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Bilberries exert an anti-atherosclerotic effect in lipid-loaded macrophages

Research Article

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Abstract: We hypothesized that the mechanism responsible for the anti-atherosclerotic action of bilberry extract (BE) is linked to its antioxidant and anti-inflammatory potential, and investigated its direct effect on the regulation of apolipoprotein E (apoE) and cholesteryl ester transfer protein (CETP) secretion from lipid-loaded macrophages. Human THP-1 macrophages were loaded with lipids by incubation with human copper-oxidized LDL (oxLDL) and then exposed to different concentrations of BE (1-5 μg mL^{-1}) obtained from bilberries (mechanically homogenized and solubilized in ethanol). Cellular and secreted proteins, the phosphorylation level of NF-κB and protein kinase A (PKA) were quantified by Western blot and gene expression was evaluated by Real-time PCR. The results showed that BE induced in lipid-loaded macrophages has: (i) an antioxidant effect by reducing the expression of NADPH oxidase subunits, p22^{phox}, p47^{phox} and NOX4, (ii) an anti-inflammatory effect by diminishing the secretion of CRP, MCP-1 and IL-1β and (iii) cholesterol efflux by increasing the secretion of apoE and CETP and by reducing cellular cholesterol content. BE exerted these effects by inhibition of NF-κB and activation of PKA signaling pathways. Our study supports BE therapeutic administration to decrease oxidative and inflammatory stress by a molecular mechanism regulated by NF-κB and PKA signaling pathways in lipid-loaded macrophages.

Keywords: *Apolipoprotein E • Atherosclerosis • Bilberry extract • Cholesteryl ester transfer protein • C-reactive protein • Inflammation • Interleukin 1-β • Macrophage • NADPH oxidase*

1. Introduction

Emerging scientific evidence indicates that consumption of a flavonoid-rich diet may prevent the risk of developing atherosclerosis, due to the ability of these compounds to inhibit oxidative stress and inflammation [1]. These processes are of major importance in atherogenesis because they stimulate oxidized LDL (oxLDL)-induced cholesterol accumulation in macrophages and the resulting foam cells in the arterial wall are the hallmark of atherosclerosis [2]. The generation of increased intracellular reactive oxygen species (ROS) was associated with oxLDL uptake by macrophages [3]. Moreover, oxLDL is known to induce the expression of NADPH oxidase subunits NOX4/p22^{phox} and NOX2/p47^{phox}/p22^{phox} in human smooth muscle cells [4] and macrophages [5,6], thus generating intracellular oxidative stress.

Macrophages are key mediators in the immune response and their physiological protective function is harmfully activated in the atherosclerotic process [7]. Macrophages contribute to plaque formation and development by internalizing oxLDL and converting into cholesterol-rich foam cells. Many inflammatory mediators, such as interleukins (IL-1β, IL-6), monocyte-chemoattractant protein (MCP)-1 and C-reactive protein (CRP), have genes targeted by the nuclear factor (NF)-κB [8], a transcription factor that is essential in orchestrating the inflammatory responses to a wide range of insults and is involved in the pathogenesis of cardiovascular diseases [9].

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Macrophage-derived foam cell development is initiated by the dysfunction of normal cholesterol metabolism, which is regulated by cellular molecular mechanisms that control the dynamic balance of cholesterol influx and efflux [7]. Apolipoprotein (apo) E has been proposed as mediator for the cholesterol efflux from macrophages in the presence/absence of extracellular acceptors, including HDL_3 and lipid-free apoA-I [10]. Macrophages secrete cholesteryl-ester transfer protein (CETP), which accounts for the enrichment of the triglyceride-rich apoB-containing lipoproteins with cholesteryl esters from HDL, but can also contribute to the reverse cholesterol efflux [11,12]. We have recently shown that lipid-free apoA-I and HDL_3 can stimulate both apoE and CETP secretion from oxLDL-loaded macrophages by the inhibition of NF-κB and activation of protein kinase A (PKA) signaling pathways [13].

Bilberry (*Vaccinium myrtillus*) is one of the richest sources of polyphenols, including anthocyanins and flavonoids [14]. Analysis of an alcoholic extract obtained from bilberries shows the presence of a high concentration of compounds with antioxidant potential [15]. Increasing evidence supports an effect of the bilberry extract (BE) on vascular protection by reduction of cholesterol and lipid peroxides levels, anti-inflammatory properties and inhibition of platelet aggregation in human subjects and animal models [1,16].

During the past four decades, basic and clinical basic and clinical studies of anthocyanin-rich BE have intensified because of its potential beneficial health effects. However, the molecular mechanisms underlying the action of BE are not completely understood and require further *in vivo* and *in vitro* research. We aimed to investigate the mechanisms that regulate the oxidative and inflammatory stress in lipid-loaded macrophages, because these cells constitute the hallmark of the atherosclerotic lesion. We hypothesized that BE might inhibit the oxidative stress through down regulation of NADPH oxidase complex and the inflammatory stress by inhibiting the secretion of some inflammatory mediators, such as CRP, MCP-1 and IL-1β. Moreover, we analyzed the role of BE in cholesterol efflux by assessing the secretion of apoE and CETP from oxLDL-loaded macrophages. We further investigated the NF-κB and PKA signaling pathways in BE-exposed lipid-loaded macrophages.

