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1,25 $(OH)_2$ VITAMIN D_3 ATTENUATES THE LOSS OF RESISTANCE ARTERY CONTRACTILE FUNCTION ASSOCIATED WITH INCUBATION IN CULTURE MEDIA

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SUMMARY: Contractile properties of rat mesenteric resistance arteries were studied immediately after isolation or after 48-hr incubation in culture medium [Dulbecco's modified Eagle's/Ham's F-12 (1:1) with insulin, transferrin and antibiotics]. Incubation in culture medium depressed active stress generating capacity, increased sensitivity to norepinephrine and ablated endothelium-dependent relaxation. The decrease in stress generation results from the loss of a releasable pool of intracellular Ca^{2+}; the enhanced sensitivity is associated with decreased neuronal amine pump function. Addition of 300 pg/ml 1,25 $(OH)_2$ vitamin D_3 to the culture medium afforded nearly complete protection against the loss of stress generating capacity and partially preserved endothelial function. It is concluded that 1,25 $(OH)_2$ vitamin D_3 partially prevents phenotypic modulation of the vascular myocyte induced by culture conditions.

Vascular smooth muscle cells in culture undergo changes in phenotype from a contractile to a proliferative synthetic state (1) and it is generally assumed that the cultured myocyte provides a model for vascular changes that occur during formation of the atherosclerotic plaque and the proliferative response to balloon injury (2,3). Among the changes that occur in culture are altered expression of isoforms of the contractile proteins actin (4) and myosin (5), an increase in free cytosolic Ca^{2+} (6), and a loss of contractile force generating ability (7).

Our laboratory and others have observed that 1,25 $(OH)_2$ vitamin D_3 enhances ^{45}Ca uptake into cultured vascular myocytes (8,9) and serves as a progression factor to initiate growth of these cells in culture (10,11). We have also recently observed that injection of the hormone into rats over a 3-day period significantly increases active stress generation by subsequently isolated resistance arteries (12). These findings suggest that 1,25 $(OH)_2$ vitamin D_3 is a long-term modulator of vascular contractility, Ca^{2+} metabolism and growth.

The present study tested the hypothesis that the inotropic and calciotropic actions of 1,25 $(OH)_2$ vitamin D_3 modulate the loss of

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contractility that has recently been reported to be associated with cell culture conditions (7).

MATERIALS AND METHODS

Male rats (12-15 weeks of age) of the Wistar Kyoto strain (Charles River, Wilmington, MA) were used. Mesenteric resistance arteries were isolated as previously described (10) and either immediately prepared for measurement of isometric force generation or placed in 60 mm sterile culture dishes containing 3 ml medium consisting of Dulbecco's modified Eagle's medium (DMEM) and Hams F-12 Nutrient mixture (1:1), insulin (5 μg/ml), transferrin (5 μg/ml), penicillin (10,000 U/ml), streptomycin (10 mg/ml) and neomycin (10 mg/ml). 1,25 $(OH)_2$ vitamin D_3 (Hoffman-LaRoche, Nutley, NJ) or vehicle (ethanol) was added at a concentration of 300 pg/ml directly to the culture dish prior to the addition of culture medium. The arteries were then placed in a humidified incubator with a 95% air/5% CO_2 atmosphere at 37°C for 48 hr, after which they were removed and mounted in the myograph.

Vessel segments were mounted in a dual channel myograph (Midori Cascade, Aloha, OR) equipped with Kulite BG-10 force transducers (Kulite Semiconductors, Leonia, NJ). The vessels were set to a diameter equivalent to 90% of that which they would have with an intraluminal pressure of 100 mmHg (14). For each vessel the contractile response to a challenge with 100 mM KCl (NaCl substituted) was determined 3 times, followed by 2 challenges with 100 mM KCl/10 μM norepinephrine. The concentration-response relationship and EC_{50} for norepinephrine were then assessed as previously described (10).

