Aggregatibacter Actinomycetemcomitans-sensitized Monocytes Induce Endothelial Cell Apoptosis

Masaaki Hirasawa, Tomomi Hashizume-Takizawa, Masanori Saito, Ryoki Kobayashi, Noriko Shinozaki-Kuwahara, and Tomoko Kurita-Ochiai

Department of Microbiology and Immunology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

Article History
Received 16 December 2018
Accepted 10 January 2019

Abstract
Periodontitis has been associated with an increased risk for atherosclerosis. Accumulating evidence suggests that endothelial dysfunction is an early marker for atherosclerosis. To determine how periodontal infection could contribute to endothelial dysfunction, we examined the ability of the major periodontal pathogen, Aggregatibacter actinomycetemcomitans (Aa) -sensitized monocytes to modulate properties of human umbilical vein endothelial cells (HUVEC) by assessment of reactive oxygen species (ROS) production and apoptosis.

Cell proliferation and apoptosis was measured by BrdU cell proliferation ELISA kit, cell death detection ELISA and caspase activities. Detection of intracellular ROS generation was evaluated fluorometrically using H2DCF-DA. Quantitative reverse transcription polymerase chain reaction was performed using primers specific for p22phox and p47phox. iNOS, p22phox and p47phox in cell lysates were detected by Western blot analysis with the respective specific antibodies.

Aa did not affect the viability of HUVEC but induced apoptosis in HUVEC cocultured with monocytes. Aa significantly induced ROS and NO productions in monocytes. Furthermore, Aa increased gene and protein expressions of p22phox, p47phoxand iNOS. Also, H2O2 induced growth inhibition, apoptosis and caspase 3/7 activities in HUVEC. These results suggest that apoptosis in HUVEC could be induced by Aa -sensitized monocytes via ROS-dependent pathway, potentially amplifying proatherogenic mechanism in the perturbed vasculature.

Keywords: A. actinomycetemcomitans, HUVEC, apoptosis, THP-1

Introduction
Recently, periodontitis was shown to increase the risk of cardiovascular disease (CVD) (1), and accumulating evidence suggests that acute and chronic infections with periodontal pathogens such as Aggregatibacter actinomycetemcomitans(Aa) and Porphyromonas gingivalis are associated with increased risk of CVD (2, 3). A potential pathway through which periodontitis may contribute to atherogenesis is through induction of oxidative stress (4). Extensive production of reactive oxygen species (ROS) has been implicated in atherosclerosis by inducing the chronic activation of the vascular endothelium and components of the immune system. Vascular endothelial ROS released from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase may play critical roles in ROS generation (5). In humans, higher expression of NADPH oxidase subunit proteins is associated with increased superoxide (O2-) production and severity of atherosclerosis. NADPH oxidase-deficient Apoe-/- mice had significantly less atherosclerosis compared with Apoe-/- mice (6). Further studies clearly demonstrated that superoxide production from both monocytes/macrophages and vascular cells plays a critical role in atherogenesis (7). Monocyte recruitment from the blood stream into the vessel wall is crucial for atherosclerosis lesion formation and progression. After endothelial
dysfunction induced by factors including LDL, hypertension, or diabetes, monocytes attach to the endothelium and migrate into the subendothelial space where they take up lipid, become foam cells and cause early lesion development (8). Monocytes induce lipid peroxidation via the generation of ROS. These modified lipids can induce the expression of adhesion molecules and mediators of inflammation in macrophages and vascular wall cells (9). Monocyte-derived ROS impair endothelial function and accelerate the progression of atherosclerotic lesion by promoting lipid oxidation, the expression of proinflammatory genes, and endothelial cell apoptosis (10). Therefore, detailed understanding of the regulation and signal transduction of ROS production in monocytes is important.

Endothelial cells are key cellular components of blood vessels, functioning as selectively permeable barriers between blood and tissues. It is believed that risk factors induce endothelial cell apoptosis, leading to the denudation or dysfunction of the intact endothelial monolayer, which causes lipid accumulation, monocyte adhesion, and inflammatory reactions that initiate atherosclerotic lesions (11, 12). Although information on risk factor-induced atherosclerosis has been accumulating, the underlying mechanism remains unclear.