2. Experimental Procedures

2.1 Reagents

RPMI-1640 medium and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase, oligo-dT_{20} primers and SYBR-Green I were obtained from Life Technologies (Carlsbad, CA, USA). Monoclonal or polyclonal antibodies to human apoE, CETP, CRP, MCP-1, NOX4, phospho-S468 and total NF-κB p65 subunit, phospho-T197 and total PKA-γ-subunit, and HRP-conjugated goat antibodies against mouse or rabbit IgG were obtained from Abcam (Cambridge, UK). Monoclonal antibodies to human $p22^{phox}$ and β-actin (C4) were purchased from Santa-Cruz Biotechnology (Dallas, Texas, USA).

2.2 Isolation and modification of human lipoproteins

LDL and HDL_3 fractions were isolated from human plasma of healthy donors from Blood Transfusion Center Bucharest by using sequential flotation ultracentrifugation on an Optima LE-80 ultracentrifuge (Beckman Coulter, CA, USA) [4]. Cu-oxidized LDL (oxLDL) was prepared and characterized as previously described [4].

2.3 Preparation of the bilberry extract

Bilberry fruits were obtained from the Romanian Fagaras Mountains, and then kept frozen at -20°C. Bilberry fruits (100 g) were mechanically homogenized and solubilized in 10 ml ethanol for 24 h. The bilberry homogenate was filtered through 0.45 μm membrane and evaporated under nitrogen. The resulting dried substance was solubilized in 50% ethanol, obtaining a stock solution of 10 g L^{-1} BE. This stock solution was then diluted 1:10 in RPMI-1640 (to obtain a 1 g L^{-1} BE solution) and freshly used in cell culture experiments.

2.4 Cell culture and experimental design

Human THP-1 monocytes were grown in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (EuroClone, Siziano, Italy) and penicillin/streptomycin. Cells were then differentiated into macrophages by exposure to 100 nmol L^{-1} PMA and seeded at a density of 10^6 cells in 1 ml for 72 h. Macrophages were loaded with lipids by 24 h-incubation with 100 μg mL^{-1} oxLDL in RPMI-1640 medium with antibiotics and without serum. The medium with oxLDL was removed after 24 h and the lipid-loaded macrophages were incubated with different concentrations of BE by adding 1, 2.5 and 5 μl mL^{-1} medium from a 10 g L^{-1} BE stock (in 50% ethanol), diluted 1:10 with RPMI-1640 medium (resulting an equivalent of 1, 2.5 and 5 μg dry extract to 1 ml medium). Control and oxLDL (without BE) cells were incubated for the same period of time with 0.25‰ ethanol. After 24 h incubation, CRP, MCP-1, IL-1β,

apoE and CETP proteins were evaluated in the culture media, and $p22^{phox}$, $p47^{phox}$, NOX4, apoE and CETP cellular expression, cellular cholesterol concentration, phosphorylation level of NF-κB and PKA were assessed in the presence or absence of BE.

2.5 Immunoblotting analysis

Culture media collected from macrophages were concentrated 50-fold with a 10 kDa - cutoff Amicon Ultracel-10 device (Milipore, Billerica, MA, USA) and used to quantify protein secretion by immunoblotting analysis. Equal amounts of protein from the cell lysates and equal volumes of concentrated media were subjected to SDS-PAGE electrophoresis. Specific primary antibodies and HRP–conjugated IgG, a chemiluminescent substrate (Pierce, Rockford, IL, USA) and an ImageQuant LAS4000 imaging system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were used to visualize proteins. Densitometric analysis of immunoblots was performed using ImageQuant TL 1D v7.0 software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). IL-1β was measured by ELISA technique with a commercial kit (Mabtech, Nacka Strand, Sweden). The cellular protein expression was normalized to cellular β-actin, while protein secretion in the culture medium was normalized to total cellular protein. All immunoblotting data were expressed relative to control values.

2.6 Gene expression analysis

Total RNA was extracted from cultured macrophages with GenElute mRNA Miniprep Kit (Sigma-Aldrich, St.Louis, MO, USA) and reverse-transcribed by using M-MLV reverse transcriptase and oligo-dT_{20} primers. Samples were amplified in triplicate by using specific primers for the genes of interest and β-actin (as housekeeping gene), as previously reported [4,13], in a StepOne Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA); relative quantification was achieved by using the Fit-Point method [17] based on SYBR-Green I detection (Invitrogen, Carlsbad, CA, USA).

2.7 Quantification of cellular cholesterol concentration

Total intracellular cholesterol was quantified using a commercial kit (Dialab, Neudorf, Austria) in the lipid extract from the cells using the Bligh and Dyer method [18].

2.8 Statistical analysis

Statistical analysis was performed using SPSS v21.0 for Windows (Chicago, IL, USA). Statistical evaluation was done using an independent two-tailed T-test and $P<0.05$ was considered statistically significant. Data were expressed as mean ± standard deviation.

