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Expression of a functional extracellular calcium-sensing receptor in human aortic endothelial cells ☆

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Abstract

Extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) regulates the functions of many cell types through a G protein-coupled $[Ca^{2+}]_o$-sensing receptor (CaR). Whether the receptor is functionally expressed in vascular endothelial cells is largely unknown. In cultured human aortic endothelial cells (HAEC), RT-PCR yielded the expected 555-bp product corresponding to the CaR, and CaR protein was demonstrated by fluorescence immunostaining and Western blot. RT-PCR also demonstrated the expression in HAEC of alternatively spliced variants of the CaR lacking exon 5. Although stimulation of fura 2-loaded HAEC by several CaR agonists (high $[Ca^{2+}]_o$, neomycin, and gadolinium) failed to increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), the CaR agonist spermine stimulated an increase in $[Ca^{2+}]_i$ that was diminished in buffer without Ca^{2+} and was abolished after depletion of an intracellular Ca^{2+} pool with thapsigargin or after blocking IP_3- and ryanodine receptor-mediated Ca^{2+} release with xestospongin C and with high concentration ryanodine, respectively. Spermine stimulated an increase in DAF-FM fluorescence in HAEC, consistent with NO production. Both the increase in $[Ca^{2+}]_i$ and in NO production were reduced or absent in HAEC transfected with siRNA specifically targeted to the CaR. HAEC express a functional CaR that responds to the endogenous polyamine spermine with an increase in $[Ca^{2+}]_i$, primarily due to release of IP_3- and ryanodine-sensitive intracellular Ca^{2+} stores, leading to the production of NO. Expression of alternatively spliced variants of the CaR may result in the absence of a functional response to other known CaR agonists in HAEC.

Keywords: Extracellular Ca^{2+} concentration; Extracellular Ca^{2+}-sensing receptor; Intracellular Ca^{2+} concentration; Endothelial cells; Spermine; Nitric oxide

Extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) affects the function of the vascular endothelium. For example, changes in $[Ca^{2+}]_o$ in the physiological range modulate the synthesis of nitric oxide by the vascular endothelium and thereby regulate vascular tone [1]. $[Ca^{2+}]_o$ directly regulates cellular functions in some cell types through a G protein-coupled $[Ca^{2+}]_o$-sensing receptor (CaR). The CaR is a 1078 amino acid cell-surface protein that was initially cloned and characterized in parathyroid cells and is activated by $[Ca^{2+}]_o$ in a physiologically relevant range (0.5–5 mM) [2]. In parathyroid cells, the receptor is involved in the response to changes in serum Ca^{2+} concentration by regulating the synthesis of parathyroid hormone [2,3]. The CaR is also expressed in cells in the kidney, gastrointestinal tract, and brain where it is involved in the regulation of a diverse set of biological responses including ion channel activity, neuronal function, and cell proliferation [4]. A recent study provided evidence of CaR mRNA and protein expression in rat mesenteric arteries and porcine coronary artery endothelial cells [5].

Many cellular functions are regulated by intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the vascular endothelium. Endothelial $[Ca^{2+}]_i$ is, in turn, controlled by Ca^{2+} entry

☆ *Abbreviations:* $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; CaR, extracellular Ca^{2+}-sensing receptor; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; HAEC, human aortic endothelial cells; IP_3, inositol 1,4,5-triphosphate; NO, nitric oxide; SOCE, store-operated calcium entry; TRPC, transient receptor potential canonical; HBS, Hepes-buffered saline; PBS, phosphate-buffered saline; BSA, bovine serum albumin; siRNA, small interference RNA; XeC, xestospongin C.

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and by intracellular Ca^{2+} release, the latter occurring chiefly from the endoplasmic reticulum. Ca^{2+} entry in vascular endothelial cells proceeds through several pathways, but the dominant mechanism in nonexcitable cells is via store-operated Ca^{2+} entry (SOCE) which occurs following intracellular Ca^{2+} store depletion and mediates capacitative Ca^{2+} entry [6]. Ca^{2+} entry via SOCE is governed by the Ca^{2+} content of agonist-sensitive intracellular Ca^{2+} stores. In addition, studies have identified mammalian homologues of the *Drosophila* transient receptor potential canonical (TRPC) gene family, several members of which are expressed in endothelial cells [7]. Knockout models of TRPC4 have shown that this channel is involved in the regulation of endothelium-dependent vasodilation and in the control of endothelial barrier function. In addition to SOCE and TRPCs, other mechanisms of Ca^{2+} entry in vascular endothelial cells include nonselective cation channels, a mechanosensitive Ca^{2+}-permeable channel, and possibly other Ca^{2+} entry pathways including a Na^+/Ca^{2+} exchanger and possibly even voltage-gated Ca^{2+} channels, as recently reviewed [7]. Given the functional significance of many Ca^{2+} entry pathways in the vascular endothelium, the possibility that Ca^{2+} itself functions as a first messenger, and not simply a second messenger, must be considered. To date, however, there is no evidence that a functional CaR is expressed in the human vascular endothelium. We therefore studied whether the CaR is functionally expressed in human aortic endothelial cells (HAEC).

Materials and methods

Cell culture and $[Ca^{2+}]_i$ measurements. HAEC were purchased from Clonetics (Walkersville, MD 21793). The maintenance of HAEC in culture and $[Ca^{2+}]_i$ measurements were performed as previously described in detail [8–10]. Briefly, HAEC were grown to passage 5–9 at ~70% confluence on gelatin-coated, 25-mm diameter circular glass coverslips (VWR Scientific, West Chester, PA 19380). HAEC $[Ca^{2+}]_i$ was measured after loading with 10 μM of the acetoxymethyl ester form of fura 2 (Invitrogen-Molecular Probes, Carlsbad, CA 92008) for 30 min at room temperature. The coverslips were washed and the cells were maintained for at least 30 min before experimentation in indicator-free HBS (Hepes-buffered saline) of the following composition (in mM): NaCl 137, KCl 4.9, $CaCl_2$ 1.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, D-glucose 15, and Hepes 20 (pH adjusted to 7.40 at room temperature with NaOH). The fluorescence of fura 2 was recorded from single HAEC on coverslips in a perfusion chamber mounted on the stage of a modified Nikon Diaphot inverted epifluorescence microscope after excitation at 340 ± 10 and 380 ± 10 nm using a xenon short arc lamp (Ushio Inc.) corresponding to the Ca^{2+}-bound and Ca^{2+}-free forms of the indicator, respectively. Bandpass interference filters (Omega Optical, Brattleboro, VT 05301) selected wavelength bands of emitted fluorescence at 510 ± 10 nm. HAEC were stimulated with a variety of agonists and inhibitors in these experiments including xestospongin C (Calbiochem, San Diego, CA 92121) and spermine, neomycin, gadolinium, histamine, thapsigargin, and ryanodine (all from Sigma, St. Louis, MO 63103). The CaR agonists spermine (10 mM) [11,12], neomycin (300 μM) [2], and gadolinium (3 mM) [2,11] were used at concentrations that stimulated an increase in $[Ca^{2+}]_i$ in other cell types. All $[Ca^{2+}]_i$ measurements were performed at room temperature.

Cells from a human medullary C cell thyroid carcinoma cell line (TT) and HEK293 cells were purchased from ATCC (Rockville, MD 20852) and cultured according to manufacturer's protocol.