The aim of this work was to study the induction of ROS and NO in Aa-sensitized monocytes followed by apoptosis in HUVEC exposed to Aa-sensitized monocytes.

**Materials and Methods**

**Bacterial Strains and Culture Methods**

*Aa* HK1651 (ATCC 700685) was grown in Todd-Hewitt broth (BBL, Cockeysville, MD, USA) supplemented with 1% yeast extract (Difco Laboratories, Detroit, MI, USA) at 37°C in 5% CO2 for 3 days until it reached an OD540nm of 0.55, corresponding to 10^9 CFU/mL. The cultured cells were then centrifuged at 8000 g for 20 min at 4°C and diluted in phosphate-buffered saline (PBS).

**Cell Line and Reagents**

HUVEC (provided by Lonza-Takara, Tokyo, Japan) were cultured at 37°C in a humidified atmosphere with 5% CO2 in endothelial cell culture medium (EGM-2 BulletKit, Lonza-Takara) according to the manufacturer’s instructions. Acute monocytic leukemia (THP-1) cells were purchased from the Health Science Research Resources Bank and cultured in RPMI1640 containing 10% fetal bovine serum, 10 mM HEPES, 100 μU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Tokyo, Japan). All experiments were performed on cells at passage 4-8 at approximately 80% confluence. N-acetyl-L-cysteine (NAC) was purchased from Wako. iNOS inhibitor 1400W was purchased from Sigma (St Louis, MO, USA).

**Cell Proliferation Assay**

Cell proliferation was determined using a BrdU cell proliferation ELISA kit (Calbiochem, Darmstadt, Germany). Briefly, cells (2.0 × 10^4 /well) were cultured in 200 μL of endothelial cell culture medium in a 48-well plate and stimulated with Aa at the indicated multiplicity of infection (MOI) or H2O2. After 16h, 40 μL of BrdU solution was added, and the cells were incubated at 37°C for 2h, followed by measurement of A450 using a spectrophotometer.

**Cell Viability assay**

Cell viability was determined using a Cell Counting Kit-8 (CCK-8; Wako, Osaka, Japan). Briefly, cells (1.0 × 10^4 /well) were cultured in 100 μL of endothelial cell culture medium in a 96-well plate and stimulated with Aa at the indicated MOI. After 19h, 10 μL of CCK-8 solution was added, and the cells were incubated at 37°C for 2h, followed by measurement of A450 using a spectrophotometer.

**Measurement of Apoptosis**

Cellular apoptosis was quantified by DNA fragmentation using the Cell Death Detection ELISA PLUS kit (Roche, Mannheim, Germany). HUVEC (2.0 × 10^4 /well) or HUVEC co-cultured with THP-1 cells (2.0 × 10^4 /well) (HUVEC/THP-1) were incubated with Aa at an MOI of 10, 100, or 1000 for 16h. HUVEC was indirectly cocultured with THP-1 cells using 4 mm pore size cell culture inserts within a 24-well plate (BD Falcon, Franklin Lakes, NJ). HUVEC was also co-cultured for 16h with tenths volume of culture supernatant of THP-1 cells sensitized with Aa for 16h. the cells were lysed in 200 μL of lysis buffer, and 20 μL of the supernatant was reacted with 80 μL of anti-DNA immunocomplex conjugated with peroxidase, which interacts with streptavidin-coated wells, in a microtiter plate for 2 h. at the end of the incubation, 100 μL of substrate was added, and color development was quantified as a wavelength of 405 nm. For the apoptosis inhibition assays, THP-1 cells were preincubated for 1h with the anti-oxidant NAC or inducible nitric-oxide synthase (iNOS) inhibitor 1400W before sensiti-
zation with *Aa*. Caspase 3/7 activity was measured using a Caspase-Glo 3/7 assay kit (Promega, Madison, USA).

