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Anti-inflammatory Effects of Proanthocyanidin-rich Cranberry Extract through the Suppression of NF-kB Pathway and Histone Acetylase in RAW 264.7 and Mouse Bone Marrow-derived Macrophages

Seok-Yeong Yu[1], Jungbae Oh[1], Justin S. Kim[1,2], Young-In Kwon[3], Emmanouil Apostolidis[4], Young-Cheul Kim[1,*]

[1]Department of Nutrition, University of Massachusetts, Amherst, MA 01003
[2]Division of Nutrition of Nutritional Sciences, University of Illinois, Urbana-Champaign, IL 61802
[3]Department of Food and Nutrition, Hannam University, Daejeon, 34054 Korea
[4]Department of Chemistry and Food Science, Framingham State University, Framingham, MA 01701
*Corresponding author: yckim@nutrition.umass.edu

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Abstract Obesity-mediated chronic inflammation promotes the progression of obesity to metabolic anti-inflammatory effect of cranberries by decreasing plasma inflammatory cytokines. However, its specific mechanisms of action remain unclear. The nuclear factor-κB (NF-κB) pathway in macrophages plays a critical role in regulating the expression of many inflammatory genes, and histone acetylation has been identified as a key epigenetic modification for the NF-κB p65-mediated inflammatory responses. The objective of the study was to investigate if proanthocyanidin (PAC)-rich cranberry extract (CBE) suppresses histone acetylation and NF-κB p65 activation in RAW 264.7 macrophages and mouse bone marrow-derived macrophages (BMDMs). Treatment with 5% and 15% PAC-containing CBEs markedly suppressed the expression of pro-inflammatory mediators (iNos, Cox-2, Tnfα, Mcp-1 and Il-6) in both RAW 264.7 macrophages and BMDMs stimulated with lipopolysaccharides (LPS). CBE significantly reduced LPS-induced phosphorylation of p65 in both cell types without changing total p65 expression levels. Moreover, 15% PAC-CBE increased the expression levels of histone deacetylase 3 (HDAC3) with a concomitant decrease in histone H4 acetylation levels. These results suggest that CBE increases HDAC3 protein expression with the subsequent inhibition of p65 phosphorylation to mediate anti-inflammatory effects in macrophages. Cranberries may serve as a dietary agent to attenuate chronic inflammation in patients with obesity and related complications.

Keywords: *cranberry extract, histone deacetylation, NF- κB activation, inflammation, RAW264.7 macrophage, mouse bone marrow-derived macrophage*

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1. Introduction

Chronic inflammation has been recognized as an important mediator for the progression of obesity to metabolic disorders such as insulin resistance, type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) [1,2,3]. Obesity-induced inflammation is primarily mediated by the accumulation of macrophages in adipose tissue (AT), which are mainly originated from bone marrow-derived macrophages (BMDMs) [2,4]. The recruitment and subsequent activation of BMDMs are a prominent source of pro-inflammatory cytokines that serve as a potential link between obesity and metabolic diseases [2,4]. Consistently, specific deletion of AT macrophages showed a striking loss of gene markers for macrophages and inflammation with improved dyslipidemia and insulin resistance in diet-induced obese mice [5]. Although anti-inflammatory agents such as nonsteroidal anti-inflammatory drugs (NSAIDs) are commercially available, clinical trials have raised safety concerns due to adverse effects related to the use of these drugs [6,7]. Increased consumption of certain fruits and vegetables rich in polyphenols has been shown to be safe and effective for managing chronic inflammatory conditions [8]. Thus, identifying food sources and bioactives with anti-inflammatory properties and potential mechanisms involved would be an important strategy to develop dietary interventions to prevent or treat obesity-related chronic inflammation and metabolic disorders.

The activation of nuclear factor-kappa B (NF-κB) signaling is a key molecular mechanism for inflammatory responses by inducing the expression of various pro-inflammatory genes such as inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNFα), monocyte chemoattractant protein-1 (MCP-1) in macrophages [9]. There are five proteins that compose NF-κB family, such as p65 (Rel-A), Rel-B, c-Rel, p50, and p52 [10]. The NF-κB p65 subunit has gained considerable attention as a major target protein for drug discovery to tackle chronic inflammation because of the disproportionate increase of p65-mediated transactivation of pro-inflammatory genes in obesity-linked metabolic diseases [10]. To induce pro-inflammatory genes, the NF-κB p65 is phosphorylated and translocated into the nucleus, followed by histone acetylation on the promoter regions of its target genes, which are constrained by histone deacetylation. This in turn allows p-p65 to bind to its specific element, regulating the expression of pro-inflammatory genes [11,12]. Among histone deacetylases (HDACs), HDAC 3 has been identified as a key regulator of histone deacetylation at inflammatory gene enhancers [11]. Therefore, suppressing p65 phosphorylation and/or interrupting histone acetylation during NF-κB activation would be a promising molecular target for preventing macrophage-induced inflammation in obesity and its related metabolic complications [13].