3. Results

3.1 Bilberry extract exerts antioxidant properties in lipid-loaded macrophages

To explore the antioxidant properties of BE we investigated the modulation of three main subunits of NADPH oxidases ($p22^{phox}$, NOX4 and $p47^{phox}$) in oxLDL-loaded macrophages. Exposure of THP-1 macrophages to oxLDL induced a 4-fold ($P<0.01$) increase of $p22^{phox}$ gene and protein expression, 1.5-fold ($P<0.01$) increase for NOX4 protein expression and 1.4-fold ($P<0.05$) for $p47^{phox}$ protein expression compared to control cells (Figure 1A-E). BE incubation with lipid-loaded macrophages exerted a dose-dependent reduction of $p22^{phox}$ (by 25%, $P<0.05$) (quantified by real-time PCR and Western Blot analysis), NOX4 (by 50 %, $P<0.01$) and $p47^{phox}$ (by 17%, $P<0.05$) (both by real-time PCR) (Figure 1A-E).

3.2 Bilberry extract dose-dependently reduces the secretion of inflammatory mediators from lipid-loaded macrophages

To investigate the anti-inflammatory properties of BE, we assessed the secretion of some well-known inflammatory mediators (CRP, MCP-1 and IL-1β) produced by exposure of macrophages to 100 μg mL^{-1} oxLDL. The lipid-loaded macrophages exhibited an upregulated gene expression of CRP (8-fold, $P< 0.01$) and MCP-1 (3.5-fold, $P< 0.01$) compared to control cells (Figure 2A). This effect was correlated with an increased secretion of CRP (60%, $P< 0.05$) and MCP 1 (2.2-fold, $P< 0.01$) compared to control cells (Figure 2D, immunoblot 2C). Moreover, IL-1β secretion from oxLDL-macrophages was increased by 2-fold ($P<0.01$) compared to control cells (assessed using ELISA, Figure 2B).

Exposure of oxLDL-loaded macrophages to BE induced a dose-dependent decrease of the secreted CRP, MCP-1 and IL-1β, with a maximum effect at 5 μg mL^{-1} BE that reduced by 50% all the assessed inflammatory mediators (Figure 2B, 2D, immunoblot 2C). This result correlated well with the dose-dependent decrease of CRP and MCP-1 gene expression in oxLDL-loaded macrophages exposed to BE (with a maximum of ~50% at 5 μg mL^{-1} BE) (Figure 2A).

3.3 Bilberry extract increases apoE and CETP secretion from lipid-loaded macrophages

We evaluated the modulation exerted by BE upon the secretion of two proteins involved in the cholesterol efflux from lipid-loaded macrophages, apoE and CETP. Exposure of THP-1 macrophages to oxLDL induced a significant increase of apoE (2.8-fold, $P< 0.01$) and CETP (2.5-fold, $P< 0.01$) gene expression (Figure 3A)