**Measurement of Intracellular ROS**

Detection of intracellular ROS generation was evaluated fluorometrically using 2’,7’-dichlorodihydrofluorescein diacetate (H2DCF-DA) (13). THP-1 cells (5 × 10^5 cells/well in 24-well plates) were treated with *Aa*, followed by the addition of 50 mM H2DCF-DA. After 30 min of incubation at 37°C, the cells were washed twice with PBS to remove any extracellular dye. The formation of the fluorescence spectrometer at excitation and emission wavelengths of 485 and 530 nm, respectively. DCFDA is deacetylated and reacts quantitatively with intracellular radicals such as H₂O₂ to produce a fluorescent product, dichlorofluorescein, which is retained within the cell and thus provides an index of cell cytosolic oxidation.

**Determination of Endothelial Nitric Oxide Production.**

THP-1 cells (5 × 10^5 cells/well in 24-well plates) were treated with *Aa*, and production of NO by THP-1 cells was measured as its stable oxidation product; nitrite, using Bioxytech nitric oxide assay kit (OxisResearch; Portland, USA). Briefly, 50 μL of the culture medium was diluted with 35 μL assay buffer and mixed with 10 μL nitrate reductase and 10 μL NADH. Following 20 minutes of incubation to convert nitrate to nitrite, total nitrite was measured at 540 nm absorbance by reaction with Griess reagents (sulfanilamide and naphthalene-ethylenediamine dihydrochloride).

**Real-Time Quantitative Reverse Transcription (RT-PCR)**

Total RNA was isolated from *Aa*-treated THP-1 cells using an RNAsol kit (Qiagen, Hilden, Germany) according to the manufacturer’s procedures. cDNA was synthesized using a PrimeScript RT Reagent Kit (Perfect Real Time; Takara Bio). Real-time quantitative RT-PCR was performed using the Thermal Cycler Dice Real Time System TP800 and SYBR Premix Ex Taq II (Perfect Real Time; Takara Bio) according to the manufacturer’s instructions. Primers specific for p22phox (forward, GGGCTTACCAGTTGCTACT; reverse, CCTCCAGGAGGCAACAAACA), p47phox (forward, GGACACTTTCATCCGTCACT; reverse, CAGG TTCCTGCCATTCTCACA), and GAPDH (forward, GCAC CGTCAAGGCTGAGAAC; reverse, TGTTGAAGACGCCAGTGGA) were designed and produced by Takara Bio.

**Antibodies and Western Blot Analysis**

Rabbit antibodies against iNOS, p22phox and p47phox were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Secondary horseradish peroxidase-HRP-horseradish peroxidase (HRP-) conjugated goat anti-rabbit antibody was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Cells were lysed in a buffer containing 10 mM Tris-HCl, 150 mM NaCl, 1% Nonidant P-40, 1 mM ethylenediamine-tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 0.1 mM phenylmethylsulfonyl fluoride, 8 μg/mL aprotinin, and 2 μg/mL leupeptin (pH 7.4). For immunoblotting, proteins resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) were transferred to polyvinylidenefluoride membranes (Millipore, Bedford, MA), which were then exposed to primary and then secondary antibodies. Chemiluminescence detection was performed with an ECL™ Western Blotting Detection Kit (Amersham). The signal intensities of the corresponding bands were measured by a Light Capture equipped with CS Analyzer software (ATTO, Osaka, Japan).

**Measurements of Oxidative Stress**

To directly monitor real time reactive oxygen/nitrogen species (ROS/RNS) a kit including an oxidative stress detection reagent (ENZO Life Sciences, Farmingdale, NY, USA) was used. THP-1 cells were infected with *Aa* at an MOI of 100 for 30 min and analyzed under fluorescence microscope.

**Statistical Analysis**

All data are presented as means ± SEM. Multiple group comparisons were made by one-way analysis of variance, followed by post hoc intergroup comparison by the Bonferroni-Dunn test. *A* value < 0.05 was considered statistically significant.