Cranberries (Vaccinium macrocarpon) are indigenous to North America and are high in micronutrients and polyphenols particularly proanthocyanidin (PAC) [14]. There are two types of PACs, A-type and B-type, based on the substitution pattern of their B-ring [15]. PACs in cranberry are predominantly composed of A-type, whereas other berries mainly contain B-type PACs [15]. The health benefits of cranberries to prevent or treat urinary tract infection are documented. Recent observations from clinical trials also suggest that increased consumption of cranberries attenuates inflammatory biomarkers, hyperlipidemia, and hyperglycemia in obese individuals [15,16]. Consistent with the observations, several lines of in vivo evidence indicate that cranberry extract (CBE) alleviates insulin resistance and inflammation in diet-induced obese mice [17,18]. Moreover, in vitro studies support the anti-inflammatory potentials of cranberries with the observations that CBEs inhibit the production of inflammatory cytokines from several immune cell lines including RAW264.7, U937, and THP-1 [19,20,21,22]. It was further revealed that CBE decreased NF-κB-dependent transcriptional activity in human T lymphocytes using luciferase reporter assay [20]. However, the underlying mechanisms by which CBE suppresses the transcriptional activity of NF-κB for inducing pro-inflammatory genes in macrophages have been still unclear.

Here we determined whether the anti-inflammatory effect of CBE is mediated by suppressing histone acetylation and NF-κB p65 phosphorylation, which are required for transactivation of pro-inflammatory genes in macrophages and whether such anti-inflammatory effects in macrophages are PAC-dependent using RAW 264.7 and primary mouse bone marrow-derived macrophages (BMDMs). The whole berry extract was used in this study rather than isolated and purified compounds given potential synergistic interactions between bioactive compounds from the crude extracts compared to single compounds in terms of bioavailability and immunomodulatory effects in vivo and, more importantly, a better representative of human consumption [23].

2. Materials & Methods

2.1. Materials

Cranberry extracts (CBEs) with two different PAC contents (5% and 15% PAC) were generously provided from Artemis International (Fort Wayne, IN, USA); The rest of the extracts was constituted with dietary sugars (60-70%). Further detailed information is available through the link (https://www.artemis-nutraceuticals.com/products/cranberry-extract/).
2-mercaptoethanol and lipopolysaccharide from E. Coli O111:B4 (LPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (high glucose, DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), penicillin/streptomycin, macrophage colony-stimulating factor (M-CSF) and Applied biosystem® SYBR® Green PCR Master Mix were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Cell Culture

RAW 264.7 cells (ATCC® TIB-71TM) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were seeded at the density of 1 x 10^5 cells/mL in 6-well plates and incubated overnight. Then, the cells were serum-starved for 1 hr and were subject to CBE-treatment. To obtain primary macrophage cells, bone marrow cells were obtained from femurs and tibias of 5-6 weeks old FVB mice and cultured in RPMI 1640 supplemented with 10% FBS, 0.05 μM 2-mercaptoethanol and 1% penicillin/streptomycin overnight. Next day, the floating bone marrow-derived macrophages (BMDMs) were collected, seeded in 6-well plates at the density of 0.2 x 10^6, and cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin in the presence of M-CSF at 10 ng/mL for 5 days. For treatments, both cell types were pretreated with CBE or a solvent, dimethyl sulfoxide (DMSO, 0.1%) for 1 hr, followed by 12-hr additional treatment with CBE in the presence of LPS (100 ng/mL). For western blot analysis, the cells were treated with CBE and LPS for 1-3 hrs. Unstimulated cells indicate the cells maintained without LPS but DMSO.

2.3. Nitric Oxide (NO-) Production from RAW 264.7 Macrophages

After 12-hr treatment with CBE in the presence of LPS, the cultured media were collected, mixed with Griess reagent (Sigma-Aldrich) at the ratio of 1:1 and incubated for 15 mins. The absorbance was then measured at 540 nm.

