Chinese Journal of Physiology 42(3): 153-159, 1999

Nω-Nitro-L-Arginine Decreases Resting Cytosolic [Ca^{2+}] and Enhances Heat Stress-Induced Increase in Cytosolic [Ca^{2+}] in Human Colon Carcinoma T84 Cells

Juliann G. Kiang and [1]David E. McClain

Department of Cellular Injury
Walter Reed Army Institute of Research
Washington DC 20307-5100
and
[1]Applied Cellular Radiobiology Department
Armed Forces Radiobiology Research Institute
Bethesda, MD 20889-5603, USA

Abstract

Nω-nitro-L-arginine (LNNA) inhibits the synthesis of heat shock proteins in animals and cultured cells exposed to heat stress. Heat shock protein synthesis is known to be Ca^{2+}-dependent. In this study, we have characterized the effect of LNNA on $[Ca^{2+}]_i$ before and after heat stress in human colon carcinoma T84 cells. In untreated cells incubated in the presence of external Ca^{2+}, the resting $[Ca^{2+}]_i$ was 201±3 nM. If these cells were exposed to 45 °C for 10 min, $[Ca^{2+}]_i$ increased by 50±2%. Preincubation with LNNA (100 μM) without subsequent heating led to a decrease in $[Ca^{2+}]_i$ in a LNNA concentration-dependent manner. Preincubation with LNNA followed by heating increased $[Ca^{2+}]_i$ to levels 88±5% greater than cells heated without LNNA pretreatment. Incubating cells in medium without external Ca^{2+} (no heating, no LNNA treatment) lowered resting $[Ca^{2+}]_i$ to 115±2 nM and greatly reduced the increase in $[Ca^{2+}]_i$ observed if cells were heated in the presence of Ca^{2+}, indicating that external Ca^{2+} plays an important role in the maintenance of $[Ca^{2+}]_i$ in T84 cells. With external Ca^{2+} absent, LNNA pretreatment further reduced $[Ca^{2+}]_i$ in unheated cells, and heating failed to enhance $[Ca^{2+}]_i$. We determined (with external Ca^{2+} present) that the heat-stress induced increase in $[Ca^{2+}]_i$ in T84 cells was blocked by dichlorobenzamil, a Na^+/Ca^{2+} exchanger inhibitor, suggesting that the exchanger mediates Ca^{2+} entry. The median inhibitory concentration (IC_{50}) in cells not treated with LNNA was 0.970±0.028 μM. With LNNA pretreatment, the IC_{50} was 5.099±0.107 μM. Heat stress of T84 cells did not affect the binding affinity of the Na^+/Ca^{2+} exchanger for external Ca^{2+}, but it increased the maximal velocity of the exchanger. In unheated cells, preincubation with LNNA decreased the binding affinity of the exchanger for Ca^{2+}, but after heat treatment, both the binding affinity and maximal velocity of the exchanger increased. Our data are consistent with the idea that LNNA affects the activity of the Na^+/Ca^{2+} exchanger. We also determined there are intracellular Ca^{2+} pools in T84 cells sensitive to thapsigargin, monensin, and ionomycin. Treatment with TMB-8, a blocker of Ca^{2+} sequestration and mobilization, or ionomycin inhibited the LNNA-induced decrease in $[Ca^{2+}]_i$ observed in the absence of external Ca^{2+}, suggesting that LNNA promotes Ca^{2+} sequestration.

Key Words: Na^+/Ca^{2+} exchanger, Ca^{2+}, NO synthase, heat stress, colon

Introduction

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is known to be regulated by the Na^+/Ca^{2+} antiporter (exchanger), Ca^{2+}-adenosine triphosphatase-regulated pumps, Ca^{2+} channels located at the cell membrane (including

Corresponding author: Dr. Juliann G. Kiang, Department of Cellular Injury, Division of Military Casualty Research, Walter Reed Army Institute of Research, Washington DC 20307-5100, USA. Tel: 202-782-1950, Fax: 202-782-3163, E-mail: Dr._Juliann_Kiang@WRSMTP-ccmail.army.mil

Received July 7, 1999; Revised July 22, 1999; Accepted August 12, 1999.

voltage-gated, second messenger-operated, and receptor-operated channels), Ca^{2+} mobilization from intracellular pools, and cytosolic Ca^{2+}-binding proteins such as calmodulin and calcineurin (22). Increases in $[Ca^{2+}]_i$ stimulate Ca^{2+}-dependent enzymes that catalyze many biochemical reactions in the cell (4, 16, 20). 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 (19).

Nitric oxide (NO) is produced by constitutive and inducible NO synthases in many cell types, including endothelial cells, neurons, and macrophages (29). It is involved in a wide range of physiological processes, including blood vessel relaxation (13), neurotransmitter transduction (23), memory functions (26), and host defense against microbial pathogens (24). Only the constitutive NO synthase is Ca^{2+}-dependent (7). Increased levels of NO (14, 33, 34) and NO synthase activity (1, 14, 25, 34, 35) have been measured during hemorrhagic shock. Inhibition of NO production reduces the damage caused by hemorrhagic shock (2, 34, 38). A number of *in vitro* studies have demonstrated that the heat stress inhibits inducible NO synthase gene expression (11, 12, 36, 37).

