma et al., 2002]. When resting $[Ca^{2+}]_i$ increases, it triggers the activity of Ca^{2+}-dependent enzymes involved in many cellular biochemical reactions [Kiang and McClain, 1993; Kiang et al., 1994;

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; cNOs, constitutive nitric oxide synthase; iNOs, inducible nitric oxide synthase; LNNA, Nω-nitro-L-arginine; L-NIL, L-N^6-(1-iminoethyl)lysine; NO, nitric oxide; XIP, exchanger inhibitory peptide, PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; HSP-70, heat shock protein 70 kDa.

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Ding et al., 1996]. This laboratory previously reported that heat stress increases $[Ca^{2+}]_i$ via a Na^+/Ca^{2+} exchange system and Ca^{2+} mobilization from an inositol 1,4,5-trisphosphate-sensitive pool in human epidermoid A-431 cells [Kiang et al., 1992] and human colon carcinoma T84 cells [Kiang and McClain, 1999].

Nitric oxide (NO) generated by nitric oxide synthase (NOs) is also involved in a wide range of physiological processes [Ignarro et al., 1987; Knowles et al., 1989; Liew and Cox, 1991; Malen and Chapman, 1997]. There are two classes of NOs; constitutive NOs (cNOs) is Ca^{2+}-dependent, whereas inducible NOs (iNOs) is not [Forstermann et al., 1998]. Both types of NOs have been detected in a number of cell types, including endothelial cells, neurons, and macrophages [Moncada et al., 1991]. cNOs plays an important role in cell homeostasis. It increases NO release within seconds of a significant elevation in $[Ca^{2+}]_i$ [Stefano et al., 2000]. Serine or tyrosine phosphorylation in the oxygenase domain of cNOs decreases its activity by an unknown mechanism [Dawson et al., 1993]. cNOs also regulates the expression of inducible heat shock protein 70 (HSP-70), a Ca^{2+}-dependent molecular chaperone, and protein kinases A and C, both of which are Ca^{2+}-dependent enzymes [Kiang et al., 2002].

Increased expression of HSP-70 resulting either from exposing human Jurkat T cells to heat stress or by gene transfection enhances the expression of the CD95/Fas antigen on the cell membrane, which makes the cells more susceptible to apoptotic death [Liossis et al., 1997]. It is reported that overexpression of Fas/CD95 desensitizes antigen receptors [Yankee et al., 2001], inhibits cell growth and impairs Ca^{2+} mobilization [Hueber et al., 2000].

For this study, we sought to determine whether heat stress alters Ca^{2+} fluxes in T cells, as it does in other cell types [Kiang et al., 1992; Kiang and McClain, 1999]. We found, using Jurkat T lymphocytes, that heat increased $[Ca^{2+}]_i$ by altering the kinetics of the cell surface membrane Na^+/Ca^{2+} exchanger. We also found that heat stress increased the phosphorylation of cNOs associated with the Na^+/Ca^{2+} exchanger to down-regulate its activity. These changes are correlated with an increased expression of Fas/CD95 on the surface of T cells, which might explain the apoptotic diathesis associated with heat stress.

MATERIALS AND METHODS

Cell Culture

T cells (Jurkat cell line, ATCC, Rockville, MD) were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 25 mM HEPES, pH 7.4 (Gibco-BRL, Gaithersburg, MD), in a humidified incubator with a 5% CO_2 atmosphere. Cells were fed every 3–4 days. Cell viability was determined using the Trypan blue exclusion assay.

Isolation of T Cells

Normal human T cells were separated from freshly collected human whole blood samples from donors. Ten milliliters of the whole blood samples were transferred to 50 ml conical tubes, diluted to 40 ml in PBS, and layered from the bottom with 10 ml Lymphocytes Separation Medium (Miltenyi Biotec, Inc., Auburn, CA), and centrifuged at 2,000*g* for 25 min at room temperature. The junctional band of cells separating the two layers containing the lymphocytes was collected. Cells were washed once with PBS and again with RPMI 1640 medium and counted. T cells were then separated using T cell isolation kit using an immuno-magnetic procedure to deplete non-T cells. Briefly, these lymphocytes were washed with cold MACS buffer (1% fetal calf serum in PBS) and resuspended in 20 μl/10^7 cells suspension of cold MACS buffer. Cells were incubated with Hapten-Antibody Cocktail (7 μl/10^7 cells, Miltenyi Biotec, Inc., Auburn, CA), washed twice with MACS buffer, resuspended in 20 μl/10^7 cells suspension of MACS buffer, and incubated for 15 min with 7 μl/10^7 cells of anti-hapten monoclonal antibody on ice. Subsequently, cells were washed twice with MACS buffer and resuspended in 100×10^6 cells/ml MACS buffer. Unlabeled T cells were separated by applying cells on top of the depletion column (MiniMACS™, Miltenyi Biotec, Inc., Auburn, CA). The column was placed within a strong magnetic field, and the unbound fraction of cells was collected (non-T cells remain bound). The column was washed with MACS buffer to ensure maximal recovery of cells. The purified T cells were washed with PBS and resuspended with RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 25 mM HEPES, pH 7.4 (Gibco-BRL, Gaithersburg, MD). Cells were counted

and stained with fluorescence coupled TCRαβ antibodies and analyzed by FACS to determine purity of separation (typically 98.5%).

Intracellular Ca^{2+} and Na^{+} Measurements

T cells were washed with Na^{+} Hanks' buffer and resuspended in Na^{+} Hanks' buffer containing 5 mM glucose and 0.05% albumin, then loaded with 5 μM fura-2AM or 10 μM SBFI-AM plus 0.2% pluronic F-127 (to make cells more permeable to the probe) at 37°C for 60 min. Cells were washed again before fluorescence measurements. The method to determine $[Ca^{2+}]_i$ has been described previously [Kiang, 1991]. Briefly, suspended cells were placed in a thermostatically controlled cuvette maintained at a constant temperature of 37°C. The fluorescence signal was measured with a DeltaScan spectrofluorometer (Photon Technology International, Inc., South Brunswick, NJ) with emission at 510 nm and dual excitation at 340 and 380 nm (slit width 4 nm). To minimize any contribution to the fluorescence signal resulting from dye in the extracellular medium, cells were washed thoroughly in Hanks' buffer before measurement of $[Ca^{2+}]$.

The method to determine $[Na^{+}]_i$ has been also described elsewhere [Kiang et al., 1992]. The fluorescence signal was measured with emission at 505 nm and dual excitation at 340 and 385 nm. For experiments performed in the absence of extracellular Ca^{2+}, cells were incubated in Ca^{2+}-free buffer containing 100 μM EGTA for 5 min prior to treatments. For experiments performed in the absence of external Na^{+}, cells were incubated in Na^{+}-free buffer containing 145 mM *N*-methyl-(+)glucamine also for 5 min prior to treatments.

