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Eotaxin augments calcification in vascular smooth muscle cells

Gayatri Raghuraman[1], **Joseph Hsiung**[1], **Mary C Zuniga**[1], **Brittanie D Baughman**[1], **Elizabeth Hitchner**[1], **Raul J Guzman**[3], and **Wei Zhou**[1,2]

[1]Department of Vascular Surgery, VAPHCS, Palo Alto, CA

[2]Department of Surgery, Stanford University, Stanford, CA

[3]Department of Surgery, Beth Israel Deaconess Hospital, Harvard Medical School, Boston, MA

Abstract

Calcification of atherosclerotic plaques in elderly patients represents a potent risk marker of cardiovascular events. Plasma analyses of patients with or without calcified plaques reveal significant differences in chemokines, particularly eotaxin, which escalates with increased calcification. We therefore hypothesize that eotaxin in circulation augments calcification of VSMCs possibly via oxidative stress in the vasculature. We observe that eotaxin increases the rate of calcification significantly in VSMCs as evidenced by increased alkaline phosphatase activity, calcium deposition, and osteogenic marker expression. In addition, eotaxin promotes proliferation in VSMCs and triggers oxidative stress in a NADPH oxidase dependent manner. These primary novel observations support our proposition that in the vasculature eotaxin augments mineralization. Our findings suggest that eotaxin may represent a potential therapeutic target in elderly for cardiovascular complication prevention.

Keywords

Eotaxin; oxidative stress; NADPH-oxidase; vascular smooth muscle cell; vascular calcification

Introduction

Arterial stiffness and vessel calcification are known to be associated with hypertension, cardiovascular disease, and atherosclerotic plaque burden and instability (Allison et al, 2004; Johnson et al, 2006; Kelly-Arnold et al, 2013). Calcification in itself is a delicate and regulated cellular process that entails trans-differentiation of SMCs into osteoblast-like cells (Lee et al, 2014). The signals that induce this transformation are multiple and overlapping. Understanding factors that provoke the calcification process may help to ameliorate disease severity and cardiovascular complications.

Corresponding author: Wei Zhou, Division of Vascular Surgery, Stanford University Medical Center, 300 Pasteur Dr., Suite H3600, Stanford, CA 94304-5642, USA, Office: (650) 849-0583, weizhou@stanford.edu.

Conflict of Interest
none declared.

Calcification is a balance between pro-mineralizing factors that stimulate VSMC dedifferentiation and inhibition. Alkaline phosphatase (ALP) is a membrane bound metalloenzyme that catalyzes the hydrolysis of organic pyrophosphate, an inhibitor of vascular calcification (Giachelli et al, 1999). Correlation with inflammation, obesity and atherosclerosis has made ALP a surrogate marker for the initiation and degree of vascular calcification (Lomashvili etal, 2008). Even inhibitors of calcification that include fetuin A, matrix gla protein and osteopontin (OPN) are known (Tonelli et al, 2009). However, at present only a limited number of studies have evaluated the pathological relationship between ALP and calcified atherosclerotic plaque. The osteogenic transcription factor Runx2 plays an important role in VSMC calcification *in vitro* (Bostrom et al, 2011). Runx2 belongs to the runt-related transcription factor family, and has been shown to induce ALP activity and the expression of bone matrix protein genes, including osteopontin (OPN), as well as mineralization in osteoblastic cells *in vitro* (Sutra et al, 2008). In normal vascular cells, the expression of Runx2 is very low, but Runx2 expression is elevated in calcified vascular tissue specimens from atherosclerotic plaques, suggesting that Runx2 may be important in vascular calcification (Byon et al, 2008; You et al, 2009). Using small interfering RNA, it has been shown that Runx2 is necessary for oxidative stress-induced VSMC calcification, and Runx2 by itself is sufficient to induce VSMC calcification *in vitro* (Tintut et al., 2012).

Eotaxin isoforms are known biomarkers for allergy-related diseases and powerful chemoattractant for eosinophils. There are three members in the human eotaxin family: CCL11 (eotaxin), CCL24 (eotaxin-2), and CCL26 (eotaxin-3). Particularly, eotaxin-1 acts via the CCR3 receptor and until recently was considered to be merely an eosinophil-specific chemoattractant (Demer, 2005). Consequently, eotaxin was studied mostly in diseases characterized by an accumulation of eosinophils in tissues, notably allergic conditions (Rankin et al, 2015). Eotaxin is currently used as a biomarker in clinical trials studying diseases of the GI tract as well as Crohn's disease (Williams, 2015). In eosinophils, eotaxin is associated with increased intracellular calcium mobilization (Tenscher et al, 1996). However, CCL11 is poorly understood outside the realm of lymphoid cells (Koc et al, 2013), particularly cells of the vasculature.

