Calcium-sensing receptor activation induces nitric oxide production in H-500 Leydig cancer cells

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1Division of Endocrinology, Diabetes, and Hypertension, Department of Medicine and Membrane Biology Program, Harvard Medical School, Boston, Massachusetts; and 2Osteoporosis and Bone Metabolic Unit, Department of Clinical Biochemistry and Endocrinology, Copenhagen University Hospital Hvidovre, Denmark

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Calcium-sensing receptor activation induces nitric oxide production in H-500 Leydig cancer cells. Am J Physiol Endocrinol Metab 288: E1206–E1213, 2005. First published January 18, 2005; doi:10.1152/ajpendo.00492.2004.—Nitric oxide (NO) is a very small, hydrophobic molecule with chemical properties that make it uniquely suited to serve as both an intra- and an extracellular messenger. In malignant tissue, the mutagenic effect of chronically high levels of NO plays a role in carcinogenesis. Reports have shown that NO also participates in cell proliferation, apoptosis, and angiogenesis in cancer cells (18).

NO is produced by three isoforms of the enzyme NO synthase (NOS). These are neuronal (n), inducible (i), and endothelial (e) NOS (1). iNOS is classically upregulated in inflammation by endotoxin, interferon-γ, TNF-α, and IL-1β (36). iNOS is constitutively active and is the major producer of NO. Importantly, its activity is independent of intracellular calcium (Ca2+) and calmodulin, which distinguishes it from n- and eNOS. iNOS acts as a homodimer, a process that can be prevented by interaction with kalirein, a peptide biosynthetic enzyme present in secretory granules (25). Currently, specific iNOS inhibitors are in clinical trials for the treatment of hypotensive shock (36).

NO production has been reported in cultured rat Leydig cells expressing iNOS mRNA, induced by IL-1β (30). In this context, NO functions as a negative regulator of Leydig cell testosterone production in vitro (10, 35). Furthermore, suppression of serum testosterone levels by agents promoting NO production and enhancement by administration of a general NOS inhibitor, l-NAME, in rats showed the role of NO as a negative modulator of steroidogenesis in Leydig cells. Immunohistochemistry of rat testis detected iNOS in both normal and inflamed Leydig cells (23). These reports reveal an important role for NO in the physiology and pathophysiology of the Leydig cell.

Extracellular calcium (Ca2+) can regulate NO production in vivo and in vitro (4, 16, 27). nNOS and eNOS are activated by opening of calcium channels, resulting in calcium influx. However, it is possible that calcium also acts by an extracellular “sensing” mechanism. One such mechanism is the calcium-sensing receptor (CaR), a G protein-coupled receptor (GPCR) that responds to very small changes in Ca2+ in tissues involved in Ca2+ homeostasis. The CaR is the mediator of calcium-induced inhibition of parathyroid hormone release and has been shown to have a variety of other functions, including the regulation of the cell cycle, peptide secretion, and apoptosis (33). We recently showed that the CaR upregulates promalignant cellular functions, such as parathyroid hormone-related protein (PTHrP) transcription and release, proliferation, protection against apoptosis, and expression of pituitary tumor transforming gene (PTTG) in rat primary Leydig cancer cells (H-500) in primary culture (8, 31, 32, 34). The H-500 model is a well-established xenotransplantable rat Leydig cell cancer model for the syndrome of humoral hypercalcemia of malignancy (24). Using this model, we tested our hypothesis that stimulating the Leydig cancer cells with high calcium would increase NO production through higher expression of iNOS.

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Furthermore, we hypothesized that the resultant high calcium-induced iNOS response would be mediated through the CaR.

MATERIALS AND METHODS

Materials

A rabbit polyclonal antiserum against iNOS was purchased from BD Transduction Laboratories (Lexington, KY). An inhibitor of NOS, l-NAME, was obtained from Calbiochem-Novabiochem (San Diego, CA), and a selective iNOS inhibitor, 1400W, was obtained from Biomol (Plymouth Meeting, PA). The enhanced chemiluminescence kit, Supersignal, was purchased from Pierce (Rockford, IL). Protease inhibitors were obtained from Boehringer Mannheim (Mannheim, Germany), and other reagents were from Sigma Chemical (St. Louis, MO).

