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Effects of bioactive ceramics on the pathogenesis of rat vascular smooth muscle cells treated with phorbol 12-myristate 13-acetate

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Abstract Vascular smooth muscle cells (VSMCs) play a pivotal role in vascular injury through proliferation and migration. Pro-inflammatory cytokines and cyclooxygenase (COX)-2 and nitric oxide synthase (NOS) are highly associated with the pathogenesis of VSMCs. We investigated the effect of bioactive ceramics on the expression of inflammatory cytokines, COX-2, and inducible NOS (iNOS) induced by phorbol 12-myristate 13-acetate (PMA) in rat VSMCs. The ceramics inhibited mRNA expression of IL-1β, TNF-α, IL-6, COX-2, and iNOS. Prostaglandin release was also diminished by the ceramics. The bioactive ceramics effect on cytokines, COX-2, and iNOS expression was achieved by inhibition of NF-κB activity. Interestingly, the ceramics-induced up-regulation of expression of endothelial NOS resulted in an increase of nitric oxide production. Thus, bioactive ceramics may have dual effects on the pathogenesis of VSMCs by regulation of NF-κB activity and NO production.

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Introduction

Biomaterials have been proposed as suitable medical and dental applications for bone graft augmentation material and as an anchorage for dental implants [1, 2]. The composition of biomaterials is variable, but the application of biomaterials is uniform, either as replacement or implantation. There are few reports regarding alternative applications for bioactive materials, such as impregnation into clinical supplies. For example, gloves impregnated with bioactive ceramics have a clinically important effect in Raynaud's syndrome [3]. Menstrual pads impregnated with ceramics have shown significantly greater pain relief than the commercial pads which are available [4]. We have focused on ceramics in menstrual pads which have an effect on pain relief. We previously reported that ceramics have anti-inflammatory effects in macrophages, which suppress the expression of inflammatory cytokines and inhibit NF-κB activity [5]. It has not been reported that ceramics have an anti-inflammatory effect. Therefore, to use ceramics as an alternative anti-inflammatory agent, it is necessary that the anti-inflammatory effects of bioactive ceramics are further studied in various types of cells and conditions.

Atherosclerosis is an inflammatory disease involving numerous cell types and inflammatory molecules. Among the numerous cell types, vascular smooth muscle cells (VSMCs) have been well-established in the study of atherosclerosis. Proliferation is an important event in the pathophysiologic course of atherosclerosis, which is mediated by inflammatory cytokines (e.g., IL-1β and TNF-α) and growth factors [6–9]. Besides cytokines and growth factors, cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS; 10, 11) are involved in the proliferation of VSMCs. NF-κB is known to be a central transcription factor which regulates gene expression in inflammatory responses. Involvement of NF-κB

in the pathogenesis of vascular diseases has become evident in a variety of studies. Activated nuclear NF-κB has been detected in smooth muscle cells after balloon-induced injury of rat carotid arteries and in the VSMCs of human atherosclerotic lesions [12, 13]. NF-κB mediates the expression of IL-1β, TNF-α, COX-2, and iNOS [14, 15].

In the present study, we investigated the anti-inflammatory effects of ceramics on VSMCs. Anti-inflammatory effects were examined by the alteration of inflammatory cytokines and NF-κB activity in rat VSMCs induced by phorbol 12-myristate 13-acetate (PMA).

Materials and methods

Reagents

ELISA kits for prostaglandins were purchased from R&D Systems (Minneapolis, MN, USA); PMA and the Griess reagent were purchased from Sigma (St. Louis, MO, USA); the luciferase assay kit was purchased from Promega (Madison, WI, USA); COX2, iNOS, endothelial NOS (eNOS), and GAPDH antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA); NF-κB antibody was purchased from abCAM (Cambridge, MA, USA); and SYBR Green Master mixture was purchased from ABI (Foster City, CA, USA).

