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Increased endothelin-1 responsiveness in human coronary artery smooth muscle cells exposed to 1,25-dihydroxyvitamin D_3

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Absi M, Ward DT. Increased endothelin-1 responsiveness in human coronary artery smooth muscle cells exposed to 1,25-dihydroxyvitamin D_3. *Am J Physiol Cell Physiol* 304: C666–C672, 2013. First published January 23, 2013; doi:10.1152/ajpcell.00349.2012.—Low blood concentrations of 25-hydroxyvitamin D_3 are associated with increased mortality, while some studies suggest improved cardiovascular outcomes with vitamin D_3 supplementation in chronic kidney disease. However, the physiological effects of vitamin D_3 on the cardiovascular system remain poorly understood making it difficult to determine whether vitamin D_3 supplementation might provide cardiovascular benefit or even cause harm. Thus here we investigated the effects of chronic 1,25-dihydroxyvitamin D_3 treatment on intracellular signaling in human coronary artery smooth muscle cells (HCASMCs) and found that 1,25-dihydroxyvitamin D_3 significantly potentiated endothelin (ET-1) signaling. Specifically, 1,25-dihydroxyvitamin D_3 (24-h pretreatment) caused a more than threefold enhancement in both ET-1-induced intracellular calcium mobilization and extracellular signal-regulated kinase (ERK) activation. This 1,25-dihydroxyvitamin D_3-elicited signaling enhancement was not observed for either vasopressin or carbachol. With the use of endothelin receptor (ETR) isoform-selective antagonists, ETRA was found to be primarily responsible for the 1,25-dihydroxyvitamin D_3-induced ET-1 responsiveness and yet ETRA mRNA expression and protein abundance were unaltered following 1,25-dihydroxyvitamin D_3 treatment. While there was an increase in ETRB mRNA expression in response to 1,25-dihydroxyvitamin D_3, the protein abundance of ETRB was again unchanged. Finally, ETRA/ETRB heterodimerization was not detected in HCASMCs in either the absence or presence of 1,25-dihydroxyvitamin D_3. Together, these data show for the first time that 1,25-dihydroxyvitamin D_3 enhances endothelin responsiveness in HCASMCs and that the effect is mediated through ETRA.

vitamin D_3; endothelin; coronary artery smooth muscle cells; intracellular calcium; extracellular signal-regulated kinase

IT IS WELL KNOWN THAT 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$ or calcitriol] elicits both calciotropic effects, including maintenance of calcium and phosphate homeostasis, as well as a wide range of noncalciotropic effects on target tissues, including the cardiovascular system (7, 11, 13, 31). Epidemiological studies suggest that low plasma vitamin D_3 concentrations predispose a person to cardiovascular events (15, 16, 27) and that oral vitamin D_3 supplementation may improve all cause mortality including by decreasing cardiovascular deaths (2). However, whether vitamin D_3 supplementation should be recommended more widely to provide cardiovascular benefit remains controversial and large-scale clinical outcome trials are currently underway to address this (22, 25). In addition, there remains insufficient understanding of the cellular and physiological effects of 1,25$(OH)_2D_3$ in the vasculature and such information is necessary to define the likely physiological or therapeutic mechanism(s) of cardiovascular action for this endogenous hormone.

Vascular smooth muscle cells (VSMCs) express vitamin D receptors the activation of which has been shown in some studies to alter vascular contractility and calcification, as well as to affect VSMC proliferation and migration, and cytosolic calcium concentration (reviewed in Ref. 31). Since 1,25$(OH)_2D_3$ is generally associated with maintaining a low intracellular Ca^{2+} ($Ca^{2+}{}_i$) concentration in cells, particularly in epithelia responsible for active Ca^{2+} transport, then it is important to understand the effect of 1,25$(OH)_2D_3$ on $Ca^{2+}{}_i$ signaling in human VSMCs.

Endothelin-1 (ET-1) is the most powerful vasoconstrictor peptide in the cardiovascular system and is produced largely by endothelial cells (32). It binds to two class-A G protein-coupled receptor (GPCR) subtypes, namely endothelin receptor A (ETRA) and B (ETRB) (21). The stimulation of ETRA leads to increased cardiac inotropy and vasoconstriction whereas ETRB stimulation results in vasodilation and ET-1 clearance (5, 10). ET-1 has been implicated in the pathogenesis of a variety of cardiovascular diseases not least atherosclerosis, the development of which involves several cell types including VSMCs (14). Acute ETRA stimulation elicits phospholipase C-mediated inositol trisphosphate formation leading to a rapid mobilization of Ca^{2+} from the sarcoplasmic reticulum, which in turn causes VSMC contraction and vasoconstriction (14). ET-1 is also associated with activation of the extracellular signal-regulated kinases (ERKs), ERK1 and ERK2, which are proline-directed protein kinases involved in proliferation and other cell functions. Thus, to better understand the action of vitamin D_3 on vascular cell physiology, our objective in the current study was to investigate the effect of 1,25$(OH)_2D_3$ pretreatment on ET-1 signaling in human coronary artery smooth muscle cells (HCASMCs).