Cell Culture

The Rice H-500 rat Leydig cell tumor was obtained from the National Cancer Institute-Frederick Cancer Research and Development Center DCT Tumor Repository (Frederick, MD). Male Fischer 344 rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 200–220 g (10 wk of age) were used for tumor implantation. A fragment of the H-500 tumor or dispersed H-500 cells (10⁶ cells) was implanted or injected subcutaneously, respectively, in each rat, and the tumors were allowed to grow for 8–14 days. The encapsulated tumor was then excised, rinsed several times with cell culture medium, minced into small pieces, and dispersed by repeated pipetting and several passages through a 22-gauge needle. Dispersed H-500 cells were subsequently plated in RPMI 1640 medium supplemented with 10% FBS and 100 U/ml penicillin-100 μg/ml streptomycin and grown at 37°C in a humidified 5% CO₂ atmosphere. Cells were passaged every 4–5 days using 0.05% trypsin-0.53 mM EDTA and used for experimentation within the first 10 passages. All cell culture reagents were purchased from GIBCO-BRL (Grand Island, NY), with the exception of FBS, which was obtained from Gemini Bio-Products (Calabasas, CA). Rats were handled in accordance with local institutional guidelines.

Western Blotting

For the determination of iNOS protein levels, monolayers of H-500 cells were grown on six-well plates. Cells were incubated for 4, 18, or 40 h in serum-free, calcium-free DMEM containing 4 mM L-glutamine, 0.2% BSA, and 0.5 mM CaCl₂. At the end of the incubation period, the medium was removed, the cells were washed two times with ice-cold PBS containing 1 mM sodium vanadate and 25 mM NaF, and then 100 μl ice-cold lysis buffer was added (20 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM sodium vanadate, 50 mM glycerophosphate, and a cocktail of protease inhibitors). The protease inhibitors were aprotonin, leupeptin, soybean trypsin inhibitor, pepstatin, and calpain inhibitor (10 μg/ml each), all from frozen stocks, and 100 μg/ml Pefabloc. The sodium vanadate, NaF, and Pefabloc were freshly prepared on the day of the experiment. The cells were scraped in the lysis buffer, sonicated for 5 s, and then centrifuged at 6,000 rpm for 5 min at 4°C. The supernatants were frozen at −20°C. After being thawed, equal amounts of supernatant protein (20 μg) were separated by SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell) and incubated with blocking solution (10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 0.25% BSA) containing 5% dry milk for at least 1 h at room temperature. iNOS protein levels were detected by immunoblotting using an 18-h incubation at 4°C with a 1:2,000 dilution of a rabbit polyclonal antiserum specific for iNOS (Transduction Laboratory). Blots were washed for five 15-min periods at room temperature (1% PBS, 1% Trition X-100, and 0.3% dry milk) and then incubated for 1 h with a secondary goat anti-rabbit, peroxidase-linked antisem (1:2,000) in blocking solution. Blots were then washed again (3 × 15 min). Bands were visualized by chemiluminescence according to the manufacturer’s protocol (Supersignal; Pierce Chemical). The same membrane was used after stripping (Restore Western Blot Stripping; Pierce) to measure β-actin. Protein concentrations were measured with the Micro BCA protein kit (Pierce).