Assessment of CaR mRNA expression by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from HAEC monolayers according to the supplier's protocol using RNAqueous (Ambion, Austin, TX 78744). To prevent DNA contamination of the RNA preparation, 2 U TURBO DNase (Ambion) was added to each RNA sample and incubated at 37 °C for 30 min. After this, 10 μl DNase Inactivation Reagent was added and incubated for 2 min at room temperature to inactivate the DNase. The mixture was then centrifuged at 10,000*g* for 1.5 min at 4 °C and the supernatant recovered. For RT-PCR, 2 μg total RNA for each treatment was used for the reverse synthesis of single-stranded cDNA using the ReactionReady™ First Strand cDNA Synthesis Kit (SuperArray, Frederick, MD 21704) under RNase-free conditions according to the manufacturer's protocol. A 2 μl sample of each completed reverse transcription reaction was used for the PCR procedure using a SingleGene PCR Kit for CaR (SuperArray) and the final PCR mixture (total volume of 25 μl) was run for 28 PCR cycles (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s).

The 5′ forward and 3′ reverse-complement primers for amplification of exon-specific sequences in CaR were 5′-GAGAGTCTGAAAGACAGA AT-3′ which is defined by cDNA sequences from nucleotide 3992 to 4011, and 5′-TACTCTGTACAGGGATAGG-3′, which is complementary to cDNA sequences from nucleotide 4528 to 4546. These primer pairs are predicted to yield a 555-base pair (bp) product. Positive and negative control experiments were also performed on 2 μg total RNA samples isolated from a human medullary C cell thyroid carcinoma cell line (TT) [13] and HEK293 cells [14], respectively, following the same procedures for CaR RT-PCR as described above. When the PCR was completed, electrophoresis was performed at 90 V for 40 min or until the orange tracking dye (at <10 bp) ran off the gel. A CCD camera-Kodak EDAS 120 imaging system (Electrophoresis Documentation and Analysis System 120, Kodak Digital Science) was used to capture the image of the gel.

Additional RT-PCR experiments were also performed using a set of primers that span the missing region of an alternatively spliced form of the CaR previously reported in human keratinocytes [14] that lack exon 5, which encodes a portion of the extracellular domain. The region encompassing exon 5 was amplified with a sense primer from exon 4 (5′-AGG AAG TCT GTC CAC AAT GG-3′) and with the antisense primer from exon 6 (5′-CAA TGA TCC CTT TCC TGG TC-3′). These primers were synthesized from Invitrogen.

CaR protein immunostaining. The fixation and the protein immunostaining of HAEC were performed according to an established protocol for cells in culture from Affinity BioReagents (Gold, CO 80403). Briefly, HAEC monolayers on coverslips were washed with phosphate-buffered saline (PBS), fixed with 4% ice-cold paraformaldehyde for 10 min at room temperature, and permeabilized with ethanol at −20 °C for >1 h. Coverslips were then washed with PBS at 4 °C and blocked with PBS containing 8% bovine serum albumin (BSA) for 1 h at room temperature. HAEC were incubated with primary antibody (rabbit anti-human CaR antiserum, Alpha Diagnostic International, San Antonio, TX 78238) at a final concentration of 1 μg/ml at 25 °C for 75 min and washed with PBS containing 2% BSA. HAEC were then incubated with secondary antibody (goat anti-rabbit fluorescein-conjugated IgG, 1:40 dilution from original unit, Calbiochem) at 25 °C for 60 min and then washed with PBS containing 2% BSA for 3 times each for 5 min. CaR immunostaining was evaluated using a fluorescence microscopy system with excitation wavelength of 490 nm and emission wavelength of 520 nm. To determine the specificity of the immunostaining using the anti-human CaR antibody, an anti-CaR blocking peptide (Alpha Diagnostic) was employed using the manufacturer's protocol. The same procedure was undertaken using the mixture of anti-human CaR antibody and the anti-CaR blocking peptide as was used for the anti-CaR antibody alone. Expression of the irrelevant protein cyclophilin B was also detected in all groups to further confirm the specificity of the siRNA using a primary antibody against cyclophilin B from Affinity BioReagents.

Western blot. In pre-treated and untreated cells, protein was simultaneously isolated from cytoplasm and membrane using CNM Compartmental Protein Extraction Kit (Biochain, Hayward, CA 94545) according to the manufacturer's protocol. The biocinchoninic acid assay was

performed to quantify cytoplasmic and membrane protein concentration using BCA Working Reagents (Pierce Biotechnology, Rockford, IL 61105).

The protein sample (6 μg) was heated at 100 °C for 10 min in SDS sample buffer and loaded in polyacrylamide gel. After electrophoresis, the separated protein was electrically transferred to Pure Nitrocellulose Membrane (Bio-Rad Laboratories, Hercules, CA 94547) in Tris–glycine SDS running buffer using a semi-dry transfer cell (Bio-Rad). After washing with water, the membrane was blocked in PBS containing 3% dried milk for 40 min with agitation and then incubated with 2 μg/ml primary monoclonal antibody against human CaR (Alpha Diagnostic) at 4 °C overnight. After washing, the membrane was further incubated with 1:2000 diluted horseradish peroxidase-conjugated secondary antibody (Upstate, Lake Placid, NY 12946) for 1 h at room temperature. The membrane was then washed with PBS three times each for 10 min and the signal was visualized by a brief incubation with ECL Western Blotting Detection Reagent (Amersham Bioscience, Piscataway, NJ 08855) and a final exposure with Hyblot CL autography Film (Amersham Bioscience). To normalize the gel loading, the membrane was also probed with polyclonal antibody against cyclophilin B. The quantitative analysis of band intensity was performed using the UN-SCAN-IT gel program (Silk Scientific, Orem, UT 84059). CaR protein levels were normalized to cyclophilin B and the ratio between CaR and cyclophilin B level were used to compare any changes of CaR protein expression.

Reducing CaR expression by siRNA. To evaluate the functional role of the CaR in HAEC, small interference RNA (siRNA) was employed to reduce the level of CaR expression. The transfection of siRNA specifically targeted to the CaR or to a nonspecific control (Dharmacon RNA Technologies, Lafayette, CO 80026) into HAEC was performed using SuperFect Transfection Reagent (Qiagen, Valencia, CA 91355) according to the manufacturer's protocol. Briefly, HAEC monolayers grown on coverslips or in 60-mm dishes (for CaR immunostaining and Western blot, respectively) were used for transfection with 1 μg siRNA against the CaR or to a nonspecific control in 100 μl DMEM containing 20 μl Qiagen Superfect reagent at 37 °C, 5% CO_2 for 2.5–3 h. To determine the effectiveness of the CaR siRNA knockdown, protein levels was monitored by immunostaining and by Western blot.

Determination of nitric oxide production. To evaluate nitric oxide (NO) production by HAEC, the membrane-permeant indicator diaminofluorescein (DAF-FM) diacetate was employed in this study [15]. HAEC monolayers on coverslips were loaded with 1 μM DAF-FM diacetate in Hepes-buffered saline at 37 °C for 30 min and then incubated with DAF-FM diacetate-free Hepes-buffered saline for an additional 20 min to allow for de-esterification of the indicator. DAF-FM fluorescence was monitored on the aforementioned fluorescence microscopy system at an excitation wavelength of 480 ± 10 nm and an emission wavelength of 510 ± 10 nm.

Statistical analysis. Data are reported as means ± SE. Statistical comparisons were made using Student's *t* test for the paired and the unpaired groups. An analysis of variance was used when multiple comparisons were performed. A difference was considered significant at $p < 0.05$.