EXPERIMENTAL PROCEDURES

Human coronary artery cell culture. HCASMCs (Life Technologies) were grown in M-231 medium including smooth muscle growth supplement (GIBCO) in the absence of antibiotics or antimycotics at 37°C in a humidified 5% CO_2 incubator. HCASMCs, used between passage 3 and 8, were grown to ~80% confluence on glass coverslips and then treated with or without 1,25$(OH)_2D_3$ for 24 h (unless otherwise stated). All chemicals were from Sigma unless otherwise stated.

Intracellular calcium assay. HCASMCs cultured on glass coverslips were loaded with fura-2/AM (10 μM for 45 min) at room temperature in the dark in Ca^{2+} assay buffer (comprising in mM: 125 NaCl, 4 KCl, 0.5 $CaCl_2$, 0.5 $MgCl_2$, 5.5 glucose, and 20 HEPES pH 7.4) supplemented with 0.1% BSA. At the beginning of the experiment, cells were transferred into a perfusion chamber and washed with Ca^{2+} assay buffer (containing 0.5 mM Ca^{2+}) before treatment. Cells were observed through a ×40 oil-immersion objective. Dual-excitation wave-length microfluorometry was performed using a Nikon Diaphot inverted microscope (Metafluor software). To estimate $Ca^{2+}{}_i$ store content, cells incubated in extracellular Ca^{2+} ($Ca^{2+}{}_o$)-free buffer (+1 mM EGTA) were exposed to 1 μM thapsigargin with the

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area under the curve of the resulting rise in $Ca^{2+}{}_i$ concentration quantified.

ERK phosphorylation assay. Cells were grown to ~80% confluence in 35-mm culture dishes and rinsed in PBS for 5 min before equilibration for 25 min in experimental buffer at 37°C. Following culture in the absence or presence of 100 nM $1,25(OH)_2D_3$, cells were then incubated for up to 10 min in buffer supplemented with various experimental treatments and then lysed on ice in the following RIPA buffer: 12 mM HEPES (pH 7.6), 300 mM mannitol, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) sodium dodecyl sulfate supplemented with 1.25 μM pepstatin, 4 μM leupeptin, 4.8 μM PMSF, 1 mM EDTA, 1 mM EGTA, 100 μM vanadate, 1 mM NaF, and 250 μM sodium pyrophosphate. Lysate was then mixed with 5× Laemmli buffer and boiled for 5 min before immunoblotting using phospho-specific anti-ERK monoclonal antibody (1:2,000 dilution; Cell Signaling). Total ERK levels were demonstrated using anti-ERK2 antibody (Santa Cruz).

Immunoblotting. Immunoblotting was performed as previously described (28). Briefly, cell lysates were prepared using RIPA buffer and boiled for 5 min in Laemmli buffer. Protein samples were then resolved by SDS-PAGE and transferred electrophoretically to nitrocellulose. Nonspecific binding sites were blocked using 2% (wt/vol) BSA or 5% nonfat milk solutions (according to antibody protocol), followed by a 1-h incubation in either anti-ETRA rabbit polyclonal antibody (1:1,000 dilution; Santa Cruz Biotechnology), anti-pERK monoclonal antibody (1:3,000 dilution; Chemicon), anti-ETRB rabbit polyclonal antibody (1:1,000 dilution; Chemicon), anti-sarco/endoplasmic reticulum Ca^{2+}-ATPase (anit-SERCA; 1:1,000 dilution; Cell Signaling), or, anti-β-actin polyclonal antibody (1:5,000 dilution; Cell Signaling). After being washed, blots were exposed to either horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:5,000) for 1 h and then developed using ECL reagents (Geneflow). All washes and incubations used Tween/TBS solution [15 mm Tris (pH 8), 150 mm NaCl, and 0.1% (vol/vol) Tween 20]. Immunoreactivity was detected using light-sensitive paper and quantified by densitometry.

Immunoprecipitation. Cell lysates were prepared as above (in the absence of SDS) and then mixed with protein A-agarose beads and incubated with rotation for 30 min at 4°C. The precleared supernatant (50 μl) was then mixed with ETRB antibody (1 μl) and incubated as before for 1 h followed by overnight incubation in the presence of protein A-agarose beads. The supernatant was preserved, and the beads were then washed three times, boiled for 5 min in Laemmli buffer and the resulting immunopreciptates and supernatants processed for immunoblotting using anti-ETRB antibody. The membranes were then stripped and reprobed using anti-ETRA antibody.

Quantitative PCR. Total RNA was collected from HCASMCs (Qiagen RNeasy mini kit) including an RNase-free DNase (Qiagen) step, followed by cDNA synthesis (Bioline). Quantitative real-time PCR (qPCR) was performed in an Applied Biosystems 7500 Real Time PCR System using gene-specific primers [ETRA, ETRB, and phospholamban (PLN)] from Primer Design (PrimerDesign, Southampton, UK). The reaction components comprised SYBR Green Master mix (10 μl), 3′-primer (final concentration 300 nM), 5′-primer (final concentration 300 nM), cDNA (5 ng/μl), and nuclease-free water (to final volume of 20 μl). The protocol included a 10-min initial activation at 95°C, 15-s denaturation at 95°C, and data collection at 60°C (50 cycles).