Ca^{2+}-Uptake Measurements

For Ca^{2+}-uptake measurements, batches of Jurkat cells (7×10^6 cells/batch) were pelleted, resuspended in 3.5 ml Na^{+} Hanks' buffer (2×10^6 cells/ml) with or without 100 μM LNNA, and then incubated for 1 h at 37°C with occasional mixing. After incubation and immediately before heat treatment, cell batches were pelleted by centrifugation and resuspended in 3.5 ml of Na^{+} Hanks' buffers containing $CaCl_2$ concentrations ranging from 0.1–1.6 mM, each with a constant specific activity of 1.32 Ci/mol $^{45}CaCl_2$ (Dupont/NEN, Boston, MA). Cells were heated by immersing the cell suspensions in a water bath held at 45°C for a time sufficient to bring the suspension to 45 °C for 10 min (~12 min). After heating, 0.5 ml aliquots of each suspension (10^6 cells) were immediately layered over 0.4 ml of silicone oil (Versilube F-50 Silicone Fluid, General Electric Co., Waterford, NY) contained in a 1.5 ml Eppendorf-style microfuge tube and centrifuged for 1 min at 13,000*g* in a table-top centrifuge to pellet the cells through the oil. After centrifugation, the radioactive medium above the oil and then the oil itself were aspirated, ensuring that no medium contacted the cell pellet. Using this silicone oil separation technique with other T lymphocytes, we have shown that entrained extracellular medium represents only 2–3% of the pellet volume. Since the different concentrations of Ca^{2+} buf- buffers that were used all contained a fixed specific activity of ^{45}Ca, radioactivity due to contamination of the pellets by the medium was a constant, small percentage of the counts measured and were ignored.

The tip of the microfuge tube containing the pellet was cut off close to the pellet and placed into a new microfuge tube containing 0.5 ml Hanks' buffer. The pellets were resuspended and disrupted by sonication (Heat Systems, Inc., fitted with microtip disrupter, power setting 4, 30 s). Two aliquots (50 μl each) were removed for protein determination using the Bio-Rad protein assay (Bio-Rad Laboratories, South Richmond, CA). The microfuge tube containing the remaining 0.4 ml of sonicate was then placed into a 20 ml glass scintillation vial and 15 ml of Ecoscint scintillation fluid (National Diagnostics, Manville, NJ) was added. The tubes were shaken vigorously to ensure thorough mixing, and radioactivity was measured (Beckman LS5801, Palo Alto, CA). Specific radioactivity of the Ca^{2+} buffers was determined by counting the DPM in a 10-μl aliquot of the 1.6 mM Ca^{2+} incubation medium. Total Ca^{2+} taken up by the cell was then calculated, correcting for the volume (20%) removed for protein measurements, and expressing the results as pmol $Ca^{2+} \times$ mg protein$^{-1} \times$ min^{-1} [Kiang et al., 1998].

Immunoprecipitation and Western Blots

Cells (5×10^6) were resuspended in lysis buffer, disrupted by sonication, centrifuged at 12,500*g* for 10 min at 4°C, and the supernatant was collected. Protein content of the supernatant was determined using the Bio-Rad assay.

Samples containing 300 μg protein were incubated with the specified antibody (5 μl), chilled on ice for 1 h, mixed with protein A/G agarose beads (50 μl; Santa Cruz Biotechnology, CA), and incubated overnight on a nutator at 4°C. The immunoprecipitate was collected by centrifugation at 12,500*g* for 10 min, washed twice with 500 μl of stop buffer, and once with 500 μl Tris wash buffer. The pellet was resuspended in the 50 μl of electrophoresis sample buffer without 2-mercaptoethanol, boiled for 5 min, then centrifuged for 30 s to remove the agarose beads. The supernatant was incubated with 5% 2-mercaptoethanol at 37°C for 1 h. Twenty-five microliters of sample was loaded onto precast 10% Tris-glycine polyacrylamide gels (Novex, San Diego, CA). After electrophoresis, proteins were transferred to a nitrocellulose membrane (MSI Micron Separations, Inc., West Borough, MA) using a Novex blotting module. Phosphotyrosine was detected using an anti-phosphotyrosine antibody and enhanced chemiluminence (Amersham Pharmacia, UK), following the manufacturer's protocols for each. Results were quantified by densitometry.

Flow Cytometry

The 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.

Nitric Oxide Measurements

Nitric oxide production indicated as nitrite was measured using Griess Reagent System with sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride under acidic condition (Promega, Madison, WI).

Solutions

Hanks' buffer contained, in mM: 145 NaCl, 4.6 KCl, 1.2 $MgCl_2$, 1.6 $CaCl_2$, and 10 HEPES (pH 7.40 at 24°C). Ca^{2+}-free Hanks' buffer was prepared by adding 100 μM EGTA to nominally Ca^{2+}-free Hanks'. In Na^+-free Hanks' solution, *N*-methyl-(+)glucamine was used to substitute for equimolar concentrations of Na^+. Cell lysis buffer contained, in mM, 10 tris, 50 NaCl, 5 EDTA, 50 NaF, 30 Na pyrophosphate, 0.1 Na orthovanadate, 0.2 phenylmethylsulfonyl fluoride (PMSF), and 1 dithiothreitol (DTT), 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 1% Triton (pH 7.4 at 24°C). Stop buffer was cell lysis buffer without triton. Tris wash buffer contained, in mM, 20 Tris-HCl, 150 NaCl, 2 EDTA (pH 7.4 at 24°C). Electrophoresis sample buffer (2×) was 0.5 M Tris-HCl, 20% glycerol, 0.1% bromophenol blue, 10% sodium dodecyl sulfate, and 5% 2-mercaptoethanol.

Statistical Analysis

All data are expressed as the mean ± SEM Analysis of variance (ANOVA), Student's *t*-test, Chi-square test, Bonferroni's inequality were used for comparison of groups with a significance level of $P < 0.05$. Curve fitting was performed with the GraphPad Inplot program (GraphPad, San Diego, CA).

Chemicals

Nω-nitro-L-arginine (LNNA), aminoquanidine, and L-N^6-(1-iminoethyl)lysine (L-NIL), *S*-nitroso-*N*-acetylpenicillamine (SNAP), and L-arginine were purchased from Sigma-Aldrich Corp. (St. Louise, MO). Dichlorobenzamil was provided by Dr. Peter K.S. Siegl (Merck & Co, Inc., West Point, PA). Fura-2AM and pluronic acid F-125 were purchased from Molecular Probes, Inc. (Eugene, OR). Antibodies against Na^+/Ca^{2+} exchanger (Affinity BioReagents, Inc., Golden, CO), nNOs (neuronal NOs; a form of cNOs), phosphorylated tyrosine, phosphorylated serine, and phosphorylated threonine were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

Presence of cNOs

Although cNOs has been presumed to exist in all cells, we are the first to demonstrate it directly in human Jurkat (Fig. 1A, lanes 1–3) and normal T cells (Fig. 1A, lanes 4–6). cNOs

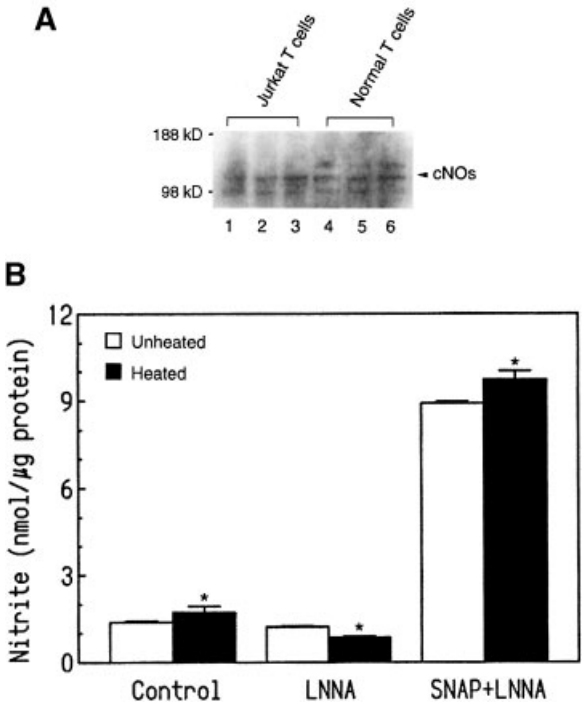

Fig. 1. Presence of cNOs and effect of cNOs inhibition on heat stress-induced increase in NO production. **A**: Cell lysates from Jurkat (**lanes 1–3**) and normal T cells (**lanes 4–6**) were used for Western blotting to detect the presence of cNOs direct against a polyclonal antibody. A representative Western blot is presented here. **B**: T cells were treated with 100 μM LNNA or SNAP for 30 min prior to heating at 45°C for 10 min. Then, cells were placed back to the incubator at 37°C for 24 h (n = 3). The medium was collected for NO measurements and cell pellets were assayed to determine the protein amount. *$P < 0.05$ vs. unheated, determined by Student's *t*-test.

was detected using an antibody directed against nNOs (Fig. 1A).