**Results**

*Aa induces HUVEC apoptosis in HUVEC/monocyte coculture system*

We examined the effects of *Aa* on the proliferative activity of HUVEC by the BrdU ELISA after 16 h of incubation. At any MOI, the addition of *Aa* had little effect on HUVEC proliferation (Fig. 1A). On the other hand, *Aa* induced apoptosis in HUVEC in the presence of THP-1 cells (Fig. 1B). Higher apoptosis rates were observed at MOI of 10^2 and 10^3 after 16 h compared to the non-treated control.
group. Next, we examined the effect of culture supernatant from THP-1 cells sensitized with Aa. THP-1 culture supernatant sensitized with Aa also induced apoptosis in HUVEC (Fig. 2A). In particular, significant apoptosis was observed when THP-1 was sensitized with MOI of $10^2$ and $10^3$. On the other hand, apoptosis of HUVEC by Aa-sensitized THP-1 culture supernatant was significantly suppressed by the addition of antioxidant (NAC) and iNOS inhibitor (1400 W) (Fig. 2B). These results suggest that active oxygen species contained in the culture supernatant of THP-1 sensitized with Aa may induce HUVEC apoptosis.

**Aa induces ROS production in monocytes**

Since apoptosis of HUVEC by Aa-sensitized THP-1 supernatant was suppressed by antioxidant (NAC) and iNOS inhibitor (1400 W), we next investigated the production of ROS and NO from THP-1 by Aa treatment. We found that in THP-1-producing ROS and NO, the amounts gradually increased in a concentration-dependent manner of Aa (Fig. 3). In particular, production of ROS and NO at MOI 100 was significantly higher than MOI 10. As shown in Fig. 4, Aa enhanced the generation of reactive species such as NO (Fig. 4A) and ROS (Fig. 4B) significantly.

**Upregulation of NADPH oxidase expression**

Real-time RT-PCR analysis showed that Aa increased gene expression levels of p22phox and p47phox in THP-1 cells (Fig. 5). Sensitization of Aa to THP-1 cells was also induced protein expressions of iNOS, p47phox, and p22phox compared to non-treated THP-1 cells (Fig. 6).

**$H_2O_2$ induces HUVEC apoptosis**

We next examined the effects of hydrogen peroxide ($H_2O_2$), a reactive oxygen species (ROS), on cell viability, apoptosis and caspase 3/7 activities in HUVEC (Fig. 7). Cell viability was significantly suppressed by high concentration (500–1000μM) of $H_2O_2$ treatment. Cell death was significantly enhanced with medium to high concentration (250–1000μM) of $H_2O_2$ treatment. Although caspase 3/7 activity was significantly enhanced by the medium concentration (250μM) of $H_2O_2$ treatment, the activity sharply decreased with high concentration (500 to 1000μM) of $H_2O_2$ treatment. These results raise the possibility that necrosis occurred at high concentrations of $H_2O_2$ treatment.
Fig. 2. *Aa*-sensitized monocyte culture supernatant induces HUVEC apoptosis. (A) HUVEC was co-cultured for 16h with tenths volume of culture supernatant of THP-1 cells which previously sensitized with *Aa* at an MOI of 1: 10-1:1000 for 16 h. Cellular apoptosis was quantified by DNA fragmentation using the Cell Death Detection ELISA<sup>®</sup> Kit as described in materials and methods. Data are expressed as the mean ± SEM of 3 different experiments. "p<0.05 vs bacteria-free control cells. (B) HUVEC was co-cultured for 16h with tenths volume of culture supernatant of THP-1 cells which previously sensitized with *Aa* at an MOI of 1:1000 for 16 h. For the apoptosis inhibition assays, THP-1 cells were preincubated for 1h with the NAC (3 or 10 mM) or 1400W (10 or 50 μM) before sensitization with *Aa*. Cellular apoptosis was quantified Data are expressed as the mean ± SEM of 3 different experiments. "p<0.05 vs bacteria-free control cells. #p<0.05 vs *Aa* sensitized cells.