Although eotaxin-3 has been previously suggested to be a trigger of calcified stenosis (Anger et al, 2007) whether and to what extent eotaxin-1 is involved in arterial stiffness and vessel calcification have not been studied. We observed significantly higher levels of circulating eotaxin-1 (referred to as eotaxin) in patients with calcified carotid plaques vs those with non-calcified plaque. We therefore hypothesize that eotaxin is intimately involved in vessel calcification by increase in oxidative stress-induced ALP activation and subsequent Runx2 expression. In this study we examined the effects of eotaxin on VSMC calcification and explore the underlying mechanisms if any discussed above.

Materials & Methods

Patient recruitment and plasma eotaxin analysis

This study adhered to IRB protocol (protocol (IRB 23476) on human subjects and was performed in compliance with the Stanford University Investigational Review Board and

with the internal Ethics Committees of the VA Medical Center at Palo Alto. Written informed consent was obtained from every individual involved in this study. Detailed demographic information including age, gender, smoking history, and body mass index (BMI = weight/height^2); and medical comorbidities, such as diabetes, coronary artery disease (CAD) were recorded.

Blood was collected from patients who underwent carotid interventions in EDTA-coated tubes. Plasma was isolated using gradient density centrifugation. Briefly, samples were centrifuged at 3000 rpm, 4°C for 15 minutes, and the plasma layer was removed, aliquoted, and stored at -80 °C prior to analysis of complete set of samples. Plasma was analyzed using the multiplex assay (Luminex magnetic bead based assay) at the Human Immune Monitoring Center facility at Stanford University. Human cytokine magnetic-plex were purchased from eBiosciences/Affymetrix and used according to the manufacturer's recommendations with modifications as described below. Briefly, antibody-linked beads were added to a 96 well plate and washed. Then, the following incubations were performed with washes in between: 1) sample were added at room temperature (RT) for 1 hr, followed by overnight incubation at 4°C; 2) followed by biotinylated detection antibody for 75 min at RT; and 3) streptavidin-PE for 30 min at RT. Finally, the reading buffer was added to the wells, and each sample was measured in duplicate. Plates were read using a Luminex 200 instrument to measure median florescence intensity (MFI). Cytokine concentration (pg/mL) was calculated with standard controls curve fitting and bead counts, using quality control cytokine samples as a reference.

Ultrasound evaluation

Carotid duplex ultrasounds were performed by experienced Registered Vascular Technologists (RVT) using a standard duplex ultrasound unit (Phillip iu-22) equipped with a linear 7.5 MHz transducer. Carotid arteries were visualized transversely and longitudinally using gray scale imaging as well as color flow Doppler. Diagnosis of carotid stenosis was determined based on carotid ultrasound velocity criteria and NASCET criteria, and confirmed with preoperative MRA evaluations. In general, end diastolic velocity of internal carotid artery >100 cm/s with either peak systolic velocity (PSV) of ICA >350cm/s and/or ICA/CCA PSV ratio>4 were interpreted as severe stenosis. A plaque was defined as calcified if hyper-echogenic plaque was visualized on gray scale imaging with concurrent hyper-echogenic shadowing on color flow Doppler that extend across the luminal diameter. Where there was disagreement between the two observers, the examination was re-evaluated by both observers and consensus was reached.

Cell calcification and in vitro treatments

Human coronary artery smooth muscle cells (Genlantis) (Clinton et al, 1993) were used for experiments. Calcification was induced by growing cells in inorganic phosphate rich medium (Pi) (2.5 mM phosphate) as mentioned by Son et al, (2006) for 11 days with and without eotaxin 0.1ng/mL. Cells were treated for various time points with or without pre-treatment by Nox specific inhibitor DPI (0.5–5 μM). Medium was changed every 2 days. Samples were collected at different time points. For Nox activity and ROS measurements, cells were pre-treated with inhibitors for 30 min before addition of eotaxin.