We then determined if the response of these cells to heat stress would alter NO production. Figure 1B shows that heat stressing of human Jurkat T cells increased NO production by 25%. Treatment with LNNA (an irreversible inhibitor of cNOs and a reversible inhibitor of iNOs) prior to heat stress reduced NO production, even lower than that found in LNNA-treated cells. Treatment of cells with SNAP (a NO donor) increased NO basal level and blocked the LNNA inhibitory effect. Similar results were observed in normal human T cells (data not shown). These results suggest that cNOs in T cells are functional.

$[Ca^{2+}]_i$ Increase in Heat-Stressed T Cells Results From Entry of Extracellular Ca^{2+}

We first established that heat stress increases $[Ca^{2+}]_i$ in human peripheral and Jurkat T lymphocytes, a response like that observed for a number of other cell types [Kiang et al., 1992; Kiang and McClain, 1999]. With Jurkat T cells, heating at 45°C for 10 min increased $[Ca^{2+}]_i$ to 194 ± 8 nM from the basal level of 112 ± 2 nM (Fig. 2A,B, $P < 0.05$ vs. unheated). With normal human T cells, heating at 45°C for 10 min increased $[Ca^{2+}]_i$ to 179 ± 44 nM from the basal level of 69 ± 3 nM (n = 4 volunteers, $P < 0.05$ vs. unheated).

We then performed experiments to determine whether the heat stress-induced increase in $[Ca^{2+}]_i$ was the result of Ca^{2+} entry from the extracellular medium or the mobilization of Ca^{2+} from intracellular pools. When Jurkat T cells were incubated in Ca^{2+}-free buffer for 5 min, resting $[Ca^{2+}]_i$ decreased to 98 ± 9 nM (n = 4) from a resting level of 119 ± 11 nM (n = 4). This suggests that the entry of external Ca^{2+} contributes to the maintenance of resting $[Ca^{2+}]_i$. When cells were heat stressed by heating at

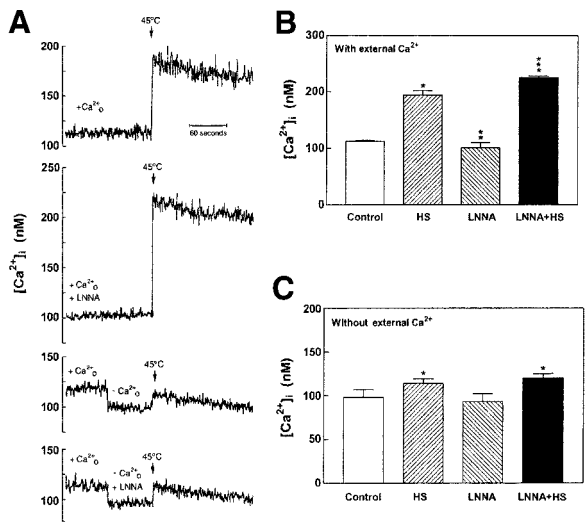

Fig. 2. Effect of cNOs inhibition on basal $[Ca^{2+}]_i$ and heat stress-induced increase in $[Ca^{2+}]_i$ in Jurkat T cells in presence and absence of external Ca^{2+}. **A**: Representative $[Ca^{2+}]_i$ tracings for the data calculated in this figure. Initial tracing is basal level in presence of external Ca^{2+}. **B**: Cells were incubated with or without 100 μM LNNA in presence of 1.6 mM external Ca^{2+} for 30 min prior to exposure to 45°C for 10 min and measurement of $[Ca^{2+}]_i$ (n = 3–6). *$P < 0.05$ vs. unheated without LNNA, unheated and heated with LNNA; **$P < 0.05$ vs. unheated and heated without LNNA and heated with LNNA; ***$P < 0.05$ vs. unheated and heated without LNNA and unheated with LNNA, determined by Chi-square test. **C**: Cells were incubated with or without LNNA in presence of 1.6 mM external Ca^{2+} for 30 min, and then transferred to Ca^{2+}-free buffer for 5 min before heating at 45°C for 10 min and $[Ca^{2+}]_i$ measurement (n = 3–6). *$P < 0.05$ vs. unheated without LNNA and unheated with LNNA, determined by Chi-square test. $+Ca^{2+}{}_o$: with external Ca^{2+}; $-Ca^{2+}{}_o$: without external Ca^{2+}; HS: heat stress at 45°C for 10 min.

45°C for 10 min in Ca^{2+}-free buffer, $[Ca^{2+}]_i$ increased, but to a level much less than that measured when external Ca^{2+} was present (Fig. 2A,C). Similar results were found in normal T cells (data not shown). These results indicate that extracellular Ca^{2+} plays a major role in the heat stress-induced increase in $[Ca^{2+}]_i$.

Inhibition of cNOs Enhances Heat-induced Increase In $[Ca^{2+}]_i$ by Promoting Entry of Extracellular Ca^{2+}

Because increases in $[Ca^{2+}]_i$ have been shown to result in a rapid increase in cNOs activity and NO release in other cells [Stefano et al., 2000], we performed experiments to determine whether cNOs is involved in the regulation of $[Ca^{2+}]_i$ in Jurkat T cells. Prior to heat stress, we incubated cells with LNNA. Treatment with 100 μM LNNA for 30 min decreased resting $[Ca^{2+}]_i$ to 101 ± 9 nM ($n = 4$) from the resting level of 127 ± 11 nM ($n = 4$, $P < 0.05$ between untreated and treated). Subsequent exposure of these cells to heat increased $[Ca^{2+}]_i$ to 250 ± 3 nM (Fig. 2A,B, $P < 0.05$ vs. heated cells without LNNA treatment), an increase greater than that normally observed after heating. Incubating normal T cells with 100 μM LNNA for 30 min reduced the baseline $[Ca^{2+}]_i$ to 47 ± 20 nM ($n = 4$ volunteers) from 63 ± 2 nM ($n = 4$ volunteers). However, heat stress increased $[Ca^{2+}]_i$ to 252 ± 2 nM ($n = 4$ volunteers), a level greater than in cells not exposed to LNNA (4-fold increase vs. 2.6-fold increase). These results suggest that cNOs plays a role in down-regulating heat-stimulated increases in $[Ca^{2+}]_i$.