Fig. 3. *Aa* induces ROS and NO Production in monocytes. *Aa* was added to nearly confluent THP-1 cells at an MOI of 1:1-1:100 and incubated at 37°C for 10 min. (A) The formation of a fluorescent product, dichlorofluorescin, was analyzed using a fluorescence spectrometer. (B) Total nitrite was measured using Bioxytech nitric oxide assay kit. Data are expressed as the mean ± SEM of 3 different experiments. "p<0.01 vs bacteria-free control cells. #p<0.01 vs *Aa*(MOI10) -sensitized cells.
Discussion

In previous studies, we demonstrated that *Aa* induced endothelial apoptosis and atherosclerosis in spontaneously hyperlipidemic mice (14). Therefore, this experiment was carried out considering that HUVEC apoptosis could be induced also in *Aa* infection. Contrary to expectation, *Aa* infection alone had no effect on the proliferation of HUVEC. Furthermore, *Aa* infection alone had no effect on HUVEC survival or apoptosis (data not shown). However, although co-culture of *Aa* and HUVEC had no effect on proliferation.
and apoptosis, co-existence of THP-1 resulted in a significant increase in apoptosis in HUVEC in proportion to MOI of Aα. On the other hand, since the enhancement of these apoptosis was significantly suppressed by the antioxidant NAC and the nitric oxide synthase inhibitor 1400 W, it was thought that HUVEC apoptosis might be induced by the active oxygen and active nitrogen contained in the culture supernatant of Aα-stimulated THP-1 cells. Indeed, Aα produced ROS and NO from THP-1 cells in a MOI-dependent manner. NADPH oxidase, an oxidoreductase, is the mechanism of superoxide generation in living organisms which is currently receiving the most attention. This enzyme present on the cell membrane or membrane of the endoplasmic reticulum membrane oxidizes NADPH and generates superoxide. NADPH oxidase receives electrons from cytoplasmic NADPH and produces superoxide by one electron reduction of oxygen molecule (15). Four kinds of

![Fig. 6. Aα increases protein expression of iNOS, p22phox and p47phox in monocytes. THP-1 cells were treated with Aα at an MOI of 1:1–1:100 for 16 h. Whole cell lysates were subjected to SDS-PAGE, followed by Western blot analysis using antibodies to iNOS, p22phox and p47phox. Levels of α-Tubrin were also detected as an internal control.](image)

![Fig. 7. ROS such as H₂O₂ induce apoptosis. (A) HUVEC treated with H₂O₂ (125–1000 μM) for 21 h to determine cell viability. (B) HUVEC treated with H₂O₂ (125–1000 μM) for 16 h to quantify cellular apoptosis. (C) HUVEC treated with H₂O₂ (125–1000 μM) for 8 h to measure caspase activities. Data are expressed as the mean ± SEM of 3 different experiments. *p<0.05 vs H₂O₂-free control cells.](image)
isoforms of NADPH oxidase in the heart. NOX 1, NOX 2, NOX 4 and NOX 5 are known (16, 17). NADPH oxidase is composed of cell membrane subunits NOX and p22phox and cytoplasmic subunits p67phox, p47phox and Noxal (NOX activator) (17). Of these, NOX 4 is a type of constitutive enzyme, and it is characterized by being always activated. Recently, the activation of NOX4 is thought to be involved in the generation of hydrogen peroxide (17). In NOX 1 and NOX 2 which is similar to the type present in granulocytes, the cytoplasmic subunits rac-GTPase, p47phox and p 67phox migrate to the membrane, form a complex with NOX and p22phox of the cell membrane subunit, and express enzyme activity (17).

In this study, increased expression of p22phox and p47phox genes, which are subunits of NADPH oxidase, was observed by the adding Aa viable bacteria to THP-1 in the range of MOI: 10–10². Furthermore, Aa enhanced expression of iNOS, P22phox and p47phox proteins in THP-1 cells. Therefore, the production of ROS and NO in Aa sensitized THP-1 and the accompanying increase in NADPH expression suggest that Aa plays a central role in oxidative stress. Furthermore, it is known that ROS induces LDL oxidation and ER stress (18, 19). Therefore, the release of ROS in HUVEC in the presence of Aa-sensitized THP-1 may induce ER stress on HUVEC. Indeed, the expression level of UPR-related genes was significantly higher in periodontitis than in gingivitis lesions (20).