In this study, we have characterized the effect of Nω-nitro-L-arginine (LNNA, an inhibitor of constitutive NO synthase) on $[Ca^{2+}]_i$ in human colon carcinoma T84 cells before and after heat stress. LNNA decreases $[Ca^{2+}]_i$ in the presence and absence of external Ca^{2+}, but it enhances the heat stress-induced increase in $[Ca^{2+}]_i$ by increasing the binding affinity of the Na^+/Ca^{2+} exchanger for external Ca^{2+} and its maximal velocity. Our data indicate that LNNA also promotes Ca^{2+} sequestration in intracellular Ca^{2+} pools that are insensitive to thapsigargin and monensin.

Materials and Methods

Cell Culture

Human colon carcinoma T84 cells (American Type Culture Collection, Rockville, MD) were grown on 75 mm^2 flasks incubated at 37 °C in a 5% CO_2 atmosphere. The tissue culture medium was Dulbecco's modified Eagle medium supplemented with 0.03% glutamine, 4.5 g/l glucose, 25 mM HEPES, 10% fetal bovine serum, 50 μg/ml penicillin, and 50 U/ml streptomycin (Gibco BRL, Gaithersburg, MD). Cells were fed every 3-4 days. Cells from passages 56-69 were used for experiments.

Intracellular Ca^{2+} Measurements

T84 cells were trypsinized from the culture flasks, washed, then loaded in suspension with 5 μM fura-2AM plus 0.2% pluronic F-127 (to make cells more permeable to the probe) at 37 °C for 60 min. Cells were washed with Na^+ Hanks' solution before fluorescence measurements. The method to determine $[Ca^{2+}]_i$ has been described previously (15). Briefly, the suspended cells were placed in a thermostatically controlled cuvette that was kept at a constant temperature of 37 °C. The fluorescence signal was measured with a PTI 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 medium, cells were washed thoroughly in Hanks' solution before measurement of $[Ca^{2+}]$. To perform experiment in the absence of extracellular Ca^{2+}, cells were incubated in the Ca^{2+}-free buffer containing 10 mM EGTA for 10 min prior to treatments with drugs.

Ca^{2+}-uptake measurements

For Ca^{2+}-uptake measurements, medium was removed from each well of six-well culture plates containing uniformly confluent cells and replaced with 3 ml Na^+ Hanks' buffer containing 100 μM LNNA. Cells were then incubated with LNNA for 1 h at 37 °C. After incubation and immediately before heat treatment, LNNA medium was removed and replaced with 2 ml Na^+ Hanks' buffers with $CaCl_2$ concentrations ranging from 0.1 to 1.6 mM, each of which contained a constant specific activity of $^{45}CaCl_2$ (1.56 Ci/mmol $CaCl_2$; Dupont/NEN, Boston, MA). Heat treatment was accomplished by floating culture dishes in a water bath held at 45 °C for sufficient time to bring medium over cells to 45 °C for 10 min. After heating, radioactive medium was immediately removed, and each well was quickly washed three times with 3 ml Na^+ Hanks' buffer containing 10 mM EGTA. Cells were removed from each well by incubating with 1 ml trypsin/EDTA, and the suspension was combined with a 1 ml Na^+ Hanks' buffer rinse. The cell suspension was sonicated and duplicate aliquots removed for protein determination. The remaining sonicated suspension was combined with Ecoscint scintillation cocktail (15 ml, National Diagnostics, Manville, NJ), and radioactivity was determined (Beckman LS5801, Palo Alto, CA). Specific radioactivity of the Ca^{2+} buffers was determined by counting the CPM 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 and expressed as pmol Ca^{2+} • mg $protein^{-1}$ • min^{-1} (17).

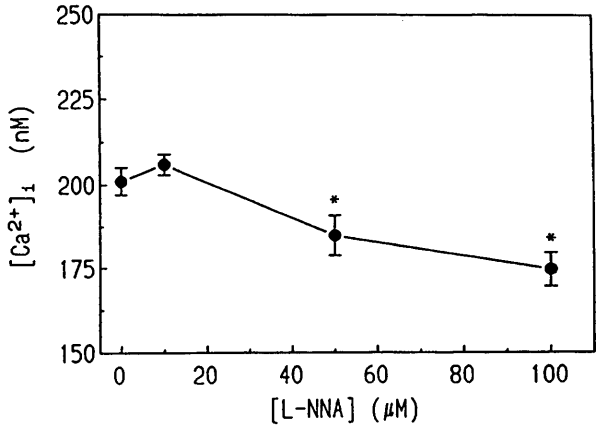

Fig. 1. Decreases in $[Ca^{2+}]_i$ by LNNA in T84 cells. Cells were treated with different concentrations of LNNA in presence of 1.6 mM external Ca^{2+} followed by $[Ca^{2+}]_i$ measurement (n=3-6). *P<0.05 vs. control, determined by one-way ANOVA and Bonferroni's inequality.