Northern Blot Analysis

To study whether high Ca²⁺ affects the expression of iNOS mRNA, we performed Northern blot analysis as described previously (7). In brief, cellular RNA was isolated (9) using the Tri-Zol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. The RNA recovered was quantitated by spectrophotometry, and aliquots of 20 μg total RNA from H-500 cells incubated at low (0.5 mM) or high Ca²⁺ (7.5 mM) concentrations in serum-free, calcium-free DMEM containing 4 mM L-glutamine, 0.2% BSA, and 0.5 mM CaCl₂ were loaded on a formamide-agarose gel after denaturation. The gel was stained with ethidium bromide to visualize RNA standards and ribosomal RNA to document equal loading of RNA from the various experimental samples. The RNA was then blotted on nylon membranes (Duralon; Stratagene, La Jolla, CA). An iNOS cDNA probe was prepared by one-step RT-PCR using 2 μg total RNA derived from H-500 cells using the following primers: 5′-TGC TAT TCC CAG CCC AAC AAC-3′ (iNOS sense, 120–140) and 5′-TTT TTC CTC TTT GTA GGA GCC-3′ (iNOS antisense, 486–466). The PCR product was then subcloned into the TOPO TA cloning kit (Invitrogen) following the manufacturer’s instructions and sequenced to confirm its homology with the corresponding region of the rat iNOS mRNA (accession no. NM_012611.1). The plasmid containing iNOS cDNA was digested with EcoRI, and the insert was 32P labeled. Blots were hybridized with a cDNA probe for iNOS and washed under high-stringency conditions as described previously (21). Equal loading was confirmed by stripping and then reprobing the membranes with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Specific radioactivity signals were analyzed on a Molecular Dynamics PhosphorImager (Sunnyvale, CA) with the ImageQuant program.

Infecting H-500 Cells with CaR Constructs in Recombinant Adeno-associated Virus

High-efficiency gene transfer into H-500 cells was accomplished using a recombinant adeno-associated virus (rAAV)-based method. The CaR sequence with a naturally occurring, dominant-negative mutation (R185Q), and the same vector containing the cDNA for the β-galactosidase gene (BG) were under the control of a cytomegalovirus-early-early promoter element and were packaged as previously described (38). The BG served as the control for nonspecific effects of rAAV infection. Cells were seeded (1,000 cells/well) in 96-well plates in 0.1 ml of growth medium and cultured overnight. About 1,000 virus particles/cell (as optimized by pilot studies) were used to infect each well. Cells were washed one time with serum-free α-minimal essential medium. Virus particles were then added, and the culture was incubated for 90 min in serum-free medium at 37°C in a cell-culture incubator. Equal volumes of RPMI 1640 containing 20% serum were added to the cells to achieve a final serum concentration of 10%. The cells were then cultured for 48 h, and experiments with low and high calcium concentrations were performed as described in subsequent sections.

Quantitative Real-time PCR

To amplify iNOS and GAPDH cDNA, sense and antisense oligonucleotide primers were designed based on the published cDNA sequences using Primer Express version 2.0.0 (Applied Biosystems, Foster City, CA). Oligonucleotides were obtained from Genosys.
(Woodlands, TX). The sequences of the primers were as follows: 5'-GAT TCA GTG GTT CCA ACC TGC A-3' (iNOS sense, 621–641), 5'-CGA CCT GAT GTT GCC ACT GTT-3' (iNOS antisense, 738–718; iNOS accession no. NM_012611.1), 5'-TTC AAT GGC ACA GTC AAG GC-3' (GAPDH sense), and 5'-TCA CCC CAT TTG ATG TTA GCC-3' (GAPDH antisense; GAPDH accession no. M17701). cDNA was synthesized with the Omniscript RT Kit (Qiagen, Valencia, CA) using 2 μg total RNA in a 20 μl reaction volume. For real-time PCR, the cDNA was amplified using an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems). The double-strand DNA-specific dye SYBR Green I incorporated in the PCR reaction buffer Quant iTech SYBR PCR (Qiagen) to allow for quantitative detection of the PCR product in a 25-μl reaction volume. The temperature profile of the reaction was 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. An internal housekeeping gene control, GAPDH, was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the RT. The size of the PCR product was first verified on a 1.5% agarose gel, followed by melting curve analysis thereafter.