Cell cultures

Rat VSMCs were obtained by the explant method, as previously described [16]. Briefly, arterial explants from the carotid arteries of 6-week-old rats were washed in PBS and subsequently cultured in DMEM supplemented with 10% fetal bovine serum and 100 U of penicillin/streptomycin/mL. After 2 weeks, cells that had migrated onto the tissue culture dish were collected by trypsinization and serially sub-cultured; cells were used after up to five passages. To evaluate the effect of bioactive ceramics on rat VSMCs, we constructed culture plate lids with the top containing bioactive ceramics.

Bioactive ceramics

Bioactive ceramics are composed of four oxidative compounds: SiO_2, Al_2O_3, TiO_2, and Fe_2O_3. Exposure of VSMCs to bioactive ceramic was performed by the method previously reported [5]. One gram of the ceramics was fixed with adhesive paper on the culture plate lid.

Luciferase assay

The NF-κB-dependent luciferase reporter (pNFkB-Luc) and its control vectors (pTA-Luc) were purchased from Panomics (Fremont, CA, USA). pNFkB-Luc is driven by three synthetic copies of the NF-κB-consensus sequence. The pTA-Luc contains the TATA box-consensus sequence. To measure the effect of silica-bioactive ceramics on PMA-induced NF-κB-dependent gene transcription, rat VSMCs were seeded onto 24-well plates at a density of 1×10^5 cells/well. Subsequently, cells were transiently transfected with pNFkB-Luc (0.5 μg) using LipofectAMINE PLUS (Invitrogen; Carlsbad, CA, USA). To normalize transfection efficiency, cells were transfected with 0.5 μg of pTA-luc control vector. After an overnight incubation, cells were treated with PMA (100 nM), followed by silica bioactive ceramics, and then harvested with 1× reporter lysis buffer (Promega). Relative luciferase activity was measured with a luciferase assay system using a POLARStar OPTIMA luminometer (BMG Labtechnologies; Mornington, Germany).

Immunoblotting

Cells were washed twice with ice-cold PBS and harvested in a lysis buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Trion X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF), including a protease inhibitor cocktail (Sigma). Cell lysates were kept on ice for 20 min and centrifuged for 15 min at 12,000 rpm at 4 °C. Total proteins (30 μg) were fractionated by 6–10% gel electrophoresis and electrophoretically transferred to PVDF membranes. The membranes were incubated at room temperature for 1 h in TBS containing 0.1% Tween 20 and 50% skim milk (TBST/milk). The membranes were then incubated with COX-2 (1:2,000), iNOS (1:500), eNOS (1:1,000), and GAPDH (1:1,000) in TBST/milk for 2–3 h at room temperature and washed three times in TBST. Peroxidase-conjugated secondary antibodies (1:2,000) were incubated for 1 h at room temperature. The membranes were developed using a chemiluminescence detection kit (Amersham Biosciences; Piscataway, NY, USA).

Real time RT-PCR

Total RNA from rat VSMCs was extracted using Trizol, according to the manufacturer's protocol (Invitrogen). Target RNA (0.5 μg) was reverse transcribed using 100 U Superscript II RT (Invitrogen) at 42 °C for 50 min and 5 μmol/L oligo$(dT)_{16}$. Real time-PCR was performed with SYBR Green Master Mix (Applied Biosystems). Rat GAPDH was used as a reference gene for normalization. The validity of GAPDH as a reference gene was confirmed experimentally by measuring the slope of the plot of the log input RNA amount versus ΔCT, the difference in thermal cycle, as suggested by the users