ALP activity and calcium deposition

ALP activity is a marker of VSMC transformation (Lomashvilli et al, 2008). Samples of cell lysates, collected at different time points of calcification, were analyzed for ALP activity (Munson and Fall, 1978) with cell lysates. Protein content was analyzed using the Bradford assay. Calcium deposition was quantified using a complexone-based assay as described (Stern and Lewis, 1957). VSMCs were analyzed for deposition of calcium phosphate using Alizarin red S staining in intact cells as a qualitative measurement, and the 6-well plate was visualized under a phase contrast microscope.

Western Blot

Cell lysate samples (15 μg of protein) separated on a 4–20% polyacrylamide gel for electrophoresis were transferred onto nitrocellulose membranes using Bio-Rad Mini-Trans-Blot system. The blocked membrane was incubated with primary antibodies for Runx2 overnight at 4°C and with secondary antibody for 1 hour at room temperature. The immunoreactive bands were detected using a BIO-RAD chemiluminescence system, and the bands were captured and intensity quantified by with BIO-RAD ChemiDoc XRS+ camera and Image Lab software respectively. For normalization, the membranes were re-probed after stripping with an anti-GAPDH antibody and quantified.

Immunofluorescence

Runx2 expression was verified by immunocytofluorescence. Briefly VSMCs were stained with anti-Runx2 antibody overnight at 4°C, followed by a fluorescence-conjugated secondary antibody (1:100 dilution in dilution buffer) for 60 min at room temperature, containing DAPI (1:1000) for nuclear staining. Stained cells were visualized with laser scanning confocal microscope (Zeiss, LSM-710).

Cytosolic ROS measurement

VSMCs grown on coverslips were either treated with or without eotaxin and Nox inhibitor for the different time points. Cells were incubated with 2 μM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen) in HBSS at 37 °C for 30 min (for cytosolic ROS) (Wu and Yotnda, 2011) and imaged using confocal microscopy (ZEISS Confocal LSM 710).

Nox activity

Nox activity was measured by the lucigenin-enhanced chemiluminescence method as described (Drummond et al, 2011). Briefly, cultured VSMCs were homogenized in lysis buffer followed by sonication and centrifugation at 8100 × g at 4°C for 10 min. Nox specific activity measured in the presence of Nox inhibitor DPI. Nox dependent superoxide anion production was expressed as relative chemiluminescence (light) units (RLU)/mg protein. Protein content was measured using the Bio-Rad protein assay reagent. Results are expressed as fold change in Nox activity compared to control.

Cell growth as detected by MTT assay

The effect of eotaxin on VSMC proliferation was detected with the MTT assay. VSMCs were transferred into the wells of 96-well plates at a density of 5×10^3 cells/ml. Cells were incubated with various treatments (resistin with or εV1–2 and VAS-2870) for 24 and 48 h, separately. A total of 10 μl MTT was added to each well and incubated under darkness for 4 h at 37°C followed by addition of solubilization buffer to each well. Then the absorbance of the colored product was detected at 570 nm using a dual-beam microplate reader.

Scratch wound migration assay

VSMCs were grown to confluence in a 6-well plate. A transverse scratch wound on each monolayer of VSMC was made using a sterilized 200 μl-tip. The scratch wounded monolayers were then stimulated with or without eotaxin (10 ng/ml) for an additional 24 h, at which point the transverse scratch wounds were re-examined for cell migration. Pictures were captured with a phase-contrast microscope and quantitated using ImageJ software.

Statistical analysis

Continuous variables were presented as mean with standard deviation (SD) and compared with Student t-test or Mann-Whitney test, where applicable. Categorical variables were presented as frequency and percentages and compared using Pearson chi-square test or Fisher's exact test, where appropriate. *In vitro* experiments were performed at least four times (n=4 to 6) in duplicate and results expressed as the mean ± SEM. Time-dependent data from ROS was compared by one-factor analysis of variance (ANOVA) followed by Dunnett's test. Effect of inhibitor data from Nox experiments on ROS and proliferation were analyzed using two-factor ANOVA followed by Tukey's test. Statistical significance was considered if the P-value was < 0.05. All statistical analyses were performed using the SPSS version 22.0 (IBM, Chicago, IL, USA).

