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Differential Modulation of *fos* and *jun* Gene Expression by 1,25-Dihydroxyvitamin D_3

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Summary: 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) modulates the mitogenic response to α-thrombin either positively or negatively in vascular smooth muscle (VSM) cells depending upon the time at which cells are exposed to 1,25-$(OH)_2D_3$. We now examine the impact of 1,25-$(OH)_2D_3$ on the induction by α-thrombin of c-*jun* and c-*fos* mRNA. When 1,25-$(OH)_2D_3$ and α-thrombin were added simultaneously to rat VSM cells, c-*jun* expression was enhanced by five-fold compared to cells exposed to thrombin alone. However, when cells were exposed to 1,25-$(OH)_2D_3$ for 48h prior to thrombin, c-*jun* expression after α-thrombin was reduced to less than 25% of the level in cells exposed to thrombin alone. c-*fos* expression after α-thrombin was unaffected by 1,25-$(OH)_2D_3$. Thus 1,25-$(OH)_2D_3$ dramatically alters the ratio of *jun*/*fos* mRNA produced during the mitogenic response and may thus modulate the response to mitogens.

In addition to its role in Ca^{2+} homeostasis, 1,25-$(OH)_2D_3$ exerts important effects on cell growth and differentiation in cells outside its traditional target tissues. Among these are tissues from the cardiovascular system, including heart (2) and vascular smooth muscle (1,3). The cellular response to 1,25-$(OH)_2D_3$ is quite variable from system to system. For example, vitamin D blocks growth and induces differentiation markers in keratinocytes (4), HL-60 cells (5,6), and mouse myeloid leukemia cells (7), while on the other hand, 1,25-$(OH)_2D_3$ stimulates the proliferation of circulating monocytes (8) and breast carcinoma cells (9). In neonatal rat aortic cells, we have defined conditions under which the effects of vitamin D on growth can be either positive or negative (1).

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Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; VSM, vascular smooth muscle; VDRE, vitamin D response element.

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1,25-$(OH)_2D_3$ exerts its effects upon cells through the action of the 1,25-$(OH)_2D_3$ receptor, a member of the steroid superfamily of receptor/transcription factors (10,11). Like the glucocorticoid (12) and retinoic acid (13) receptors, the vitamin D receptor may be either a positive or a negative regulator of transcription, and the direction of its effects may be altered by the level of AP-1 components (11,14,15). To help understand the opposite effects of 1,25-$(OH)_2D_3$ on mitogenic responses under these two different conditions, we asked if 1,25-$(OH)_2D_3$ alters the induction by thrombin of the AP-1 components, c-*jun* and c-*fos*.

MATERIAL AND METHODS

Materials Unless otherwise specified, all chemicals were purchased from Sigma. Human α-thrombin was generously supplied by John W. Fenton II (New York State Department of Health, Albany, NY). 1,25-$(OH)_2D_3$ was a gift of Dr. M. Uskokovic of Hoffman-LaRoche (Nutley, New Jersey). ^{3}H-thymidine (6.7 Ci/mmol) was obtained from Dupont (Boston, MA), and ^{32}P-dCTP (400 Ci/mmol and 3000 Ci/mmol) from Amersham (Arlington Heights, IL). Nick translation and random primers kits were from BRL (Bethesda, MD).

Cell Culture Primary cultures of VSM cells from newborn rat were established by Peter Jones, University of Southern California (16). From these primary cultures, the R22 D clone was established and passaged as previously described (17); cells from passages 15-22 were used for the present studies. Cells were growth-arrested by placing them into quiescence medium containing minimal essential medium, 20 mM HEPES (pH7.4), 5 μg/ml transferrin, 0.5 mg/ml bovine serum albumin (Miles, Inc.), penicillin (50 U/ml) and streptomycin (50 U/ml). Quiescence medium was changed daily for 3 days.

Thymidine Incorporation Cultured VSM cells were harvested, plated onto 24- or 96-well plates and cultured to confluence. The confluent monolayers were then placed in quiescence medium for an additional 3 days. Cells were then exposed to mitogens. After 24 hours, cells were pulsed for 6 hours with 1 μCi/ml ^{3}H-thymidine, fixed with 1 ml of 15% trichloroacetic acid (TCA). TCA-insoluble material was dissolved in 1M NaOH, neutralized with 1 M HCl, and counted in a liquid scintillation counter.

Northern Blots After two washes with PBS, cells were lysed with guanidine thiocyanate and RNA was separated on CsCl gradients (18). 15 μg total RNA was separated on 1% agarose/formaldehyde gels and transferred to nylon filters. c-*jun*, c-*fos*, and β_2 microglobulin probes were radiolabeled with α^{32}P-dCTP using random primers following linearization with BamH1 (c-*jun*, c-*fos*) or EcoR1 (β_2-microglobulin). 10^7 CPM of probe per ml of hybridization mixture (1 M NaCl, 1% SDS, 10% dextran sulfate, 100 μg/ml salmon sperm DNA) was incubated in a shaking water bath overnight at 65° C and washed with 2X SSPE at 65° C. The blots were stripped by boiling twice for 15 min. in 0.1X SSPE, 0.1% SDS and then rehybridized with each of the other probes being examined. Autoradiography was from 4 to 36 hours at -70° C using a Kodak X-Omatic screen.