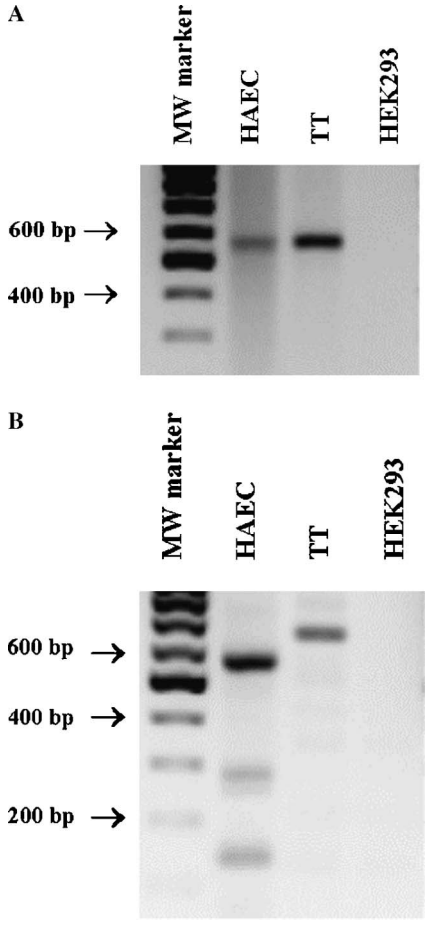

Fig. 1. (A) RT-PCR confirmation of Ca^{2+}-sensing receptor (CaR) expression in human aortic endothelial cells (HAEC). Reverse transcription and PCR amplification of HAEC RNA with CaR-specific primers yielded the expected 555-bp product corresponding to the CaR gene. The RT-PCR product from a human medullary C cell thyroid carcinoma cell line (TT) RNA sample and that from HEK293 are shown as a positive and a negative control, respectively. Representative findings of at least three separate RT-PCR experiments are shown. (B) RT-PCR analysis of alternatively spliced variants of CaR in HAEC. The full-length CaR from TT cells and alternatively spliced forms from HAEC were detected as products with different molecular sizes from RT-PCR using a primer set spanning exon 5 as described in Materials and methods. The band in TT cells is consistent with the expected full-length (650 bp) CaR. The bands between 500–600 and 100–200 bp are consistent with the alternatively spliced variant. The middle band(s) (200–300 bp) are likely heterodimer(s) of the above two forms. Representative findings of three separate RT-PCR experiments are shown.

Results

RT-PCR analysis of CaR mRNA expression in HAEC

To determine whether the CaR is present in HAEC, RT-PCR was performed using specific primers for the CaR. As shown in Fig. 1A, reverse transcription and PCR amplification of HAEC RNA with CaR-specific primers yielded the expected 555-bp product corresponding to the CaR gene detected in RT-PCR product agarose gel. As a positive control, under the identical conditions of reverse transcription and amplification, a RT-PCR product from a human medullary C cell thyroid carcinoma cell line (TT) [13] RNA sample using the same primers revealed a band of the same size. HEK293 cells, which do not express endogenous CaR [14], did not show any product of the same size. These findings confirm that the CaR is genetically expressed in HAEC.

Since it has been reported that human keratinocytes express both the full-length CaR and an alternatively spliced form lacking 231 (1378–1608) nucleotides encoding exon 5 [14], RT-PCR was performed using a set of primers previously shown to detect this previously documented

spliced variant. As shown in Fig. 1B, RT-PCR demonstrated that in contrast to the expected 650-bp band corresponding to the full-length CaR detected in TT cell line [13], a band between 500 and 600 bp was clearly noted in HAEC. Two additional faint bands between 200–300 and 100–200 bp were also demonstrated in HAEC. These results suggest that in HAEC, at least two alternatively spliced variants of the CaR are expressed in association with exon 5 deletion. The faint band(s) between 200 and 300 bp is either an additional splicing variant or the heterodimers of the other two splicing variants.

Immunostaining and Western blot for CaR protein expression

To determine whether CaR protein is expressed in HAEC, immunostaining was performed using a monoclonal antibody against human CaR. As shown in Fig. 2A, CaR protein is expressed in HAEC. Of note is the major localization of the fluorescence in the cytosol in this cell type, a finding reported in several other cell types as well [16–18] despite the presumed plasma membrane localization of the mature functional protein. To confirm the specificity of this immunostaining, a specific blocking peptide was used in parallel experiments under identical conditions. Also shown in Fig. 2B, the specific blocking peptide completely abolished CaR immunostaining.

To confirm CaR protein expression and cellular distribution in HAEC, Western blot experiments were also performed using cell lysate simultaneously isolated from cytoplasm and membrane. As shown in Fig. 2C, a band between 100 and 130 kDa, corresponding to the mature CaR, was revealed in the HAEC cytosolic fraction. A band of the same size was isolated from TT cell cytosolic fraction as a positive control [13], but not from the HEK293 cytosolic fraction (negative control) [14], confirming the specificity of CaR expression in HAEC. Another band between 55 and 70 kDa was also detected in the lysate from the HAEC membrane. The different band intensity may be related to differences between the relative distribution of CaR in the total cytosolic protein and in the total membrane protein fractions. These results suggest that post-translational modification of CaR protein may occur during translocation from the cytoplasm to the membrane in HAEC. Western blotting also detected a band between 130 and 170 kDa in both the HAEC and TT cell cytosolic fractions (not shown). This is consistent with the existence of a glycosylated form of the CaR, as previously reported in human keratinocytes [14]. There also appears to be additional post-translational modification of CaR protein in HAEC, since the molecular weight of CaR in the membrane fraction is smaller than that in the cytosolic fraction as shown in Fig. 2C.

Effects of CaR stimulation by known CaR agonists on HAEC $[Ca^{2+}]_i$

To determine whether the presence of CaR mRNA and protein is associated with the presence of functional receptors, fura 2-loaded HAEC were stimulated by several known CaR agonists. Increasing $[Ca^{2+}]_o$ (extracellular Ca^{2+} concentration) throughout the physiologic (and supraphysiologic) range is commonly used as a method for stimulating the CaR [2]. A 10-min elevation

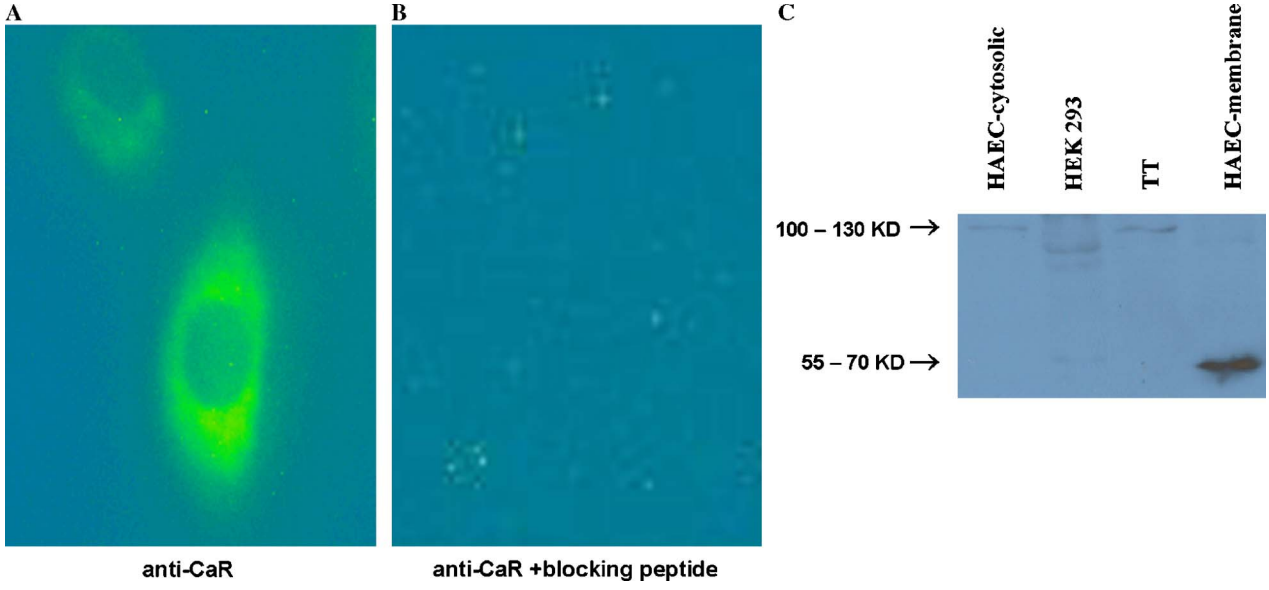

Fig. 2. Confirmation of Ca^{2+}-sensing receptor (CaR) protein expression in human aortic endothelial cells (HAEC) by immunostaining and Western blot. (A) Immunostaining of an HAEC shows that the CaR protein is expressed in HAEC and mainly localized in the cytosol (600× magnification). (B) Immunostaining of an HAEC using an anti-human CaR monoclonal antibody is completely abolished by a specific blocking peptide (600×). (C) Western blot showing different distributions of CaR with different molecular sizes between cytoplasm and membrane. Positive and negative control from a human medullary C cell thyroid carcinoma cell line (TT) and HEK293 are also shown.

