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Regulatory effects of 1,25-dihydroxyvitamin D_3 on vascular smooth muscle cells

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Inflammatory response has been recognized as a central feature in the development and progression of atherosclerosis, and VSMCs (Vascular Smooth Muscle Cells) — the main cellular component of *media,* play an important role in this process. Many reports indicate that the biologically active vitamin D metabolite — 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$ = calcitriol), besides its well established role in calcium homeostasis, plays an essential role in the regulation of the inflammation process. The aim of this study was to determine the regulatory effects of calcitriol, applied at two supra-physiological doses (10 nM and 100 nM), in VSMC culture. Secretion of the pro-inflammatory cytokines, IL-6 and TNF-α, was significantly attenuated in calcitriol-treated VSMC culture, but the level of anti-inflammatory TGF-β was generally unchanged. Since in advanced atherosclerosis lesions several cell types, including VSMCs, overproduce the HSP70 chaperone protein, we also checked the effects of calcitriol on its synthesis. The presence of $1,25(OH)_2D_3$ did not affect HSP70 synthesis under physiological conditions but the synthesis of HSP70 in VSMCs exposed to heat shock was significantly inhibited by calcitriol (=100 nM). We observed that $1,25(OH)_2D_3$ induced SOD 1 activity, stimulated the expression of IκB-α, and did not influence the level of NF-κB-p65 in VSMCs. The results of our study suggest that $1,25(OH)_2D_3$ may serve as a natural anti-inflammatory agent and may therefore play a beneficial role in the physiology of VSMC in some contexts of atherosclerosis.

Key words: HSP70; TNF-α; IL-6; VSMC; vitamin D; calcitriol; NF-κB; IκB-α

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INTRODUCTION

Vascular Smooth Muscle Cells (VSMCs) constitute the most common cell type in the *medial* layer of the arterial wall. Under pathological conditions many external factors alter the homeostatic balance, causing VSMCs to change their physiology and participate in atherosclerotic plaque formation, via proliferation and migration from the *media* to the *intima* (Ross, 1999; Doran *et al.*, 2008).

Although the etiology of atherosclerosis seems to be multi-factorial, strong evidence exists that chronic inflammation and elements of innate and adaptive immune response are involved in its pathogenesis (Bielecka-Dabrowska *et al.*, 2009). VSMCs can actively contribute to the inflammatory response by the production of a broad array of cytokines e.g. IL-6, TNF-α, or IL-1 (Raines & Ferri, 2005; Tedgui & Mallat, 2006). IL-6, in particular, reveals multiple biological activities and its important role as an autocrine or paracrine factor activating VSMCs has been shown (Viedt *et al.*, 2002). VSMCs are able to secrete this "messenger" cytokine into the intracellular space thus contributing to the initiation of an inflammatory process (Zampetaki *et al.*, 2005).

One of the promising regulatory factor in atherosclerotic plaque formation may be the biologically active vitamin D metabolite — 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$ — calcitriol) (Tukaj, 2008; Tukaj *et al.*, 2010). Although vitamin D has traditionally been associated with systemic calcium homeostasis, there are now increasing lines of evidence that $1,25(OH)_2D_3$ regulates cell proliferation, differentiation, apoptosis and immune responses (Takeda *et al.*, 2010; Bobryshev, 2010). The biological effects of $1,25(OH)_2D_3$ are usually mediated by the vitamin D receptor (VDR), a member of the superfamily of nuclear hormone receptors, which is present in many cells, including cardiomyocytes, VSMCs, and endothelial cells (Wu-Wong *et al.*, 2006). It is generally believed that VDR is directly involved in the regulation of nuclear factor-κB (NF-κB) activation (Sun *et al.*, 2006) by a direct involvement in the regulation of IκB-α expression (NF-κB inhibitor) (Wu *et al.*, 2010). NF-κB belongs to a family of transcription factors that play an essential role in the inflammation process (Bonizzi & Karin, 2004) and is thought to be involved in the pathogenesis of atherosclerosis (Wilson *et al.*, 2002).