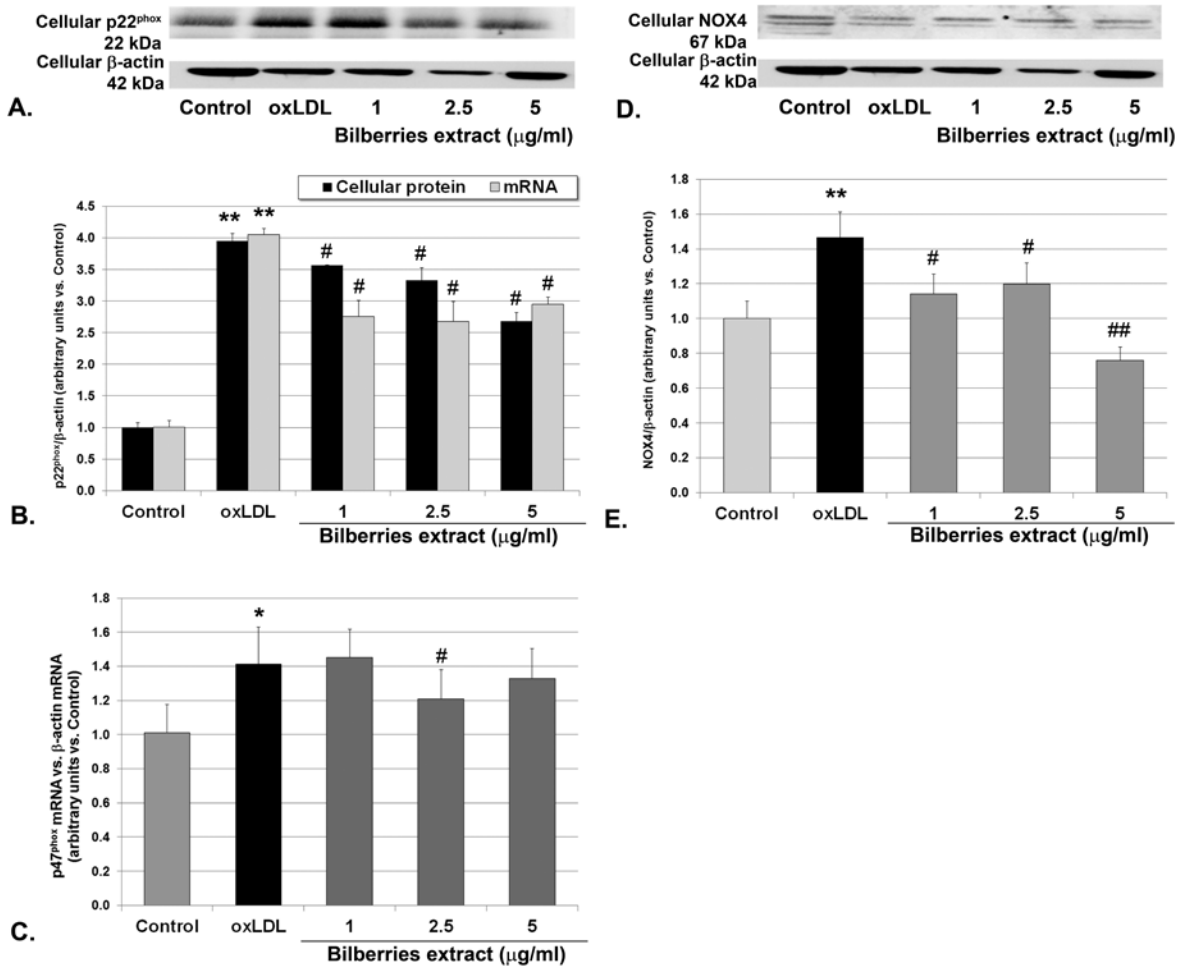

Figure 1. Gene and protein expression of NADPH oxidase subunits p22^{phox} (A-B), p47^{phox} (C) and NOX4 (D-E) in oxLDL-loaded macrophages following the incubation with bilberries extract (1-5 μg mL^{-1}). The gene expression of p22^{phox} and p47^{phox} was determined by real-time PCR and expressed relative to β-actin mRNA (B, C). The protein expression of p22^{phox} (A - representative immunoblot, B - histograms) and NOX4 (D - representative immunoblot, E - histograms) was assessed by Western blot and normalized to cellular β-actin. All data were normalized to Control values and the experiments were done in triplicate (*P< 0.05, **P< 0.01 *vs*. Control; #P< 0.05, ##P< 0.01 *vs*. oxLDL).

that correlated with an increased secretion of apoE (40%, P< 0.05) and CETP (60%, P< 0.01), as compared to control cells (Figure 3B, immunoblot 3D). Moreover, oxLDL induced a significant intracellular accumulation of cholesterol (by 2-fold, P<0.001) compared to control cells (Figure 3C).

Incubation of oxLDL-loaded macrophages with BE induced a dose-dependent increase of both apoE (2.5-fold, P< 0.01) and CETP (2-fold, P< 0.01) gene expression, with a maximum effect obtained for 5 μg mL^{-1} BE (Figure 3A). BE incubation with oxLDL-loaded macrophages stimulated a significant, dose-dependent increase of the secreted apoE (up to 50 %, P< 0.01 for 5 μg mL^{-1} BE) and CETP (up to 40 %, P< 0.05 for 5 μg mL^{-1} BE) as revealed by Western blot analysis of the culture media (Figure 3B, immunoblot 3D). This effect was associated with a similar dose-dependent increase of apoE and CETP mRNA levels in oxLDL-loaded cells, with a maximum for 5 μg mL^{-1} BE (Figure 3A). Addition of BE also induced a dose-dependent reduction of intracellular cholesterol concentration of lipid-loaded macrophages with a maximum for 5 μg mL^{-1} BE (Figure 3C).

3.4 Bilberry extract modulates both NF-κB and PKA signaling pathways in oxLDL-loaded macrophages

We searched for the molecular mechanisms by which BE induces the anti-oxidant and anti-inflammatory effects, as well as the increase of apoE and CETP secretion from oxLDL-loaded macrophages. To do this we evaluated the phosphorylation levels of p65 subunit of NF-κB and