of $[Ca^{2+}]_o$ from 0.5 to 2.5 mM did not stimulate any detectable change in $[Ca^{2+}]_i$ in fura 2-loaded HAEC (ratio 0.40 ± 0.01 to 0.40 ± 0.01, $n = 12$, $p =$ NS). When $[Ca^{2+}]_o$ was increased above 2.5 mM, to 15 and 30 mM, no detectable change in $[Ca^{2+}]_i$ was observed over at least 10 min (ratio 0.48 ± 0.06 to 0.50 ± 0.07 for 15 mM $[Ca^{2+}]_o$ and ratio 0.50 ± 0.07 to 0.50 ± 0.07 for 30 mM $[Ca^{2+}]_o$, $n = 5$, $p =$ NS for each, Fig. 3A). Thus, although the CaR is expressed in HAEC, it does not appear to respond to extracellular Ca^{2+} as an agonist under these experimental conditions.

Additional experiments were performed using three other known CaR agonists, neomycin, gadolinium, and spermine [2]. To evaluate the effects of these CaR agonists, fura 2-loaded HAEC were first exposed to 0.5 mM $[Ca^{2+}]_o$ and then HAEC $[Ca^{2+}]_i$ was continuously monitored for 10 min after $[Ca^{2+}]_o$ was increased to 2.5 mM to determine the response to an increase in $[Ca^{2+}]_o$. $[Ca^{2+}]_o$ was then decreased again to a physiologic level (1.5 mM) and HAEC were then exposed to 300 μM neomycin, 3 mM gadolinium (Gd^{3+}), or 10 mM spermine for 10 min. Finally, cells were exposed to 1 μM histamine to verify that the $[Ca^{2+}]_i$ response to agonist stimulation was intact.

As shown in Fig. 3B, neomycin, at a concentration that stimulated the CaR and increased $[Ca^{2+}]_i$ in other cell types [2], failed to trigger any increase in HAEC $[Ca^{2+}]_i$ (ratio 0.37 ± 0.01 to 0.37 ± 0.01, $n = 3$, $p =$ NS). In these same cells, the response to histamine was preserved; an abrupt increase in the fura 2 ratio (mean increase of 0.79 ± 0.03, $n = 3$) was observed after stimulation by histamine.

As shown in Fig. 3C, Gd^{3+} at a concentration that stimulated the CaR and increased $[Ca^{2+}]_i$ in other cell types [2,11] did not increase HAEC $[Ca^{2+}]_i$ (ratio 0.41 ± 0.02 to 0.41 ± 0.02, $n = 7$, $p =$ NS). Again, in these same cells, the response to histamine was preserved; an abrupt increase in the fura 2 ratio (mean increase of 0.55 ± 0.10, $n = 7$) was observed.

As shown in Fig. 4A, spermine at a concentration that stimulated the CaR and increased $[Ca^{2+}]_i$ in other cell types [11,12] stimulated an increase in $[Ca^{2+}]_i$ (ratio 0.41 ± 0.01 to 1.32 ± 0.16, $n = 3$, $p < 0.05$). Heterogeneity was noted in the $[Ca^{2+}]_i$ response stimulated by spermine; in two of three HAEC examined, $[Ca^{2+}]_i$ returned to a level slightly above the baseline during the experimental period, whereas in the remaining HAEC, $[Ca^{2+}]_i$ returned to the baseline level. Fig. 4A shows an example of the former response. To determine the Ca^{2+} source of this response, HAEC were exposed to Hepes-buffered saline without added Ca^{2+} and

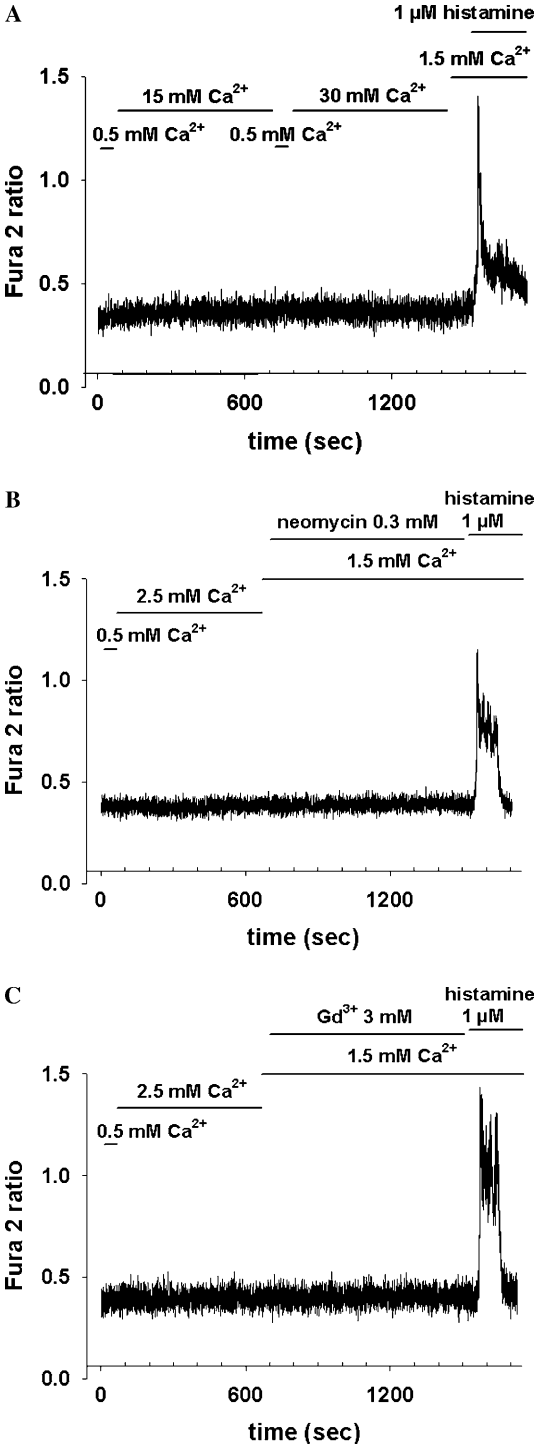

Fig. 3. Effects of Ca^{2+}-sensing receptor (CaR) agonists on $[Ca^{2+}]_i$ in human aortic endothelial cells (HAEC). (A) Representative tracing of five similar experiments from fura 2-loaded HAEC initially in buffer with an extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$) of 0.5 mM and subsequently exposed to buffer with 15 and 30 mM $[Ca^{2+}]_o$ for 10 min each. The change in $[Ca^{2+}]_o$ did not initiate any detectable change in $[Ca^{2+}]_i$. The subsequent exposure to 1 μM histamine in buffer with 1.5 mM $[Ca^{2+}]_o$ stimulated an increase in $[Ca^{2+}]_i$ in all cells studied (mean increase in fura 2 ratio $= 0.79 \pm 0.13$, $n = 5$). (B) Representative tracing of three similar experiments from fura 2-loaded HAEC exposed to 300 μM neomycin. No increase in $[Ca^{2+}]_i$ was observed when $[Ca^{2+}]_o$ was initially increased from 0.5 to 2.5 mM. In buffer with 1.5 mM $[Ca^{2+}]_o$, neomycin failed to trigger any increase in HAEC $[Ca^{2+}]_i$. In these same cells, the response to 1 μM histamine was preserved. (C) Representative tracing of seven similar experiments from fura 2-loaded HAEC exposed to 3 mM gadolinium (Gd^{3+}). No increase in $[Ca^{2+}]_i$ was observed when $[Ca^{2+}]_o$ was initially increased from 0.5 to 2.5 mM. In buffer with 1.5 mM $[Ca^{2+}]_o$, Gd^{3+} did not affect HAEC $[Ca^{2+}]_i$. In these same cells, the response to 1 μM histamine was preserved.