RESULTS

When cells were deprived of 1,25-$(OH)_2D_3$ and serum for 72 hours, and then 10 nM 1,25-$(OH)_2D_3$ was returned to the medium, the growth response to α-thrombin was significantly enhanced. However, when cells were exposed to 10 nM 1,25-$(OH)_2D_3$ for 48 h prior to thrombin, the subsequent mitogenic response to α-thrombin was blunted (Table 1).

Like many growth factors, α-thrombin induced c-*jun* and c-*fos* mRNA coordinately in cultured rat aortic smooth muscle cells. c-*jun* mRNA first appeared at 15 min, peaked at 30 min.

Table 1. *Effects of 1,25-$(OH)_2D_3$ on response to α-thrombin*

Condition	^{3}H-thymidine incorporation
Experiment 1	
Control	2818 ± 176
1,25-$(OH)_2D_3$	25056 ± 2014
Thrombin	30553 ± 4211
Thrombin + 1,25-$(OH)_2D_3$	140815 ± 9703
Experiment 2	
Control	1971 ± 209
1,25-$(OH)_2D_3$	9589 ± 785
Thrombin	29813 ± 3152
Thrombin + 1,25-$(OH)_2D_3$	19024 ± 2417

Experiment 1: Cultures of neonatal rat VSM cells were washed daily for 3 days with serum-free medium and then exposed simultaneously to 1,25-$(OH)_2D_3$ (10 nM) and α-thrombin (3 nM) for 24h. Results are mean ± SE for an experiment representative of 3 similar experiments. Experiment 2: VSM cells were exposed to 1,25-$(OH)_2D_3$ (10nM) for 48h prior to addition of α-thrombin but were otherwise treated as in Experiment 1.

and disappeared one hr. after exposure to thrombin, while c-*fos* mRNA appeared at 15 min. and disappeared by 45 min (Fig. 1a). Consistent with its mitogenic activity in vascular smooth muscle cells (1,19), 1,25-$(OH)_2D_3$ also induced both genes, but not until 1-2h after application (Fig. 1b). In single blots where the response to both 1,25-$(OH)_2D_3$ and α-thrombin was compared (not shown), maximal induction of both genes by 1,25-$(OH)_2D_3$ and α-thrombin was quantitatively similar.

We next examined expression of c-*jun* and c-*fos* after simultaneous administration of 10 nM 1,25-$(OH)_2D_3$ and 3 nM α-thrombin in cells deprived of serum for 72h, the condition where 1,25-$(OH)_2D_3$ enhances mitogenicity (Fig. 2). Under these conditions, 1,25-$(OH)_2D_3$ markedly enhanced the appearance of c-*jun* message after α-thrombin. Of note, 1,25-$(OH)_2D_3$ did not significantly alter the induction of c-*fos* after α-thrombin. When these data were quantitated by densitometry and normalized for expression of the β_2 microglobulin gene, 1,25-$(OH)_2D_3$ enhanced the c-*jun* response to thrombin by 4.7 fold at 30 min, but the effect on c-*fos* expression was insignificant.

Lastly, we examined the effects of 1,25-$(OH)_2D_3$ on transcription of c-*jun* and c-*fos* when the same concentration of 1,25-$(OH)_2D_3$ was added 48 h before addition of the α-thrombin (Fig. 3). These are the conditions under which 1,25-$(OH)_2D_3$ blunts the mitogenic response to α-thrombin (Table 1). Under these conditions, 1,25-$(OH)_2D_3$ markedly blunted the appearance of c-*jun* mRNA by α-thrombin (Fig. 3), the opposite of what was observed with simultaneous

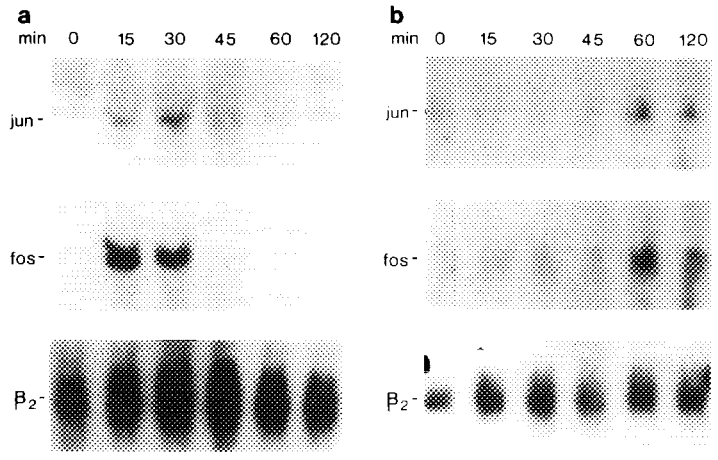

Figure 1. Time course of expression of c-*fos* and c-*jun* in response to α-thrombin and 1,25-$(OH)_2D_3$. After deprivation of serum for 72 h, cells were exposed to a) α-thrombin (3 nM) or b) 1,25-$(OH)_2D_3$ (10 nM) for the indicated times. Total RNA was electrophoresed, blotted, and sequentially probed for c-*jun*, c-*fos*, and β_2-microglobulin as indicated.

exposure to 1,25-$(OH)_2D_3$ in Fig. 2. Quantitation of this data revealed a reduction of c-*jun* mRNA by 83% compared to the level seen with thrombin alone. As in Fig. 2, the effect on c-*fos* expression was insignificant.