Statistical analysis. Data are presented as means ± SE. Statistical significance was determined by one-way or repeated-measures ANOVA or by unpaired *t*-test, using GraphPad Prism.

RESULTS

Effect of 1,25(OH)$_2$D$_3$ on endothelin responsiveness in HCASMCs. Pretreatment of HCASMCs with $1,25(OH)_2D_3$ (100 nM) for 24 h significantly increased the responsiveness of the

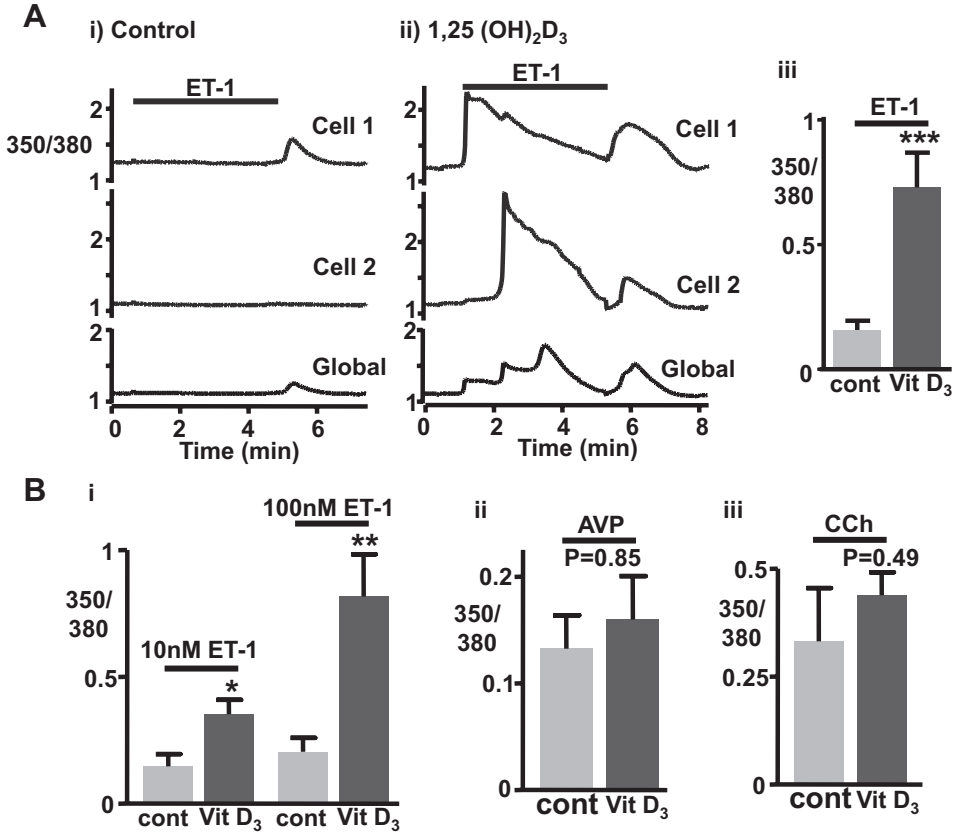

Fig. 1. *A*: representative traces showing the intracellular Ca^{2+} ($Ca^{2+}{}_i$) responses to stimulation with 100 nM endothelin [ET-1; in the presence of 0.5 mM extracellular Ca^{2+} ($Ca^{2+}{}_o$)] in individual human coronary artery smooth muscle cells (HCASMCs; Cell 1 and Cell 2) and "Global" increase in $Ca^{2+}{}_i$ in a cluster of cells, following 24-h culture in the absence (*i*) or presence (*ii*) of 100 nM 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. Quantification of the increased ET-1 response in $1,25(OH)_2D_3$-treated cells as represented by area under the curve/min (*iii*). ***$P < 0.001$ vs. control (n = 12–18 coverslips). *B*: quantification of the changes in $Ca^{2+}{}_i$ concentration in HCASMCs pretreated as before but then exposed to either 10 or 100 nM ET-1 (*i*), vasopressin (100 nM AVP; *ii*), or carbachol (100 μM CCh; *iii*; n = 5–8 coverslips).

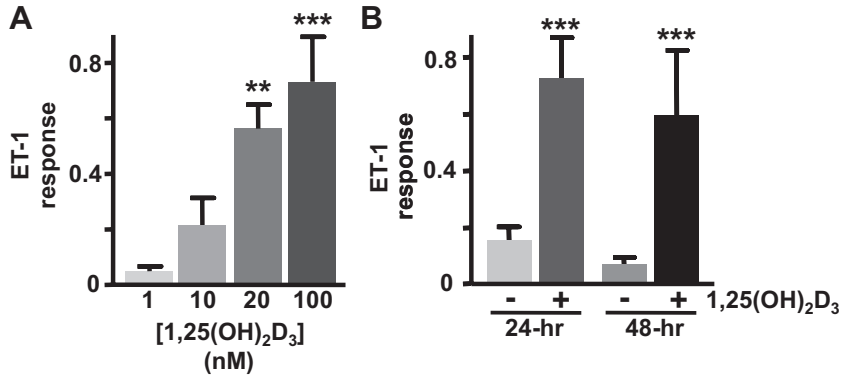

Fig. 2. *A*: changes in $Ca^{2+}{}_i$ in response to 100 nM ET-1 (in the presence of 0.5 mM $Ca^{2+}{}_o$) in cells pretreated with either 1, 10, 20, or 100 nM $1,25(OH)_2D_3$ for 24 h. *B*: quantification of the $Ca^{2+}{}_i$ changes in response to ET-1 in HCASMCs treated with 100 nM $1,25(OH)_2D_3$ for 24 or 48 h. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the respective control; $n = 4$–10 coverslips.