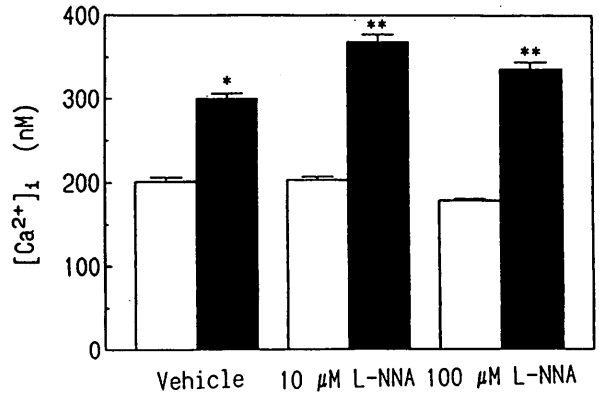

Fig. 2. Enhancement of heat-induced increase in $[Ca^{2+}]_i$ by LNNA in T84 cells. Cells were treated with 100 μM LNNA in presence of 1.6 mM external Ca^{2+} for 1 h prior to exposure to 45 °C for 10 min and measurement of $[Ca^{2+}]_i$ (n=5). *P<0.05 vs. unheated cells; **P< 0.05 vs. unheated cells and vehicle/heated cells, determined by two-way ANOVA and studentized-range test. : unheated; ■: heated

Measurements of Cell Viability

Cell viability was determined by trypan blue exclusion assay. Twenty microliters of cell suspension were mixed with 20 μl of 0.4% trypan blue solution (Sigma Chemical Co., St. Louis, MO). A drop of the mixture was placed on the hemocytometer and cells were counted under the microscope. Cells turned blue represented the unviable cells, whereas others represented the viable cells. The viability was calculated according to the following equation: Viability (%) = [number of viable cells/ (number of viable cells + number of unviable cells)]×100%.

Solutions

Hanks' solution 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' solution was prepared by adding 10 mM EGTA to nominally Ca^{2+}-free Hanks' solution.

Statistical Analysis

All data are expressed as the mean±S.E.M. Analysis of variance, Student's t-test, Studentized Range-test, and Bonferroni's inequality were used for comparison of groups. Curve fitting was determined using the GraphPad Inplot program (GraphPad, San Diego, CA).

Chemicals

Nω-nitro-L-arginine, aminoquanidine, and caffeine were purchased from Sigma Chemical Co. (St Louise, MO). Dichlorobenzamil was provided by Dr. Peter K.S. Siegl (Merck & Co, Inc., West Point, PA). Fura-2AM, trimethoxybenzoate (TMB-8), thapsigargin, monensin, and ionomycin were purchased from Molecular Probes, Inc. (Eugene, OR). Bradykinin was obtained from Peninsula Laboratories (Belmont, CA).

Results

Resting $[Ca^{2+}]_i$ in human colon carcinoma T84 cells was 201±3 nM (n=9). Treatment of cells with LNNA, an inhibitor of constitutive NO synthase, significantly decreased resting $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 1). Exposure of cells to 45 °C for 10 min increased $[Ca^{2+}]_i$ to 150±2 % of controls ("Vehicle", Fig. 2). Figure 2 shows that if cells were pretreated with 100 μM LNNA for 1h, the heat stress-induced increase in $[Ca^{2+}]_i$ was enhanced. Aminoguanidine, an inhibitor of inducible NO synthase, altered neither the basal level of $[Ca^{2+}]_i$ nor the increase in $[Ca^{2+}]_i$ after heating (data not shown), indicating that the LNNA effect is specifically associated with constitutive NO synthase. Data from cell viability studies showed that pretreatment with LNNA or aminoguanidine before or after exposure of cells to heat stress did not alter cell viability (Fig. 3).

Removal of external Ca^{2+} decreased the resting $[Ca^{2+}]_i$ in T84 cells to 115±2 nM (n=18), suggesting that Ca^{2+} entry contributes to the maintenance of resting $[Ca^{2+}]_i$. Treatment of these cells with LNNA significantly decreased $[Ca^{2+}]_i$ (Fig. 4), and the relative magnitude of the decrease was similar to that measured in cells treated with LNNA in the presence of 1.6 mM Ca^{2+}. This result suggests that action of LNNA on

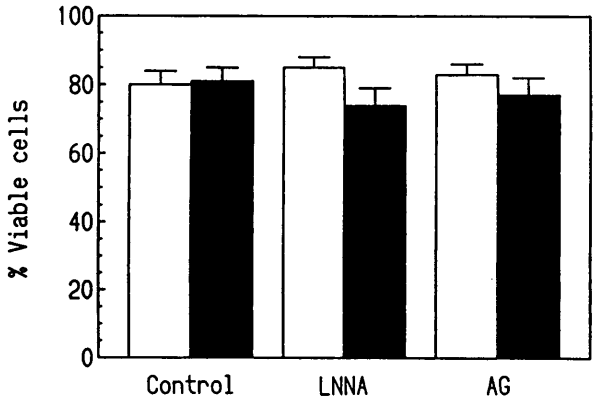

Fig. 3. No changes in cell viability after treatment with LNNA or aminoguanidine followed by heat stress in T84 cells. Cells were treated with 100 μM LNNA or aminogaunidine (AG) for 1 h prior to exposure of cells to heat stress (HS) at 45 °C for 10 min (n=3). □: unheated; ■: heated