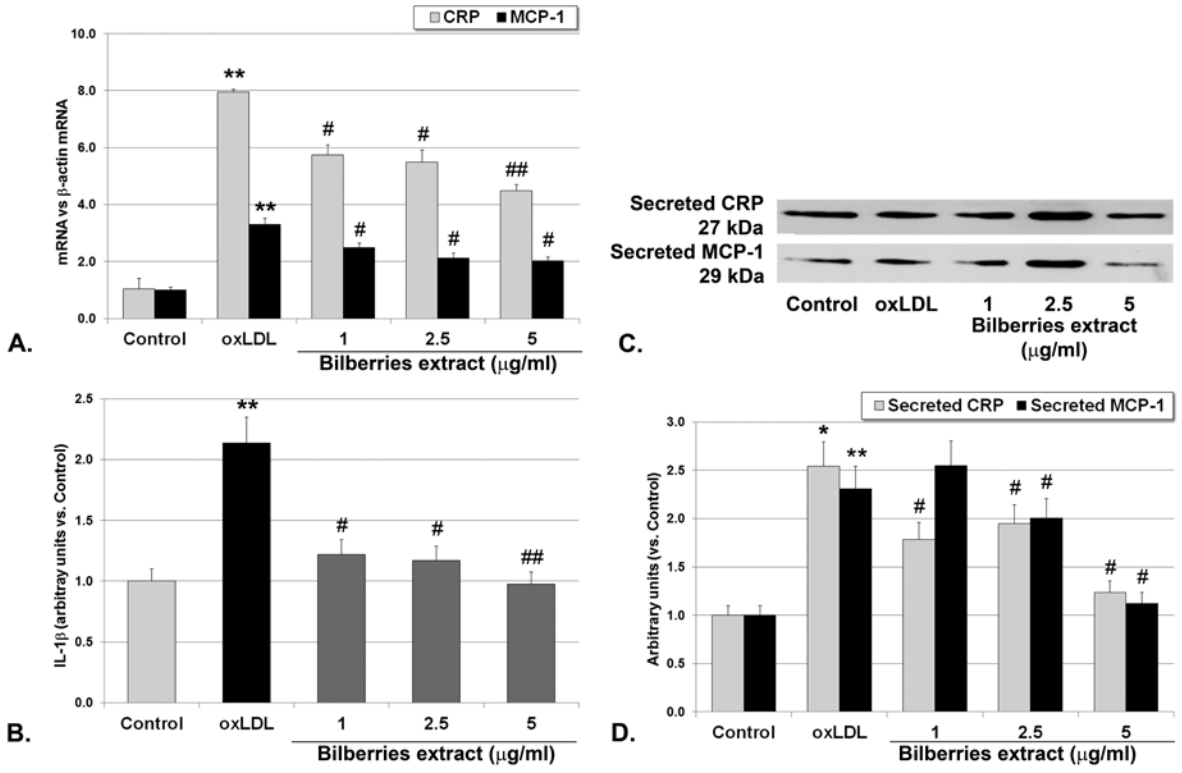

Figure 2. Gene expression and secretion of the inflammatory mediators CRP, MCP-1 and IL-1β in oxLDL-loaded macrophages following the incubation with bilberries extract (1-5 μg mL^{-1}). The gene expression of CRP and MCP-1 was evaluated by real-time PCR and expressed relative to β-actin mRNA (A). Secreted levels of CRP and MCP-1 were assessed by Western blot and normalized to total cellular protein (C – representative immunoblot, D - histograms). Secreted IL-1β was measured by ELISA technique and the resulting values are expressed as ng/total cellular protein (B). All data were normalized to Control values and the experiments were done in triplicate (*P< 0.05, **P< 0.01 *vs*. Control; #P< 0.05, ##P< 0.01 *vs*. oxLDL).

of γ-subunit of PKA in oxLDL-loaded macrophages. We found a dose-dependent decrease of the phospho-p65/total-p65 ratio in oxLDL-loaded macrophages incubated with BE, a decrease equivalent with the inhibition of NF-κB-mediated signaling pathway which reached a maximum at 5 μg mL^{-1} BE (Figure 4A immunoblot, 4B). In contrast with NF-κB signaling, the PKA signaling pathway was activated in a dose-dependent manner in BE-exposed lipid-loaded macrophages, as expressed by the increase in the phosphorylation levels of the PKA γ-subunit. The increase in phospho-PKA-γ/total-PKA-γ ratio had a maximum at 5 μg mL^{-1}, similar to the maximum inhibition observed for the NF-κB pathway (Figure 4A immunoblot, 4B).

4. Discussion

In this study we have demonstrated that BE exerts its effect in oxLDL-loaded macrophages in a dose-dependent manner by: (i) reducing the expression of NADPH oxidase subunits, p22^{phox}, NOX4 and p47^{phox}, (ii) diminishing the secretion of inflammatory molecules, CRP, MCP-1 and IL-1β, and (iii) stimulating the cholesterol efflux expressed by the increase of secreted apoE and CETP and by the decrease of cellular cholesterol accumulation. Moreover, we hypothesize that these anti-atherosclerotic effects produced by BE might be induced by the modulation of NF-κB and PKA signaling pathways in parallel with other pathways (like LXR's). Polyphenols, including flavonoids, and especially anthocyanins, are the main components of bilberries [19]. The anthocyanin-rich BE has been used as a pharmaceutical product and popular treatment for vision health problems worldwide [20,21].

Oxidative stress is a major contributor to the etiology of all severe vascular pathologies, including atherosclerosis. The inhibition of the oxidative stress is considered an important therapeutic approach and many efforts have been made to identify the antioxidant potential of various compounds, including medicinal plants, vitamins, plant polyphenols or probiotics [22]. Unfortunately, all tested antioxidants to date have