cells to subsequent endothelin (ET-1; 100 nM) exposure (Fig. 1*A*). Specifically, ET-1 elicited significantly greater $Ca^{2+}{}_i$ mobilization following 24-h $1,25(OH)_2D_3$ pretreatment than in its absence. This increased endothelin responsiveness appeared independent of $Ca^{2+}{}_o$ concentration, being observed in either 0.5, 1.2, or 2 mM $Ca^{2+}{}_o$ (data not shown) and was also observed using only 10 nM ET-1 (Fig. 1*Bi*). Next, to assess whether the $1,25(OH)_2D_3$-induced increase in endothelin responsiveness is specific to this particular G protein-coupled receptor agonist or represents a more general phenomenon, the cells were alternatively stimulated acutely with either vasopressin (100 nM; Fig. 1*Bii*) or carbachol (100 μM; Fig. 1*Biii*). In these experiments, $1,25(OH)_2D_3$ pretreatment failed to increase vasopressin or carbachol responsiveness significantly.

Effect of 1,25(OH)$_2$D$_3$ on endothelin responsiveness as a function of concentration and time. The concentration of $1,25(OH)_2D_3$ commonly used in cell studies is 100 nM, for example, to change global gene expression in HCASMCs (31). Thus we next determined the concentration dependency of the $1,25(OH)_2D_3$ response. Exposure of the cells to increasing concentrations of $1,25(OH)_2D_3$ (up to 100 nM, 24 h) did result in a concentration-dependent increase in ET-1 responsiveness (Fig. 2*A*), with 20 nM $1,25(OH)_2D_3$ being sufficient to elicit a significant effect. Next, cells were incubated with $1,25(OH)_2D_3$ (100 nM) for either 24 or 48 h and then stimulated with ET-1 (100 nM; Fig. 2*B*). Treatment with $1,25(OH)_2D_3$ for 24 h elicited the greatest effect although even at 48 h; the endothelin responsiveness was still elevated.

Identification of the receptor subtype mediating endothelin-induced calcium mobilization. To determine whether ETRA and/or ETRB are responsible for the $1,25(OH)_2D_3$-induced endothelin responsiveness, HCASMCs were stimulated with endothelin (100 nM) in the absence or presence of antagonists to ETRA (BQ123) and ETRB (BQ788). Cells were pretreated with either 100 nM or 3 μM of the antagonists for 10 min and then cotreated with the endothelin. The ETRA antagonist BQ123 largely abolished the response to ET-1 in $1,25(OH)_2D_3$-treated cells (Fig. 3*A*). In contrast, the ETRB antagonist BQ788 failed to significantly inhibit the same ET-1 response (Fig. 3*B*). Consistent with this, the ETRB-selective agonist sarafotoxin S6c (100 nM) was without effect, despite subsequent ET-1 treatment eliciting a similar response to before (Fig. 3*C*). Together, these results suggest that ETRA represents the main contributor to the stimulatory effect of $1,25(OH)_2D_3$ on ET-1 responsiveness.

Effect of 1,25(OH)$_2$D$_3$ pretreatment on endothelin-induced ERK activation. Next, it was necessary to determine whether the increased endothelin responsiveness reported here is limited to changes in $Ca^{2+}{}_i$ mobilization or whether other downstream signaling pathways are similarly affected. To address this, the effect of $1,25(OH)_2D_3$ on endothelin-induced ERK (extracellular signal-regulated) activation was then investigated. ET-1 produced