Biological NO Imaging by 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate

Cells were plated on coverslips in a six-well plate. After 72 h at 70–80% confluence, the cells were challenged with 0.5 or 7.5 mM calcium for 18 h in serum-free, calcium-free DMEM containing 4 mM l-glutamine and 0.2% BSA. The cells were then washed twice with RPMI 1640 medium without phenol red. The cells were then loaded for 1 h with 1 ml RPMI 1640 (without phenol red) containing 10 μM 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate; Molecular Probes, Eugene, OR), 0.1% pluronic acid, and 1 mM probenecid at 37°C in a 5% CO2 incubator with no calcium. Finally, the coverslips were washed two times in RPMI 1640 medium without phenol red.

Direct visualization of NO production. The coverslips incubated in 37°C RPMI 1640 without phenol red were placed horizontally under the microscope lens. Photomicrographs with the fluorescent NO indicator were acquired with a laser scanning confocal microscope (Leica TCS-NT, Heidelberg, Germany) equipped with an argon-krypton laser at an excitation wavelength of 488 nm and a bandpass filter for 500–550 nm (29). Simultaneous visualization of cell morphology by differential interference contrast microscopy was performed to confirm equal cell numbers on the coverslips. The fluorescence images were obtained as a 1,024 × 1,024 pixel frame. All other settings, including scanning speed, pinhole diameter, and voltage gain, remained the same for all experiments, the images were stored on magneto-optical storage devices.

Measurement of NO by fluorometry in cell populations. The coverslips were placed diagonally in thermostatted quartz cuvettes containing 37°C RPMI 1640 medium without phenol red. Excitation monochrometers were centered at 490 nm, and emission light was collected at 520 ± 40 nm through a wide-band emission filter.

Microarray

Total RNA was quantified by measuring ultraviolet absorption ratio at 260/280 nm and checked for quality using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Preparation of the biotin-labeled cRNA target was performed using the BioRobot 9604 (Qiagen) and a PTC-225 DNA Engine Tetrad Cycler (MJ Research, Boston, MA). Single-stranded cDNA was prepared from 2 μg total RNA using a T7-(dT) 24-oligonucleotide primer and Superscript II RNaseH-RT (200 U/μl). Included in this reaction was a mixture of six bacterial RNAs of known concentration for use as positive controls (2.5 pg/ml arab/entF, 8.33 pg/ml fixB/gnd, and 25 pg/ml hisB/leucB). Double-stranded cDNA was then generated with Escherichia coli DNA polymerase I (10 U/μl) and RNase H (2 U/μl). After purification using a Qiagen QIAquick purification kit, the double-stranded cDNA served as a template to prepare biotin-labeled cRNA via in vitro transcription, performed in the presence of the biotinylated nucleotides. The labeled cRNA transcripts were purified using RNEasy columns (Qiagen) and assessed for quantity and quality using the same methods described above. The biotin-labeled cRNA was then randomly fragmented by incubating 2 μg of the sample in the presence of manganese for 20 min at 94°C. This resulted in fragmented target with a size range between 100 and 200 bases.

Hybridization and scanning. The biotinylated cRNA target was hybridized to two ADME-Rat Bioarrays (Motorola Life Sciences). For each array, 2 μg of the fragmented target cRNA was added to 260 μl of hybridization buffer, denatured, injected in hybridization chambers, sealed, and incubated for 18 h at 37°C while shaking at 300 rpm. Each array was rinsed in a stringent 46°C wash in 0.75× TNT TrisHCl, NaCl, and Tween-20 solution for 60 min, followed by streptavidin labeling for 30 min (RT) and then the 0.75× TNT and 0.05% TNT in series. The slides were spin-dried in an Eppendorf 5810R centrifuge (2,000 rpm for 3 min; swinging bucket rotor). Processed arrays were scanned using an Axon GenePix Scanner, and array images were acquired and analyzed using CodeLink Expression Analysis Software.