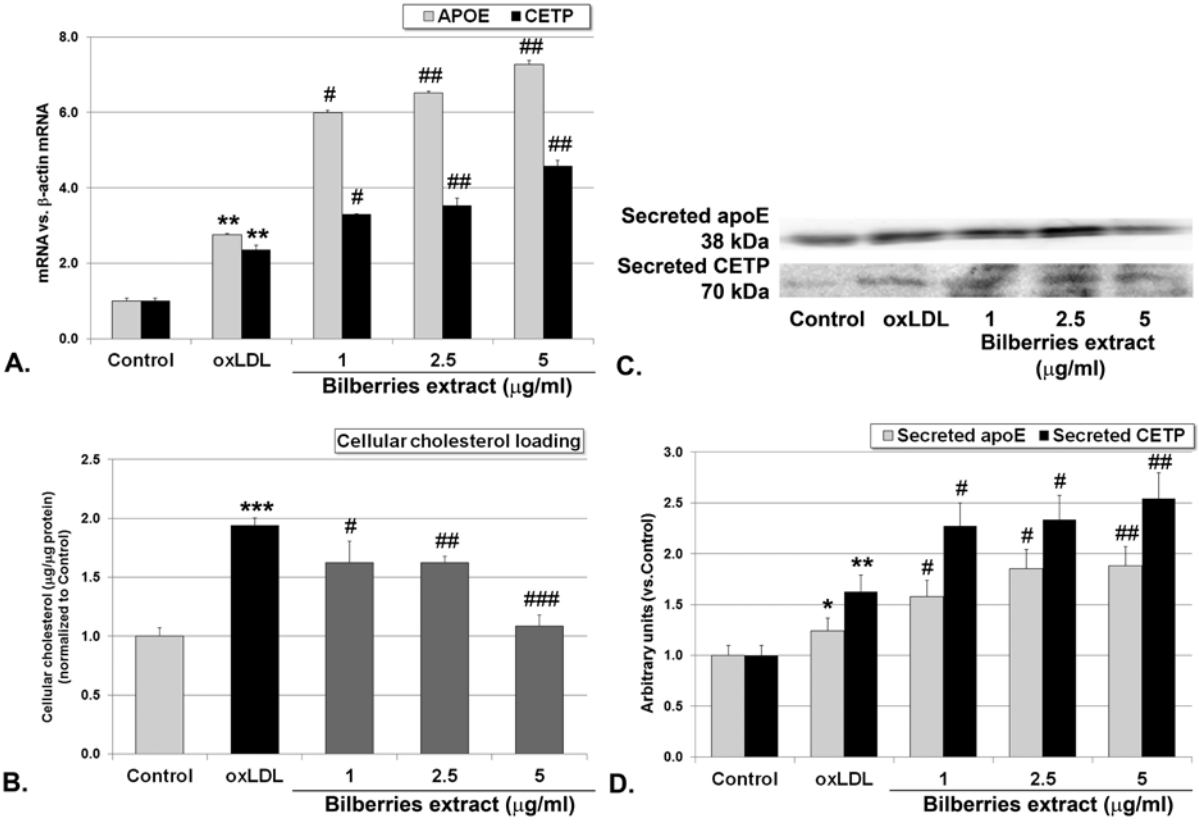

Figure 3. Gene expression and secretion of apoE and CETP and cellular cholesterol loading in oxLDL-loaded macrophages following the incubation with the bilberries extract (1-5 μg mL^{-1}). ApoE and CETP mRNA were determined by real-time PCR and expressed relative to β-actin mRNA (A). The levels of secreted apoE and CETP were assayed in concentrated media by Western blot and normalized to total cellular protein (C – representative immunoblot, D - histograms). Cellular cholesterol concentration was measured in cell lysates after lipid extraction in organic solvents and expressed as μg cholesterol to μg cellular protein (B). All data were normalized to Control values and the experiments were done in triplicate (*P<0.05, **P< 0.01, ***P< 0.001 *vs*. Control; #P< 0.05, ##P< 0.01, ###P< 0.001 *vs*. oxLDL).

failed to show a significant anti-atherosclerotic effect in humans [23].

NADPH oxidases (Nox) are a complex of multicomponent enzymes that have as their unique function the generation of ROS in the cells of the vascular wall or in circulating immune cells [24]. We have demonstrated that the antioxidant effect of BE is exerted through the reduced expression of two main Nox subunits, p22^{phox} and NOX4, in oxLDL-loaded macrophages. The p22^{phox} subunit is essential for Nox activity and it is known that p22^{phox} is more abundant in the advanced atherosclerosis plaque than in non-atherosclerotic areas, suggesting a correlation between p22^{phox} expression, superoxide production and the severity of atherosclerotic lesions [25]. Recent studies report that NF-kB regulates p22^{phox}, NOX4 and p47^{phox} gene expression in human aortic smooth muscle cells [26]. Published data show that oxLDL, but not native LDL, upregulate the expression of NOX4/p22^{phox} in human macrophages, mediated by MEK1/ERK pathway, upstream messengers of NF-κB signaling, but the upstream components of this signaling mechanism in macrophages have not yet been clearly identified [5]. Our experiments showed that BE inhibits NF-κB in oxLDL-loaded macrophages, a signaling pathway responsible for the inhibition of p22^{phox} and NOX4 expression, similar to the one described in human aortic smooth muscle cells [4]. Unlike the other subunits of Nox complex, NOX1 and NOX2, which demand regulatory subunits for their activation, NOX4 generates ROS constitutively, and changes in its levels may directly affect Nox activity [26]. Therefore, the regulation of NOX4 expression by BE represents an important modulation in human lipid-loaded macrophages.

BE has been found to inhibit the pro-inflammatory cytokine production in humans [1]. Exposure of human microglial cells in culture to BE has also been shown to reduce lipopolysaccharides-induced pro-inflammatory