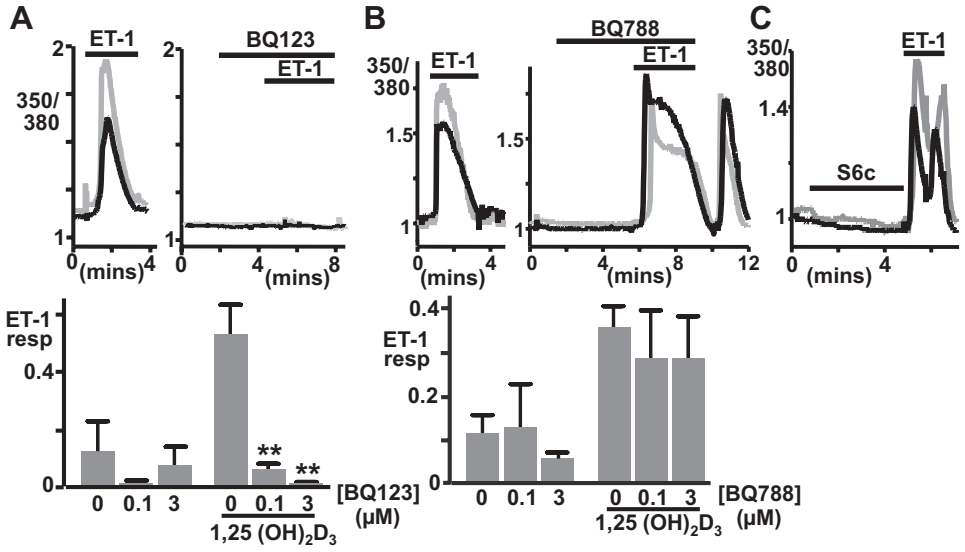

Fig. 3. Representative traces showing the $Ca^{2+}{}_i$ responses to stimulation with 100 nM ET-1 (in the presence of 0.5 mM $Ca^{2+}{}_o$) in individual HCASMCs in the absence or presence of endothelin receptor A (ETRA) antagonist BQ123 (*A*; $N = 3$–16 coverslips) or endothelin receptor B (ETRB) antagonist BQ788 (*B*; $n = 4$–31 coverslips) in HCASMCs pretreated with 100 nM $1,25(OH)_2D_3$ for 24 h. Grey trace is a result from a single cell while the black trace shows the "global" response of the entire cell cluster. Quantification of the total $Ca^{2+}{}_i$ changes in the cluster are also shown for *A* and *B*. $^{**}P < 0.01$ vs. ET-1 alone. Addition of 100 nM sarafotoxin 6c (S6c) to cells pretreated with $1,25(OH)_2D_3$ failed to elicit any response whereas subsequent treatment with ET-1 induced robust $Ca^{2+}{}_i$ mobilization as before (*C*; $n = 6$).

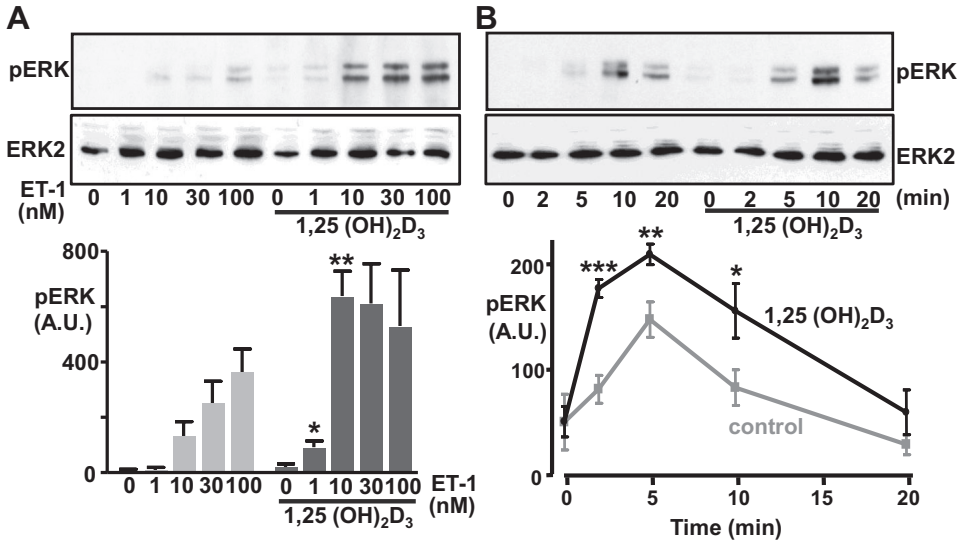

Fig. 4. Extracellular signal-regulated kinase (ERK) phosphorylation assay in HCASMCs pretreated with vehicle or 100 nM $1,25(OH)_2D_3$ for 24 h and then exposed to increasing concentrations of ET-1 up to 100 nM (*A*; 5 min; $n = 4$) or 100 nM ET-1 for various time points up to 20 min (*B*; $n \geq 5$). *A* and *B* include representative immunoblots for ERK phosphorylation (pERK) and total ERK2 (ERK2) as well as pERK quantified by densitometry. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by repeated-measures ANOVA with Dunnett's posttest.

a concentration-dependent increase in ERK phosphorylation in HCASMCs pretreated either with or without $1,25(OH)_2D_3$; however, endothelin responsiveness was significantly greater in the $1,25(OH)_2D_3$-pretreated cells (Fig. 4*A*). In particular, the responses to 1 and 10 nM ET-1 were markedly elevated following 24-h $1,25(OH)_2D_3$ pretreatment. The time dependence of the ERK responses to ET-1 was similar in both control and $1,25(OH)_2D_3$-treated cells, that is, they both peaked at 5 min (Fig. 4*B*) and declined to baseline by 20 min; however, again, the cells treated with $1,25(OH)_2D_3$ exhibited significantly larger responses to ET-1 at 2, 5, and 10 min.