Results

Patients' clinical characteristics and plasma analyses of patients with calcified plaques vs non-calcified plaques:

We first examined the role of eotaxin in atherosclerotic plaque calcification. Calcification was measured using ultrasound and patients were grouped as those with or without calcified plaques. Clinical criteria with regard to key demographics and cardio vascular risk factors were similar in both groups as shown in Table 1. Carotid lesions from both groups were assessed and showed features of advanced atherosclerosis. A total of 82 subjects were analyzed including 61 with calcified plaques and 21 with non-calcified plaques. The mean ± standard deviation (SD) age was 70 ± 1.2 years in the non-calcified and 66.5 ± 1.4 years in the calcified group, with no significant difference among the two groups ($p = 0.11$). Preexisting conditions and clinical values show no significant difference between the groups as seen in Table 1. Plasma was collected from patients who underwent carotid intervention. We found a significantly higher level of chemokine, eotaxin, in patients with calcified plaques. The results shown in Figure 1 suggest that eotaxin shows significant association with calcification. Several other plasma cytokines including IL-12p70, IP-10, IFNα and

IL-17A were also higher in patients with calcified plaques compared to the non-calcified ones (Supplementary Figure S1**)**.

Effects of eotaxin on migration and calcium deposition in VSMCs:

The effects of eotaxin on functions of VSMCs, particularly calcification of VSMCs, were examined. We show that eotaxin dose-dependently affects VSMC proliferation (Figure 2A) and migration (Figure 2B). Mineralization was observed using Alizarin red staining, as well as measuring calcium accumulation. More granular deposits developed in cells grown in phosphate medium with eotaxin than without as demonstrated by Alizarin Red staining shown in (Figure 2C) and quantified in Supplementary Figure S2 Eotaxin was found to significantly increase calcium accumulation over time, as shown in Figure 2D, with significance at days 5, 7, 9, and 11. Calcium complexes with *o*- cresolphthalein complexone as a purple-colored complex measured colorimetrically and normalized with protein estimation. The data suggests that the deposition of calcium over time is increased in the presence of eotaxin.

Effect of eotaxin on ALP activity and Runx2 expression in VSMCs:

Increased alkaline phosphatase activity is thought to precede initiation of calcification. ALP activity was monitored by the rate of the formation of *para*-nitrophenol and inorganic phosphate. ALP activity was found to be significantly higher in VSMCs treated with eotaxin, with maximum activity at day 5. Figure 3A shows that eotaxin increased ALP activity compared with controls. ALP activity as an early marker of VSMC transformation was increased much earlier after exposure of human VSMC to phosphate and eotaxin than in the absence of eotaxin. Runx2 is a nuclear transcription factor found in developing bone. Maintaining human VSMC in calcification medium in the presence of eotaxin for 7 days resulted in a significant increase in Runx2 levels compared with cells grown in the absence of eotaxin (Figure 3B&C). VSMCs calcified in the presence of eotaxin revealed an increase in Runx2 expression as visualized by ICC using confocal microscopy. The increased green fluorescence in the presence of eotaxin represents the increased levels of Runx2 protein expression. Western blotting confirmed that eotaxin upregulates Runx2 expression. Protein levels of the transcription factor Runx2 were visualized by western blotting and normalized to the housekeeper protein GAPDH. Figure 3B shows eotaxin-mediated upregulation of Runx2 protein expression at days 3, 6, and 9, as quantified in Figure 3B **(lower panel)**. Confocal images of Runx2 expression are shown in Figure 3C. Real time PCR data reveals that both ALP and Runx2 mRNA levels were significantly higher in the presence of eotaxin as shown in Supplementary Figure S3.

Eotaxin induce ROS in VSMCs via activation of NADPH oxidase:

Increased oxidative stress has been associated with augmented calcification via Runx2 (Al-Aly, 2011), and NADPH oxidase (Nox) activation (Shao et al, 2007) has been shown to promote Runx2/Cbfa1 expression and the osteogenic mineralization of vascular smooth muscle (Byon et al, 2008). We found that VSMCs treated with eotaxin exhibited a time dependent increase in ROS levels as observed under a confocal microscope (Figure 4A), which persisted up to 18h. Inhibition studies point to NADPH oxidase as a possible source

of eotaxin-provoked oxidative stress (Figure 4B). Time dependent increase in Nox activity was observed in VSMCs after eotaxin treatment (Figure 4C). Nox inhibitor, DPI completely inhibited eotaxin mediated increase in ALP activity (Figure 4D). These findings suggest that ROS production and subsequent ALP expression may represent the underlying mechanistic basis for eotaxin-induced VSMC calcification as ROS inhibition blocks eotaxin mediated increase in ALP activation.