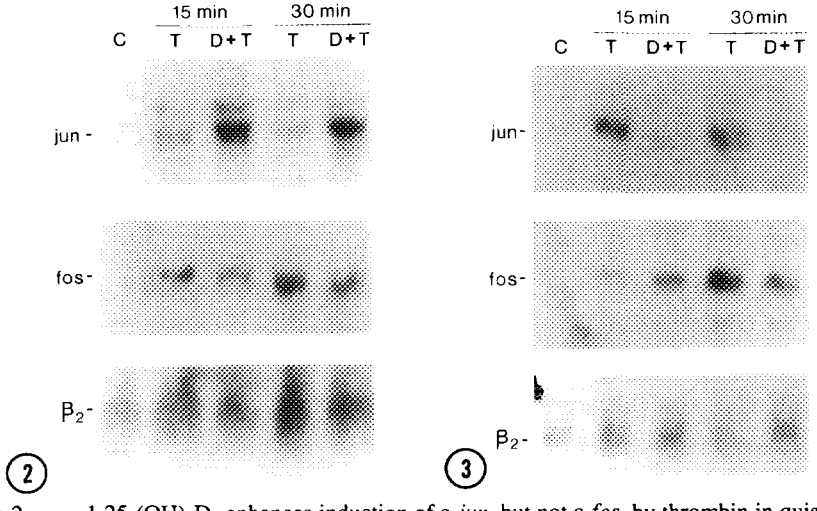

Figure 2. 1,25-$(OH)_2D_3$ enhances induction of c-*jun*, but not c-*fos*, by thrombin in quiescent cells. Confluent cultures of VSM cells were deprived of serum for 72 h and 1,25-$(OH)_2D_3$ (10 nM) and α-thrombin (3 nM) were added simultaneously. After the indicated times, total RNA was isolated and probed as in Fig. 1.

Figure 3. Prolonged incubation with 1,25-$(OH)_2D_3$ blunts induction of c-*jun*, but not c-*fos*, by α-thrombin. Confluent cultures of VSM cells were treated as in Fig. 2 except that 1,25-$(OH)_2D_3$ (10 nM) was added 48h before thrombin. At the indicated times after addition of α-thrombin, total RNA was isolated and probed as in Fig. 1.

DISCUSSION

Under conditions in which 1,25-$(OH)_2D_3$ is growth-stimulatory, it enhances the induction of c-*jun*, but not c-*fos*, mRNA by α-thrombin. Strikingly, under conditions where 1,25-$(OH)_2D_3$ is growth-inhibitory, it blunts induction of c-*jun* mRNA, but not c-*fos*. This differential effect of vitamin D under the two conditions would lead to a greater than 20-fold difference in the ratio of *jun/fos* mRNA after stimulation by α-thrombin. Since the *jun/fos* protein ratio has important implications for the function of the transcription factor AP-1 (12), the finding that 1,25-$(OH)_2D_3$ can potentially impact this ratio may have significant physiologic consequences. Of note, another member of the steroid superfamily, retinoic acid, is also capable of differential regulation of the transcription of *jun* and *fos* mRNA. 1-2 h exposure of mouse melanoma cells to 10^{-10} M retinoic acid dramatically reduced serum-stimulated transcription of c-*fos*, but not c-*jun*, mRNA (20). Thus multiple members of the steroid superfamily are capable of altering the *fos/jun* ratio after mitogenic stimulation.

The mechanism by which 1,25-$(OH)_2D_3$ impacts so strongly on c-*jun* transcription remains obscure. A vitamin D response element has been identified in a region between -513 and -493 bp in the osteocalcin promoter (11,21), but there is no obvious homologous region in the c-*jun* promoter. With regards to the long term effect of 1,25$(OH)_2D_3$ to depress the c-*jun* response, this probably involves the intermediary action of another protein induced by 1,25-$(OH)_2D_3$.

Finally, it is intriguing that an AP-1 site is embedded in the VDRE in the osteocalcin promoter, and AP-1 dramatically alters the transcriptional response to vitamin D (14,15,22). Thus, not only can the presence or absence of vitamin D alter the production of AP-1 components as shown here, but AP-1 can alter the cellular response to vitamin D_3. It seems plausible that both of these mechanisms contribute to the decision made by cells to differentiate or divide, depending upon the environmental conditions.

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