Next, to determine which receptor subtypes are responsible for the ET-1-induced ERK activation, cells were cotreated with either BQ123 or BQ788. Unlike in the experiments investigating $Ca^{2+}{}_i$ mobilization, neither antagonist inhibited ET-1-induced ERK activation when used at 100 nM concentration (Fig. 5). However, in the presence of 1 μM BQ123 the ERK response to ET-1 was completely ablated whereas BQ788 was still without effect. Therefore, for both $Ca^{2+}{}_i$ mobilization and ERK activation, the $1,25(OH)_2D_3$-enhanced ET-1 responses in HCASMCs appear to be mediated via ETRA.

Effect of $1,25(OH)_2D_3$ on ETR expression in HCASMCs. Next, total RNA and protein lysates were collected from HCASMCs cultured in the absence or presence of $1,25(OH)_2D_3$ (100 nM, 24 h) and the relative expression of ETRA and ETRB determined by qPCR and immunoblotting. Despite the apparent increase in ETRA-mediated endothelin responsiveness elicited by $1,25(OH)_2D_3$, no change in ETRA expression was observed either at the transcriptional level (Fig. 6*A*) or in terms of protein abundance (Fig. 6*B*). In contrast, despite the apparent lack of ETRB involvement in endothelin responsiveness (Figs. 3 and 5), qPCR analysis revealed a 150% increase in ETRB expression in the $1,25(OH)_2D_3$-treated HCASMCs (Fig. 6*A*). However, the ETRB protein abundance was unchanged by the $1,25(OH)_2D_3$ pretreatment (Fig. 6*B*). Next, to determine whether the apparent increase in ETRA responsiveness is due to altered ETRA/ETRB heterodimerization, ETRB was immunoprecipitated from HCASMCs pretreated with or without $1,25(OH)_2D_3$ and then probed with anti-ETRA antibodies. However, no ETRA was detected in the ETRB precipitates under either condition, suggesting that (altered) heterodimerization does not explain $1,25(OH)_2D_3$-enhanced ET-1 responsiveness. Finally, to investigate whether $1,25(OH)_2D_3$ may be modulating the $Ca^{2+}{}_i$ stores more directly, we examined the effect of $1,25(OH)_2D_3$ pretreatment on the expression in HCASMCs of the SERCA pump and of its negative regulator PLN as well as on the relative amounts of thapsigargin-mobilizable stored $Ca^{2+}{}_i$ within the cell. $1,25(OH)_2D_3$ (100 nM, 24 h) failed to alter protein expression of SERCA (Fig. 6*Di*) but did inhibit PLN gene expression by 80% (Fig. 6*Dii*), consistent with a previous microarray study (30); however, this could not be confirmed at the protein level. If PLN downregulation did occur in the presence of unaltered SERCA expression, then we might expect to see increased $Ca^{2+}{}_i$ store filling (due to decreased SERCA inhibition) and interestingly the total amount of stored $Ca^{2+}{}_i$ in the HCASMCs estimated using thapsigargin treatment (in the ab-

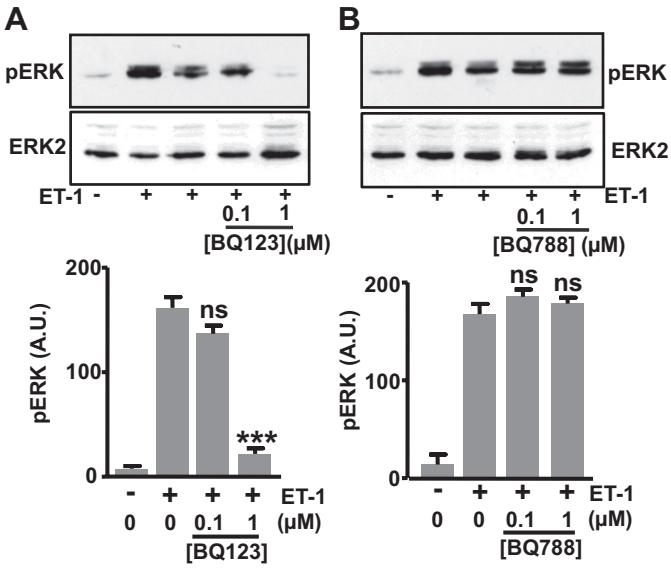

Fig. 5. Quantification of pERK activity in protein samples prepared from HCASMCs treated with 100 nM $1,25(OH)_2D_3$ for 24 h and then exposed to 100 nM ET-1 alone for 5 min in the absence and in the presence of ETRA antagonist BQ123 (*A*) or ETRB antagonist BQ788 (*B*) ($n = 4$ experiments). $^{***}P < 0.001$.