Discussion

Although eotaxin has been originally identified as important eosinophil-specific chemokine evidence is mounting that eotaxin could have functions other than eosinophil recruitment and/or activation (Jose et al, 1994; Kitaura et al, 1996). Even though high levels of eotaxin mRNA and protein have been reported in human atherosclerotic plaques (Haley et al, 2000) alluding to its expression by VSMCs, the functions of eotaxin in atherosclerosis have not yet been fully elucidated. It is known that eotaxin acts via CCR3 in endothelial cells (Cheng et al, 2002) which is markedly up-regulated by TNF-α (Atasoy et al, 2003), a cytokine that is actively involved in the progression of atherosclerosis (Salcedo et al, 2001; Branen et al, 2004).

A further contribution of eotaxin in atherogenesis may lie in the fact that eotaxin could not only promote inflammation on endothelial cells, but also support the necessary vascularity at inflammatory sites through its proosteogenic action on human vascular smooth muscle (Salcedo et al, 2001). This is the first study to systemically examine the effect of eotaxin on VSMCs and we demonstrated that eotaxin increases the rate VSMC mineralization and osteoblastic transformation.

Differences in plasma eotaxin levels of control subjects vs CAD patients have been reported (Economou et al, 2001; Mosedale et al, 2005). Our plasma analyses from patients with severe carotid disease revealed that eotaxin levels are higher in patients with calcified vs non-calcified plaques. In a calcifying environment of elevated phosphate (Pi), eotaxin increases ALP activity, enhances calcium deposition, and upregulates osteogenic markers in VSMCs. These experiments confirmed previous reports identifying elevated Pi as an inducer of calcification of VSMC Giachelli et al, 2001). Normally VSMCs do not express ALP but they can transdifferentiate into calcified VSMCs that show increased ALP activity (Shanahan et al, 1999; Reynolds et al, 2004). Similar to others, we showed that eotaxin induced migration of VSMCs (Kodali et al, 2004). In addition, we also demonstrated that eotaxin promoted VSMC proliferation, as well as increased *ALP activity and Runx2 expression*. These results coupled with our human plasma analyses support the concept that eotaxin exacerbates vascular smooth muscle calcification.

Oxidative stress is commonly observed in patients with cardiovascular and kidney disease. Although the association between oxidative stress and vascular calcification has been noted in clinical epidemiology for a while, evidence linking oxidative stress to activation of osteogenic programs and vascular calcification has started to emerge only relatively recently. Our show also that in the presence of a Nox inhibitor eotaxin mediated increase in ALP activation is lacking. Hydrogen peroxide, a ROS (pro-inflammatory second messenger), was

shown to induce osteogenic gene expression, suppress SMC molecular signature, and induce calcification (Byon et al, 2008); this was Runx2-dependent and involved phosphatidylinositol-3′-kinase–AKT signaling. A recent study showed that H_2O_2 is increased and co-localizes with the osteogenic transcription factors Msx2 and Runx2 in calcified human aortic valves (Miller et al, 2008). Other inducers like β-glycerophosphate via the nuclear factor-κB (NF-κB) pathway in bovine aortic smooth muscle cells seem to affect calcification via specifically superoxide (O_2^-) (Zhao et al, 2008), while TNF-α induces calcification by stimulating peroxide production through NADPH oxidases activation and NF-κB signaling. Our results add valuable information to this limited literature (Ardhanari et al, 2014). We find that eotaxin promotes reactive oxygen species generation through NADPH oxidase activation, which leads to trans-differentiation of VSMCs and further exacerbation in the rate of calcification.

Nevertheless, it should be noted the plasma analysis has important limitations. Firstly, the size of the patient cohort used in the study is relatively small and lacks a group of age-matched healthy controls. As we did not perform a clinical follow-up of the present cohort, we evaluated associations, not prediction or causation. Hence, we believe that future prospective studies are needed to clarify whether these differences in eotaxin levels have implications for asymptomatic subjects, as well as in predicting future cardiovascular events. Despite the strength of the association between eotaxin levels and calcification found in our present cohort, it should be acknowledged that the link between eotaxin and atherosclerosis is at present far less explored compared to other chemokines such as MCP-1 (Ikeda et al, 2002; Sheikine and Hansson, 2004) to allow us to draw any definitive conclusion. We acknowledge that the eotaxin levels in the systemic circulation are lower than the concentrations used in the study. We believe that the localized eotaxin levels in tissues are much higher than the systemic levels (Kodali et al, 2004)