To assess Ca^{2+} sensitivity, intracellular Ca^{2+} stores were depleted by washing 3 times with Ca^{2+}-free physiologic isotonic salt solution (PSS) containing 0.1 mM EGTA over a 4-min period, followed by a wash with Ca^{2+}-free PSS containing 5 mM EGTA for 15 sec. The vessels were then challenged with 10 μM norepinephrine in Ca^{2+}-free PSS containing 0.1 mM EGTA until no response was observed (generally 3-4 times). Phentolamine (1 μM) was then added and the vessels depolarized with PSS containing 40 mM KCl (NaCl substituted) and $CaCl_2$ was cumulatively added from 10 μM to 10 mM.

When the effect of endothelial denudation was assessed, paired vessels were examined for each condition; one had a functionally intact endothelium, the other had the endothelium disrupted by inserting a human hair into the lumen immediately after dissection. To assess the success of endothelial denudation, vessel segments were contracted with 10 μM norepinephrine and the relaxation response to acetylcholine determined.

All values are reported as mean$\pm$SEM. Active force generated by each vessel was normalized to cross-sectional area (product of media thickness and axial length) and reported as active stress (mN/mm^2), or as percent of the maximal response to 100mM K^+/10 μM norepinephrine. Differences between treatments were assessed using analysis of variance (ANOVA) using the SYSTAT software package (SYSTAT, Evanston, IL). Results were considered to be significantly different at $p<0.05$.

RESULTS

The lumenal diameters of the arterial segments maintained for 48-hr in culture medium were significantly less than the other two groups (Table 1). It seems unlikely that this decrease was the result of a random selection since 32 separate vessels were included in each group. The difference likely represents an actual narrowing of the lumen over the 48-hr period. Incubation for 48-hr also resulted in a significant increase in media thickness which was reflected as an increased wall to lumen ratio (Table 1). Vessel segments that

Table 1. Physical properties of vessels

	Fresh	48-hr	48-hr+vit D
lumen diameter (μm)	237±6.3	211±6.0*	219±6.8
wall thickness (μm)	36.5±0.69	39.8±1.07*@	36.7±0.74
wall:lumen ratio	0.16±0.01	0.19±0.01*	0.17±0.01

Properties were measured as described in the text. Values are mean ± SEM and 32 vessels were studied in each group. "Fresh" indicates freshly isolated vessels, "48-hr" indicates vessels incubated for 48-hr with culture medium, "48-hr+vit D" indicates the addition of 300 pg/ml 1,25 $(OH)_2$ vitamin D_3 to the culture medium. *Indicates a significant difference between fresh and 48-hr groups, @ indicates a difference from the vitamin D treated group at $p<0.05$.

were treated with 1,25 $(OH)_2$ vitamin D_3 showed no changes in any of the structural properties compared with the fresh vessels (Table 1).

Incubation with culture medium resulted in a significant attenuation of active stress generating capacity in response to norepinephrine (Fig. 1). Addition of 300 pg/ml 1,25 $(OH)_2$ vitamin D_3 to the medium resulted in nearly complete protection against this loss (Fig. 1). Vessels incubated for 48-hr were significantly more sensitive to norepinephrine with a decrease in the EC_{50} value from 1.24±0.15 μM to 0.44±0.05 μM ($n=8$, $p<0.05$). Addition of 1,25 $(OH)_2$ vitamin D_3 to the medium had no effect on the shift in sensitivity (EC_{50} = 0.47±0.07 μM, $p<0.05$ compared with fresh vessels). When the dose-response of incubated vessels was repeated in the presence of 1 μM cocaine, no further shift in the EC_{50} was detected (control EC_{50} = 0.47±0.04 μM vs cocaine treated EC_{50} = 0.39±0.05 μM ($n=6$, ns). The latter observation indicates that the increase in sensitivity was the result of deterioration of the neuronal amine pump.