At times of cellular stress, including infection, fever, mechanical damage and chronic inflammation, the expression of various heat shock proteins — HSPs is markedly elevated (van Eden *et al.*, 2005). HSPs are a family of evolutionarily conserved proteins, which play an important role in cell physiology under normal and stress conditions. The main risk factors for atherosclerosis, such as hyperlipidemia, hypertension or smoking, cause ROS elevation in the cells and oxidative stress is known as an inducer of HSPs in VSMC (Pockley *et al.*, 2010). In advanced atherosclerosis lesions, several cell types, including monocytes, macrophages, dendritic cells and SMCs, overproduce the HSP70 chaperone protein (Hamel *et al.*, 2000; Bobryshev *et al.*, 2002; Svensson *et al.*, 2006). The intensity of HSP expression positively correlates with the severity of atherosclerosis, and serum HSP70 level predicts the development of atherosclerosis in subjects with established hypertension (Pockley *et al.*, 2003). On the other hand, there is clinical evidence that supports the protective function of HSP70 in the

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Abbreviations: NF-κB, nuclear factor-κB; VSMCs, vascular smooth muscle cells

development of atherosclerosis, perhaps through its cytoprotective role as well as anti-inflammatory properties (Bielecka-Dabrowa *et al.*, 2009; van Eden *et al.*, 2005; Hamel *et al.*, 2000; Pockley *et al.*, 2009). It has also been suggested that circulating HSP70 (HSPA1A) has atheroprotective effects (Pockley *et al.*, 2009; Martin-Ventura *et al.*, 2007).

Several autoimmune conditions have been linked to a deficiency in vitamin D_3 and epidemiological data indicate that vitamin D_3 deficiency is associated with cardiovascular events (Bobryshev, 2010). Calcitriol supplementation led to a marked reduction in atherosclerotic lesion formation in an animal model, and it is presumed that immune components, such as the $CD4^+CD25^+FoxP3^+$ cells and dendritic cells are main calcitriol targets (Takeda *et al.*, 2010).

In the present study we have observed that calcitriol could influence the activity of NFκB via stimulation of a natural inhibitor of NFκB (IκB-α). The action of calcitriol was also associated with down-regulation of pro-inflammatory mediators. We also show for the first time that calcitriol induces SOD 1 activity in VSMCs and thus can potentially reduce vascular cell-mediated oxidation of the low-density lipoprotein (LDL).

MATERIAL AND METHODS

Cell culture. VSMCs were obtained from aortic media of neonatal Wistar rats. Rats were purchased from an animal breeding company (Medical University of Gdansk). VSMCs were isolated, purified and cultured according to a method described previously, with minor modifications (Tukaj, 2008). Cells were cultured in Eagle's Medium (MEM) supplemented with 10% FCS at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The purity and identity of the VSMCs were confirmed by immunocytochemical staining for α-smooth muscle actin (Sigma, USA) as shown in Fig. 1, and described previously (Tukaj *et al.*, 2010). Viable cells were identified by the exclusion of trypan blue and their number was higher than 95%. VSMCs were used between passages 2 and 4. The culture medium was changed every second day. VSMCs were inoculated into Petri dishes (3 cm diameter) to 0.5×10^6 cell/ml 24 h prior to the experiment. The procedures were performed in accordance with institutional requirements of the Ethics Committee for Animal Care at the Medical University of Gdansk, Poland.

Figure 1. The rat VSMC actin cytoskeleton.
The purity and identity of the VSMC cultures were confirmed by immunocytochemical staining for α-smooth muscle actin, as described previously (Tukaj *et al.,* 2010).