We performed experiments to determine which Ca^{2+} source—intracellular or extracellular—is responsible for the enhanced increase in $[Ca^{2+}]_i$ that was observed after treatment with LNNA. When cells were treated with 100 μM LNNA in the absence of external Ca^{2+}, we did not observe the enhancement of $[Ca^{2+}]_i$ that was observed after heat stress in the presence of external Ca^{2+} (Fig. 2A,C). This indicates that cNOs regulates the entry of extracellular Ca^{2+} after heat stress, not internal Ca^{2+} stores.

Although LNNA is recognized primarily as an irreversible cNOs inhibitor, it is also a reversible inhibitor of iNOs. We therefore determined whether iNOs inhibition might contribute to the decrease in $[Ca^{2+}]_i$ response that occurs after incubation with LNNA. We incubated cells with 100 μM L-N^6-(1-iminoethyl)lysine (L-NIL), a specific inhibitor of iNOs. L-NIL altered neither the basal level of $[Ca^{2+}]_i$ nor the increase in $[Ca^{2+}]_i$ after heating (data not shown). This indicates that cNOs primarily regulates the entry of extracellular Ca^{2+} into the cytoplasm.

We performed additional experiments to determine how cNOs might be regulating $[Ca^{2+}]_i$, or more specifically, whether NO produced by cNOs plays a role in regulating increases in $[Ca^{2+}]_i$. To do this we artificially increased levels of NO in the cells by treating cells with L-arginine, which is both a stimulator of NOs synthases and a substrate for NO production. Simply incubating cells with 100 μM L-arginine had no effect on either the resting level of $[Ca^{2+}]_i$ or the size of the increase in $[Ca^{2+}]_i$ measured after heat treatment. However, if cells were treated with L-arginine prior to treatment with LNNA, the enhanced increase in $[Ca^{2+}]_i$ after heat stress did not occur (Table I). The elevated levels of NO stimulated by L-arginine treatment apparently overcame the inhibition of cNOs activity that was exerted by LNNA, and regulation of the $[Ca^{2+}]_i$ response was restored. Table I also shows that treatment with SNAP (a potent NO donor) markedly reduced the heat stress-induced increase in $[Ca^{2+}]_i$ and the LNNA-induced enhancement on $[Ca^{2+}]_i$ responses to heat stress. These results indicate that NO production plays an important role in regulating the $[Ca^{2+}]_i$ response to heat stress.

cNOs Is Not Involved in CD3-mediated Increase in $[Ca^{2+}]_i$ in Jurkat T Cells

Cross-linking of CD3 in T cells is known to lead to a significant increase in $[Ca^{2+}]_i$, which

TABLE I. LNNA Enhancement of Heat Stress-Induced Increase in $[Ca^{2+}]_i$ in Jurkat T Cells

	$[Ca^{2+}]_i$ (nM)		
Treatment	Baseline	Heated	%Increase
Control	222 ± 1	464 ± 27*	242 ± 27
LNNA	180 ± 6	471 ± 19*	297 ± 19**
L-arginine	207 ± 2	438 ± 17*	231 ± 17
L-arginine + LNNA	211 ± 3	425 ± 19*	214 ± 19
SNAP	233 ± 11	316 ± 12*	136 ± 9**
SNAP + LNNA	221 ± 17	343 ± 15*	155 ± 7**

Cells were treated with 100 μM LNNA, 100 μM L-arginine, 100 μM SNAP alone or combination with LNNA for 30 min prior to heat stress at 45°C for 10 min ($n = 4–7$). Treatments with LNNA, L-arginine, or SNAP before or after exposure of cells to heat stress did not alter cell viability.

*$P < 0.05$ vs. Respective baseline;
**$P < 0.05$ vs. Increases in control, determined by Student's *t*-test.

results from the release of Ca^{2+} from intracellular pools and Ca^{2+} influx from the extracellular space via receptor-mediated Ca^{2+} channels [Liossis et al., 1997]. To investigate if cNOs is involved in suppressing this response, we performed experiments with Jurkat T cells treated with the anti-CD3 antibody OKT3, which normally triggers the Ca^{2+} response. Incubating cells with OKT3 alone (10 μg/ml) increased $[Ca^{2+}]_i$ by 490 ± 19%. In the presence of 100 μM LNNA, OKT3 induced a similar increase in $[Ca^{2+}]_i$, indicating that cNOs is not involved in the CD3-induced $[Ca^{2+}]_i$ response.

Heat Stress and cNOs Effects on Na^+/Ca^{2+} Exchanger Activity

It has been previously shown that the heat stress-induced increase in $[Ca^{2+}]_i$ in human epidermoid A-431 cells is mediated by the Na^+/Ca^{2+} exchanger [Kiang et al., 1992]. To determine whether a similar mechanism is involved in human Jurkat T cells, we tested the effect of heat stress while inhibiting the Na^+/Ca^{2+} exchanger by incubating cells in Na^+-free Hanks' solution. Under these conditions, heat stress induced a greater increase in $[Ca^{2+}]_i$ than was measured with Na^+ present. Treatment with LNNA did not induce an additional increase in $[Ca^{2+}]_i$ (Fig. 3A,B). These data indicate that LNNA activity in Jurkat T cells uses a pathway that involves Na^+.

We also measured $[Na^+]_i$ in Jurkat T cells. With the use of the Na^+-specific fluorescent dye, SBFI, in unheated cells, the resting $[Na^+]_i$ was 17.16 ± 0.94 mM (n = 5). Figure 3B shows that heat stress decreased $[Na^+]_i$. Treatment with LNNA further decreased $[Na^+]_i$. In Na^+-free Hanks' solution, heat stress also decreased $[Na^+]_i$ more, but treatment with LNNA failed to decrease further. The results reinforce the view that LNNA uses the pathway involves in Na^+.

We also incubated cells with dichlorobenzamil, a known inhibitor of the Na^+/Ca^{2+} exchanger. Figure 4 shows that treatment with dichlorobenzamil inhibited the heat stress-induced increase in $[Ca^{2+}]_i$ in a concentration-dependent manner, with a median inhibitory concentration (IC_{50}) of 3.24 ± 0.06 μM, suggesting that Ca^{2+} entry occurs through the Na^+/Ca^{2+} exchanger. In the presence of 100 μM LNNA, the IC_{50} of dichlorobenzamil increased to 10.9 ± 0.2 μM ($P < 0.05$ vs. that observed in LNNA-untreated cells, Student's *t*-test), suggesting that LNNA treatment alters the function of the Na^+/Ca^{2+} exchanger.