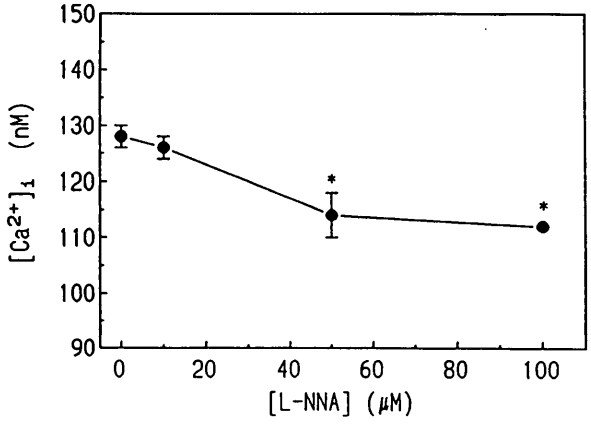

Fig. 4. Decreases in $[Ca^{2+}]_i$ by LNNA in unheated T84 cells in absence of external Ca^{2+}. Cells were treated with different concentrations of LNNA in absence of external Ca^{2+} and $[Ca^{2+}]_i$ was measured (n= 3-6). *P<0.05 vs. control, determined by one-way ANOVA and Bonferroni's inequality.

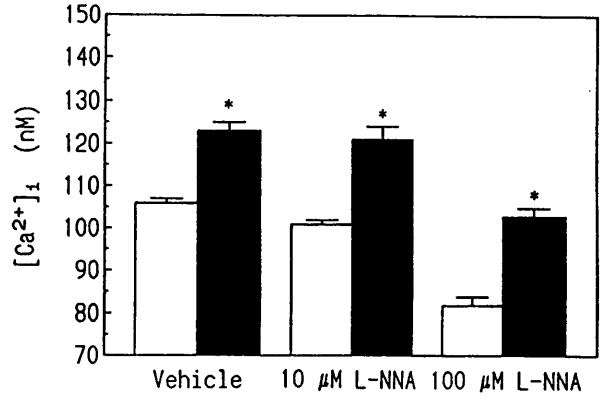

Fig. 5. No enhancement by LNNA of heat-induced increase in $[Ca^{2+}]_i$ in T84 cells in absence of external Ca^{2+}. Cells were treated with 100 μM LNNA for 1 h in absence of external Ca^{2+} prior to exposure of cells to 45 °C for 10 min and measurement of $[Ca^{2+}]_i$ (n=5). *P< 0.05 vs. unheated cells, determined by two-way ANOVA and studentized-range test. □: unheated; ■: heated

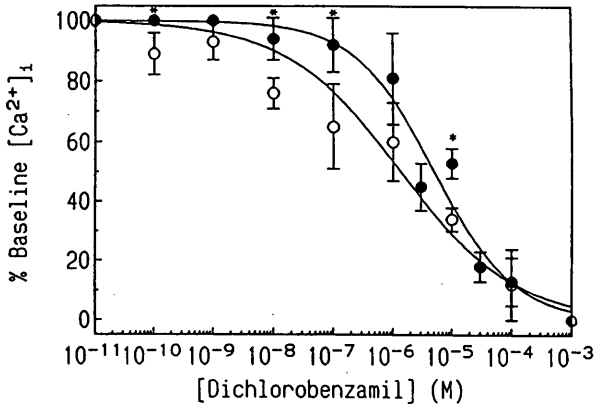

Fig. 6. Dichlorobenzamil inhibition of heat stress-induced increase in $[Ca^{2+}]_i$ after LNNA pretreatment. Cells were preincubated without or with 100 μM LNNA for 1 h prior to exposure to 45 °C for 10 min in presence of different concentrations of dichlorobenzamil followed by measurement of $[Ca^{2+}]_i$ (n=3-5). IC50 of dichlorobenzamil in untreated and LNNA-treated cells are 0.97±0.03 μM and 5.1±0.1 μM, respectively. P<0.05 vs. control, determined by two-way ANOVA and Boferroni's inequality. -○-: control; -●-: LNNA-treated

T84 cells is not related to external Ca^{2+}.

In the absence of external Ca^{2+} and with no LNNA treatment, heating cells at 45 °C for 10 min still increased $[Ca^{2+}]_i$ but to a much lower level than that measured in the presence of external Ca^{2+}, indicating that extracellular Ca^{2+} makes a major contribution to the heat stress-induced increase in $[Ca^{2+}]_i$. With no external Ca^{2+} present, pretreatment with 100 μM LNNA for 1 h did not further enhance the heat stress-induced increase in $[Ca^{2+}]_i$ (Fig. 5).

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 (19). To determine whether a similar mechanism is involved in T84 cells, we tested the effect of incubating the cells with dichlorobenzamil, a specific inhibitor of Na^+/Ca^{2+} exchangers. Figure 6 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 0.97±0.03 μM, suggesting that Ca^{2+} entry is via the Na^+/Ca^{2+} exchanger. In the presence of 100 μM LNNA, the IC_{50} of dichlorobenzamil was 5.1±0.1 μM (P<0.05 vs. that observed in LNNA-untreated cells, Student's t-test), suggesting that LNNA exerts its effect on the Na^+/Ca^{2+} exchanger.