Given the study caveats, we nonetheless believe that our results could have two major implications. First, our finding that eotaxin is associated with the extent of calcification indicates that elevated plasma levels of this chemokine could represent a novel marker of calcified plaque. Second, and perhaps more importantly, our *in vitro* data strongly support that eotaxin could play an important role in non-eosinophil inflammation in atherogenesis. Haley et al (2000) showed increased expression of eotaxin and its receptor in human plaques. Recently, we observed higher plasma eotaxin level patients with diabetes compared to non-diabetics. Targeting eotaxin in circulation in diabetic patients may prove to be novel approach to reduce plaque burden and subsequent complications. Extending mechanistic investigations on eotaxin may raise enticing therapeutic opportunities across several diseases. This is the first time that eotaxin has been shown to affect cells of the vasculature, particularly, calcification of VSMCs.

Conclusions

Thus a novel link between Pi, eotaxin and oxidative stress (because of their implications in the pathobiology of vascular calcification) could have important clinical and therapeutic implications. These findings suggest that increased cardiovascular complications in elderly adults may be in part due to eotaxin induced aggressive vascular calcification. We believe

that regulation of eotaxin on VSMCs may potentially ameliorate cardiovascular consequences in elderly individuals by delaying calcification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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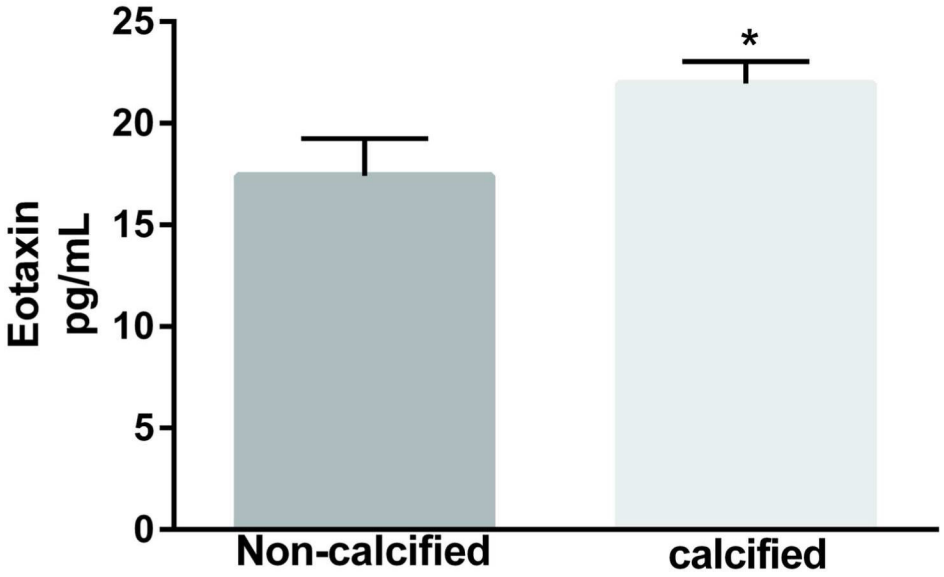

Figure 1: Eotaxin levels are significantly higher in patients with calcified vs non-calcified plaques.

Results shown represent plasma levels of eotaxin in 82 patients undergoing carotid interventions. Eotaxin levels in patients with calcified carotid plaques were compared to those that have non-calcified plaques using a Luminex bead based assay and data analyzed using the Mann Whitney test (*$p<0.05$).