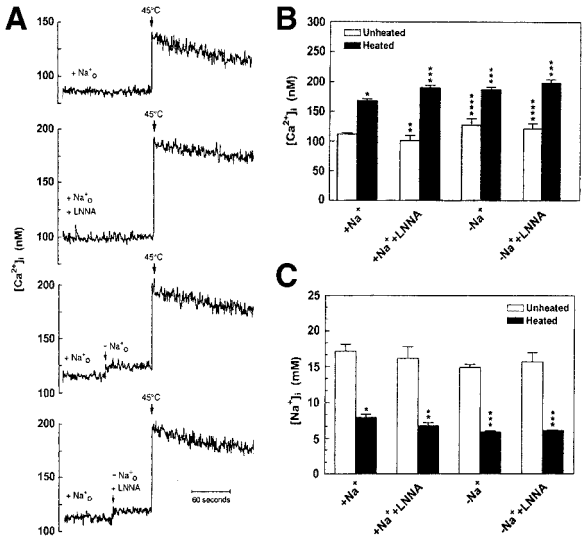

Fig. 3. Effect of cNOs inhibition on heat stress-induced increase in $[Ca^{2+}]_i$ and decrease in $[Na^+]_i$ in Jurkat T cells in presence and absence of external Na^+. **A**: Representative $[Ca^{2+}]_i$ tracings for the data calculated in this figure. Initial tracing is basal level in presence of external Na^+. **B**: Cells were incubated with or without 100 μM LNNA in Na^+-free Hanks' solution for 30 min prior to exposure to 45°C for 10 min. $[Ca^{2+}]_i$ was measured (n = 5–7 for each group). The $[Ca^{2+}]_i$ baselines for cells in presence of external Na^+ ($+Na^+$), in the presence of external Na^+ and LNNA ($+Na^+$+LNNA), in absence of external Na^+ ($-Na^+$), or in absence of external Na^+ but with LNNA ($-Na^+$+LNNA) were 112 ± 2 nM, 101 ± 9 nM, 127 ± 11 nM, and 121 ± 9 nM, respectively. $^*P < 0.05$ vs. unheated plus Na^+ and all other groups; $^{**}P < 0.05$ vs. LNNA plus Na^+ and heated and all other groups; $^{***}P < 0.05$ vs. all other groups; $^{****}P < 0.05$ vs. all other groups, determined by two-way ANOVA and Bonferroni's inequality. **C**: $[Na^+]_i$ was measured (n = 3–5 for each group). $^*P < 0.05$ vs. unheated+Na^+, unheated and heated without Na^+, and unheated and heated without Na^+ but with LNNA; $^{**}P < 0.05$ vs. unheated and heated with Na^+, unheated without Na^+, and unheated without Na^+ but with LNNA, $^{***}P < 0.05$ vs. all other groups, determined by two-way ANOVA and Bonferroni's inequality.

Heat Stress and cNOs Effects on Na^+/Ca^{2+} Exchanger Kinetics

The activity of the Na^+/Ca^{2+} exchanger is determined by both its binding affinity (K_m) for extracellular Ca^{2+} and its maximal velocity (V_{max}) for transporting Ca^{2+}. Changes in either K_m or V_{max} or both can alter the activity of this exchanger. To measure the kinetics of Ca^{2+} uptake, we incubated cells in medium containing various concentrations of Ca^{2+}, with and without heating at 45°C for 10 min. Table II lists the K_m and V_{max} values obtained from each

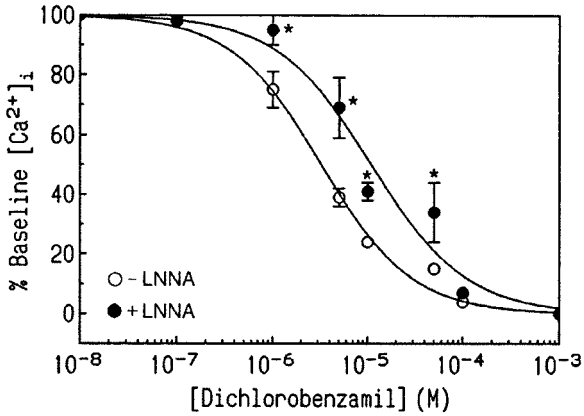

Fig. 4. Dichlorobenzamil inhibition of heat stress-induced increase in $[Ca^{2+}]_i$ after LNNA treatment in Jurkat T cells. Cells were incubated with or without 100 μM LNNA for 30 min prior to exposure to 45°C for 10 min in presence of different concentrations of dichlorobenzamil; $[Ca^{2+}]_i$ was then measured (n = 3–5). IC_{50} of dichlorobenzamil in untreated and LNNA-treated cells are 3.24 ± 0.06 μM and 10.9 ± 0.2 μM, respectively. *$P < 0.05$ vs. −LNNA, determined by two-way ANOVA and Bonferroni's inequality.

group. Heat stress decreased K_m for extracellular Ca^{2+} by 43% and V_{max} by 10%. A new kinetic steady state was established when cells were treated with LNNA alone. Nevertheless, treatment with LNNA prior to heat stress decreased K_m by 138% and V_{max} by 27%, suggesting the vastly increased binding affinity to extracellular Ca^{2+} contributes enhancement of the heat stress-induced $[Ca^{2+}]_i$ increase.

cNOs Associates With Na^+/Ca^{2+} Exchanger

Because the above experiments suggest that cNOs regulates the activity of the Na^+/Ca^{2+} exchanger, we asked whether these two molecules physically associate with one another. Immunoprecipitation experiments detected a cNOs–Na^+/Ca^{2+} exchanger complex in lysates

TABLE II. Association Constant (K_m) and Maximal Velocity (V_{max}) of Ca^{2+} Entry in Jurkat T Cells

Treatment	K_m (mM)	V_{max} (pmol Ca^{2+}/mg protein/min)
Unheated	0.534 ± 0.150	348 ± 26
Heated	0.373 ± 0.037*	315 ± 9
LNNA, unheated	0.693 ± 0.152	423 ± 21
LNNA, heated	0.291 ± 0.042**	332 ± 12***

Cells were treated with 100 μM LNNA for 30 min prior to heat stress at 45°C for 10 min (n = 4).
*$P < 0.05$ vs. unheated K_m;
**$P < 0.05$ vs. LNNA plus unheated K_m;
***$P < 0.05$ vs. LNNA plus unheated V_{max}, determined by Student's *t*-test.

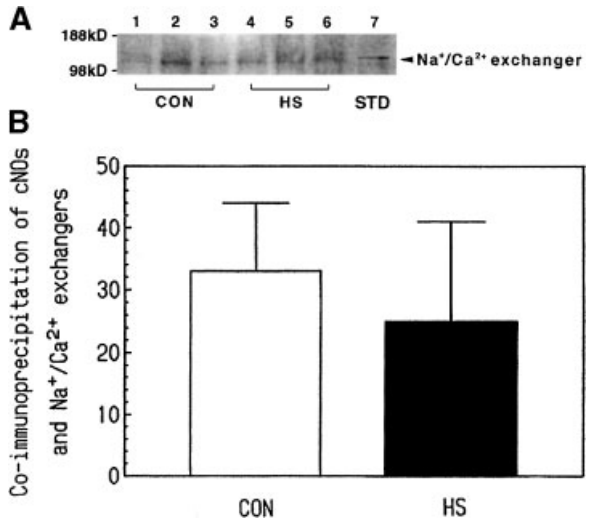

Fig. 5. Co-immunoprecipitation of cNOs and Na^+/Ca^{2+} exchanger in Jurkat T cells. Cells were incubated at 37°C (CON) or 45°C for 10 min (HS). Lysates were collected and immunoprecipitated with anti-cNOs antibody followed by immunoblotting antibody for Na^+/Ca^{2+} exchanger (n = 3): (**A**) representative immunoblot shows the presence of Na^+/Ca^{2+} exchanger in lysates from control cells (**lanes 1–3**) and HS-treated cells (**lanes 4–6**); (**B**) densitometric quantitation of Na^+/Ca^{2+} exchanger. $P > 0.05$ vs. unheated, determined by Student's *t*-test. STD: standard protein from rat heart lysate (**lane 7**); CON: control; HS: heat stress.

from non-heat stressed cells (Fig. 5A, lanes 1–3), but the amount of the complex did not change after exposure of cells to heat stress (Fig. 5A, lanes 4–6 and Fig. 5B). This suggests that mere association of cNOs with the Na^+/Ca^{2+} exchanger does not account for its functional effect. Western blots failed to detect heat shock protein 70 kDa (HSP-70), 90 kDa (HSP-90), or TCR-ζ chain in the co-immunoprecipitated complex (data not shown), indicating that the association between cNOs and the Na^+/Ca^{2+} exchanger is specific.