To determine the binding affinity (K_m) of the Na^+/Ca^{2+} exchanger for external Ca^{2+} and the maximal velocity (V_{max}) of this exchanger, we measured the kinetics of Ca^{2+} uptake in cells incubated in medium containing different concentrations of Ca^{2+} with and

Table 1. Association Constant (K_m) and Maximal Velocity (V_{max}) of Ca^{2+} Entry in T84 Cells

Treatment	K_m (mM)	V_{max} (pmol Ca^{2+}/mg protein/min)
Unheated	0.430±0.075	362±57
Heated	0.489±0.071	826±10[c]
LNNA, unheated	0.700±0.168[a]	506±116
LNNA, heated	0.340±0.037[b]	869±81[c]

[a] P<0.05 vs. unheated, heated, and LNNA plus heated;
[b] P<0.05 vs. heated, and LNNA plus unheated;
[c] P<0.05 vs. unheated and LNNA plus unheated, determined by Student's t-test.

without heating to 45 °C for 10 min. Table 1 lists the K_m and V_{max} obtained from each group. Heat stress (no LNNA pretreatment) did not affect K_m, but it increased V_{max} by 128%, indicating that heat stress did not affect the affinity of the Na^+/Ca^{2+} exchanger for external Ca^{2+}, but it did increase the exchanger velocity. Preincubation of cells with 100 μM LNNA for 1 h altered the kinetics of the exchanger even without heating. The affinity of the exchanger for Ca^{2+} decreased by 63%, though its velocity did not change. Exposure of these LNNA-treated cells to heat stress resulted in a decreased Km by 52% as well as an increased Vmax. These results suggest that the constitutive NO synthase may directly or indirectly regulate the Na^+/Ca^{2+} exchanger. It has been shown in a number of different cell types that there are various intracellular Ca^{2+} pools (3, 5, 8, 10, 18, 30). To characterize the different intracellular Ca^{2+} pools in T84 cells, cells were treated sequentially with 10 μM bradykinin, 10 μM thapsigargin, 40 mM caffeine, 100 μM monensin, and 10 μM ionomycin. The concentration used of each of these agents was optimized for T84 cells by selecting the concentration that just failed to elicit an additional response after a second treatment of the cells.

Incubated in the absence of external Ca^{2+}, T84 cells were found to have intracellular Ca^{2+} pools sensitive to thapsigargin, monensin, and ionomycin, but not bradykinin or caffeine (Fig. 7). Treatment with thapsigargin, monensin, and ionomycin increased $[Ca^{2+}]_i$ by 16±2% (n=7), 19±3% (n=7), and 66±20 % (n=5), respectively. After the thapsigargin-sensitive Ca^{2+} pool was depleted by thapsigargin, treatment of these cells with 100 μM LNNA still decreased $[Ca^{2+}]_i$ (Fig. 8). A similar result was obtained after cellular Ca^{2+} pools were depleted by monensin. When cells were depleted sequentially with thapsigargin, monensin, and ionomycin, LNNA failed to decrease $[Ca^{2+}]_i$. This indicates that LNNA decreases $[Ca^{2+}]_i$ by promoting Ca^{2+} sequestration to an intracellular

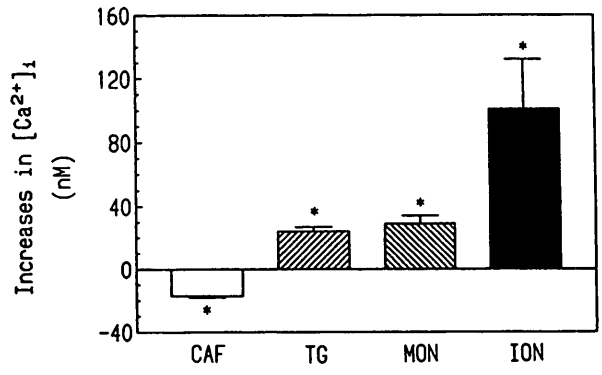

Fig. 7. Detection of intracellular Ca^{2+} pools in T84 cells. Cells were treated sequentially with 40 mM caffeine (CAF), 10 μM thapsigargin (TG), 100 μM monensin (MON), and 10 μM ionomycin (ION) in absence of external Ca^{2+} (n=4-9). *P<0.05 vs. Control baseline, determined by Student's t-test.

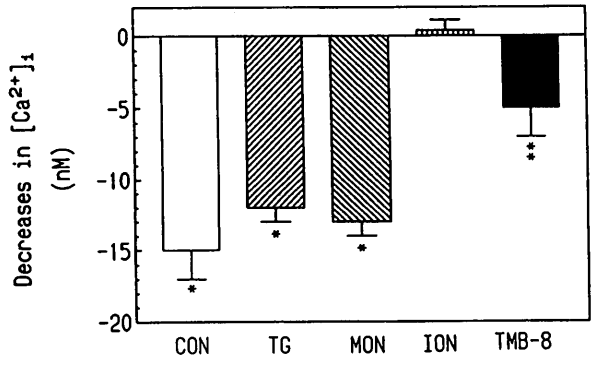

Fig. 8. Inhibition of LNNA-induced decrease in $[Ca^{2+}]_i$ by ionomycin and TMB-8. Cells were treated with 10 μM thapsigargin (TG), 100 μM monensin (MON), 10 μM ionomycin (ION), and 100 μM TMB-8 in absence of external Ca^{2+} (n=4-9) before treatment with 100 μM LNNA. *P<0.05 vs. control, determined by student's t-test. CON: Control.