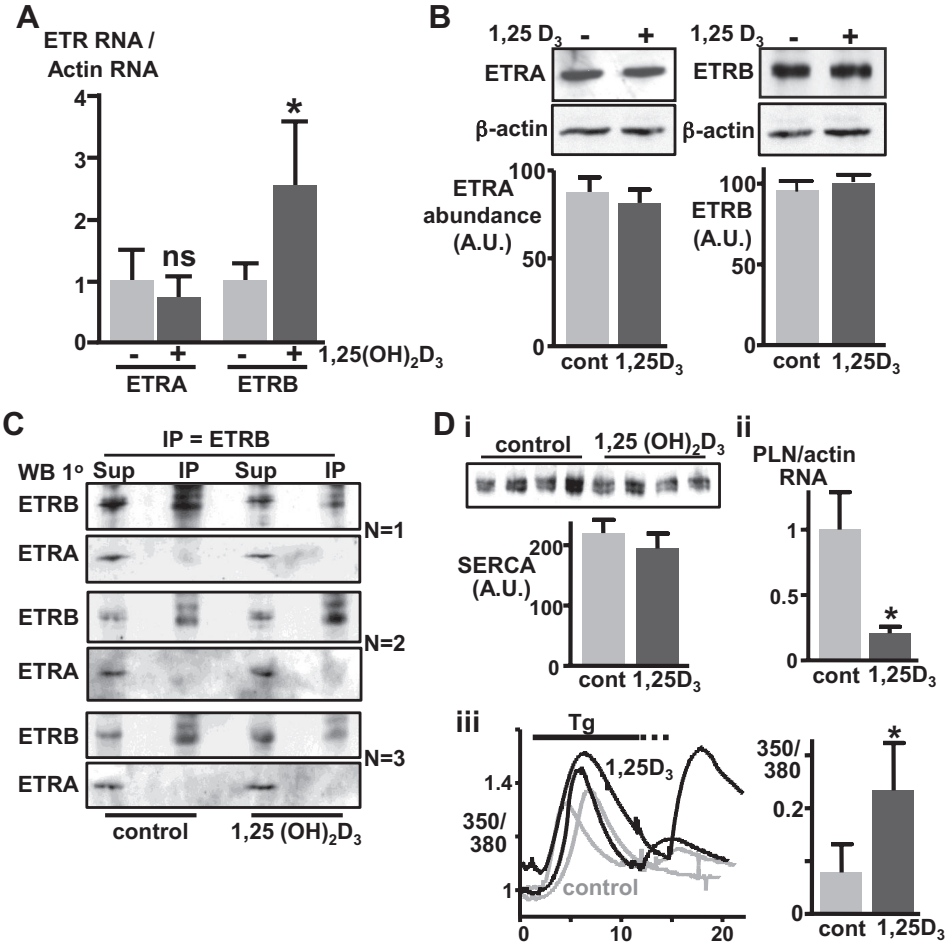

Fig. 6. *A*: quantitative PCR of ETRA and ETRB mRNA isolated from HCASMCs treated with vehicle or 100 nM $1,25(OH)_2D_3$ for 24 h. *$P < 0.05$ vs. control. *B*: Western blot analysis of protein samples obtained from HCASMCs in the absence and in the presence of 100 nM $1,25(OH)_2D_3$ for 24 h and probed with anti-ETRA or anti-ETRB antibodies ($n = 7$). *C*: ETRB content of lysates of HCASMCs pretreated with or without $1,25(OH)_2D_3$ (100 nM for 24 h) was collected by immunoprecipitation. The resulting immunoprecipitates (IP) and supernatants (Sup) were probed with anti-ETRB antibody to confirm (partial) pull down and the membrane then stripped and reprobed with anti-ETRA antibody. All 3 replicates are shown. An intermediate step confirming the complete removal of anti-ETRB antibody from the blot was also conducted (not shown). *D*: pretreatment with $1,25(OH)_2D_3$ failed to significantly alter SERCA protein abundance (*i*; $n = 6$) but did inhibit phospholamban (PLN) gene expression (*ii*; $n = 4$) and also increase the amount of $Ca^{2+}{}_i$ mobilizable using thapsigargin (Tg; $n = 4$). Traces show the global responses for 2 independent sets of representative experiments [Control, grey traces; $1,25(OH)_2D_3$, black traces].

sence of extracellular Ca^{2+}) was indeed significantly greater in cells pretreated for 24 h with $1,25(OH)_2D_3$ (Fig. 6*Diii*).

DISCUSSION

In HCASMCs, ET-1 elicited increases in $Ca^{2+}{}_i$ concentration and ERK phosphorylation consistent with activation of ETRs on the cell membrane. Specifically, endothelin exerts its vasoactive effects via two GPCRs, namely ETRA and ETRB both of which can couple to $G\alpha_{q/11}$-induced PLC activation leading to $Ca^{2+}{}_i$ mobilization and also to the Raf/MEK/ERK pathway (see 20). In the current study, the presence of both ETRA and ETRB in the HCASMCs was confirmed both by qPCR and immunoblotting. Furthermore, with the use of isoform-selective antagonists, BQ123 for ETRA and BQ788 for ETRB, it was found that ETRA mediated the responses of ET-1 on $Ca^{2+}{}_i$ mobilization and ERK activation in the HCASMCs.

Next, 24-h exposure to $1,25(OH)_2D_3$ significantly and consistently enhanced ET-1-induced ERK activation and $Ca^{2+}{}_i$ mobilization in HCASMCs. To our knowledge, this represents the first report of $1,25(OH)_2D_3$-mediated stimulation of endothelin responsiveness. In contrast, $1,25(OH)_2D_3$ failed to alter ET-1-induced ERK activation in neonatal rat VSMCs (4), while in rat osteoblastic osteosarcoma cells, $1,25(OH)_2D_3$ actually lowered ET-1-induced inositol phosphate metabolism (18). Interestingly, the $1,25(OH)_2D_3$-induced increase in ET-1-mediated $Ca^{2+}{}_i$ mobilization seen here in HCASMCs appeared relatively specific to endothelin since it failed to increase the responsiveness of vasopressin or carbachol, vasoactive substances that also induce $G\alpha_{q/11}$-coupled GPCRs to increase $Ca^{2+}{}_i$ concentration (19, 26).