Heat Stress Induces Tyrosine Phosphorylation of cNOs

Since cNOs is apparently closely associated with the Na^+/Ca^{2+} exchanger, yet the number of complexes does not change after heating, we performed experiments to determine how cNOs might regulate the exchanger. It is known that the activity of many proteins is regulated by phosphorylation and dephosphorylation [Kiang and Tsokos, 1998]. To determine whether this is true for our system, we blotted anti-cNOs precipitates with antibodies against phosphorylated serine, threonine, or tyrosine residues. Figure 6 shows that heat stress increased

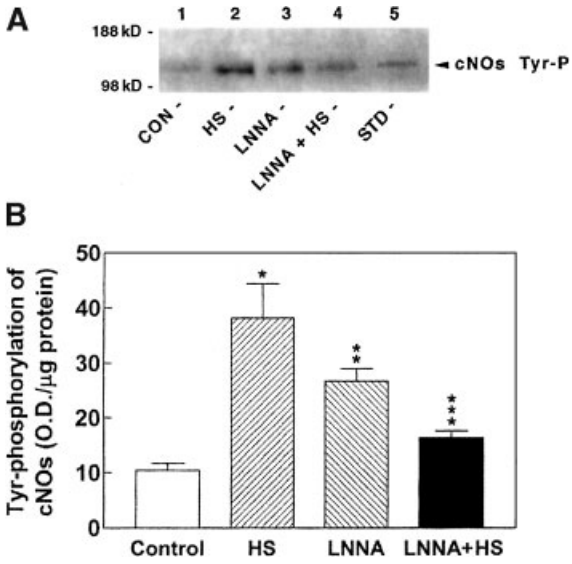

Fig. 6. Effect of LNNA inhibition on heat stress-induced tyrosine phosphorylation of cNOs in Jurkat T cells. Cells were treated with or without 100 μM LNNA 30 min prior to exposure to 37 or 45°C for 10 min. Lysates were collected and immunoprecipitated with anti-cNOs, followed by immunoblotting with tyrosine phosphorylation antibody (n = 3). **A**: Representative immunoblot; (**B**) densitometric quantitation of tyrosine phosphorylation. $^{*}P < 0.05$ vs. control, LNNA, and LNNA + HS groups; $^{**}P < 0.05$ vs. control, HS, and LNNA+HS groups; $^{***}P < 0.05$ vs. control, HS, and LNNA groups, determined by Chi-square test. STD: standard protein from rat heart lysates.

phosphorylation of cNOs at tyrosine residues by 4-fold (Fig. 6A, lane 2 vs. lane 1) but not at serine or threonine residues (data not shown).

Incubating cells with LNNA alone significantly increased the basal level of the tyrosine phosphorylation of cNOs in unheated cells, but LNNA treatment prior to heat stress completely inhibited the phosphorylation induced by heat stress (Fig. 6B). This suggests that the heat stress-induced tyrosine phosphorylation of cNOs inhibits the Na^{+}/Ca^{2+} exchanger.

Inhibition of cNOs Enhances Fas/CD95 Expression in Heat Stressed Cells

We next determined whether heat stress alters Fas/CD95 expression on the cell membrane and whether expression is affected by cNOs inhibition. Jurkat cells were first incubated either with or without LNNA. Cells were subsequently heat stressed at 45 °C for 10 min, then returned to 37°C for different periods to monitor changes in surface Fas/CD95 expression. Histograms derived from flow cytometry data (Fig. 7A) indicate that heat stress increased the percentage of cells expressing Fas/CD95, regardless of whether the cells had been treated with LNNA or not. Heat stress without LNNA treatment stimulated an increase in Fas/CD95 expression in the total population beginning at 12 h that reached a maximum at 24 h. By 48 h, two distinct peaks of Fas/CD95-expressing cells appeared, representing populations expressing high and low densities of Fas/CD95. Expression returned to basal levels at 120 h (Fig. 7A,B).

Working with data from cells not treated with LNNA before heating, we compared the number of cells in the two subpopulations (see Fig. 7C,D). Twenty-four hours after heat stress, 58% of the cells displayed low-density Fas/CD95 expression 9% showed high-density expression. At 48 h, 41% displayed low-density expression while 31% exhibited high-density expression. At 72 h, 40% displayed low-density expression while 16% showed high-density expression. Ninety-six hours after heat stress, no cells were expressing a high-density of Fas/CD95 (Fig. 7C,D).

Treatment of cells with LNNA alone without heat stress did not alter the Fas/CD95 basal expression (data not shown). When total Fas/CD95 expression in the cell population was measured in cells treated with LNNA prior to heat stress, the profile was very similar to the expression measured in cells heat stressed without LNNA treatment until 120 h, at which point the LNNA-treated cells exhibited higher levels of total expression (Fig. 7A,B). More pronounced differences were observed when the low- and high-density Fas/CD95 expressing cells in this experiment were analyzed independently. Twenty-four hours after LNNA-treated cells were heated, 58% of cells displayed low-density Fas/CD95 expression while 10% displayed high-density expression. At 48 h, 18% cells displayed low-density expression while 56% exhibited high-density expression. At 72 h, 28% cells displayed low-density expression while 32% showed high-density expression. No high-density Fas/CD95 expression was observed 96 h after heat stress (Fig. 7D), but low-density expressing cells were still present (21% at 96 h and 18% at 120 h; Fig. 7C). These results suggest that cNOs down-regulates Fas/CD95 expression on the cell membrane of cells exposed to heat stress.

DISCUSSION

The purpose of this study was to characterize regulation of $[Ca^{2+}]_i$ and Fas/CD95 expression by cNOs in human Jurkat T cells exposed to