Calcitriol addition. $1,25(OH)_2D_3$ (Sigma, USA) was dissolved in 95% ethanol and stored at –80°C. Calcitrol was added to the culture to a final concentration of 10 or 100 nM. Two doses of $1,25(OH)_2D_3$ were prepared in such a manner to add the same volumes (5 μL/ml of medium) of ethanol to the culture.

Annexin/Propidium Iodide Apoptosis Assay. Apoptotic cells were assessed by binding Annexin V-FITC to exposed phosphatidylserine residues and propidium iodide exclusion to confirm the integrity of the cell membrane. We used the Annexin V-FITC Apoptosis Detection Kit II from BD Pharmingen (Cat. No. 556570) and the assay was performed following the manufacturer's procedure. The cells were detached from the dishes using trypsin solution and the soft rubber scraper was used to collect the cells. Cells were washed twice with cold PBS then 1×10^6 cells were resuspended in 1 ml of binding buffer. Next 100 μl of solution with 1×10^5 cells were transferred to a cytometer tube. After the addition of 5 μl of Annexin V-FITC, 5 μl of PI and 15-minute incubation at 20°C to 25°C (room temperature) in the dark, 400 μl of binding buffer were added. Samples were analyzed (typically 15 000 cells per sample) with a LSRII cytometer (Becton-Dickinson) with a laser with an emission wavelength of 488 nm.

HSP70 induction. The VSMC cultures at the initial density (0.5×10^6 cell/ml) were heated at 41°C in a tissue culture incubator (in a 5% CO_2 atmosphere). After 2 h of heat shock treatment the cells were allowed to recover for 22 h at 37°C, with or without calcitriol.

Preparation of cell extracts. After 24-hour treatment, rat VSMCs were scraped from the culture plate with a soft rubber scraper and washed twice in PBS buffer. The extraction of the cells was performed by Extraction Reagent provided by the Hsp70 ELISA Kit (Stressgen, USA) containing protease inhibitor cocktail (Sigma, USA). After 30-min incubation at 4°C with occasional mixing, the homogenate was spun (20 min, 20 000×*g*) at 4°C and for further analysis supernatant was used. The protein content was assayed as described by (Bradford 1971) using BSA as a standard.

Western blot analysis of IκB-α and NFκB. An equal amount of protein extract was loaded onto 12.5% polyacrylamide gel (SDS/PAGE), separated in a Mini-Protean apparatus (Bio-Rad) and transferred onto nitrocellulose in a buffer containing 200 mM glycine, 25 mM Tris and 20% methanol at RT by 2 h (80 V). The membrane was blocked for 2 hours at 4°C in TBS (20 mM Tris/HCl (pH 7.5), 0.5 M NaCl) containing 3% w/v nonfat dry milk. After the washing step (3×10 min in TBS), the membrane was incubated overnight with primary antibodies — rabbit polyclonal IgG anti-IκB-α (Santa Cruz, 1:200), or anti-NFκB (Santa Cruz, 1:200) at 4°C. Next, the membrane was incubated with secondary antibodies (1:2000) — goat anti-IgG rabbit coupled with HRP (Sigma, USA) for 1 h in RT. The membrane was washed and signals were detected using the Lumi-Light Western Blotting Substrate (Roche). The intensity of bands was measured densitometrically. Results for protein levels were expressed relative to β-actin levels detected by immunoblotting with monoclonal Anti-β-Actin-Peroxidase antibodies (Sigma, USA).

HSP70 quantification. HSP70 levels in VSMC extracts or supernatants were measured using a sandwich Enzyme-Linked Immunosorbent Assay — ELISA (Stressgen, USA) according to the manufacturer's in-

structions. The detection sensitivity was 0.2 ng/ml. The antibodies (Abs) used in ELISA were specific for HSP70 and did not cross-react with HSC70. The amount of intracellular HSP70 was calculated according to a standard curve prepared from the recombinant HSP70 included in the kit. 100 μL of the culture medium (24 h after the start of the experiment) were taken to determine the level of extracellular HSP70 concentration. The optical density was measured at 450 nm using an ELISA plate reader (Asys Hitech GmbH, Austria).