Ca^{2+} pool not sensitive to thapsigargin and monensin. Treatment with TMB-8, a blocker that inhibits Ca^{2+} sequestration and mobilization, 30 min prior to LNNA treatment significantly diminished the LNNA-induced decrease in $[Ca^{2+}]_i$ (Fig. 7). This observation supports the concept that constitutive NO synthase regulates Ca^{2+} sequestration.

Discussion

Treatment of T84 cells with LNNA, an inhibitor of constitutive NO synthase, in the presence of external Ca^{2+}, decreased the basal level of $[Ca^{2+}]_i$ in a LNNA concentration-dependent fashion. If these LNNA-treated cells were heated, the $[Ca^{2+}]_I$ increase observed was greater than in cells not treated with LNNA. Removal of external Ca^{2+} greatly diminished the heat stress-induced increase in $[Ca^{2+}]_I$ in cells not treated

with LNNA, and LNNA treatment failed to enhance the increase in $[Ca^{2+}]_i$ that was observed when external Ca^{2+} was present.

As has been shown in other types of cells (19), heat stress of T84 cells apparently increases $[Ca^{2+}]_i$ by activating the Na^+/Ca^{2+} exchanger. When T84 cells were heated (but not treated with LNNA), the exchanger's affinity for binding external Ca^{2+} did not change, but the maximal velocity of the exchanger increased by 128%. LNNA treatment alone reduced the binding affinity of the exchanger for external Ca^{2+} by 63% but did not significantly change V_{max}. Heating the LNNA-treated cells led to an increase in the affinity of the exchanger for Ca^{2+} and its maximal velocity, which explains the capability of LNNA enhancing the heat stress-induced increase in $[Ca^{2+}]_i$. Since LNNA is known to exert its effect through constitutive NO synthase, this suggests that either constitutive NO synthase (or perhaps NO) may be regulating the activity of the exchanger.

How this regulation might occur is not clear. In unpublished studies with other cell types, we have shown that HSP-70 immunoprecipitates with the Na^+/Ca^{2+} exchanger after exposure to heat or low levels of hypoxia (see review, 21, 22). This suggests that formation of constitutive NO synthase/exchanger complex might be responsible for activating the exchanger in those cells, thereby increasing the exchanger's affinity for external Ca^{2+} and its maximal velocity. It is possible that in these T84 cells the constitutive NO synthase could bind the Na^+/Ca^{2+} exchanger and likewise down-regulate the sensitivity of the exchanger. NO has been shown to be involved in not only S-nitrosylation reactions which have critical physiological relevance to mammalian neurotransmission, ion channel function, intracellular signalling, and anti-microbial defense (see review 9) but also in protein interactions (see review 32). Therefore, the possibility that cellular levels of NO might also control the function of the Na^+/Ca^{2+} exchanger cannot be ruled out.

We detected three different intracellular Ca^{2+} pools in T84 cells, ones sensitive to thapsigargin, monensin, and ionomycin. Surprisingly, Ca^{2+} pools sensitive to inositol 1,4,5-trisphosphate were not found, which differentiates these cells from a number of other cell types. Inositol 1,4,5-trisphosphate-, monensin- and ryanodine-sensitive intracellular Ca^{2+} pools are observed in GH3 (8) and A-431 cells (18). In rat PC 12 cells, inositol 1,4,5-trisphosphate-, monensin- and caffeine-sensitive intracellular Ca^{2+} pools are detected (5). Jurkat cells possess inositol 1, 4,5-trisphosphate-, thapsigargin-, caffeine-, ryanidine- and ionomycin-sensitive pools (10, 30). In Lewis rat splenic T lymphocytes inositol 1,4,5-trisphosphate, thapsigargin- and ionomycin-sensitive Ca^{2+} pools have been identified (3).

In the absence of external Ca^{2+}, treatment of T84 cells with LNNA resulted in a decrease in the basal level of $[Ca^{2+}]_i$. This decrease was not observed when intracellular Ca^{2+} pools were depleted by ionomycin. Treatment with monensin or thapsigargin alone did not affect the LNNA-induced decrease in $[Ca^{2+}]_I$. Furthermore, blockade of Ca^{2+} sequestration and mobilization by treatment with TMB-8 also effectively inhibited the LNNA-induced decrease in $[Ca^{2+}]_i$. These results suggest that the LNNA-induced decrease in $[Ca^{2+}]_i$ is not mediated by Ca^{2+} entry from the external sources. The decrease in $[Ca^{2+}]_i$ may be due to the capacity of either constitutive NO synthase or NO to regulate Ca^{2+} sequestration into intracellular Ca^{2+} pools not sensitive to monensin or thapsigargin. It is not likely that either constitutive NO synthase affect $[Ca^{2+}]_i$ by altering Ca^{2+} efflux, because, if so, the LNNA-induced decrease in $[Ca^{2+}]_i$ would have been measured even after the intracellular Ca^{2+} pools had been depleted by ionomycin or blocked by TMB-8.