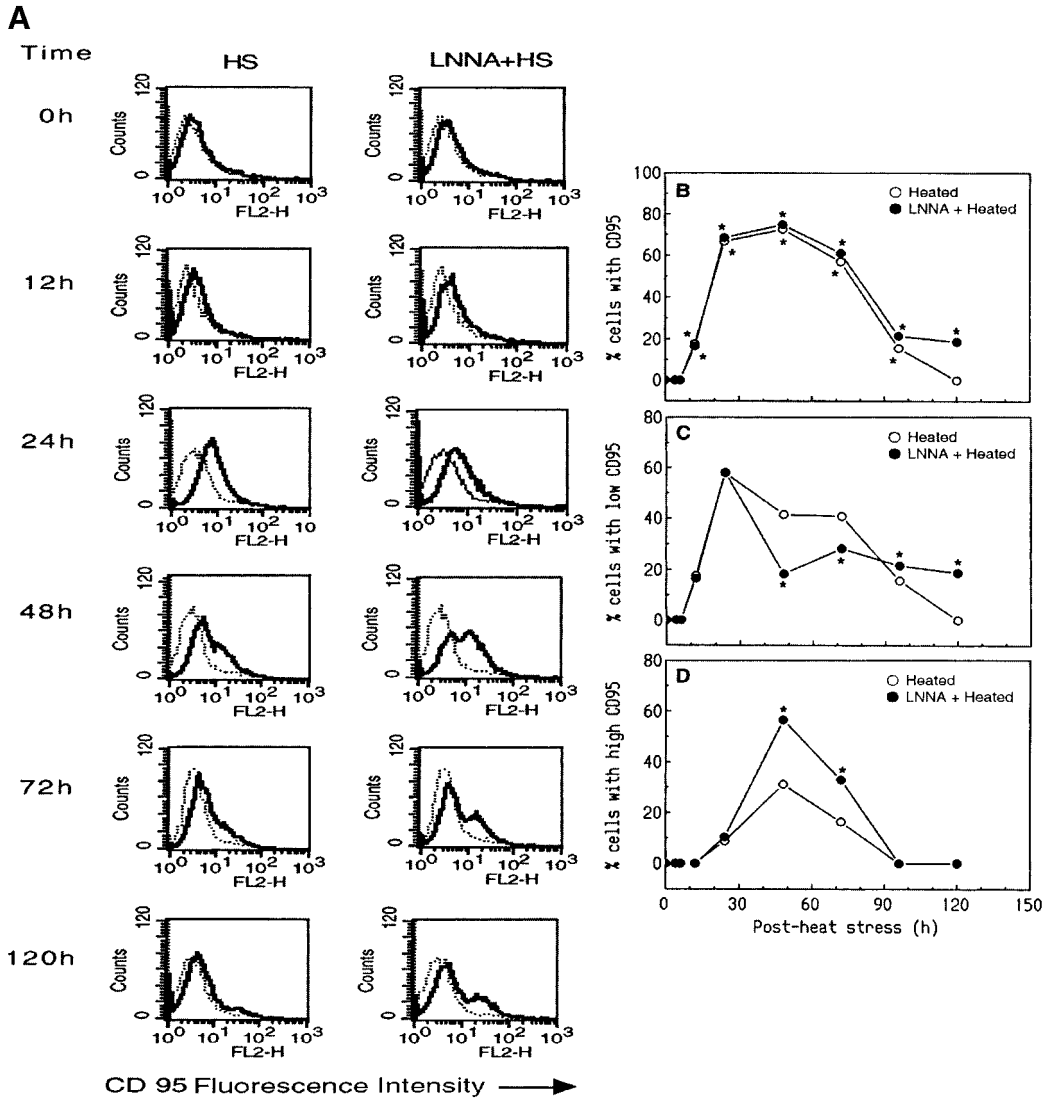

Fig. 7. Enhancement of heat stress-induced Fas/CD95 expression by LNNA in Jurkat T cells. Cells were treated with or without 100 μM LNNA 30 min prior to exposure to 45°C for 10 min in the presence of external Ca^{2+}. Treated cells were collected immediately, 12, 24, 48, 72, 96, and 120 h after heat stress. Cells were then stained with PE-conjugated anti-CD95 antibody for 20 min and expression of Fas/CD95 was assessed by flow cytometry. Three independent experiments were conducted. **A**: Histograms showing time courses of expression in heat-stressed cells without LNNA treatment (HS) and with LNNA treatment (LNNA + HS). Dotted lines represent data from control cells not heat stressed; solid lines represent data from heat stressed cells. **B**: Percentage of total cells that express Fas/CD95, $*P < 0.05$ vs. percentage immediately after heat stress, determined by Student's *t*-test. **C**: Percentage of cells expressing Fas/CD95 in the low-density population. $*P < 0.05$ vs. no LNNA treatment at same time point, determined by Student's *t*-test. **D**: Percentage of cells expressing Fas/CD95 in the high-density population. $*P < 0.05$ vs. no LNNA treatment at same time point, determined by Student's *t*-test.

heat stress. Our results have demonstrated that there is an association between cNOs and the Na^+/Ca^{2+} exchanger located at the cell membrane. Heat stress increased both the kinetic activity of the Na^+/Ca^{2+} exchanger and the level of cNOs phosphorylation at tyrosine residues.

We detected the presence of cNOs in human normal and Jurkat T cells. This cNOs was capable of increasing the NO production induced by heat stress. The increase was significantly inhibited by cNOs inhibitor LNNA, indicating cNOs catalyzes NO production. The inhibitory effect of LNNA was reversed by L-arginine (a NO substrate) and SNAP (a powerful NO donor). These results are consistent with observations found in human T84 intestinal epithelial cells [Kiang et al., 2002].

Exposure of Jurkat T cells to heat stress increased $[Ca^{2+}]_i$ by promoting the entry of Ca^{2+} from the extracellular space. Similar to what has been shown in other types of cells [Kiang et al., 1992; Kiang and McClain, 1999], we have shown here that the increases in $[Ca^{2+}]_i$ resulted from activation of the Na^+/Ca^{2+} exchanger. This is because the increases were stimulated by external Na^+ removal and inhibited by external Ca^{2+} removal or treatment with dichlorobenzamil, a known inhibitor of the Na^+/Ca^{2+} exchanger. This view is further supported by the data of $[Na^+]_i$ measurements in cells in Na^+-free Hanks' solution (Fig. 3B).

Heat stress alone altered the affinity (K_m) and the maximal velocity (V_{max}) of the exchanger for extracellular Ca^{2+}. This response is different from that observed in human epidermoid A-431 cells [Kiang et al., 1992] and colon carcinoma T84 cells [Kiang and McClain, 1999], a difference that may be a reflection only of the cell types used. Inhibition of cNOs increased the K_m and the V_{max} of the Na^+/Ca^{2+} exchanger for extracellular Ca^{2+}. Heating cNOs-inhibited cells resulted in a vastly decreased K_m, which explains why the inhibition of cNOs enhances the heat stress-induced increase in $[Ca^{2+}]_i$.

These results suggest cNOs regulates the kinetic activity of the exchanger. The Na^+/Ca^{2+} exchanger in mammalian heart [Reeves et al., 1994] is known to have 11 putative transmembrane segments and a large hydrophilic domain of 520 amino acids between the fifth and sixth transmembrane segments. The transmembrane segments are responsible for regulating K_m and V_{max}. Iwamoto et al. [2000] reported that the second and third transmembrane segments (i.e., the α-1 repeat) as well as the seventh transmembrane segment (the α-2 repeat) contain amino acid residues that regulate the K_m of the exchanger for extracellular Ca^{2+}. The hydrophilic domain also has regulatory potential; the 219-RRLLFYKYVYKRAGKQRG region binds the exchanger inhibitory peptide (XIP) and the 446-DDDIFEEDE and 498-DDDHAGIFTFE regions represent the Ca^{2+} binding domains for intracellular Ca^{2+}. Cytosolic Na^+ in the absence of ATP and absence of cytosolic Ca^{2+} controls exchanger activity [Reeves et al., 1994; Ottolia et al., 2001]. In human epidermoid A-431 cells, heat stress results in an opening of Na^+ channels at the cell membrane, and the resulting increase in $[Na^+]_i$ activates the Na^+/Ca^{2+} exchanger and increases V_{max} [Kiang et al., 1992].

We have shown that Jurkat T cells contain several intracellular Ca^{2+} stores that are sensitive to monensin, OKT3, thapsigargin, and ionomycin (Kiang, unpublished data), which may be mediated by store-operated channels [Cho et al., 1997; Waldron et al., 1997; Ma et al., 2002]. In this study, the enhanced $[Ca^{2+}]_i$ increase induced by heat stress plus LNNA disappeared completely in the absence of external Ca^{2+}, implying that intracellular Ca^{2+} stores do not play a role in this process.