manual (Bio-Rad Laboratories; Hercules, CA, USA). The primers for real-time PCR were as follows: GAPDH (forward: 5′-gtcttcaccaccatggagaaggc-3′; reverse: 5′-atgccagtgagcttcccgttcagc-3′), COX-2 (forward: 5′-actcactcagtttgttgagtcatt-3′; reverse: 5′-tttgattagtactgtagggttaatg-3′), iNOS (forward: 5′-atggaacagtataaggcaaac-3′; reverse: 5′-gtttctggtcgatgtcatgag-3′), IL-1β (forward: 5′-atggcaactgttcctgaactcaact-3′; reverse: 5′-caggacaggtatagattctttccttt-3′), IL-6 (forward: 5′-tgctgg tgacaacaacggcc-3′; reverse: 5′-gtactccagaagaccagagg-3′), TNF-α (forward: 5′-cctgtagcccacgtccgtagc-3′; reverse: 5′-ttgacctcagcgctgagttg-3′), and eNOS (forward: 5′-ggctgcc tgtgaaactttctgtgt-3′; reverse: 5′-ttgctgctctgtaggttctccaca-3′). PCR amplification was performed in triplicate wells using the following cycle conditions: 40 cycles at 90 °C for 30 s, 55–60 °C for 45 s, and 72 °C for 1 min.

Prostaglandin ELISA

Prostaglandin determinations were performed with commercial kits (R&D Systems) following the manufacturer's recommended protocol. The OD was read at 450 nm in an ELISA reader (BMG Labtechnologies; Mornington, Germany).

Statistical analyses

Data are expressed as the mean ± SD. Statistical analyses were performed using one-way ANOVA and the Duncan method for multiple comparisons. A $p < 0.05$ was considered to be statistically significant. SPSS Windows, version 12.0 was used for statistical analysis.

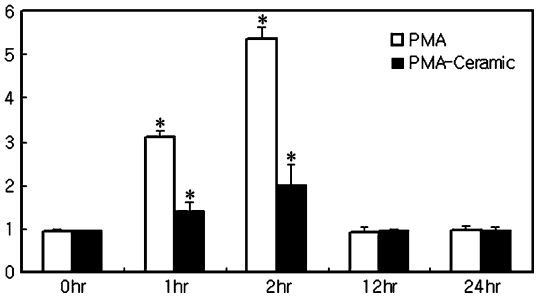

Fig. 1 Effect of bioactive ceramics on NF-κB activity. NF-κB activity was measured by the luciferase assay. Rat VSMCs were cultured with no PMA (–), PMA (100 nM), or PMA under the ceramics (PMA-Ceramic). Cells were collected 2 h after PMA treatment and total cell extracts were obtained by adding lysis buffer to the assay. The *y*-axis represents luciferase activity of NF-κB normalized to that of control. Data are expressed as mean ± SD of three independent experiments

Results

Analysis of NF-κB activity

We previously reported that NF-κB activity was regulated by bioactive ceramics in LPS-activated macrophages [5]. To attempt to clarify the suppressive effect of bioactive