Cytokine detection. The concentration of all cytokines studied was measured in the VSMC culture supernatant after 24 h treatment using a Rat IL-6 ELISA kit (Abnova), Rat TNF-α ELISA kit (Abnova), Rat TGF-β ELISA kit (Abnova), or Rat IL-1-α ELISA kit (Abnova) as directed by the manufacturer. The detection limit was 12 pg/ml for IL-6, 11 pg/ml for TNF-α and 4 pg/ml for IL-1-α. The amount of cytokines was calculated according to a standard curve prepared from the recombinant proteins included in the kit. The optical density was measured at 450 nm using an ELISA plate reader (Asys Hitech GmbH, Austria).

Superoxide dismutase activity. Cytoplasmic superoxide dismutase SOD1 (Cu/Zn SOD) activity was assayed by a modified technique combining PAGE and densitometry according to Beauchamp and Fridovich, 1971. After electrophoresis on native polyacrylamide gels, negative bands corresponding to the SOD activity were visualized by soaking the gels in nitroblue tetrazolium (Sigma), next in riboflavin, and, finally, exposing to light. The intensity of bands was measured densitometrically.

Statistical analysis. All data are expressed as mean ± SEM. Statistical analyses were performed using Microsoft Office Excel 2003. Data were analyzed using Student's t-test for paired samples or analysis of variance (ANOVA). The level of statistical significance was set at $P<0.05$.

RESULTS

Cytoprotective action of calcitriol in VSMC culture

Annexin/propidium iodide staining of VSMCs demonstrated a significant decrease in the numbers of necrotic/late apoptotic cells treated with calcitriol after a 7-day culture (Fig. 2). There was no significant effect of calcitriol on the number of necrotic/late apoptotic cells after 24 h (not presented).

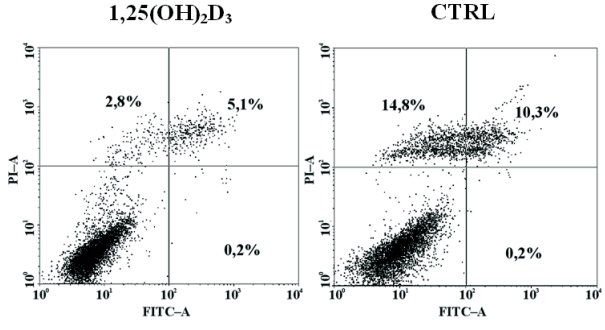

Figure 2. Representative dot-plots of FITC-Annexin V/propidium iodide (PI) dual color flow cytometry for vascular smooth muscle cells grown in the presence of calcitriol (100 nM) — $1,25(OH)_2D_3$, or absence of the vitamin — CTRL.
Bottom left quadrant of each dot plot shows viable cells, which exclude PI and are negative for FITC-Annexin V binding. Bottom right quadrant represents the early apoptotic cells (FITC-Annexin V positive and PI negative), demonstrating cytoplasmic membrane integrity. Top right quadrant contains the late apoptotic cells (positive for FITC-Annexin V binding and for PI uptake) and the upper left quadrant shows dead cells.