There are various kinds of ROS in the living body, among them O₂−, H₂O₂ and hydroxyl radical (OH) are important (21). Each of these reactive oxygen species has different reactivity and half-life, and each has its own characteristic. Hydrogen peroxide is produced not only by metabolic processes of superoxide but also by enzymatic reactions in vivo. H₂O₂ has weak toxicity, but its half-life is long, so once it is produced, it has a characteristic of staying relatively long, and when converted to OH, it causes cell damage. Therefore, given the half-life, ROS produced in the THP-1 culture supernatant may have existed in the form of H₂O₂. Alternatively, it may have induced endothelial cell apoptosis in the form of exosomes containing NADPH oxidase (22).

Significant apoptosis of luminal endothelial cells has been reported in advanced human atherosclerotic plaques (23), a finding that is compatible with the onset role of endothelial apoptosis in plaque erosion. Our findings suggest that HUVEC apoptosis mediated by ROS (24) and NO (25) which were produced by monocytes sensitized with Aa may be partly involved in the development of atherosclerotic lesions by Aa.

In conclusion, our data suggest that oxidative stress such as ROS and NO produced by Aa-sensitized monocytes may induce apoptosis in HUVEC. NADPH oxidase such as p22phox and p47phox, and iNOS were involved in the induction mechanisms of these oxidative stress. Therefore, our data also suggests to provide antioxidants as a potential strategy for the prevention of Aa-induced endothelial dysfunction.

References
1. Scannapieco FA, Bush RB, Paju S: Associations between periodontal disease and risk for atherosclerosis, cardiovascular disease, and stroke. A systematic review. Ann Periodontol, 8: 38–53, 2003.
2. Desvarieux M, Demmer RT, Rundek T, Boden-Albala B, Jacobs DR, Sacco RL, Papapanou PN: Periodontal microbiota and carotid intima-media thickness: the Oral Infections and Vascular Disease Epidemiology Study (INVEST). Circulation, 111: 576–582, 2005.
3. Spar A, Klein E, Khuseyinova N, Boecckh C, Muche R, Kunze M, Rothenbacher D, Pezeski G, Hoffmeister A, Koenig W: Periodontal infections and coronary heart disease: role of periodontal bacteria and importance of total pathogen burden in the Coronary Event and Periodontal Disease (CORODONT) study. Arch Intern Med, 166: 554–559, 2006.
4. Kurita-Ochiai T, Jia R, Cai Y, Yamaguchi Y, Yamamoto M: Periodontal disease-induced atherosclerosis and oxidative stress. Antioxidants, 4: 577–590, 2015.
5. Stocker R, Keeaney Jr, JF: Role of oxidative modifications in atherosclerosis. Physiol Rev, 84: 1381–1478, 2004.
6. Craige SM, Kant S, Reif M, Chen K, Pei Y, Angoff R, Sugamura K, Fitzgibbons T, Keeaney JF: Endothelial NADPH oxidase 4 protects ApoE/- mice from atherosclerotic lesions. Free Radic Biol Med, 89: 1–7, 2015.
7. Vendrov AV, Hakim ZS, Madamanchi NR, Rojas M, Madamanchi C, Runge MS: Atherosclerosis is attenuated by limiting superoxide generation in both macrophage and vessel wall cells. Arterioscler Thromb Vasc Biol, 27: 2714–2721, 2007.
8. Cekkova S, Kralova-Lesna I, Polemis R: Monocyte adhesion to the endothelium is an initial stage of atherosclerosis development. Cor et Vasa, 58: e419-e425, 2016.
9. Soo JW, Yang EJ, Yoo KH, Choi IH: Macrophage differentiation from monocyte is influenced by the lipid oxidation degree of low density lipoprotein. Mediators Inflammation, 2015, 235797. Doi: 10.1155/2015/235797, 2015.
10. Tavakoli S, Asmis R: Reactive oxygen species and thiol redox...
10. Tsuchihashi N, Honda T, Nakajima T, Tabelo K, Amiko Y, Yamazaki K: Up-regulation of the endoplasmic reticulum stress-response in periodontal disease. Clinica Chirurca, 401: 134–140, 2009.