Nitric oxide overproduction has been implicated in disease pathogenesis in a variety of autoimmunoinflammatory syndromes including type I diabetes (6), inflammatory arthritis (27, 28), inflammatory bowel disease (31), and hemorrhagic shock (14, 33, 34). Although NO overproduction can damage tissues, it also appears in certain cases to play beneficial roles such as helping to maintain immunological homeostasis. It would be of interest to determine whether LNNA might prove to be therapeutically useful for preventing tissue damage caused by NO overproduction.

In summary, LNNA decreases the resting $[Ca^{2+}]_i$ in a concentration-dependent manner, which was due to its promoting Ca^{2+} sequestration to intracellular $Ca^{2+}]_i$ pools. LNNA enhanced the heat stress-induced increase in $[Ca^{2+}]_i$ by increasing the binding affinity of Na^+/Ca^{2+} exchangers for external Ca^{2+}.

Acknowledgements

This work was supported by the Department of the Army RAD II STO C and AFRRI Work Unit 00150. Views presented in this paper are those of the authors; no endorsement by the Department of the Army or the Department of Defense has been given or should be inferred.

References

1. Adcock, I.M., Liu, S., Brown, C.R., Evans, T.W. and Barnes, P.J. Endotoxin and steroid effects of nitric oxide synthase mRNA expression in rat lung and other tissues. *Biochem. Soc. Trans.* 22:

188S, 1994.
2. Amezcura, J.L., Villanueva, C., Pearce, F.J. and Wiesmann, P.W. N-nitro-L-arginine prolongs the survival time during the decompensatory phase of haemorrhagic shock in rats. In: The Biology of Nitroc Oxide: Physiological and Clinical Aspects, edited by Moncada, Marletta, Hibbs Jr. and Higgs. London and Chapel Hill : Portland Press, p. 37-39, 1992.
3. Clementi, E., Martino, G., Grimaldi, L.M., Brambilla, E. and Meldolesi, J. Intracelluar Ca^{2+} stores of T lymphocytes: changes induced by *in vitro* and *in vivo* activation. *Eur. J. Immunol.* 24: 1365-1371, 1994.
4. Ding, X.Z., Smallridge, R.C., Galloway, R.J. and Kiang, J.G. Increases in HSF1 translocation and synthesis in human epidermoid A-431 cells: role of protein kinase C and $[Ca^{2+}]_i$. *J. Investig. Med.* 44: 144-153, 1996.
5. Fasolato, C., Zottini, M, Clementi, E., Zacchetti, D., Meldolesi, J. and Pozzan, T. Intracellular Ca^{2+} pools in PC12 cells. Three intracellular pools are distinguished by their turnover and mechanisms of Ca^{2+} accumulation, storage, and release. *J. Biol. Chem.* 266: 20159-20167, 1991.
6. Fehsel, K., Kroncke, K.D. and Kolb-Bachofen, V. The action of NO and its role in autoimmune diabetes mellitus. *Res. Immunol.* 146: 711-715, 1995.
7. Forstermann, U., Boissel, J. and Kleinert, H. Expressional control of the constitutive isoforms of nitric oxide synthase (NOS1 and NOSIII). *FASEB J.* 12: 773-790, 1998.
8. Galloway, R.J., Fein, H.G. and Smallridge, R.C. The sodium ionophore, monensin, raises cytosolic free calcium from both intracellular and extracellular sources in GH_3 pituitary cells. The 72nd Annu. *Meet. Endocr. Soc.* Atlanta GA, 1990; P.261, (Abstract).
9. Gaston, B. Nitric oxide and thio groups. *Biochim. Biophys. Acta* 1411: 323-333, 1999.
10. Guse, A.H., Roth, E. and Emmrich, F. Intracellular Ca2+ pools in Jurkat T-lymphocytes. *Biochem. J.* 291: 447-451, 1993.
11. Hauser, G.I., Dayao, E.K., Wasserloos, K., Pitt, B.R. and Wong, H.R. HSP induction inhibits iNOS mRNA expression and attenuated hypotension in endotoxin-challenged rats. *Am. J. Physiol.* 271: H2529-H2535, 1996.
12. Hirvonen, M.R., Brune, B. and Lapetina, E.G. Heat shock proteins and macrophage resistance to the toxic effects of nitric oxide. *Biochem. J.* 315: 845-849, 1996.
13. Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E. and Chaudhuri, G. Endothelium-derived relaxing factor produced and released from artery and vein is nitrc oxide. *Proc. Natl. Acad. Sci. USA* 84: 9265-9269, 1987.
14. Kelly, E., Shah, N.S., Morgan, N.N., Watkins, S.C., Peitzman, A.B. and Billiar, T.R. Physiologic and molecular characterization of the role of nitric oxide in hemorrahgic shock: evidence that type II nitric oxide synthase does not regulate vascular decompensation. *Shock* 7: 157-163, 1997.
15. Kiang, J.G. Effect of intracellular pH on cytosolic free $[Ca^{2+}]$ in human epidermoid A-431 cells. *Eur. J. Pharmacol.* (Molecular Pharmacol Section) 207: 287-296, 1991.
16. Kiang, J.G., Carr, F.E., Burns, M.R. and McClain, D.E. HSP-72 synthesis is promoted by increase in $[Ca^{2+}]_i$ or activation of G proteins but not pHi or cAMP. *Am. J. Physiol.* 265: C104-C114, 1994.
17. Kiang, J.G., Ding, X.Z. and McClain, D.E. Overexpression of HSP-70 attenuates increases in $[Ca^{2+}]_i$ and protects human epidermoid A-431 cells after chemical hypoxia. *Toxicol. Appl. Pharmacol.* 149: 185-194, 1998.
18. Kiang, J.G. and Koenig, M.L. Characterization of intracellular calcium pools and their desensitization in thermotolerant cells. *J. Investig. Med.* 44: 352-361, 1996.
19. Kiang, J.G., Koenig, M.L. and Smallridge, R.C. Heat shock increases cytosolic free Ca^{2+} concentration via Na^+-Ca^{2+} exchange in human epidermoid A-431 cells. *Am. J. Physiol.* 263: C30-C38, 1992.
20. Kiang, J.G. and McClain, D.E. Effect of heat shock, $[Ca^{2+}]_i$, cAMP on inositol trisphosphate in human epidermoid A-431 cells. *Am. J. Physiol.* 264: C1561-C1569, 1993.
21. Kiang, J.G. and Tsokos, G.C. Cell signaling and heat shock protein expression. *J. Biomed. Sci.* 3: 379-388, 1996.
22. Kiang, J.G. and Tsokos, G.C. Heat shock protein 70 kDa: Molecular Biology, Biochemistry, and Physiology. *Pharmacol. Ther.* 80: 183-201, 1998.
23. Knowles, R.G., Palacios, M., Palmer, R.M.J. and Moncada, S. Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc. Natl. Acad. Sci. USA* 86: 5159-5162, 1989.
24. Liew, F.Y. and Cox, F.E. Nonspecific defense mechansim: the role of nitric oxide. *Immunol. Today* 12: A17, 1991.
25. Liu, S.F., Barnes, P.J. and Evans, T.W. Time course and cellular localization of lipopolysaccharide-induced inducible nitric oxide synthase messenger RNA expression in the rat in vivo. *Crit. Care. Med.* 25: 512-518, 1997.
26. Malen, P.L. and Chapman, P.F. Nitric oxide facilitates long-term potentiation, but not long-term depression. *J. Neurosci.* 17: 2645-2651, 1997.
27. McCartney-francis, N., Allen, J.B., Mizel, D.E., Albina, J.E., Xie, Q.W, Nathan, C.F. and Wahl, S.M. Suppression of arthritis by an inhibitor of nitric oxide synthase. *J. Exp. Med.* 178: 749-754, 1993.
28. McInnes, I.B., Leung, B.P., Field, M., Wei, X.Q., Huang, F.P., Sturrock, R.D, Kinninmonth, A., Weidner, J., Mumford, R. and Liew, F.Y. Production of nitric oxide in the synovial membrane of rheumatoid and osteoarthritis patients. *J. Exp. Med.* 184: 1519-1524, 1996.
29. Moncada, S., Palmer, R.M.J. and Higgs, A. Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* 43: 109-142, 1991.
30. Ricard, I., Martel, J., Dupuis, L., Dupuis, G. and Payet, M.D. A caffeine/ryanodine-sensitive Ca^{2+} pool is involved in triggering spontaneous variations of Ca^{2+} in Jurkat T lymphocytes by a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism. *Cell Signal.* 9: 197-206, 1997.
31. Singer, I.I., Kawka, D.W., Scott, S., Weidner, J.R., Mumford, R.A., Riehl, T.E. and Stenson, W.F. Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. *Gastroentorology* 111: 871-885, 1996.
32. Stuehr, D.J. Mammalian nitric oxide synthases. *Biochim. Biophys. Acta* 1411: 217-230, 1999.
33. Szabo, C. and Thiemermann, C. Invited opinion: role pf nitric oxide in hemorrhagic, traumatic, and anaphylactic shock and thermal injury. *Shock* 2: 145-155, 1994.
34. Thiemermann, C., Szabo, C., Mitchell, J.A. and Vane, J.R. Vascular hyporeactivity to vasoconstrictor agents and hemodynamic decompensation in hemorrhagic shock is mediated by nitric oxide. *Proc. Natl. Acad. Sci. USA* 90: 261-271, 1993.
35. Wizemann, T.M., Gardner, C.R., Lakin, J.D., Quinones, S., Durham, S.K, Goller, N.L., Ohnishi, S.T. and Laskin, D.L. Production of nitric oxide and peroxynitrite in the lung during acute endotoxemia. *J. Leukoc. Biol.* 56: 759-768, 1994.
36. Wong, H.R., Finder, J.D., Wasserloos, K. and Pitt, B.R. Expression of iNOS in cultured rat epithelial pulmonary artery smooth muscle cells is inhibited by the heat shock response. *Am. J. Physiol.* 269: L843-L848, 1995.
37. Wong, H.R., Ryan, B. and Wispe, J.R. The heat shock response inhibits inducible nitric oxide synthase gene expression by blocking I -B degradation and NF- B nucleus translocation. *Biochem. Biophys. Res. Comm.* 231: 257-263, 1997.
38. Zingarelli, B., Squadrito, F., Altavilla, D., Calapai, G., Campo, G.M., Calo, M., Saitta, A. and Caputi, A.P. Evidence for a role of nitric oxide in hypovolemic hemorrhagic shock. *J. Cardiovasc. Pharmacol.* 19: 982-986, 1992.