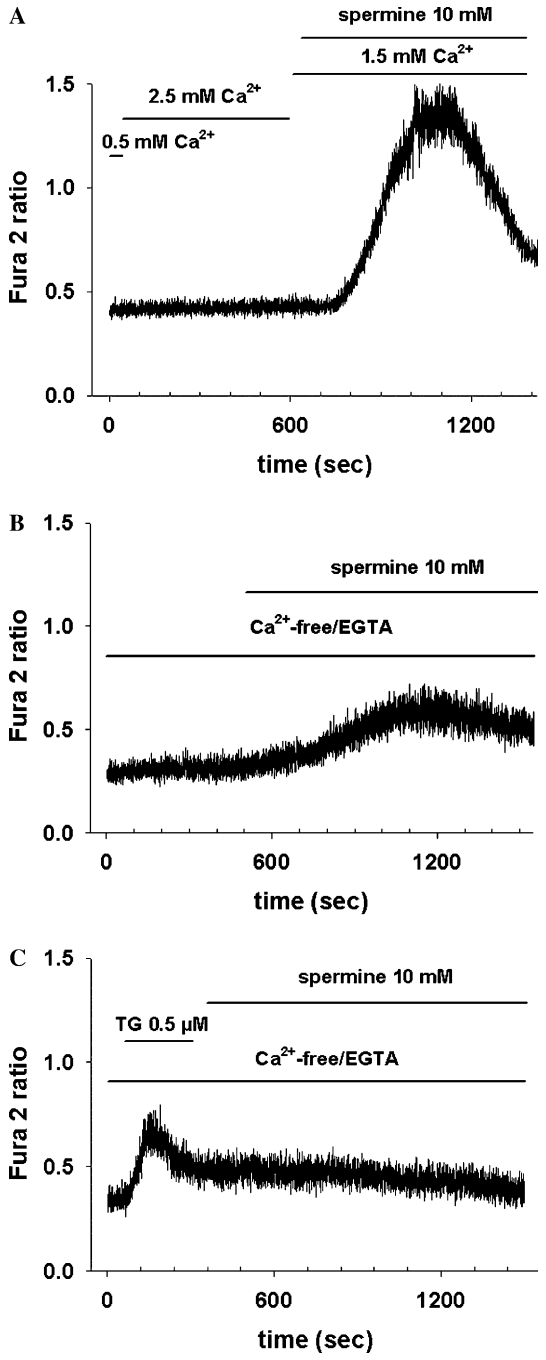

Fig. 4. Effect of spermine on $[Ca^{2+}]_i$ in human aortic endothelial cells (HAEC). (A) Representative fura 2 fluorescence from an HAEC monolayer exposed to 10 mM spermine for at least 10 min in the presence of buffer with 1.5 mM Ca^{2+}. No increase in $[Ca^{2+}]_i$ was observed when an extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$) was initially increased from 0.5 to 2.5 mM. Spermine stimulated an increase in HAEC $[Ca^{2+}]_i$ (ratio 0.41 ± 0.01 to 1.32 ± 0.16, $n = 3$, $p < 0.05$). (B) Representative fura 2 fluorescence from an HAEC monolayer showing that in buffer without added Ca^{2+} and with 1 mM EGTA, 10 mM spermine stimulated an increase in HAEC $[Ca^{2+}]_i$ that was smaller than that observed in the presence of 1.5 mM $[Ca^{2+}]_o$. (C) Representative tracing of three similar experiments from fura 2-loaded HAEC showing that after depletion of an intracellular Ca^{2+} store by thapsigargin (TG) in Ca^{2+}-free/EGTA buffer, the $[Ca^{2+}]_i$ response to spermine was abolished.

with 1 mM EGTA, and were then stimulated by spermine in Ca^{2+}-free/EGTA buffer. As shown in Fig. 4B, 10 mM spermine stimulated an increase in HAEC $[Ca^{2+}]_i$ in the nominal absence of buffer Ca^{2+} (ratio 0.38 ± 0.01 to 0.55 ± 0.02, $n = 3$) that was smaller than that observed in the presence of 1.5 mM $[Ca^{2+}]_o$ (Δ ratio $= 0.17 \pm 0.03$ vs. 0.91 ± 0.16, $p = 0.01$). These results suggest that spermine induced both intracellular Ca^{2+} release and extracellular Ca^{2+} influx in HAEC. When the intracellular Ca^{2+} store was first depleted by exposure to the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin in Ca^{2+}-free/EGTA buffer, the $[Ca^{2+}]_i$ response to spermine was abolished. In these experiments, 0.5 μM thapsigargin itself increased $[Ca^{2+}]_i$ from 0.34 ± 0.02 to 0.62 ± 0.06 ($n = 3$, $p < 0.05$); after this, spermine failed to induce a detectable change in $[Ca^{2+}]_i$ (ratio from 0.48 ± 0.04 to 0.51 ± 0.05, $n = 3$, $p =$ NS, Fig. 4C).

Experiments were also performed to assess the contribution of inositol 1,4,5-trisphophate (IP_3)- and ryanodine-sensitive Ca^{2+} stores to the increase in $[Ca^{2+}]_i$ stimulated by spermine in HAEC. In these experiments, fura 2-loaded HAEC in buffer with 1.5 mM Ca^{2+} were stimulated with 10 mM spermine after a 30-min pretreatment with either the specific, membrane-permeable IP_3 receptor blocker xestospongin C (XeC, 25 μM) [9,19], 100 μM ryanodine, or both. Although ryanodine acts as a ryanodine receptor agonist at low concentrations, ryanodine also blocks ryanodine receptors at high concentrations and has previously been used at this concentration in endothelial cells for this purpose [20]. After pretreatment with XeC alone, exposure to spermine in the continued presence of XeC resulted in an increase in $[Ca^{2+}]_i$ of lower amplitude than that induced by spermine in the absence of XeC (Δ ratio $= 0.31 \pm 0.03$, $n = 3$, $p < 0.05$ vs spermine in the absence of XeC). Similarly, after ryanodine pretreatment and in the continued presence of ryanodine, the increase in $[Ca^{2+}]_i$ stimulated by spermine was diminished compared to that in the absence of ryanodine (Δ ratio $= 0.28 \pm 0.01$, $n = 3$, $p < 0.05$ vs. spermine in the absence of ryanodine). XeC and ryanodine together completely blocked the spermine-induced $[Ca^{2+}]_i$ increase (Δ ratio $= 0.01 \pm 0.01$, $n = 3$, $p < 0.05$ vs. spermine alone).

Dependence of spermine-stimulated $[Ca^{2+}]_i$ signaling on CaR in HAEC

In order to determine the dependence of spermine-stimulated $[Ca^{2+}]_i$ signaling on CaR in HAEC, siRNA specifically targeted to CaR was introduced into HAEC to decrease CaR expression. Semiquantitative Western blot and immunostaining were performed to determine whether siRNA specifically knocks down the expression of CaR.