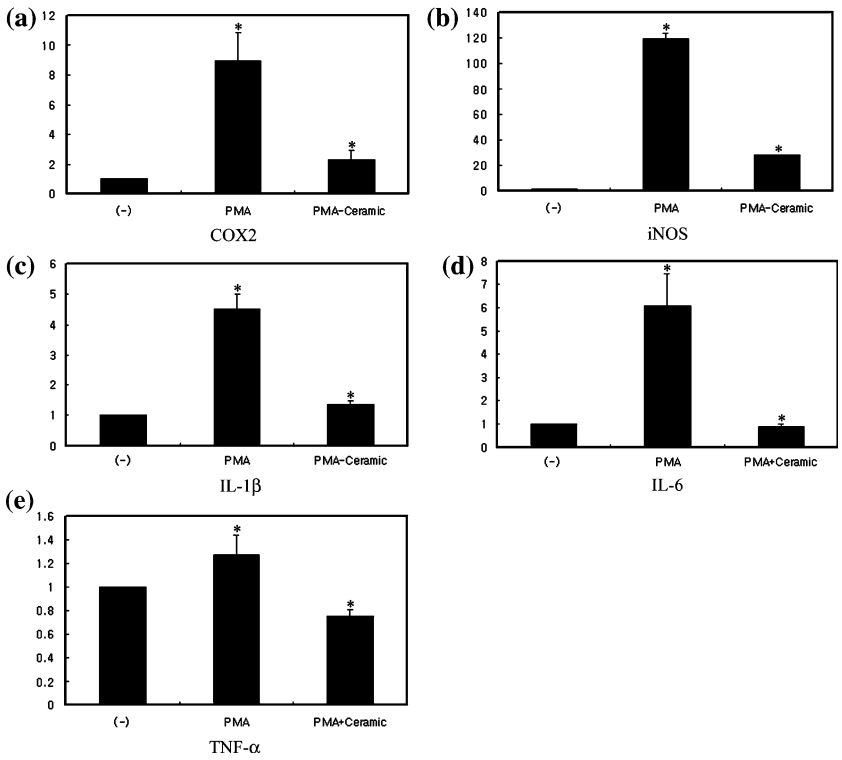

Fig. 2 Effect of bioactive ceramics on mRNA expression of PMA-induced target genes of NF-κB in rat VSMCs. Real-time RT-PCR was performed using total RNA isolated from rat VSMCs treated with no PMA (–), PMA (PMA), or PMA under the ceramics (PMA-Ceramic). GAPDH was used as an internal control. The *y*-axis represents the PCR value to be normalized to the internal control GAPDH, $2^{-\text{ddct of target genes}}/2^{-\text{ddct of GAPDH}}$. Each value represents the mean ± SD of three independent experiments performed in duplicate