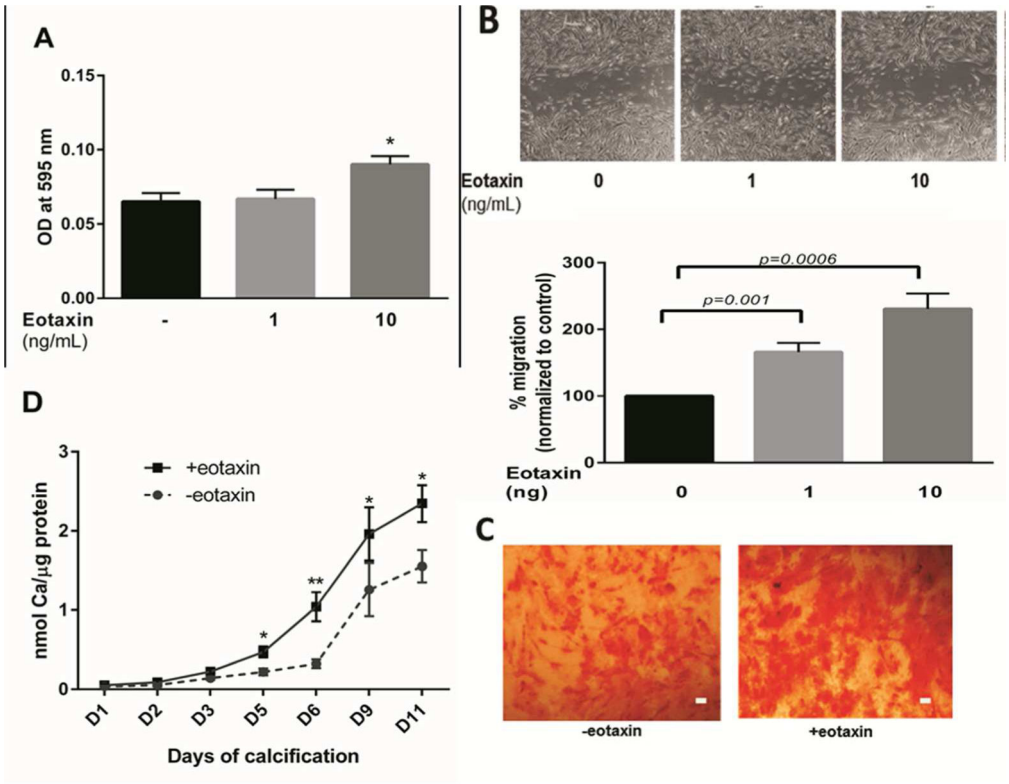

Figure 2: Eotaxin increases VSMC proliferation and migration as well calcification.

VSMCs were treated With increasing concentration of eotaxin (1,10,100 ng/mL) for 24h and cell proliferation monitored in MTT assay (**A**) and migration using the scratch wound assay and quantitated (**B**). VSMCs calcified in the presence or absence of eotaxin were analysed for calcium phosphate deposition shown by Alizarin Red staining as in (**C**) and calcium deposition shown in (**D**).Results shown are representative of at least 5 independent experiments in duplicate.

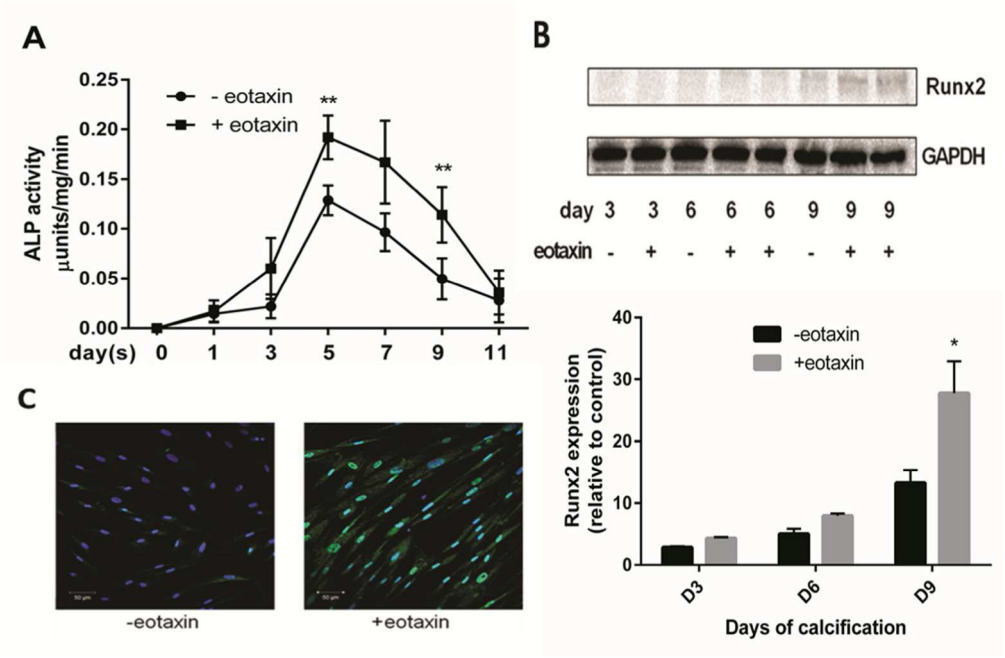

Figure 3: Eotaxin activates ALP and upregulates Runx2 in VSMCs under calcifying conditions.