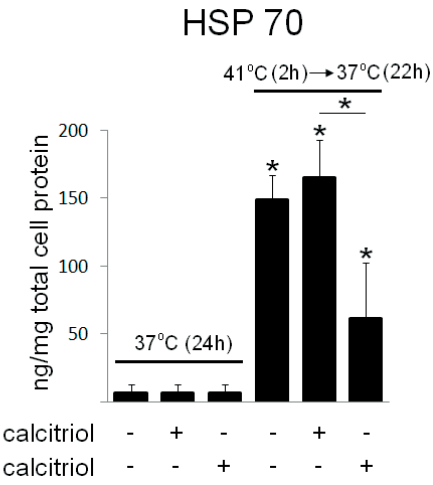

Figure 3. HSP70 was determined by ELISA, as described in Materials and Methods, in extracts from VSMCs treated for 24 h.
VSMCs were exposed to heat shock (2 h/41°C) followed by exposure to two doses of $1,25(OH)_2D_3$ for the next 22 hours at 37°C. The results are presented as mean (±SD) values (n = 4). For statistical analysis ANOVA was used. VSMC responses to $1,25(OH)_2D_3$ and/or heat shock are compared to those of the untreated cells, unless otherwise indicated *$P\leq0.05$.

Calcitriol down-regulates thermally induced HSP70 synthesis

We found no significant influence of $1,25(OH)_2D_3$ on HSP70 synthesis after 24 hours (37°C) in VSMC culture (Fig. 3). After pre-incubation (41°C, 2 h), VSMCs were subjected to two doses of $1,25(OH)_2D_3$ (10 nM or 100 nM) or allowed to recover in an agent-free medium for the next 22 h at 37°C. Thermally treated VSMCs overproduced HSP70 approximately 20-fold during the next 22 hours at 37°C (Fig. 3). Depending on the dose, the presence of $1,25(OH)_2D_3$ in the culture inhibited the synthesis of thermally induced HSP70. There was no detectable level of extracellular HSP70 protein in VSMC culture treated with calcitriol or heat shock after 24 hours of incubation.

IL-6 and TNF-α secretion is attenuated in VSMC treated with calcitriol

Three pro-inflammatory cytokines (IL-6, TNF-α, or IL-1-α), and anti-inflammatory — TGF-β were assayed in culture medium of the VSMC culture exposed to calcitriol. The presence of 10 nM of $1,25(OH)_2D_3$ considerably inhibited the secretion of IL-6 ($P<0.05$) (Fig. 4A). The level of IL-6 in the culture exposed to a higher dose of $1,25(OH)_2D_3$ (=100 nM) was below detection (Fig. 4A). Interestingly, both concentrations of calcitriol inhibited the secretion of TNF-α, but the lower dose of vitamin was a stronger inhibitor, and TNF-α detection was impossible (Fig. 4B). The level of anti-inflammatory TGF-β was generally unchanged in the culture treated with calcitriol (Fig. 4C). There was no detectable level of IL-1-α in the culture medium.

Calcitriol stimulates the expression of NFκB inhibitor, IκB-α, and does not influence the level of NF-κB

Calcitriol (100 nM) statistically significantly stimulated (about 3-fold) the synthesis of the natural inhibi-

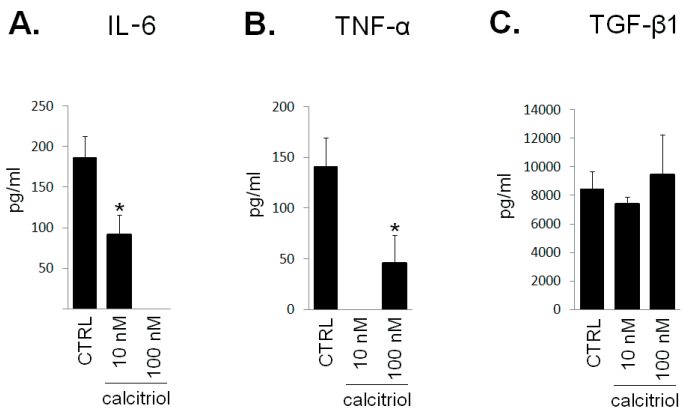

Figure 4. Cytokines were evaluated by ELISA, as described in Materials and Methods, in the culture media from VSMCs treated for 24 h.
VSMCs were exposed to two doses of 1,25(OH)$_2$D$_3$ (24 h/37°C). The results are presented as mean (±S.D.) values (n = 4). For statistical analysis Student's *t*-test was used. VSMC responses to 1,25(OH)$_2$D$_3$ are compared to those of the untreated cells (CTRL). *P≤0.05.