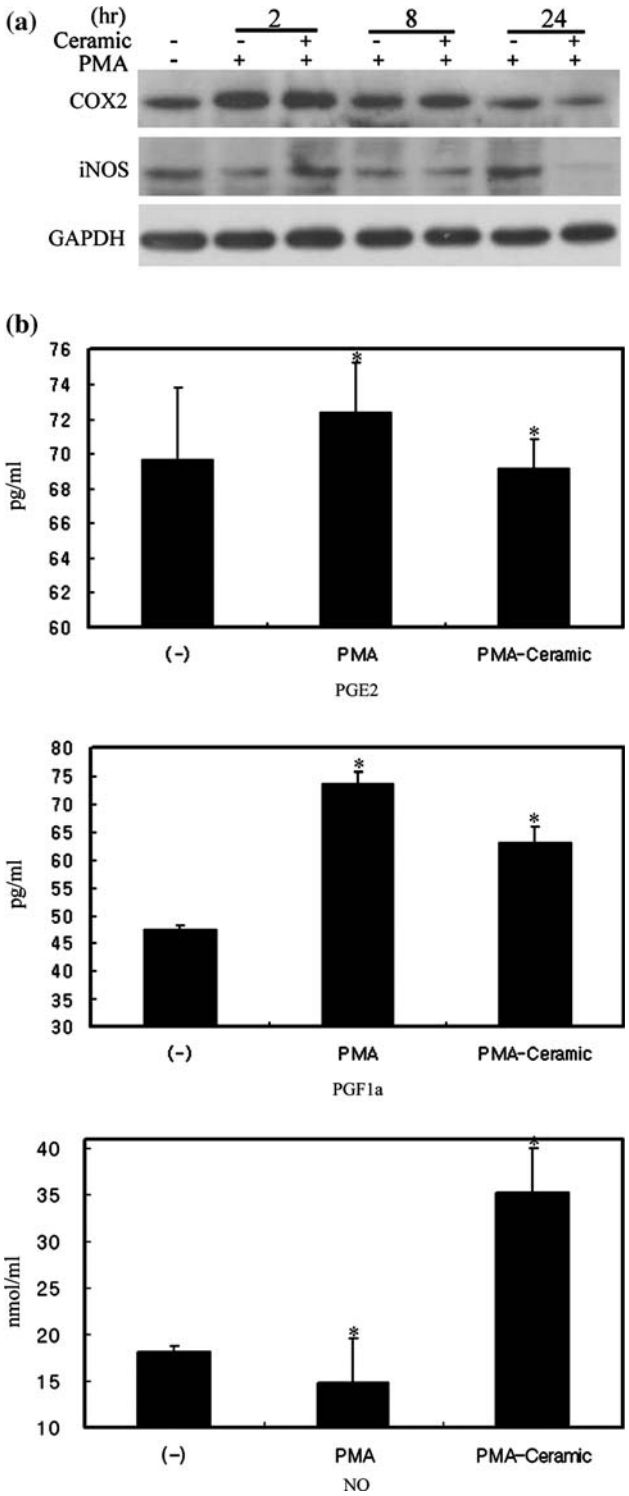

Fig. 3 Suppressive effect of bioactive ceramics on protein expression and activity of COX2 and iNOS. (**a**) COX2 and iNOS protein expression was examined with total protein extract obtained from cells treated with no PMA (–), PMA (PMA), and PMA under the ceramics (PMA-Ceramic) by Western blotting. GAPDH was used as an internal control. (**b**) Production of prostaglandin E2 (PGE2), prostaglandin F1a (PGF1α), and NO was determined with culture media obtained from cells treated with no PMA (–), PMA (PMA), or PMA under ceramics (PMA-Ceramic) by ELISA assay. The *y*-axis represents the amount of PGs (pg/mL) and NO (nM/mL). Each value represents the mean ± SD of three independent experiments performed in duplicate

ceramics on NF-κB activity, a rat VSMCs system was used. NF-κB is an important transcription factor in the expression of several inflammatory mediators, such as IL-1 and TNF-α. The effect of bioactive ceramics on NF-κB activity was examined with a reporter assay using a construct containing three artificial NF-κB binding sites. As shown in Fig. 1, compared with PMA treatment at time 0, NF-κB activity was gradually increased up to 5-fold 2 h after PMA treatment; however, bioactive ceramic attenuated PMA-induced NF-κB activity.

Analysis of target gene expression regulated by NF-κB

In rat VSMCs stimulated by PMA, a transcriptional factor, NF-κB, was activated and it induced target genes, such as IL-1β, TNF-α, IL-6, COX-2, and iNOS. The influence of bioactive ceramics on target gene expression was investigated at the transcriptional level by real-time RT-PCR (Fig. 2a–e). Compared with PMA alone, down-regulation of IL-1β, IL-6, TNF-α, COX-2, and iNOS was shown in cells cultured under ceramics (PMA-Ceramic). The results showed that bioactive ceramics could down-regulate IL-1β, IL-6, TNF-α, COX-2, and iNOS mRNA levels in rat VSMCs stimulated by PMA. We focused on and examined the expression of COX-2 and iNOS at the protein level. Although COX-2 and iNOS proteins did not seem to be affected early on, both proteins were significantly decreased by 24 h in the PMA-ceramic group (Fig. 3a).

Analysis of COX-2 and iNOS activities

COX-2 and iNOS activities were detected as prostaglandin and NO release 24 h after treatment with PMA. Prostaglandin F1α (PGF1α) production was increased by PMA (Fig. 3b), whereas prostaglandin E2 (PGE_2) and NO release were not significantly different between the no PMA (–) and PMA treatments (Fig. 3b). Bioactive ceramics had a 20% decrease of PGF1α production, but had no significant decrease in PGE_2. When we treated with NS-398, a COX-2 inhibitor, PGF1α production decreased but there was no effect on PGE_2 production (data not shown). On the other hand, ceramics increased NO release (Fig. 3 b), which did not coincide with mRNA and protein results of iNOS.

Nitric oxide was produced by iNOS and eNOS in VSMCs. Thus, we examined whether up-regulation of eNOS by bioactive ceramics caused a NO elevation in PMA treated rat VSMCs. As shown in Fig. 4, bioactive ceramics elicited eNOS protein as well as the expression of its transcript. In contrast, PMA diminished eNOS expression in rat VSMCs.

We wondered whether a common NF-κB inhibitor also increased NO production. Pyrrolidinedithiocarbamate (PDTC) is a well-known NF-κB inhibitor. We compared the suppressive effect of bioactive ceramics with PDTC on the expression and activity of COX-2 and iNOS. PDTC inhibited the expression of not only COX-2 but also iNOS mRNA, which was similar to the bioactive ceramics. Interestingly, PDTC had no effect on NO release, but increased the production of PGF1α (Fig. 5).