VSMCs cultured in the presence or absence of eotaxin were analyzed for ALP activity (**A**) and Runx2 expression by western blotting **(B)** and confocal microscopy **(C)**. Data are shown as mean ± S.E.M of at least 5 independent experiments in duplicate. *p<0.05, **p<0.001, by non-parametric Mann Whitney test.

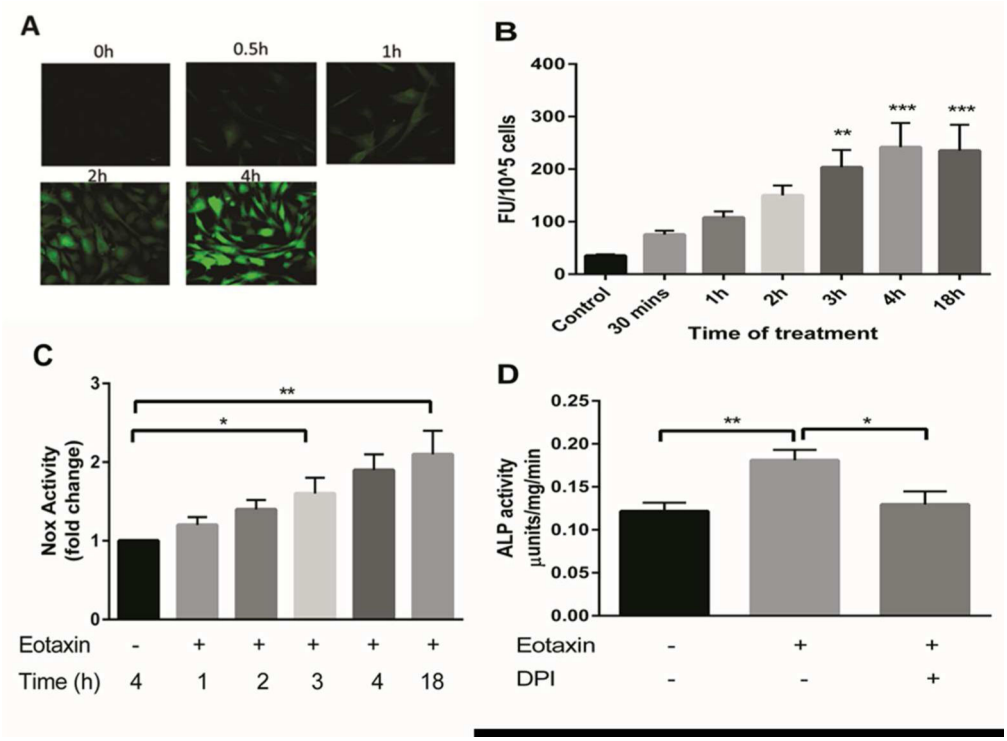

Figure 4. Eotaxin induces ROS in VSMCs under normal conditions via NADPH oxidase activation.

VSMCs treated with eotaxin 10 ng/mL for different time points and ROS visualized by DCFDA staining shown in (A) and quantitated on a fluorescent plate reader shown in (**B**). NADPH oxidase (Nox) activity measured using a chemiluminescence assay is shown in (**C**). Effect of Nox inhbition on ALP activity at day 5 of calcification is shown in (**D**).

Table 1:

Baseline characteristics of patients without and with calcification.

	No calcification (n = 22)	calcification (n = 66)	P value
Age (y)	66.5 ± 1.4	70.03 ± 1.2	0.11[a]
Diabetes mellitus	10 (45%)	29 (43.9%)	0.97[c]
CAS	45%	59.1%	0.27[c]
Cholesterol (mg/dL)	162.8 ± 34.5	157.6 ± 43.52	0.53[b]
Hemoglobin (g/dL)	13.9± 1.5	13.4± 1.5	0.2[b]
Obesity	40%	39.3%	0.47[c]
CAD	30%	50%	0.12[c]
Current smokers	40%	28.8%	0.86[c]
Formerly smokers	40%	53%	0.6[c]
Hypertension	90%	92%	0.96[c]

Data are shown as mean ± S.D., median (interquartile range), or frequency counts (percentages), as appropriate.

[a]Student's t test,

[b]Mann–Whitney U test,

[c]$\chi 2$ test were used.