Motorola cDNA chip data analysis. The “normalized intensity” probe data generated by the CodeLink Expression Analysis Software (Amersham) were exported into Microsoft Excel. The data were then separated into the following two classes: high dose and low dose. The Excel function TTEST (low-dose data, high-dose data, 2, 2) was applied to each gene probe. Probes with P values greater than the P value threshold (PVAL-THRESH) of 0.05 were eliminated. The Excel function AVERAGE (high dose data)/AVERAGE (low dose data) was applied next. Probes with ratios between the RATIO-THRESH of 2.0 and 1/RATIO-THRESH of 0.5 were removed. The remaining probes were candidates for significantly changed mRNAs. These genes demonstrated acceptable P values and exhibited at least a twofold change between the high- and low-dose calcium treatments.

Statistics

Data are presented as means ± SE of the indicated number of experiments. Data were analyzed by one-way ANOVA followed by Dunnett’s multiple comparison test or Student’s t-test when appropriate. A P value of <0.05 was taken to indicate a statistically significant difference.

RESULTS

Reports on the effects of calcium on NOS are plentiful, although such effects have been thought to be mediated solely through changes in Ca2+ levels mediated via alterations in calcium channel or calcium pump activity (36). With this in mind, we first wanted to investigate whether calcium had any effect on the machinery needed for NO production in a rat model of humoral hypercalcemia of malignancy. Therefore, we performed a high-quality, oligonucleotide microarray analysis on four samples of total RNA subsequently made into cDNA from cells treated with either 3.5 or 0.5 mM calcium (41). The microarray data showed that H-500 cells treated with 3.5 mM calcium for 18 h exhibited an upregulation of iNOS mRNA on four samples of total RNA subsequently made into cDNA from cells treated with either 3.5 or 0.5 mM calcium (41). The microarray data showed that H-500 cells treated with 3.5 mM calcium for 18 h exhibited an upregulation of iNOS mRNA on four samples of total RNA subsequently made into cDNA from cells treated with either 3.5 or 0.5 mM calcium (41). The microarray data showed that H-500 cells treated with 3.5 mM calcium for 18 h exhibited an upregulation of iNOS mRNA on four samples of total RNA subsequently made into cDNA from cells treated with either 3.5 or 0.5 mM calcium (41). The microarray data showed that H-500 cells treated with 3.5 mM calcium for 18 h exhibited an upregulation of iNOS mRNA on four samples of total RNA subsequently made into cDNA from cells treated with either 3.5 or 0.5 mM calcium (41).
secretion in the H-500 cells is 3–4 mM calcium (26). Therefore, we chose 0.5 and 7.5 mM Ca\(^{2+}\) to be our low and high calcium concentrations, respectively. In Fig. 1, it can be seen that iNOS protein is upregulated at 4, 18, and 40 h in cells treated with high calcium compared with cells treated with low calcium. β-Actin in the lower panel is used to show equal loading of the protein samples.

We then addressed whether the induction of iNOS transcript seen in the Chip data was reproducible. We measured iNOS mRNA utilizing the "gold standard" method of Northern blotting, as well as by quantitative real-time PCR analysis. First, mRNA from H-500 cells treated for 18 h with low or high calcium were used to investigate whether high calcium would upregulate iNOS transcript. Northern blotting confirmed that calcium indeed upregulated iNOS mRNA at 18 h using a rat-specific iNOS probe made in our laboratory. The blot was reprobed with GAPDH to show equal loading (Fig. 2). To characterize the time course and dose response for the effect of calcium on iNOS mRNA, we next utilized quantitative real-time PCR. iNOS mRNA levels peaked at 18 h, exhibiting a 6.1 ± 1.4-fold increase when the cells were treated with high calcium compared with cells treated with low calcium (\(P < 0.05\); Fig. 3A). GAPDH was used to normalize differences in RNA isolation, RNA degradation, and/or the efficiency of RT reaction.