Treatment with LNNA prior to heat stress enhanced the heat stress-induced increase in $[Ca^{2+}]_i$, which was not observed if cells were treated with L-NIL (a selective iNOS inhibitor), but prevented by L-arginine, SNAP (Table I), or removal of external Ca^{2+}, suggesting that this enhancement is mediated by a Ca^{2+}-dependent cNOs. Furthermore, this observation is consistent with that of NO production being significantly lower in the group of LNNA plus heat stress than the group of LNNA alone (Fig. 1B), indicating that the heat stress-induced increase in NO production is probably related to the heat stress-induced increase in $[Ca^{2+}]_i$. The results reinforce the view that a Ca^{2+}-dependent cNOs plays a critical role in the process.

Results of our immunoprecipitation experiments demonstrate the existence of cNOs/exchanger complexes in Jurkat T cells, but exposing cells to heat stress did not affect the degree of complex formation. Complex formation has been observed in human epidermoid A-431 cells that overexpress HSP-70 (Kiang, unpublished data), a system in which a desensitization of the Na^+/Ca^{2+} exchanger has been shown to occur [Kiang et al., 1998]. A complex between the cardiac Na^+/Ca^{2+} exchanger and the cytoskeletal protein ankyrin has also been shown [Li et al., 1993].

Heat stress increased the phosphorylation of the cNOs at tyrosine residues, but not at serine and threonine residues, suggesting that cNOs negatively regulates the basal activity of the Na^+/Ca^{2+} exchanger by altering the level of tyrosine phosphorylation. Blocking tyrosine phosphorylation of cNOs terminates the enzyme's ability to down-regulate the Na^+/Ca^{2+} exchanger, which in turn potentiates the kinetic activity of the Na^+/Ca^{2+} exchanger and enhances the heat stress-induced increase in $[Ca^{2+}]_i$. This finding is in agreement with that found in primary cortical cell cultures, in which the catalytic activity of NOs was diminished by

phosphorylation and enhanced by dephosphorylation [Dawson et al., 1993].

Our preliminary data suggest that protein tyrosine kinase inhibitors such as genistein and herbimycin A can regulate $[Ca^{2+}]_i$ responses to heat stress. Genistein decreases the resting $[Ca^{2+}]_i$ and its responses to heat stress, whereas herbimycin A increases the resting $[Ca^{2+}]_i$ and its responses to heat stress [Kiang, 2003]. These observations reinforce the view that phosphorylation of cNOs at tyrosine residues plays an important role in regulating the activity of the Na^+/Ca^{2+} exchanger.

NO has been shown to be involved not only in *S*-nitrosylation reactions, which are relevant to mammalian neurotransmission, ion channel function, intracellular signaling, and antimicrobial defense [Gaston, 1999], but also in protein interactions [Stuehr, 1999]. It has been shown in brain that cNOs binds to the dystrophin-glycoprotein complex [Chang et al., 1996], peptides ending with Asp-X-val or Thr/Ser-X-Val present on glutamate and melatonin receptors [Stricker et al., 1997], protein inhibitor of neuronal NOs [Tochio et al., 1998], and caveolins [Venema et al., 1997]. Moreover, HSP-90 has been shown to bind endothelial cell NOs and enhance its activation. HSP-90 also facilitates signaling mediated by the growth factor, G-protein, and mechanotransduction pathways that lead to the activation of NOs in endothelial cells [Garcia-Cardena et al., 1998]. Our data therefore extend the list of proteins to which cNOs binds and regulates their activity. The association of cNOs with the Na^+/Ca^{2+} exchanger is a selective one in that it failed to bind to HSP-70 and TCR-ζ chain.

Inhibition of cNOs increased the number of cells expressing a high density of Fas/CD95 after heat stress. The increase is likely Ca^{2+}-dependent because a significant enhancement of the heat stress-induced increase in $[Ca^{2+}]_i$ was observed prior to the increased Fas/CD95 expression. The possibility that this enhancement is related to the overexpression of heat shock protein 72 kDa (HSP-72) cannot be ruled out, because the time course of HSP-72 production [Kiang et al., 1994] parallels that of Fas/CD95. In Fig. 8, we present a schematic representation of a model for the interaction between cNOs and the Na^+/Ca^{2+} exchanger. Based on our data, cNOs and the exchanger are normally associated with each other in some way. Heat stress activates the reversed mode of

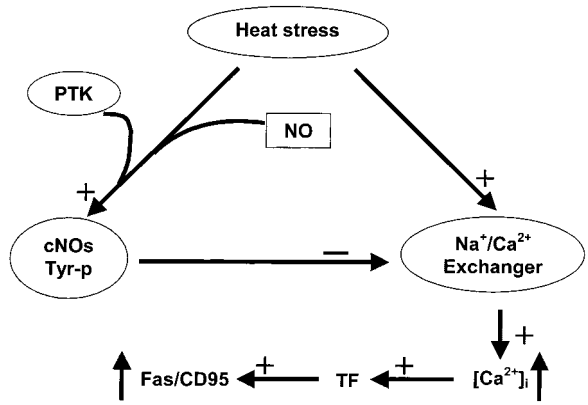

Fig. 8. Proposed schematic for cNOs regulation of Na^+/Ca^{2+} exchanger. cNOs associates with Na^+/Ca^{2+} exchanger. Heat stress simultaneously activates reversed mode of exchanger to allow external Ca^{2+} to enter cell and stimulates phosphorylation of cNOs at tyrosine residues, probably by protein tyrosine kinase (PTK) and increases NO production. Phosphorylated cNOs negatively regulates activity of exchanger and reduces level of Ca^{2+} entry. Increase in $[Ca^{2+}]_i$ is sufficient to activate an as-yet unidentified transcriptional factors (TF) to increase expression of Fas/Apo-1/CD95 (+ stimulation; − inhibition; ↑ increase).

the exchanger to allow extracellular Ca^{2+} into the cell while increasing NO production and simultaneously stimulating phosphorylation of cNOs at tyrosine residues. The phosphorylated cNOs negatively regulates the kinetic activity of the exchanger and diminishes the level of Ca^{2+} entry. However, the increase in $[Ca^{2+}]_i$ is enough to turn on as yet unidentified transcriptional factors that may lead to increased expression of Fas/CD95. Inhibition of cNOs by compounds such as LNNA enhances the heat stress-induced increase in $[Ca^{2+}]_i$, which in turn may lead to additional expression of Fas on the cell surface membrane.

Increases in NO production are observed in various diseases such as autoimmune diabetes mellitus [Fehsel et al., 1995], arthritis [McCartney-Francis et al., 1993], rheumatoid [McInnes et al., 1996], inflammatory bowel disease [Singer et al., 1996], hemorrhagic shock [Thiemermann et al., 1993; Szabo and Thiemermann, 1994; Kelly et al., 1997], and injuries with trauna, anaphylactic shock and heat [Szabo and Thiemermann, 1994]. Reduction of NO production under these pathological conditions may ameliorate the progress of pathogenesis. Therefore, NOs inhibitors such as LNNA may have potential therapeutic use for treating diseases.

In summary, inhibition of cNOs decreased the resting $[Ca^{2+}]_i$ in the presence or the absence of

external Ca^{2+}, but enhanced the heat stress-induced increases in $[Ca^{2+}]_i$ and Fas/CD95 expression by increasing the binding affinity and the maximal velocity of Na^+/Ca^{2+} exchangers for extracellular Ca^{2+}. cNOs was found to associate with the Na^+/Ca^{2+} exchanger and when phosphorylated at tyrosine residues it decreases the kinetic activity of the Na^+/Ca^{2+} exchanger.

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