As shown in Fig. 5, immunostaining experiments demonstrated that siRNA targeted to the CaR decreased the expression of CaR protein to an undetectable level (Fig. 5D). Nonspecific siRNA (Fig. 5C) or vehicle (Fig. 5B) did not affect CaR immunostaining in HAEC.

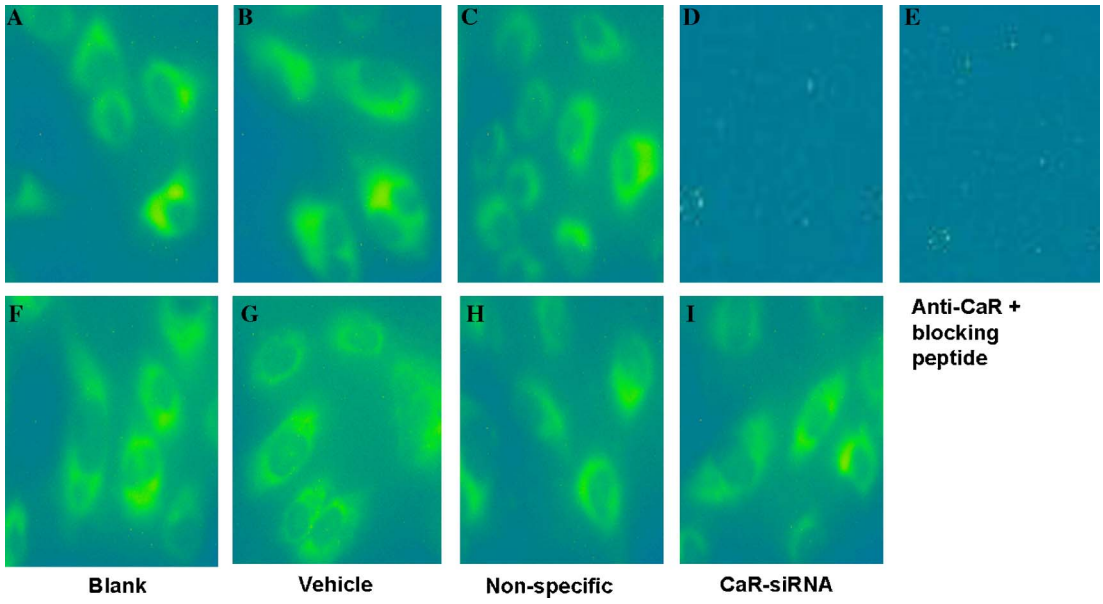

Fig. 5. Effect of siRNA targeted to the Ca^{2+}-sensing receptor (CaR) on CaR protein expression in human aortic endothelial cells (HAEC)-immunostaining studies. CaR immunostaining was no longer detected in HAEC transfected with siRNA targeted to the CaR (D) as compared to HAEC control (A), HAEC exposed to vehicle (B), or HAEC transfected with nonspecific siRNA (C). Immunostaining was no longer detected when a specific blocking peptide was used (E). Expression of the irrelevant protein cyclophilin B was constant in all groups. Representative findings of three separate experiments are shown.

A specific blocking peptide was also used to confirm the specificity of the staining (Fig. 5E). Expression of the irrelevant protein cyclophilin B was constant in all groups (Figs. 5F–I). Western blot was also performed to confirm and semi-quantify the effects of siRNA targeted to the CaR on CaR protein expression. As shown in Fig. 6A, siRNA against the CaR resulted in a significant decline in CaR expression (with no change in cyclophilin B expression). By contrast, no effect on CaR expression was found in HAEC transfected with nonspecific siRNA. Semi-quantitative analysis shows that CaR-targeted siRNA decreased CaR expression by more than 86% (CaR/cyclophilin B ratio $= 0.06 \pm 0.02$ for CaR siRNA-transfected HAEC, $p < 0.05$ vs. 0.44 ± 0.06 and 0.49 ± 0.11 for blank and nonspecific siRNA control HAEC, respectively, $n = 3$ for each, Fig. 6B).

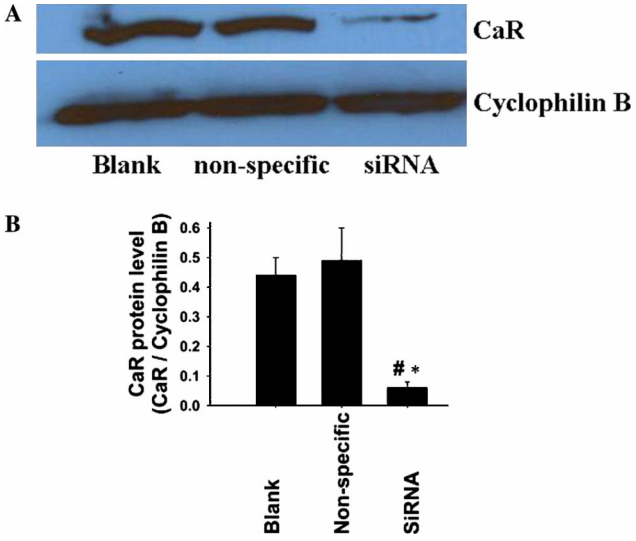

Fig. 6. Effect of siRNA targeted to the Ca^{2+}-sensing receptor (CaR) on CaR protein expression in human aortic endothelial cells (HAEC)-Western blot. (A) Representative semi-quantitative Western blot results demonstrating that siRNA targeted to the CaR specifically decreases the expression of CaR protein (100–130 kDa band shown); cyclophilin B level is unaffected. (B) Averaged data showing that siRNA targeted to the CaR decreases CaR protein levels by >86% in HAEC. $^{\#*}p < 0.05$ vs. blank and nonspecific control, respectively, $n = 3$ for each.

HAEC transfected with either siRNA to the CaR or to a nonspecific control were loaded with fura 2 and the $[Ca^{2+}]_i$ signal was observed after 10 mM spermine stimulation in Hepes-buffered saline for 10 min. In CaR siRNA-transfected HAEC (Fig. 7A), 10 mM spermine failed to increase $[Ca^{2+}]_i$ in 6 out of 7 experiments (ratio 0.44 ± 0.04 to 0.45 ± 0.04, $p =$ NS); a small $[Ca^{2+}]_i$ increase was noted in the remaining CaR siRNA-transfected HAEC. The $[Ca^{2+}]_i$ response to 1 μM histamine was still observed in the 6 HAEC that did not respond to spermine (ratio 0.42 ± 0.04 to 1.18 ± 0.29, $n = 6$, $p < 0.05$). In HAEC transfected with a nonspecific control siRNA (Fig. 7B), 10 mM spermine increased $[Ca^{2+}]_i$ in 10 of 12 experiments (ratio 0.44 ± 0.03 to 1.25 ± 0.05, $n = 10$, $p < 0.001$); there was no obvious $[Ca^{2+}]_i$ response in the remaining two HAEC. These results suggest that spermine increases $[Ca^{2+}]_i$ through the CaR in HAEC.