After determining the time course of iNOS mRNA induction, we studied the dose-response relationship for the effect of Ca\(^{2+}\) on the expression of the gene at 18 h. Using real-time PCR, we observed a dose-dependent increase in iNOS/GAPDH mRNA from 2 to 10 mM Ca\(^{2+}\). The increases were significant at 5, 7.5, and 10 mM Ca\(^{2+}\) (5.2 ± 1.2-, 8.3 ± 1.9-,

### Table 1. iNOS mRNA is upregulated in cells treated overnight with 0.5 or 3.5 mM calcium for 18 h in 60-mm plates

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<th>Ca(^{2+}) (mM)</th>
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<th>3.5</th>
<th>3.5</th>
<th>P Value</th>
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<td>0.020</td>
<td>0.065</td>
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mRNA was prepared and used for the synthesis of cDNA and was then analyzed on a Motorola cDNA chip, as described in MATERIALS AND METHODS.

![Fig. 1. Calcium induces inducible nitric oxide synthase (iNOS) protein upregulation in H-500 cells.](image)

![Fig. 2. Calcium induces iNOS mRNA upregulation.](image)

![Fig. 3. Time course for calcium-induced increase in iNOS mRNA and dose-response curve at 18 h.](image)
to be an agonist that is linked to GPCRs, a purinergic receptor. We have previously shown ADP inducing this effect, we used ADP, the ligand of another deed CaR mediated. To assess the specificity of the CaR in Cai phosphoinositide-phospholipase C (PLC) system, which elevates cells express a functional ADP receptor linked to the phosphoinositide-phospholipase C (PLC) system, which elevates

The cells were then starved for 4 h in serum-free medium, and mRNA was prepared after incubation of the cells with 0.5, 3.5, or 7.5 mM Ca^{2+} in serum-free medium for 18 h. The results represent the degree of increase compared with basal iNOS expression and are pooled from 3 independent experiments. Calcium-mediated iNOS expression (3.5 and 7.5 mM) was 5.9 ± 3.7- and 19.6 ± 4.4-fold greater at 3.5 and 7.5 mM Ca^{2+}, respectively, than basal iNOS expression in the H-500 cells infected with BG. In cells infected with dominant negative CaR, the effect was abolished at 3.5 and 7.5 mM calcium, a result that is significantly different from the response seen in the cells infected with BG at 7.5 mM calcium (*P < 0.05 compared with 7.5 mM in cells infected with BG). GAPDH was used to normalize differences in RNA isolation, RNA degradation, and/or efficiency of the RT reaction.

and 9.0 ± 2.3-fold, respectively) compared with the mRNA iNOS-to-GAPDH ratio at 0.5 mM Ca^{2+} (P < 0.05; Fig. 3B).

We next sought to determine whether the Ca^{2+}-induced increase in iNOS mRNA was mediated by the CaR. In cells infected with rAAV expressing BG, Ca^{2+} stimulated iNOS mRNA in a dose-responsive fashion, with 5.87 ± 3.67- and 19.62 ± 4.37-fold increases at 3.5 and 7.5 mM, respectively, compared with 0.5 mM calcium (P < 0.05). Infecting H-500 cells with a dominant-negative CaR (R185Q) via rAAV substantially reduced the stimulation of iNOS mRNA by Ca^{2+} compared with cells infected with BG. The difference between BG and dominant negative was significant at 7.5 mM calcium (P < 0.05; Fig. 4).