Table 1. The list of primer sequences used

Gene	Forward (5'-3')	Reverse (5'-3')
iNos	TACGCCTTCAACACCAAGG	CTTGTCACCACCAGCAGTAG
Cox2	GGCCATGGAGTGGACTTAAA	GATACACCTCTCCACCAATGAC
Tnfα	AAGCATGATCCGCGACGTG	ATAGGCACCGCCTGGAGTTC
Mcp-1	CTCACCTGCTGCTACTCATTC	ACTACAGCTTCTTTGGGACAC
Il-6	TGCCTTCTTGGGACTGATGC	TTCTGCAAGTGCATCATCGTT
18s	CGCTTCCTTACCTGGTTGAT	GAGCGACCAAAGGAACCATA

iNos, inducible nitric oxide synthase; **Cox2**, cyclooxygenase 2; **Tnfα**, tumor necrosis factor alpha; **Mcp-1**, monocyte chemoattractant protein-1; **Il-6**, interleukin 6.

2.4. Quantitative Real-time-PCR (qRT-PCR)

Total RNAs were isolated using TRIzolTM reagent (Thermo Fisher Scientific) by following the manufacturer's protocol, and cDNAs were synthesized using RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific). qRT-PCR was performed using ViiATM 7 RT-PCR system (Life technologies, Grand Island, NY) to analyze the mRNA expression levels of genes of interest. The target genes were normalized by β-actin or 18S gene expression, and the relative expression was calculated using the $2^{-(\Delta\Delta nd)}$. Primers used were mouse-specific and listed in Table 1.

2.5. Western Blotting

Cell lysates were prepared using RIPA buffer (Thermo Fisher Scientific) supplemented with a protease and phosphatase inhibitor tablet and deacetylase inhibitor cocktail (Thermo Fisher Scientific). For Western blot analysis, 20 μg of lysates was separated on 10% SDS polyacrylamide gel, transferred to PVDF membranes, and then incubated with primary antibody overnight at 4°C, followed by secondary antibody incubation for 1 to 1.5 hr at room temperature. Primary antibodies including p65, p-p65 (Ser536), acetylated histone H3 (Lys9/Lys14) and acetylated histone H4 (Lys8), acyl-H3 and acyl-H4, were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies to HDAC3 and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Then, the membrane was incubated in ClarityTM Western ECL Blotting substrate (Bio-Rad, Hercules, CA) for 5 min and exposed to X-ray film. The band intensities were determined using ImageJ software [National Institutes of Health (NIH), Bethesda, MD, US].

2.6. Statistics

The data were presented as the means ± S.D of three independent experiments (n=3). To evaluate statistically significance, unpaired t-test was used, and the differences were considered statistically significant at $P < 0.05$. All data were plotted and analyzed using SigmaPlot version 11.0 (SPSS, Chicago, IL, USA)

3. Results

3.1. CBE Decreases NO Production and Tnfα mRNA Expression in LPS-stimulated RAW 264.7 Macrophages

A pharmacokinetic study has reported that cranberry PAC showed 12% recovery from the plasma when healthy adults were given a single-dose of cranberry juice cocktail [23]. Given the estimated range of PACs in cranberry juices which is between 30 to 150 μg/mL [24], we assume that the circulating PACs from the juices would be between 3.6 and 18 μg/mL in humans. Therefore, we chose 5% PAC- and 15% PAC-CBEs at 50 and 100 μg/mL concentrations so as to yield PACs between 2.5 and 15 μg/mL in the current study. Furthermore, we found no cytotoxicity at 50 and 100 μg/mL concentrations of 5% PAC- and 15% PAC-CBEs on RAW 264.7 macrophages (data not shown). First, we investigated whether CBE suppresses macrophage inflammation and whether such anti-inflammatory effects of CBE are PAC-dependent. We treated cells with 5% PAC- and 15% PAC-CBEs at 50 and 100 μg/mL concentrations 1-hr before and also during the stimulation of RAW 264.7 macrophages with LPS for 12 hrs to measure NO production and TNFα mRNA expression. Compared to unstimulated RAW 264.7 macrophages (no LPS), LPS significantly increased NO production (Figure 1 A). In CBE-treated cells, both 5% PAC- and 15% PAC-CBEs considerably decreased LPS-induced NO production at 100 μg/mL concentration. Similar to NO production, LPS greatly upregulated the expression of Tnfα mRNA in RAW 264.7 macrophages when compared to unstimulated ones (Figure 1 B). Treatment of 5% or 15% PAC-CBEs at concentrations of 50 and 100 μg/mL significantly decreased LPS-induced upregulation of Tnfα mRNA level. 15% PAC-CBE treatment showed a stronger inhibitory effect on Tnfα mRNA expression when compared to 5% PAC-CBE. The inhibitory effects of CBE on NO production and Tnfα gene expression were PAC concentration-dependent and also in agreement with previous studies [19,20,21,22]. We selected 50 μg/mL of both 5% PAC- and 15% PAC-CBEs for the further experiments to address the anti-inflammatory effects on macrophages and potential molecular mechanisms involved.