Given the cytosolic localization of CaR immunofluorescence in HAEC (Fig. 2) and in other cell types [16–18], experiments were performed to determine whether translocation of the CaR to the plasma membrane might occur after stimulation by spermine. Immunostaining experiments

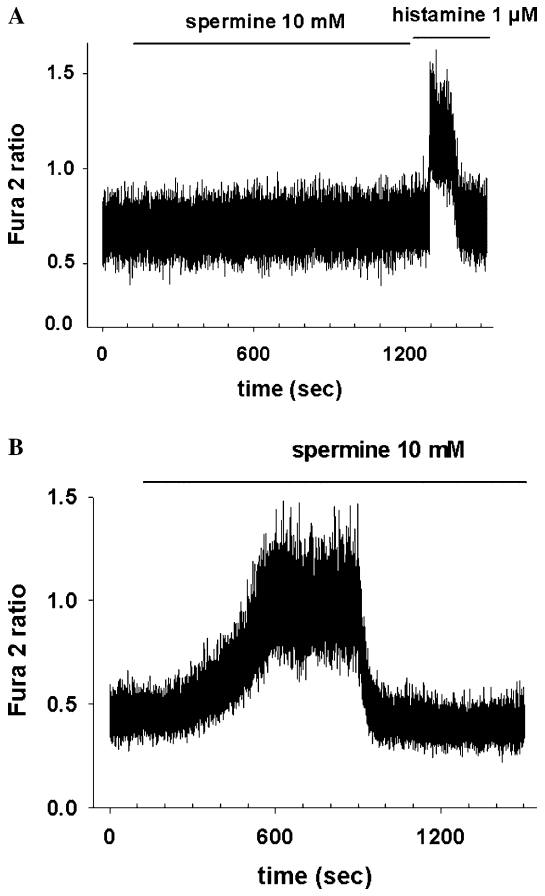

Fig. 7. Dependence of spermine-stimulated $[Ca^{2+}]_i$ increase on the Ca^{2+}-sensing receptor (CaR) activation in human aortic endothelial cells (HAEC). (A) Representative tracing of six similar experiments from fura 2-loaded HAEC transfected with siRNA targeted to the CaR and exposed to 10 mM spermine for at least 10 min in buffer with 1.5 mM Ca^{2+}. Spermine did not increase $[Ca^{2+}]_i$ in 6 of 7 siRNA-transfected HAEC examined; a small $[Ca^{2+}]_i$ increase was noted in the other. Exposure of these cells to 1 μM histamine stimulated an increase in HAEC $[Ca^{2+}]_i$ in all cells examined ($n = 6$). (B) Representative tracing of 10 similar experiments from fura 2-loaded HAEC transfected with siRNA targeted to a nonspecific control and exposed to 10 mM spermine for at least 10 min in buffer with 1.5 mM Ca^{2+}. Spermine increased HAEC $[Ca^{2+}]_i$ in 10 of 12 nonspecific siRNA-transfected HAEC examined.

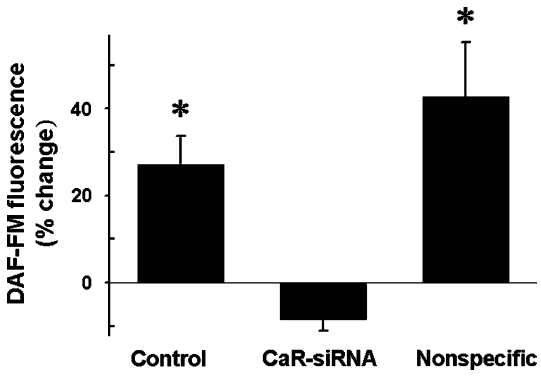

Fig. 8. Spermine stimulates nitric oxide production via the Ca^{2+}-sensing receptor (CaR) activation in human aortic endothelial cells (HAEC). Averaged data showing that 10 mM spermine stimulated a $27.1 \pm 6.7\%$ increase in DAF-FM fluorescence in HAEC. No increase in DAF-FM fluorescence was observed in HAEC transfected with siRNA targeted to the CaR and exposed to 10 mM spermine, whereas spermine stimulated a $42.8 \pm 12.4\%$ increase in DAF-FM fluorescence in nonspecific siRNA-transfected HAEC ($n = 6$ for each, $^*p < 0.05$ vs. baseline).

in HAEC fixed at different time points (from 30 s to 1 h) after spermine stimulation show no evidence of translocation of the CaR protein in this cell type (data not shown).

Spermine stimulates CaR-dependent nitric oxide production in HAEC

To examine a potential functional role of CaR activation in HAEC, the production of nitric oxide (NO) was determined in HAEC stimulated by spermine using the fluorescent NO indicator DAF-FM. NO was examined since its biosynthesis from endothelial cells stimulated by vasoactive agents is Ca^{2+}-dependent [1,21]. As shown in Fig. 8, 10 mM spermine stimulated a $27.1 \pm 6.7\%$ increase in DAF-FM fluorescence intensity; this was noted after approximately 15 min and reached a plateau after 41.4 ± 11.0 min ($n = 6$, $p < 0.05$). In HAEC transfected with siRNA against the CaR, 10 mM spermine failed to increase DAF-FM fluorescence intensity after more than 1 h observation (change in DAF-FM fluorescence $-8.4 \pm 2.7\%$, $n = 6$, $p =$ NS vs. baseline). A response to 1 μM histamine was still observed in these cells ($27.0 \pm 9.9\%$ increase in DAF-FM fluorescence, $n = 6$, $p < 0.05$, not shown in figure). In HAEC transfected with nonspecific control siRNA, spermine stimulated a $42.8 \pm 12.4\%$ increase in DAF-FM fluorescence ($n = 6$, $p < 0.05$). These data show that the effect of spermine on NO production is mediated through the CaR. Of note, the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 1 mM) completely inhibited both the spermine- and the histamine-induced increase in DAF-FM fluorescence intensity in HAEC (change in fluorescence intensity of $-1.7 \pm 1.6\%$ and $-1.4 \pm 2.4\%$, for spermine and histamine, respectively, $n = 3$, $p =$ NS vs. baseline for each).

Discussion

The present study shows that CaR mRNA and protein are expressed in HAEC and that when the CaR is stimulated by spermine, $[Ca^{2+}]_i$ increases primarily due to intracellular Ca^{2+} release and leads to the synthesis and release of nitric oxide. Spermine was the only known CaR agonist examined in this study that stimulated an increase in $[Ca^{2+}]_i$ in this cell type. Of the polyamines, spermine contains the highest number of free amino groups and is therefore the most potent agonist [22]. In the presence of physiologic $[Ca^{2+}]_o$ of around 1.5 mM, the concentration of spermine necessary to activate the receptor is reported to be around 100 μM [23]. The effect of spermine to release intracellular Ca^{2+} in HAEC in this study is similar to the effects reported by Canaff et al. [11] in hepatocytes. These authors noted that spermine (1.25–10 mM) increased

$[Ca^{2+}]_i$ in a dose–response manner in this cell type, with the effect reaching a plateau at 10 mM spermine. As with HAEC in the present report, prior depletion of intracellular Ca^{2+} pools by thapsigargin in Ca^{2+}-free buffer prevented the increase in $[Ca^{2+}]_i$ induced by spermine in hepatocytes [11]. These results, as well as those in which IP_3- and ryanodine receptor-mediated Ca^{2+} release were inhibited by XeC and high concentration ryanodine, respectively, indicate that activation of the CaR by spermine releases both IP_3- and ryanodine-sensitive Ca^{2+} stores in HAEC. This is the first time, to our knowledge, that the expression of the CaR and evidence of its potential functional significance have been reported in human endothelial cells. A recent study provided evidence of CaR mRNA and protein expression in rat mesenteric arteries and in porcine coronary artery endothelial cells [5]. Activation of rat and porcine vascular endothelial CaR opened Ca^{2+}-sensitive K^+ channels with subsequent vascular smooth muscle hyperpolarization [5], but effects on $[Ca^{2+}]_i$ were not reported.

When $[Ca^{2+}]_o$ was increased from subphysiologic concentrations (although not Ca^{2+}-free conditions) to supraphysiologic concentrations, no increase in $[Ca^{2+}]_i$ was observed in HAEC, whereas a similar protocol resulted in a large increase in $[Ca^{2+}]_i$ in other cell types known to express a functional CaR [22,24,25]. Likewise Gd^{3+} did not increase HAEC $[Ca^{2+}]_i$ even at a concentration of 3 mM, which is higher than the concentration of Gd^{3+} that has been shown to activate the receptor in other cell types [2]. We used this high concentration because in preliminary experiments, 1 mM Gd^{3+} did not increase $[Ca^{2+}]_i$ in this cell type (data not shown) and because a dose–response relationship of Gd^{3+} from 1 to 3 mM on $[Ca^{2+}]_i$ was previously reported in hepatocytes [11] with no response observed at concentrations of 500 μM in hepatocytes [7] or 600 μM in pancreatic acinar cells [17].