Thus high Ca^{2+}-induced iNOS mRNA upregulation is indeed CaR mediated. To assess the specificity of the CaR in inducing this effect, we used ADP, the ligand of another GPCR, a purinergic receptor. We have previously shown ADP to be an agonist that is linked to Go_{q,11}, like the CaR, through its receptor in H-500 cells, thereby eliciting Ca^{2+} release in H-500 cells loaded with the calcium-sensitive dye fura 2. Treatment of H-500 cells with ADP produced a rapid and transient increase in Ca^{2+} (32), thus confirming that the H-500 cells express a functional ADP receptor linked to the phosphoinositide-phospholipase C (PLC) system, which elevates Ca^{2+} in an agonist-dependent manner. Because ADP is degraded within 15–30 min by ectonucleotidases, we used adenosine 5’-O-(2-thiodiphosphate) (ADPβS), a nondegradable agonist of the ADP receptor, in the 18-h stimulation. We next used ADPβS to determine whether the stimulation of iNOS mRNA by high Ca^{2+}, acting via the CaR, is a generalized effect of activation of any GPCR linked to Go_{q,11}. Our data showed that, whereas high Ca^{2+} significantly increased iNOS mRNA (5.1 ± 1.9-fold increase), in contrast, cells treated with ADPβS (10^{-6} M) did not show any change in iNOS mRNA expression (0.8 ± 0.1, Fig. 5A). Because the microarray did not have oligonucleotides corresponding to mRNA sequences of eNOS, we assessed whether high calcium would have any effect on eNOS mRNA expression by real-time PCR. Stimulation of the cells for 18 h had no effect on the level of the transcript for eNOS compared with low calcium (Fig. 5B).

NO is a short-lived molecule, and in most publications NO measurements have been indirect. The discovery of a group of NO-specific dyes, the diaminofluorescines, allowed us to assess NO production in real time using fluorescent measurements (15). Thus we investigated whether CaR-induced iNOS upregulation also leads to augmented NO production. Figure 6A shows confocal micrographs of H-500 cells on coverslips that were treated with low or high calcium overnight and then loaded with 10 μM DAF-FM as described in MATERIALS AND METHODS. The cells treated with high calcium had higher fluorescence intensity with excitation at 488 nm and emission at 500–550 nm. To quantify this increase, we performed spectrophotometry in five independent experiments under similar conditions. The value of emission at 520 nm in cells treated with low calcium overnight was set to be one for each experiment, and the degree of change between cells treated with high

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DISCUSSION

We report here that iNOS is upregulated by calcium in a model of humoral hypercalcemia of malignancy. The cDNA Chip data confirmed our hypothesis that calcium induced changes in the machinery for NO production by increasing iNOS production. Neither eNOS nor nNOS was found to be altered by real-time PCR and microarray analysis, respectively. Determination of iNOS protein and transcript, respectively, by Western blotting or Northern blotting and real-time PCR showed that the effect of calcium was similar for all three parameters.

The mediatory role of the CaR in our study was proved by infecting the H-500 cells with the dominant-negative R185Q CaR and comparing the effect of high Ca\(^{2+}\) on iNOS concentration-response curve, similar to the effect of this mutant CaR on the response of the wild-type CaR to Ca\(^{2+}\) in transiently transfected HEK 293 cells (2). Similar inhibitory effects of the dominant-negative CaR results were seen in H-500 cells when examining PTHrP release and PTTG, a protooncogene, in response to calcium (32, 34). The effect of the CaR on iNOS is not a nonspecific event caused by activation of any GPCR, since activation of a functional G\(_{q,11}\)-coupled purinergic receptor in these cells by ADPβS (a nondegradable form of ADP) failed to alter iNOS expression in the H-500 cells. Activation of ADP receptors (like the CaR, a GPCR coupled to G\(_{q,11}\)) has been reported to upregulate the level of iNOS mRNA in human gingival epithelial cells (22). In the same cells, additional studies showed that IL-15 enhanced iNOS expression at both the mRNA and protein levels, similar to our study (39). However, another study found that IL-4 and interferon (IFN)-γ increased iNOS enzyme activity and protein expression in B cell chronic lymphocytic leukemia (B-CLL) cells during in vitro culture (19). IFN-γ, like calcium in our study, but not IL-4, increased iNOS mRNA expression in the cultured B-CLL cells, suggesting that IL-4-mediated changes of iNOS protein expression occurred at the posttranscriptional level. In macrophages, endotoxin-mediated NO synthesis has been shown to be dependent on heterotrimeric G\(_q\) protein signal transduction (17). In addition, another GPCR agonist, thrombin, whose cognate receptor is linked to G\(_{q,11}\), has been found to regulate iNOS in endothelial cells (14). Therefore, multiple hormones, including those activating GPCRs, regulate iNOS mRNA expression in a complex and tissue-specific manner.