3.2. CBE Mitigates LPS-induced Upregulation of Pro-inflammatory Mediators in RAW 264.7 Macrophages and BMDMs

Next, we investigated the anti-inflammatory effects of CBE on macrophages in RAW264.7 macrophage cell line as well as mouse BMDMs, which are the major source of tissue macrophages and inflammation in obesity and metabolic disorders [4,25] (Figure 2). LPS stimulation markedly upregulated the mRNA levels of pro-inflammatory mediators such as iNos, Cox-2, Tnfα, Mcp-1 and Il-6 in both RAW 264.7 macrophages and BMDMs compared to unstimulated cells (Figure 2 A-D).

Treatment of cells with 5% and 15% PAC-CBEs at a concentration of 50 μg/mL significantly inhibited LPS-induced upregulation of iNos, Cox-2, Tnfα, Mcp-1 and Il-6 in both cell types. The inhibitory effects of CBE on LPS-induced expression of pro-inflammatory mediators in RAW264.7 macrophages were statistically greater in the cells treated with 15% PAC-CBE than 5% PAC-CBE, whereas no statistically significant difference between CBE-treated groups was observed in BMDMs.

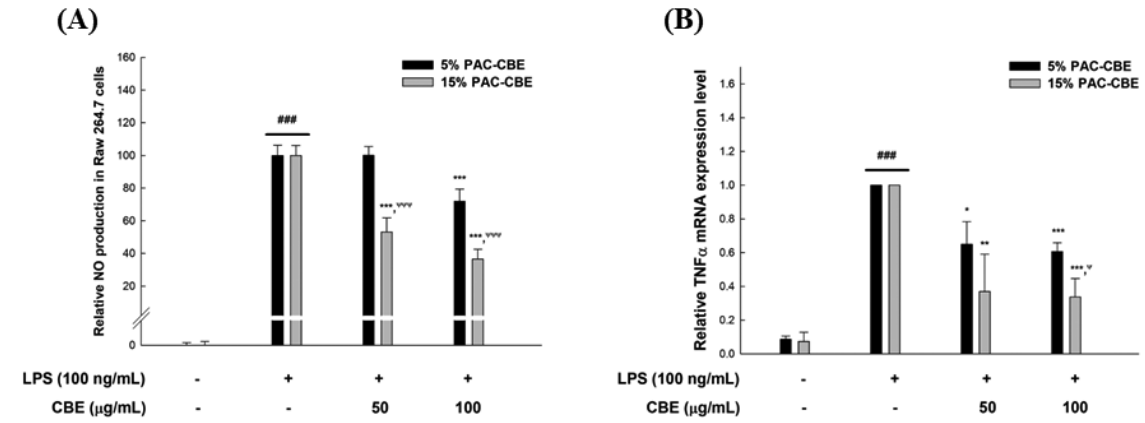

Figure 1. The effects of CBE with either 5% or 15% PAC on NO production and Tnfα expression in LPS-stimulated RAW 264.7 macrophages. Cells were preincubated with CBE for 1 hour and co-treated with LPS for 12 hours. The media were collected for NO production (A) and cells were harvested for RT-PCR (B). # P<0.05, ## P<0.01, ### P<0.001 as compared to control (unstimulated cells), * P<0.05, ** P<0.01, *** P<0.001 as compared to only LPS-stimulated cell. Ψ P<0.05, ΨΨ P<0.01, ΨΨΨ P<0.001 as compared to 5% PAC-CBE-treated cells. n = 3 of independent experiments, bars represent mean and S.D