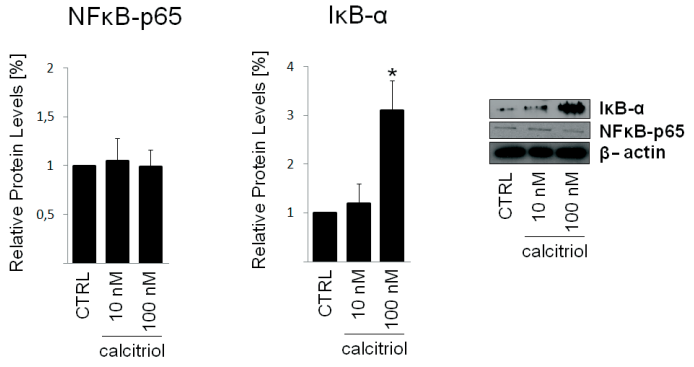

Figure 5. The levels of NF-κB — Nuclear Factor κB, or IκB-α — Inhibitor κB-α proteins were determined by western-blotting as described in Materials and Methods, in extracts from VSMCs treated for 24 h with two concentrations of calcitriol-1,25(OH)$_2$D$_3$.
An example of the blots shows NF-κB, and IκB-α protein level in a single representative experiment. Protein concentration is expressed relative to β-actin level. For statistical analysis Student's *t*-test was used. The results are presented as mean (±S.D.) values (n = 4) from the densitometry measurements. *P≤0.05.

tor (IκB-α) of NF-κB (Fig. 5). The levels of NF-κB-p65 were unchanged in cell extracts (Fig. 5).

The activity of Superoxide Dismutase 1 — SOD1 is enhanced in VSMCs treated with calcitriol

Calcitriol (10 nM or 100 nM) statistically significantly induced SOD1 (Cu/Zn SOD) activity in VSMCs (Fig. 6). We did not detect other isoforms of SOD in cell extracts.

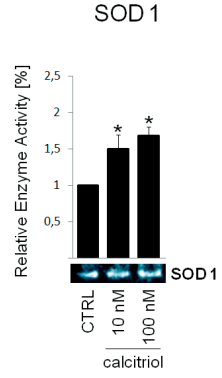

Figure 6. SOD1 activity was determined by a modified technique combining PAGE and densitometry, as described in Materials and Methods, in extracts from VSMCs treated with 1,25(OH)$_2$D$_3$ for 24 h.
The results are presented as mean (±S.D.) values (n = 4) from the densitometry measurements. For statistical analysis Student's *t*-test was used. VSMC responses to 1,25(OH)$_2$D$_3$ are compared to those of the untreated cells (CTRL). *P≤0.05. An example of the gel presenting SOD1 activity in a single representative experiment is shown below the graph.

DISCUSSION

It is well known that inflammatory response plays a major role in the development and progression of atherosclerosis. Inflammation is partly mediated through the ability of VSMCs and other cells (endothelial cells, macrophages and dendritic cells) involved in the formation of atherosclerotic plaque to secrete pro-inflammatory cytokines (Tedgui & Mallat, 2006). Unfortunately, so far, there is no effective cure for atherosclerosis and there is no way to reverse the damage inflicted by it. Therefore, one of the therapeutic goals is to slow down the progression of the disease by attenuating the inflammation process.

Our study was designed to test the hypothesis that an active metabolite of vitamin D exhibits regulatory properties in VSMC culture. The main finding of this study is that calcitriol attenuated the pro-inflammatory interleukin 6 (IL-6) secretion. It is believed that VSMCs are the major source of this cytokine in the vascular wall (Maret *et al.*, 1999). Another main pro-inflammatory mediator, TNFα, was also diminished in VSMC culture treated with calcitriol. The level of anti-inflammatory TGF-β was generally unchanged in the culture treated with calcitriol. We conclude that the regulatory effects of vitamin D are not caused by cell death induction, since we have observed that calcitriol acts as anti-apoptotic or anti-necrotic agent in VSMC culture. Moreover we also observed previously that calcitriol induced proliferation of VSMCs labeled with CFSE (Tukaj *et al.*, 2007).