The apparent specificity of the $1,25(OH)_2D_3$ effect for ET-1 responsiveness could be potentially explained by increased expression of ETRA or perhaps ETRB or other downstream signaling mediators. However, there was no change in the gene expression of ETRA and neither was there a change in the protein abundance of either ETRA or ETRB. Interestingly, there was a significant increase in ETRB gene expression, but in the absence of a change in the resulting protein abundance, this may then reflect increased protein turnover rather than a sustained rise in the number of ETRB molecules present at any time. Therefore, the heightened responsiveness to ET-1 induced by $1,25(OH)_2D_3$ cannot be explained by gross changes in ETRA expression. Another possible explanation is that $1,25(OH)_2D_3$ somehow influences heterodimerization of ETRA/ETRB such that it alters ET-1 responsiveness but without changing receptor expression levels. Indeed, heterodimerization of ETRA and ETRB has been demonstrated by fluorescence resonance energy transfer analysis (9), while in ETRA-transfected HEK-293 cells, coexpression with ETRB causes the ETRA signaling to become more sustained (8). However, in the current study, there was no evidence of ETRA/ETRB heterodimerization in either control or $1,25(OH)_2D_3$-

treated cells, possibly consistent with the differential subcellular localization of ETRA and ETRB seen recently in human VSMCs (3). Therefore, we were unable to identify the mechanism by which $1,25(OH)_2D_3$ increased ET-1 responsiveness although we were at least able to rule out changes in ETRA expression, ETRA/ETRB heterodimerization, or a general increase in GPCR responsiveness. However, we were further able to clarify that $1,25(OH)_2D_3$ does not alter SERCA expression but we did observe decreased gene expression of PLN together with an apparent increase in the amount of thapsigargin-mobilizable $Ca^{2+}{}_i$ stored within the cells. It should be noted that the area under the curve measurements used herein approximate to, although are not definitive for, $Ca^{2+}{}_i$ store content as they are also affected by plasma membrane Ca^{2+}-ATPase activity. Nevertheless, these data do point to a possible effect of $1,25(OH)_2D_3$ pretreatment on the $Ca^{2+}{}_i$ handling machinery in HCASMCs.

Although the concentration of $1,25(OH)_2D_3$ used here in vitro is higher than its plasma concentration in vivo, it should be noted that VSMCs express 1α-hydroxylase and can actually synthesize $1,25(OH)_2D_3$ (1, 12, 17, 24, 33), in addition to that delivered via the bloodstream. Therefore, it remains to be determined whether the $1,25(OH)_2D_3$-induced increase in endothelin responsiveness reported here represents the physiological consequence of vitamin D_3 repletion or is instead more indicative of the pharmacological consequence of $1,25(OH)_2D_3$ therapy. It is interesting in this regard that the therapeutic use of calcitriol and its analogs is associated with a bimodal, U-shaped relationship between plasma $1,25(OH)_2D_3$ concentration and its subsequent vascular effects (23), such that both low and high $1,25(OH)_2D_3$ concentrations can result in increased intima-media thickness and coronary artery calcification (23). Similarly, with regards to ET-1 sensitivity in the HCASMCs, $1,25(OH)_2D_3$ pretreatment increased responsiveness to 1 nM ET-1 by ERK assay and to 10 nM ET-1 by $Ca^{2+}{}_i$ assay. This is consistent with most vascular studies in vitro, which require low-to-moderate nM concentrations of ET-1 to elicit functional responses (6, 29) despite circulating ET-1 levels being in the low-to-moderate pM range in vivo. It is unclear whether this consistent discrepancy is due to the lack of endogenous positive allosteric modulators in the simple salt buffers used in vitro (not least ET-2) or to the absence of signal crosstalk from other circulating growth factors, neurotransmitters or integrins.

Currently, the potential cardiovascular benefit of vitamin D analog therapy remains controversial (22) with a recent study finding that it failed to alter left ventricular mass index or improve certain measures of diastolic dysfunction in chronic kidney disease patients (25). However, a number of large-scale clinical outcome trials are underway to resolve this issue conclusively. In any case, as an endogenous circulating hormone it is important to understand the physiological cellular consequences of $1,25(OH)_2D_3$ on the vasculature. Thus, in light of our observation that $1,25(OH)_2D_3$ heightens HCASMC responsiveness to the endogenous vasoactive peptide endothelin, it will now be necessary to determine whether these effects can also be observed in vivo.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.A. and D.T.W. conception and design of research; M.A. performed experiments; M.A. analyzed data; M.A. interpreted results of experiments; M.A. drafted manuscript; M.A. and D.T.W. approved final version of manuscript; D.T.W. prepared Figs.; D.T.W. edited and revised manuscript.

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