The CaR is considered promiscuous with respect to its ligand specificity and is able to be stimulated by polyvalent cations (Ca^{2+}, Mg^{2+}, and Gd^{3+}) [2], polycationic pharmacologic agents like neomycin and polylysine, and endogenous polycationic molecules like the polyamines (spermine, spermidine, and putrescine) [22]. Stimulation of the CaR in HAEC produced very different effects on $[Ca^{2+}]_i$ from those reported in other cell types known to express a functional CaR. For example, Bapty et al. [26] showed that the CaR is expressed in an immortalized MDCT (mouse distal convoluted tubule) cell line and that when a Ca^{2+}-free perfusion solution is changed to one with 1 mM Ca^{2+}, $[Ca^{2+}]_i$ increased rapidly and transiently from 92 ± 15 to 732 ± 92 nM. Exposure of MDCT cells to 0.1 mM Gd^{3+} or to 10 μM neomycin induced a similar, if not greater, increase in $[Ca^{2+}]_i$. These authors noted that these responses were not dependent on the presence of extracellular Ca^{2+} and therefore were due to intracellular Ca^{2+} release. McNeil et al. [27] reported that rat fibroblasts respond to Gd^{3+} with an increase in IP_3 production and intracellular Ca^{2+} release. Gama et al. [28] studied the $[Ca^{2+}]_i$ response of the human intestinal epithelial cell line (HT-29-18-C1) to various CaR agonists, including Ca^{2+}, which produced an increase in $[Ca^{2+}]_i$ that was blocked by inhibiting phosphatidylinositol-phospholipase C and by prior depletion of intracellular Ca^{2+} stores with thapsigargin, suggesting that CaR stimulation in this cell type resulted in the mobilization of intracellular Ca^{2+} in this cell type.

Of note, though, the $[Ca^{2+}]_i$ response of cells following CaR stimulation reported by other investigators is also highly variable and unpredictable. McNeil et al. [27] reported that only 25–33% of cells in a given field responded to Gd^{3+} with intracellular Ca^{2+} release. Bruce et al. [17] showed that pancreatic acinar cells responded to 1 mM Gd^{3+} with one of five types of $[Ca^{2+}]_i$ responses, the most common of which was no response. Of note, though, when these authors exposed cells to 600 μM Gd^{3+}, which is often the maximum concentration used in heterogonous expression systems [2], no change in $[Ca^{2+}]_i$ was noted. Exposure of acinar cells to 8 mM $[Ca^{2+}]_o$ produced four types of $[Ca^{2+}]_i$ responses, again the most common of which was no response. Neomycin, at concentrations of 500 μM, and 1 mM, did not stimulate any change in $[Ca^{2+}]_i$ [17].

Bapty et al. [26] also reported heterogeneous responses to CaR stimulation in MDCT cells; of a total of 108 individual cells studied, 24 (22.2%) failed to respond to polyvalent cations. Of note, these authors found that the response of cells to agonists that stimulate the CaR was all-or-none, since very high concentrations of extracellular cations did not stimulate increases in $[Ca^{2+}]_i$ in cells that were initially nonresponsive. The responses in various cell types, including distal tubular cells, fibroblasts, and intestinal epithelial cells, suggest that stimulation of the CaR in cells other than those of the parathyroid produces a rapid, transient, but unpredictable, increase in $[Ca^{2+}]_i$ that, when present, is primarily due to release of an intracellular store. These findings, including the unpredictable nature of the response, are consistent with our findings in human endothelial cells. What is unique in HAEC, however, is that no $[Ca^{2+}]_i$ response was noted when cells were stimulated by polyvalent cations or by neomycin and that only spermine elicited a $[Ca^{2+}]_i$ response. The reason why the CaR demonstrates such an unusual pattern of response to known agonists in HAEC and why it exhibits such a variable response in other cell types as well is unclear. However, experiments in this study suggest some clues. First, our RT-PCR experiments detected several exon 5-associated mRNA alternatively spliced variants (Fig. 1B). Second, Western blot experiments comparing CaR expression in the cytosolic and membrane fractions suggest that post-translational modification of the CaR may occur in HAEC during translocation of the protein from the cytoplasm to the membrane (Fig. 2C). The unique CaR agonist response in HAEC may also be related to low level expression of the CaR in cell types like HAEC that do not play a role in Ca^{2+} homeostasis [3]. The receptor may be heterogeneously distributed [17] or coupled less efficiently to G proteins [11] in cell types like HAEC which respond unusually or inconsistently to known CaR agonists. Finally, the unusual

response in HAEC may relate to the localization of the receptor primarily intracellularly, as suggested by the intracellular staining noted in Figs. 2A–C. Cytosolic localization of the receptor has been observed in other cell types [16–18] and may relate to membrane receptor recycling, true intracellular localization of the receptor, a high rate of receptor biosynthesis, or extensive post-translational modification [17]. Cytosolic-to-plasmalemmal translocation of the CaR upon activation was not observed in HAEC in this study, despite the presumed membrane localization of the mature functional protein. It is possible, however, that if most of the receptor is located in the cytosol both in the unstimulated state and after spermine stimulation, the translocation of a fraction of the total protein might have been missed by immunofluorescence staining or even Western blot. Thus, we cannot exclude the possibility that some of the protein translocated to the plasma membrane upon activation by spermine, but these experiments suggest that even if this were true, such translocation could only involve a very small fraction of the total protein.

The unusual response pattern of the HAEC CaR to known agonists of this receptor may relate to the expression in HAEC of a splice variant of the receptor, a situation previously reported in human keratinocytes [14]. As shown in Fig. 1B, a 500–600 bp product, smaller than the 650 bp product from the full-length CaR in human medullary C cell thyroid carcinoma cells, was noted in HAEC. Additionally, a 100–200 bp or a 200–300 bp product was also demonstrated in HAEC associated with, but not restricted to, the spliced variant lacking exon 5, since the observed molecular size is much less than the size of the splice variant missing exon 5 (420 bp) reported in human keratinocytes [14]. It has previously been reported that keratinocytes or HEK293 cells transfected with cDNA for the spliced variant of CaR lacking exon 5 do not respond to $[Ca^{2+}]_o$, and coexpression of the alternatively spliced form with the full-length CaR reduces the function of the full-length receptor [14]. It has been suggested that a change in binding affinity of the receptor to Ca^{2+} may be caused by the deletion of specific acidic amino acids in the region of the CaR encoded by exon 5 [29].

In summary, HAEC express a functional CaR that responds to spermine with an increase in $[Ca^{2+}]_i$ but does not respond with an increase in $[Ca^{2+}]_i$ to other known CaR agonists under the conditions studied. The presence of the CaR in HAEC suggests the possibility that this receptor may be involved in mediating some of the effects of $[Ca^{2+}]_o$ on the function of the vascular endothelium like the synthesis of nitric oxide [1,21], particularly if the receptor's sensitivity to Ca^{2+} as a ligand differs in certain vascular beds or under certain physiological conditions. The development of calcimimetic agents that increase the sensitivity of the CaR to activation by extracellular Ca^{2+} for the treatment of hyperparathyroidism [30] and the ongoing development of calcilytic agents to treat osteoporosis [31] have drawn attention to the CaR as a therapeutic target in clinical disorders of Ca^{2+} regulation. Clearly, unanticipated effects of these drugs might occur if functional CaRs are widely expressed, as suggested by our findings. Further studies especially the molecular cloning the full-length of CaR in vascular endothelial cells are needed to determine the factors that regulate the expression, localization, and biological activity of the CaR in the vascular endothelium.

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