Discussion

In this study, we investigated the anti-inflammatory effects of bioactive ceramics on PMA-induced VSMCs. We found significant differences in the expression of inflammatory cytokines (IL-1β, TNF-α, and IL-6) and mediators (COX-2 and iNOS) between groups that were and were not exposed to the ceramics. In addition, the differences were caused by an alteration of NF-κB activity. As mentioned above, we previously reported that the ceramics used in present study

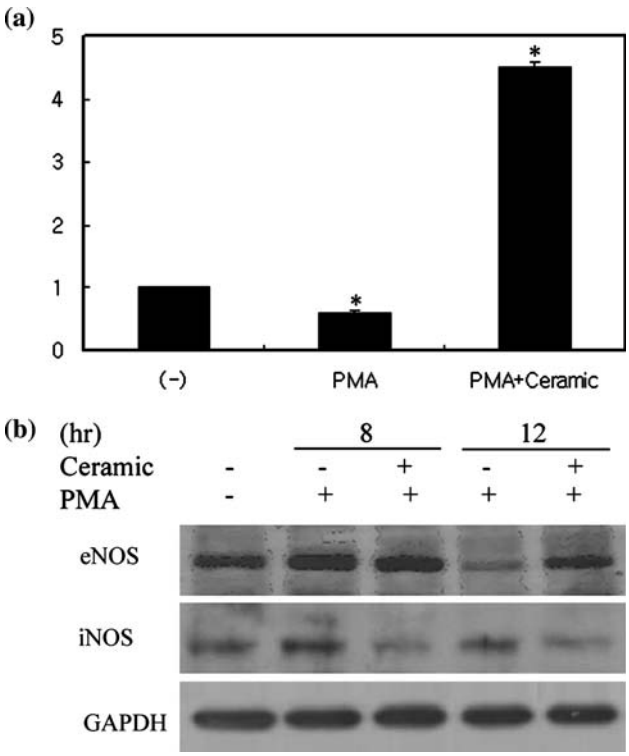

Fig. 4 Effect of bioactive ceramics on expression of eNOS in rat VSMCs. (**a**) eNOS mRNA expression and (**b**) protein expression were determined by RT-PCR and Western blotting, respectively. Total RNA and total protein were extracted from rat VSMCs cultured with no PMA (–), PMA treatment (PMA), or PMA with ceramics (PMA-Ceramic). The *y*-axis represents the PCR value to be normalized to the internal control GAPDH, $2^{-\text{ddct of eNOS}}/2^{-\text{ddct of GAPDH}}$. Each value represents the mean ± SD of three independent experiments performed in duplicate