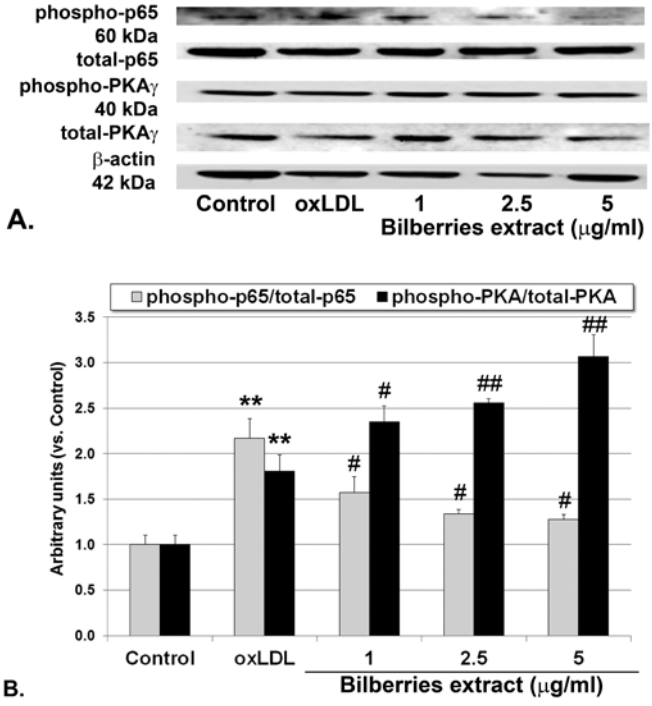

Figure 4. The signaling pathways modulated by the bilberries extract (1-5 μg mL^{-1}) in oxLDL-loaded macrophages. The phosphorylation levels of NF-κB p65 subunit and PKA were assessed by Western blot. Modulation of the signaling pathways was evaluated by using monoclonal and polyclonal antibodies to phospho- and total-p65, and respectively, phospho- and total-PKA-γ (A – representative immunoblot, B – histograms). Data were expressed as the ratio of phospho/total- p65 and PKA and normalized to Control cells and the experiments were done in triplicate (**P<0.01 *vs*. Control; #P< 0.05, ##P< 0.01 *vs*. oxLDL).

mediators, such as tumor necrosis factor (TNF)-α and IL-1β [27]. To our knowledge, we demonstrate for the first time that BE reduces in a dose-dependent manner the production of the pro-inflammatory mediators, CRP, MCP-1 and IL-1β from lipid-loaded macrophages. The data are in good agreement with a recent study showing that the consumption of bilberry juice modulates the plasma concentration of NF-κB related inflammatory markers, like CRP, IL-6, IL-15, and monokine induced by interferon-γ (MIG) in human subjects at risk for cardiovascular disease [28].

Cholesterol removal from lipid-loaded macrophages requires the activation of molecular mechanisms that stimulate the movement of cholesterol from cellular pools to the cell membrane, together with the efflux pathways through increased expression, stability and cell surface localization of the scavenger receptor (SR)-BI, the transporter ABCA1 and the secretion of apoE [29]. To date, there are no data indicating that BE can modulate cholesterol efflux from lipid-loaded macrophages. We demonstrate that BE induces a significant dose-dependent increase of secreted apoE associated with CETP secretion from lipid-loaded macrophages, a process that might be regulated by the inhibition of NF-κB and stimulation of PKA signaling in a dose-dependent manner. This effect is similar to the one demonstrated for lipid-free apoA-I and HDL_3 that stimulate both apoE and CETP secretion from lipid-loaded macrophages by the modulation of NF-κB and PKA signaling pathways [10,13,30].

In conclusion, our study supports the therapeutic administration of BE to decrease oxidative and inflammatory stress by a molecular mechanism that might be also regulated by NF-κB and PKA signaling pathways in lipid-loaded macrophages.

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Conflict of interest

The authors declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

References

[1] Zafra-Stone S., Yasmin T., Bagchi M., Chatterjee A., Vinson J.A., Bagchi D., Berry anthocyanins as novel antioxidants in human health and disease prevention, Mol. Nutr. Food Res., 2007, 51, 675-683

[2] Kaplan M., Aviram M., Oxidized low density lipoprotein: atherogenic and proinflammatory characteristics during macrophage foam cell formation. An inhibitory role for nutritional antioxidants and serum paraoxonase, Clin. Chem. Lab. Med., 1999, 37, 777-787

[3] Seimon T., Tabas I., Mechanisms and consequences of macrophage apoptosis in atherosclerosis, J. Lipid Res., 2009, 50 Suppl, S382-387

[4] Sima A.V., Botez G.M., Stancu C.S., Manea A., Raicu M., Simionescu M., Effect of irreversibly glycated LDL in human vascular smooth muscle cells: lipid loading, oxidative and inflammatory stress, J. Cell. Mol. Med., 2010, 14, 2790-2802

[5] Lee C.F., Qiao M., Schroder K., Zhao Q., Asmis R., Nox4 is a novel inducible source of reactive oxygen species in monocytes and macrophages and mediates oxidized low density lipoprotein-induced macrophage death, Circ. Res., 2010, 106, 1489-1497

[6] Bae Y.S., Lee J.H., Choi S.H., Kim S., Almazan F., Witztum J.L., et al., Macrophages generate reactive oxygen species in response to minimally oxidized low-density lipoprotein: toll-like receptor 4- and spleen tyrosine kinase-dependent activation of NADPH oxidase 2, Circ. Res., 2009, 104, 210-218

[7] McLaren J.E., Michael D.R., Ashlin T.G., Ramji D.P., Cytokines, macrophage lipid metabolism and foam cells: implications for cardiovascular disease therapy, Prog. Lipid Res., 2011, 50, 331-347

[8] Bunting K., Rao S., Hardy K., Woltring D., Denyer G.S., Wang J., et al., Genome-wide analysis of gene expression in T cells to identify targets of the NF-kappa B transcription factor c-Rel, J. Immunol., 2007, 178, 7097-7109

[9] Libby P., Inflammation in atherosclerosis, Arterioscler. Thromb. Vasc. Biol., 2012, 32, 2045-2051