The time course for the effect of calcium on iNOS upregulation was sustained, suggesting that iNOS upregulation could be functionally important. Therefore, we evaluated real-time NO production after stimulating the cells with low or high calcium overnight. Our photomicrographs and the spectrophotometric measurements of DAF-FM-loaded H-500 cells on coverslips treated overnight with 0.5 or 7.5 mM calcium. The cells were excited at 490 nm, and emission was measured at 520 nm. Pooled data from 5 independent experiments (*P < 0.05).

and low calcium was thus calculated. Figure 6B shows that the cells treated with high calcium overnight had a 2.7 \pm 0.7-fold higher fluorescence intensity compared with cells treated with low calcium (P < 0.05).

**Fig. 6.** Calcium stimulates NO production in H-500 cells. Cells were plated on coverslips and, after 72 h, at 80–90% confluence, were treated with 0.5 or 7.5 mM calcium for 18 h and washed and loaded with 4-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate (DAF-FM) dye as described in MATERIALS AND METHODS. A: NO production is upregulated in H-500 cells on coverslips treated with 7.5 mM calcium. Top left: fluorescence photomicrograph of the cells; top right: light photomicrograph; bottom left: overlay of the two fluorescent and light photomicrograph pictures. Representative confocal micrographs from 4 independent experiments. B: spectrophotometric measurements of DAF-FM-loaded H-500 cells on coverslips treated overnight with 0.5 or 7.5 mM calcium. The cells were excited at 490 nm, and emission was measured at 520 nm. Pooled data from 5 independent experiments (*P < 0.05).
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omeometric data show that NO production is upregulated. The DAF dyes were developed using the same principle as the fura dyes, i.e., the dye is bound to an ester group that is cleaved off by cytosolic esterases (15). Intracellular DAF bound to NO and not to other substrates changes the DAF to aromatic dimines, thereby inducing a chemical transformation and a resultant change in the fluorescence of the dye, which is measured by exciting the cells at 490 nm and measuring emission at 500–550 nm. Shortly after the discovery of the DAF dyes, a report indicated that calcium changed the fluorescence of the DAF-NO complex (5). This was later amended by Suzuki et al. (28), who showed that calcium changes NO release by the NO donors and not the DAF-NO complex fluorescence. In our study, we washed and incubated H-500 cells treated with low and high calcium in RPMI 1640 media without phenol red containing the same low level of Ca$_{c}^{2+}$ (0.5 mM), so that the calcium levels were the same at the time of the experimental readings.

Immunohistochemistry and mRNA data obtained using RT-PCR have shown that iNOS is constitutively present in the Leydig cells of adult rats (23). iNOS expression was upregulated by lipopolysaccharide, an inflammation-inducing substance. An NOS inhibitor decreased serum testosterone and testicular interstitial fluid formation without changing the level of luteinizing hormone. Therefore, those authors suggested that iNOS was important in the autocrine or paracrine regulation of the testicular vasculature, Leydig cell steroidogenesis, and spermatogenesis in normal rat testis. We report here that high Ca$_{c}^{2+}$ substantially increased NO production in Leydig cancer cells. We speculate, therefore, that the increase in iNOS production caused by high Ca$_{c}^{2+}$ will be important in regulating testosterone production and growth of the tumor. In H-500 cells, we have shown that the CaR induces proliferation and protection against apoptosis (31). However, high calcium-induced proliferation in H-500 cells was not altered by the competitive NOS inhibitor L-NAME or the iNOS inhibitor induced proliferation in H-500 cells was not altered by the protection against apoptosis (31). However, high calcium in RPMI 1640 media without phenol red containing the same low level of Ca$_{c}^{2+}$ (0.5 mM), so that the calcium levels were the same at the time of the experimental readings.

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REFERENCES