In contrast to the response to norepinephrine, maximal active stress generation induced by the addition of Ca^{2+} to K^+-depolarized vessels was significantly lower in freshly isolated vessels than vessels incubated with culture medium (Fig. 2). Maximal stress generation for the vessels treated with 1,25 $(OH)_2$ vitamin D_3 was intermediate between the other two groups (Fig. 2). When the magnitude of stress development induced by Ca^{2+} was normalized to the maximal response obtained in medium containing 100 mM K^+/10 μM norepinephrine and 1.25 mM Ca^{2+}, the response of vessels incubated with culture medium was also significantly greater than that of freshly isolated vessels (Table 2). Vessels exposed to 1,25 $(OH)_2$ vitamin D_3 again had intermediate responses (Table 2). Incubation of arteries in culture medium also resulted in a significant decrease in the apparent sensitivity of the contractile elements to Ca^{2+} which was enhanced by the addition of 1,25 $(OH)_2$ vitamin D3 (Table 2).

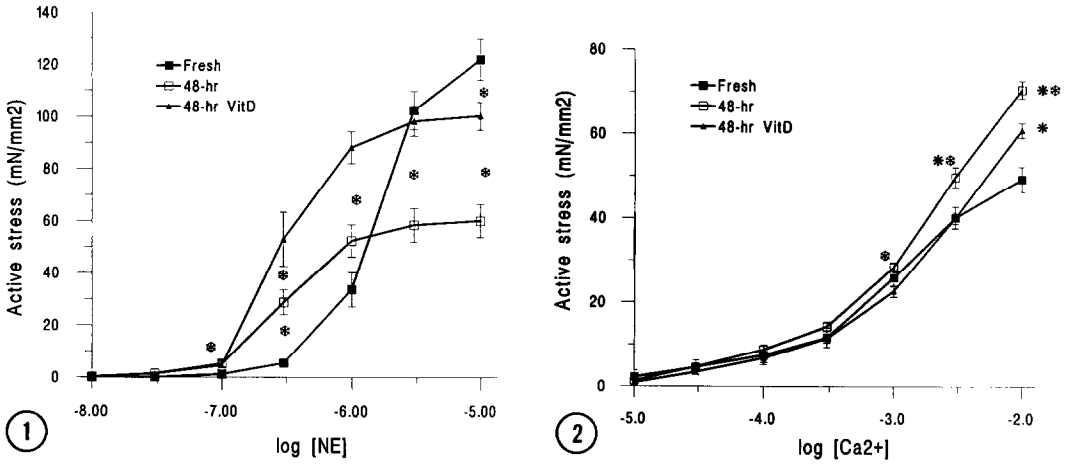

Figure 1. Active stress response of isolated mesenteric resistance arteries to norepinephrine. "Fresh" indicates freshly isolated vessels, "48-hr" indicates vessels incubated in culture medium for a 48-hr period and "48-hr VitD" indicates the addition of 300 pg/ml 1,25 $(OH)_2$ vitamin D_3. All values are mean ± SEM; n=8 for each group. Snowflakes indicate a significant difference between points at $p<0.05$.

Figure 2. Stress induced by addition of Ca^{2+} to K^+-depolarized mesenteric resistance arteries. See legend to Fig. 1 for explanation of symbols. All values are mean ± SEM; n=8 for each group. Asterisks indicate a significant difference from fresh vessels, snowflakes indicate a difference from vitamin D treated vessels at $p<0.05$.

Removal of the endothelium had no effect on the loss of contractile force generating capacity induced by incubation in culture medium. In the absence of endothelium, incubated vessels showed a suppressed maximal force response to norepinephrine and addition of 300 pg/ml 1,25 $(OH)_2$ vitamin D_3 again afforded protection against the loss (Fig. 3). In addition, the sensitivity of the incubated vessels to norepinephrine remained elevated (Fig. 3). When the effect of incubation in culture medium on endothelium dependent relaxation of the vessel segments was examined, it was observed that incubated vessels lost their ability to relax in response to acetylcholine (Fig. 4).