According to our observation, the regulatory action of 1,25(OH)$_2$D$_3$ is probably mediated by the inhibition of NF-κB activity, since we noted that IκB was significantly over-expressed in treated cells. Vallabhapurapu and Karin (2009) suggested that the activity of NF-κB was regulated by IκB, which bound to NF-κB to block its nuclear translocation. Szeto *et al.* (2007) postulated that VDR appeared to regulate the NF-κB activation by targeting NF-κB-p65 and IκB-α. It is speculated that VDR-p65 interaction helps suppress NF-κB-p65 activity. Moreover, Wu *et al.* (2010) suggested that VDR regulated inhibitor κB (IκB), since VDR deletion affected the expression of IκBα, reducing the level of IκBα protein in mouse embryonic fibroblast culture. VDR may participate in the regulation of inflammation, and its regulatory properties may be enhanced in the presence of 1,25(OH)$_2$D$_3$. Although the mechanism related to the anti-inflammatory effect of calcitriol has not been clearly elucidated, previous studies in mammalian cell types have provided evidence for a role of the NF-κB pathway in IL-6 induction (Libermann & Baltimore, 1990). Vitamin D analogues, 1,24(OH)$_2$D$_3$ or 20(OH) $_2$D$_3$, have been reported to inhibit NF-κB activity (Sun *et al.*, 2006; Janjetovic *et al.*, 2009).

Since in advanced atherosclerosis lesions, several cell types, including monocytes, macrophages, dendritic cells and VSMCs, overproduce the HSP70 chaperone protein (Hamel *et al.*, 2000; Bobryshev *et al.*, 2002; Svensson *et al.*, 2006) we checked the effects of calcitriol on HSP70 synthesis in VSMCs. Although there was no significant

influence of $1,25(OH)_2D_3$ on HSP70 synthesis under physiological conditions, our results demonstrate that $1,25(OH)_2D_3$ regulated heat-induced HSP70. HSP70 has been shown to improve viability of stressed VSMCs and its cytoprotective properties allow the cells to adapt and survive lethal conditions (Johnson *et al.*, 1995). On the other hand, overproduction of HSP70 is an indicator of the physiological state of smooth muscle cells. It is therefore tempting to speculate that $1,25(OH)_2D_3$ may participate in the recovery of smooth muscle cells.

Another approach may point to the regulatory action of superoxide dismutase - SOD. Indeed, using the SOD activity assay we have found for the first time that two supra-physiological doses of $1,25(OH)_2D_3$ (10 nM or 100 nM) induced SOD1 activity. It has been shown that the increase in superoxide dismutase (SOD) activity reduces vascular cell-mediated oxidation of the low-density lipoprotein (LDL), a process mediated by aorta segments and VSMCs (Yang *et al.*, 2004; Tang *et al.*, 2007). In addition to this, it is suggested that SOD exhibits anti-inflammatory properties via inactivation of the NF-$\varkappa$B pathway (Huang *et al.*, 2001) and therefore may play an important role in the prevention of atherosclerosis (Lin *et al.*, 2005). We assume that the enhanced activity of SOD1 in VSMC treated with $1,25(OH)_2D_3$ may attenuate the activation of NF-$\varkappa$B-p65 by stimulating the expression of I$\varkappa$B-α.

CONCLUSION

The results of our study indicate that $1,25(OH)_2D_3$ may serve as a natural anti-inflammatory agent and may therefore play a beneficial role in the physiology of VSMC in some contexts of atherosclerosis.

Acknowledgments

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