11. Xu Q: The impact of progenitor cells in atherosclerosis. Nat Clin Pract Cardiovasc Med, 3: 94–101, 2006.

12. Dardik A, Chen L, Frattini J, Asada H, Aziz F, Kudo FA. Sumpio BE: Differential effects of orbital and laminar shear stress on endothelial cells. J Vasc Surg, 41: 869–880, 2005.

13. Cominacini L, Pasini AF, Garbin U, Davoli A, Tosetti ML, Campagnola M, Rigoni A, Pastorino AM, Lo Cascio V, Sawamura T: Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-I in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species. J Biol Chem, 275: 12633–12638, 2000.

14. Zhang T, Kurita-Ochiai T, Hashizume T, Oguchi S, Abiko Y, Yamamoto M: Aggrecitacitact ometrytecomitosines leads to endothelial apoptosis and atherosclerosis development in spontaneously hyperlipidemic mice. Int J Oral-Med Sci, 8: 132–141, 2010.

15. Konior A, Schramm A, Czesnikiewicz-Guzik M, Guzik TJ: NADPH oxidases in vascular pathology. Antioxid Redox Signal, 20: 2794–2814, 2014.

16. Akki A, Zhang M, Murdoch C, Brewer A, Shah AM: NADPH oxidase signaling and cardiac myocyte function. J Mol Cell Cardiol, 47: 15–22, 2009.

17. Schulz E, Munzel T: NOX5, a new “radical” player in human atherosclerosis? J Am Coll Cardiol, 52: 1810–1812, 2008.

18. Yokouchi M, Hiramatsu N, Hayakawa K, Okamura M, Du S, Kasai A, Takano Y, Shitamura A, Shimada T, Yao J, Kitamura M: Involvement of selective reactive oxygen species upstream of proapoptotic branches of unfolded protein response. The Journal of Biological Chemistry, 283: 4252–4260, 2008.

19. Tagawa Y, Hiramatsu N, Kasai A, Hayakawa K, Okamura M, Yao J, Kitamura M: Induction of apoptosis by cigarette smoke via ROS-dependent endoplasmic reticulum stress and CCAAT/enhancer-binding protein-homologous protein (CHOP). Free Radic Biol Med, 45: 50–59, 2008.

20. Donon H, Takahashi N, Honda T, Nakajima T, Tabelo K, Amiko Y, Yamazaki K: Up-regulation of the endoplasmic reticulum stress-response in periodontal disease. Clinica Chirurca, 401: 134–140, 2009.

21. Redza-Dutordoir M, Averill-Bates DA: Activation of apoptosis signaling pathways by reactive oxygen species. Biochim Biophys Acta, 1863: 2977–2992, 2016.

22. Gambhir MH, Carmo AO, Marti L, Verissimo-Filho S, Lopes LR, Janiszewski M: Platelet-derived exosomes induce endothelial cell apoptosis through peroxynitrite generation: experimental evidence for a novel mechanism of septic vascular dysfunction. Critical Care, 11: R107. doi: 10.1186/cc6133, 2007.

23. Tricot O, Mallat Z, Heymes C, Belmin J, Leseche G, Tedgui A: Relation between endothelial cell apoptosis and blood flow direction in human atherosclerotic plaques. Circulation, 101: 2420–2423, 2000.

24. Kobayashi N, DeLano FA, Schmid-Schonbein GW: Oxidative stress promotes endothelial cell apoptosis and loss of microvessels in the spontaneously hypertensive rats. Arterioscler Thromb Vasc Biol, 25: 2114–2121, 2005.

25. Walford GA, Moussignac RL, Scribner AW, Lescalzo J, Leopold JA: Hypoxia potentiates nitric oxide-mediated apoptosis in endothelial cells via peroxynitrite-induced activation of mitochondria-dependent and -independent pathways. J Biol Chem, 279: 4425–4432, 2004.