Table 2. Effect of culture incubation on sensitivity to Ca^{2+}

	Fresh	48-hr	48-hr+vit D
EC_{50} (mM)	0.96±0.08	1.55±0.1*	1.87±0.2*
% Response	49.3±2.98	70.8±2.1*	60.6±1.9*

Values are mean±s.e.m. and n=8 for each group. See legend for Table 1 for explanation of abbreviations. EC_{50} values were determined as described in the text. "% Response" indicates the maximal response to Ca^{2+} in depolarizing solution normalized to the maximal response obtained in 100 mM K^+/10 μM norepinephrine. * Indicates a significant difference from freshly isolated vessels at $p<0.05$.

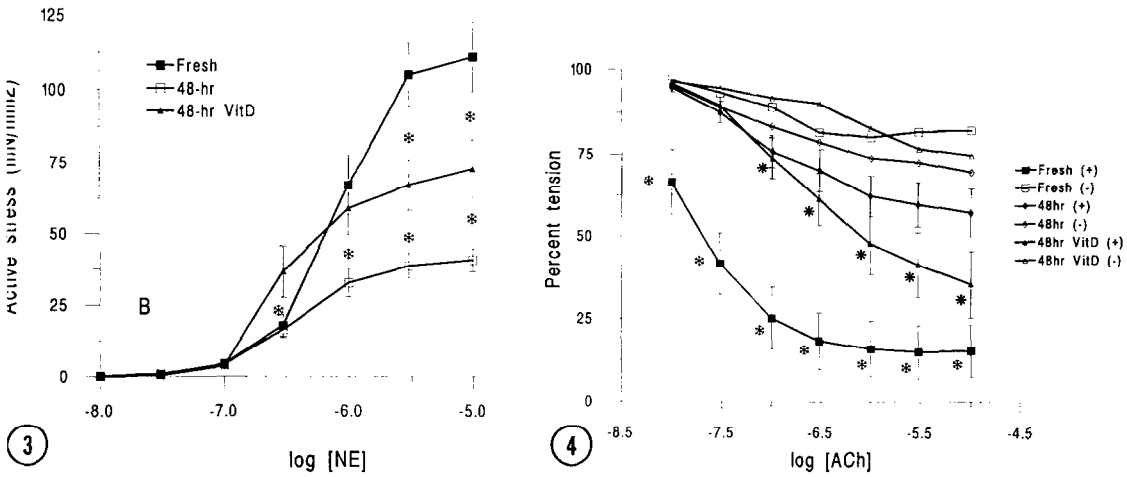

Figure 3. Active stress response to norepinephrine of endothelium denuded resistance arteries. See legend to Fig. 1 for explanation of symbols. All values are mean ± SEM; n=8 for each group. Snowflake indicates a significant difference between points at $p<0.05$.

Figure 4. Acetylcholine-induced relaxation of intact (closed symbols) and endothelium denuded (open symbols) resistance arteries. See legend to Fig. 1 for additional explanation of symbols. Asterisks indicate a significant relaxation of vitamin D-treated vessels, snowflakes indicate relaxation of freshly isolated vessels at $p<0.05$.

1,25 $(OH)_2$ vitamin D_3 significantly reduced the loss of endothelial function over the 48-hr period (Fig. 4).

DISCUSSION

The present study demonstrates that incubation of resistance arteries in culture medium supplemented with insulin and transferrin induces a loss of contractile force generating capacity and that 1,25 $(OH)_2$ vitamin D_3 protects against these changes. We chose to employ DMEM:Ham's F-12 medium supplemented with insulin and transferrin because it has been used by several laboratories including our own (5,8,15) to maintain cultured vascular smooth muscle cells in a quiescent state. Although insulin is a modulator of vascular reactivity (16,17), we do not believe that the loss of contractile force generating ability is a direct result of exposure to this hormone, since incubation of vessels in DMEM alone results in an even greater fall in contractile force generating capacity over the same time period (Li and Bukoski, unpublished observation).