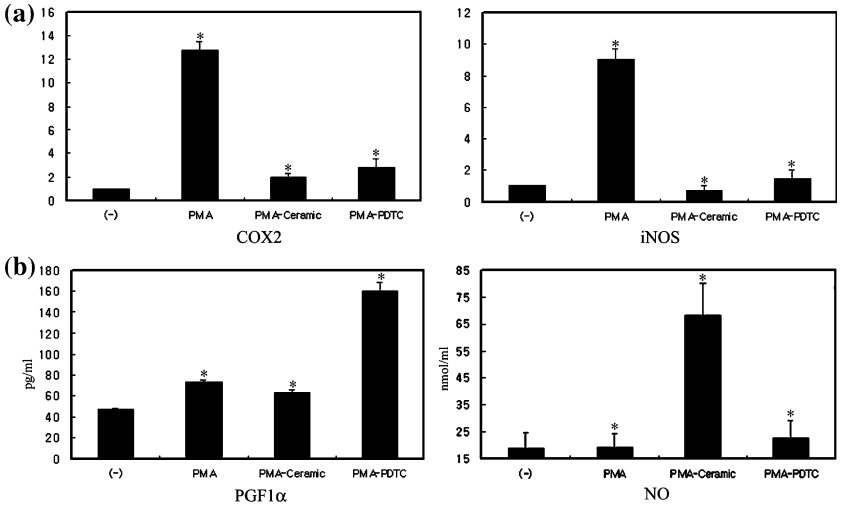

Fig. 5 Comparison of the effect of bioactive ceramics with that of PDTC, an inhibitor of NF-κB, on the regulation of COX2 and iNOS. (**a**) COX2 and iNOS mRNA expression was determined by RT-PCR using total RNA extracted from cells cultured with no PMA (–), PMA, PMA under ceramics, and PDTC (10 μM). PDTC was treated for 1 h prior to PMA. The *y*-axis represents the PCR value to be normalized to the internal control GAPDH, $2^{-\text{ddct of COX2 or iNOS}}/2^{-\text{ddct of GAPDH}}$. (**b**) PGF1α and NO release were determined by ELISA assay. The *y*-axis represents the amount of PGF1α (pg/mL) and NO (nM/mL)

reduced the NF-κB activity leading to a decrease in expression of inflammatory cytokines in macrophages [5]. Therefore, the results of present study were in agreement with those of a previous report [5]. The suppression of NF-κB activity by the ceramics was found in LPS-stimulated macrophages and PMA-treated VSMCs. NF-κB is an essential transcription factor in inflammatory processes and regulates inflammatory cytokines, such as IL-1β and TNF-α [17]. Thus, ceramics decreased the expression and production of IL-1β and TNF-α in VSMCs as well as macrophages. We repeated the same procedure at least three times, and obtained the same results with both types of cells. As a result, the reproducibility of the effect of the ceramics on the regulation of NF-κB activity and cytokine expression was confirmed.

However, there are a few differences between the present study and a previous study [5]. First, with respect to the expression and activity of COX2, one of the inflammatory mediators regulated by NF-κB, there were no significant changes in COX-2 mRNA expression and activity in macrophages by bioactive ceramics [5], whereas significant differences were detected in VSMCs. Second, mRNA and protein expression of iNOS and eNOS were not mentioned in the previous study [5], but the expression of both were examined in the present study. Ceramics regulate the expression of eNOS as well as iNOS in PMA-stimulated VSMCs. Third, NO production is due to an increase in eNOS expression. Nitric oxide is one of the most important inflammatory mediators. However, the biological effect of NO has been controversial. Some have suggested that NO induces various harmful responses, including tissue injury, septic shock, and apoptosis [18–20], which are attributed to the iNOS-mediated production of NO and the associated generation of potent reactive radicals, like peroxynitrite [21, 22]. On the other hand, NO serves many important functions in the cardiovascular system, such as vasodilation [23], inhibition of platelet adhesion, leukocyte adhesion to vascular endothelium [24], anti-proliferation [25], and scavenging superoxide anion [26]. Several studies have shown that impaired NO synthesis is often present in atherosclerosis [27, 28]. Accumulated data indicate that NO release compounds that may provide added therapeutic value, especially in those pathologic conditions, such as diabetes and atherosclerosis, where an impairment of endothelial function causes the inability to produce endogenous NO [29]. Specifically, anti-oxidant reagents have been used as NO donor agents and the effect on inhibition of VSMCs proliferation by an influence on eNOS protein expression, eNOS catalytic activity, and eNOS gene promoter activity [30]. Bioactive ceramics use has an effect on eNOS protein expression and NO release, which is similar to the inhibitory effect of anti-oxidant reagents on VSMCs proliferation.

In conclusion, bioactive ceramics down-regulated the expression of inflammatory cytokines (IL-1β, TNF-α, and IL-6) and mediators (prostaglandin F2a) which were downstream to NF-κB signaling in PMA-stimulated VSMCs. Moreover, the ceramics stimulated NO production by the up-regulation of eNOS expression. Taken together, we propose that bioactive ceramics, as used in the present study might have dual effects (i.e., anti-inflammatory and anti-oxidant) in PMA-stimulated VSMCs.

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