[10] Kockx M., Jessup W., Kritharides L., Regulation of endogenous apolipoprotein E secretion by macrophages, Arterioscler. Thromb. Vasc. Biol., 2008, 28, 1060-1067

[11] Barter P.J., Brewer H.B. Jr., Chapman M.J., Hennekens C.H., Rader D.J., Tall A.R., Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis, Arterioscler. Thromb. Vasc. Biol., 2003, 23, 160-167

[12] Lakomy D., Rebe C., Sberna A.L., Masson D., Gautier T., Chevriaux A., et al., Liver X receptor-mediated induction of cholesteryl ester transfer protein expression is selectively impaired in inflammatory macrophages, Arterioscler. Thromb. Vasc. Biol., 2009, 29, 1923-1929

[13] Niculescu L.S., Robciuc M.R., Sanda G.M., Sima A.V., Apolipoprotein A-I stimulates cholesteryl ester transfer protein and apolipoprotein E secretion from lipid-loaded macrophages; the role of NF-kappaB and PKA signaling pathways, Biochem. Biophys. Res. Commun., 2011, 415, 497-502

[14] Mauray A., Felgines C., Morand C., Mazur A., Scalbert A., Milenkovic D., Nutrigenomic analysis of the protective effects of bilberry anthocyanin-rich extract in apo E-deficient mice, Genes Nutr., 2010, 5, 343-353

[15] Laplaud P.M., Lelubre A., Chapman M.J., Antioxidant action of Vaccinium myrtillus extract on human low density lipoproteins in vitro: initial observations, Fundam. Clin. Pharmacol., 1997, 11, 35-40

[16] Mauray A., Felgines C., Morand C., Mazur A., Scalbert A., Milenkovic D., Bilberry anthocyanin-rich extract alters expression of genes related to atherosclerosis development in aorta of apo E-deficient mice, Nutr. Metab. Cardiovasc. Dis., 2012, 22, 72-80

[17] Livak K.J., Schmittgen T.D., Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods, 2001, 25, 402-408

[18] Bligh E.G., Dyer W.J., A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol., 1959, 37, 911-917

[19] Faria A., Oliveira J., Neves P., Gameiro P., Santos-Buelga C., de Freitas V., et al., Antioxidant properties of prepared blueberry (Vaccinium myrtillus) extracts, J. Agric. Food Chem., 2005, 53, 6896-6902

[20] Bagchi D., Sen C.K., Bagchi M., Atalay M., Anti-angiogenic, antioxidant, and anti-carcinogenic properties of a novel anthocyanin-rich berry extract formula, Biochemistry (Mosc.), 2004, 69, 75-80

[21] Miyake S., Takahashi N., Sasaki M., Kobayashi S., Tsubota K., Ozawa Y., Vision preservation during retinal inflammation by anthocyanin-rich bilberry extract: cellular and molecular mechanism, Lab. Invest., 2012, 92, 102-109

[22] Ozkan O.V., Yuzbasioglu M.F., Ciralik H., Kurutas E.B., Yonden Z., Aydin M., et al., Resveratrol, a natural antioxidant, attenuates intestinal ischemia/reperfusion injury in rats, Tohoku J. Exp. Med., 2009, 218, 251-258

[23] Reiner Z., Catapano A.L., De Backer G., Graham I., Taskinen M.R., Wiklund O., et al., ESC/EAS Guidelines for the management of dyslipidaemias: the Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS), Eur. Heart J., 2011, 32, 1769-1818

[24] San Jose G., Bidegain J., Robador P.A., Diez J., Fortuno A., Zalba G., Insulin-induced NADPH oxidase activation promotes proliferation and matrix metalloproteinase activation in monocytes/macrophages, Free Radic. Biol. Med., 2009, 46, 1058-1067

[25] Manea A., Simionescu M., Nox enzymes and oxidative stress in atherosclerosis, Front. Biosci. (Schol Ed.), 2012, 4, 651-670

[26] Manea A., Tanase L.I., Raicu M., Simionescu M., Transcriptional regulation of NADPH oxidase isoforms, Nox1 and Nox4, by nuclear factor-kappaB in human aortic smooth muscle cells, Biochem. Biophys. Res. Commun., 2010, 396, 901-907

[27] Lau F.C., Bielinski D.F., Joseph J.A., Inhibitory effects of blueberry extract on the production of inflammatory mediators in lipopolysaccharide-activated BV2 microglia, J. Neurosci. Res., 2007, 85, 1010-1017

[28] Karlsen A., Paur I., Bohn S.K., Sakhi A.K., Borge G.I., Serafini M., et al., Bilberry juice modulates plasma concentration of NF-kappaB related inflammatory markers in subjects at increased risk of CVD, Eur. J. Nutr., 2010, 49, 345-355

[29] Mulay V., Wood P., Rentero C., Enrich C., Grewal T., Signal transduction pathways provide opportunities to enhance HDL and apoAI-dependent reverse cholesterol transport, Curr. Pharm. Biotechnol., 2012, 13, 352-364

[30] Robciuc M.R., Metso J., Sima A., Ehnholm C., Jauhiainen M., Human apoA-I increases macrophage foam cell derived PLTP activity without affecting the PLTP mass, Lipids Health Dis., 2010, 9, 59