The loss of contractile force generating capacity associated with incubation in the culture medium is consistent with a recent report by DeMey and colleagues who examined renal artery segments maintained in DMEM (7). Force generation in these segments declined between days 2 and 6 and was accompanied by a small increase in the percent of cells in a proliferative

phase. Our observation that the lumen diameter of the vessel decreases while the thickness of the media increases indicates that vessel wall restructuring is taking place. Although we currently have no insight into the mechanism of action, it is clear that 1,25 $(OH)_2$ vitamin D_3 significantly attenuates these changes.

The results of experiments that examined Ca^{2+} sensitivity (Fig. 2, Table 2) may provide insight into the cellular changes that were taking place during the 48-hr period and the mechanism of action of 1,25 $(OH)_2$ vitamin D_3. It is accepted that contraction induced in K^+ depolarized vessels segments is the result of an influx of extracellular Ca^{2+} while tone induced by norepinephrine is the result of both an influx of extracellular Ca^{2+} and a release of internal stores of Ca^{2+} (18).

Table 2 shows that in freshly isolated vessels, the contraction induced by influx of Ca^{2+} into K^+-depolarized vessels was 50% of maximal induced by combined K^+/norepinephrine. It follows therefore, that 50% of the maximal response is the result of a release of an intracellular Ca^{2+} store. In contrast to the fresh vessel, in the vessels incubated with culture medium for 48-hr, 70% of maximal force generation can be explained by influx of Ca^{2+} and only 30% by the release of an intracellular pool. We propose, therefore, that the loss of active stress generating capacity is the result of a loss of a releasable pool of intracellular Ca^{2+}. Furthermore, the protective effect of 1,25 $(OH)_2$ vitamin D_3 may result from the ability of the hormone to maintain this releasable pool of Ca^{2+}. It was also observed that incubation of the vessels in culture medium suppressed the apparent Ca^{2+} sensitivity of the vascular smooth muscle cells and that addition of 1,25 $(OH)_2$ vitamin D_3 caused a further shift to the right. These latter results indicate that 1,25 $(OH)_2$ vitamin D_3 does not exert its protective actions by increasing the sensitivity of the myofilaments for Ca^{2+}.

The observation that removal of endothelium has no effect on either the loss of contractile force generating ability or on the protective ability of 1,25 $(OH)_2$ vitamin D_3 indicates that the effect of the culture medium and the protective action of 1,25 $(OH)_2$ vitamin D_3 are exerted directly on the vascular smooth muscle cell.

The results of the experiments illustrated in Fig. 4 indicate that endothelial-dependent relaxation was ablated by the 48-hr incubation in culture medium. Furthermore, 1,25 $(OH)_2$ vitamin D_3 exerted a significant protective effect against the endothelial changes. The loss of endothelial function could result from either a loss of functional integrity of the endothelial cell layer or a disruption in pathways that generate endothelium derived relaxing factors (19). Given recent evidence that endothelial cells contain receptors specific for 1,25 $(OH)_2$ vitamin D_3 it is possible that the protective action of the hormone is a direct one on the endothelial cell (20).

The present results confirm the recent report of DeMey and coworkers (7) that significant changes in contractile function occur within 48-hr of placing an isolated blood vessel in culture medium, suggesting that modulation of the vascular smooth muscle occurs in media used to maintain the vascular myocyte in a quiescent and non-contractile state. The results also indicate that the changes in contractility are associated with structural changes in the vessel wall and demonstrate a protective action of the calciotropic hormone, 1,25 $(OH)_2$ vitamin D_3. It will be important to determine whether dietary maneuvers that modulate the circulating levels of 1,25 $(OH)_2$ vitamin D_3 alter the progress of vascular lesions observed in atherosclerosis in response to balloon injury.

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