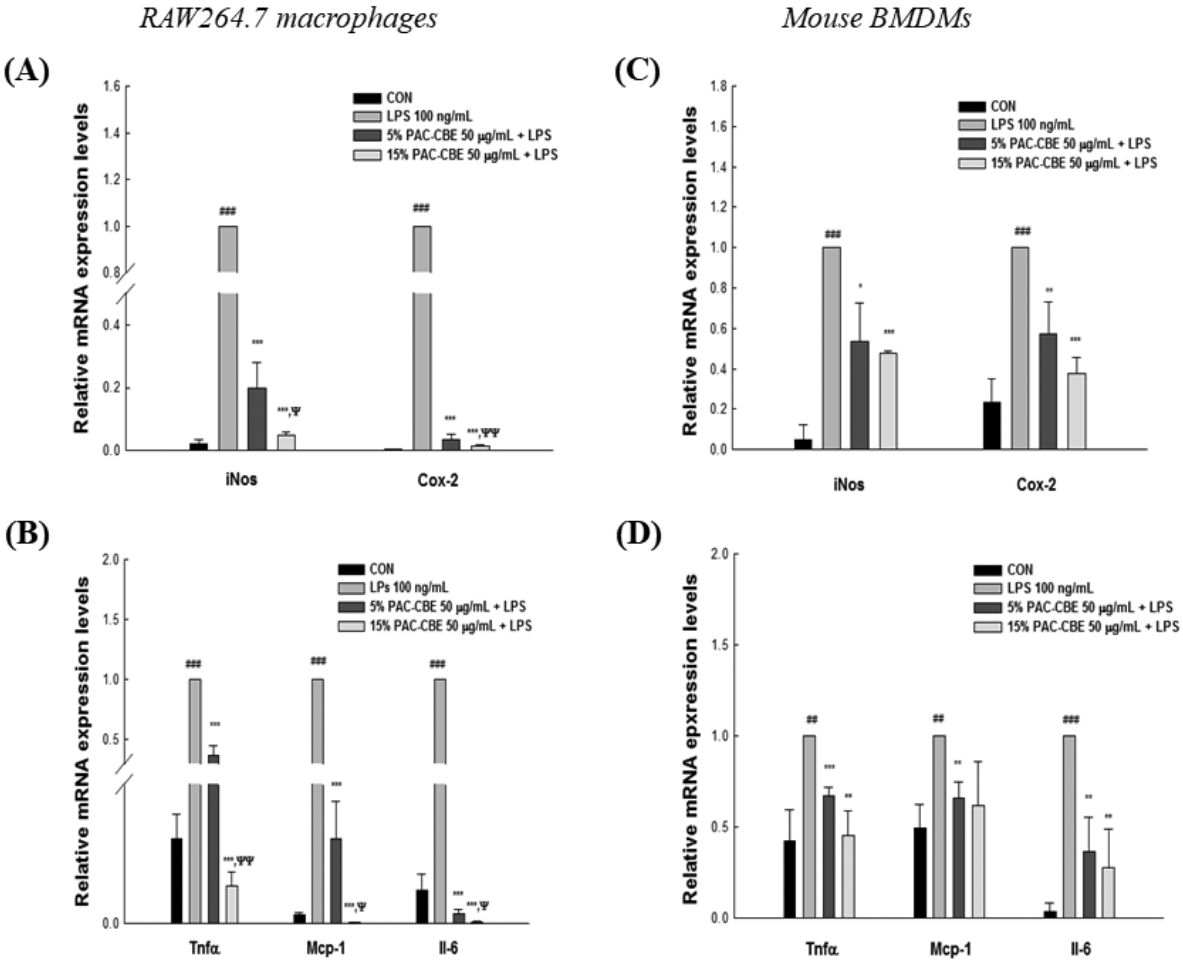

Figure 2. CBE inhibited LPS-induced expression of proinflammatory genes in RAW264.7 macrophages and mouse BMDMs. Cells were preincubated with CBEs for 1 hour and co-treated with LPS for 12 hours. The data were expressed as the fold change in gene expression normalized to a housekeeping gene (18S) and relative to the LPS-stimulated cells. Gene expression from RAW 264.7 macrophages (A & B) and mouse BMDMs (C & D). # P<0.05, ## P<0.01, ### P<0.001 as compared to control (unstimulated cells), * P<0.05, ** P<0.01, *** P<0.001 as compared to only LPS-stimulated cell. Ψ P<0.05, ΨΨ P<0.01, ΨΨΨ P<0.001 as compared to 5% PAC-CBE-treated cells. n = 3 of independent experiments, bars represent mean and S.D

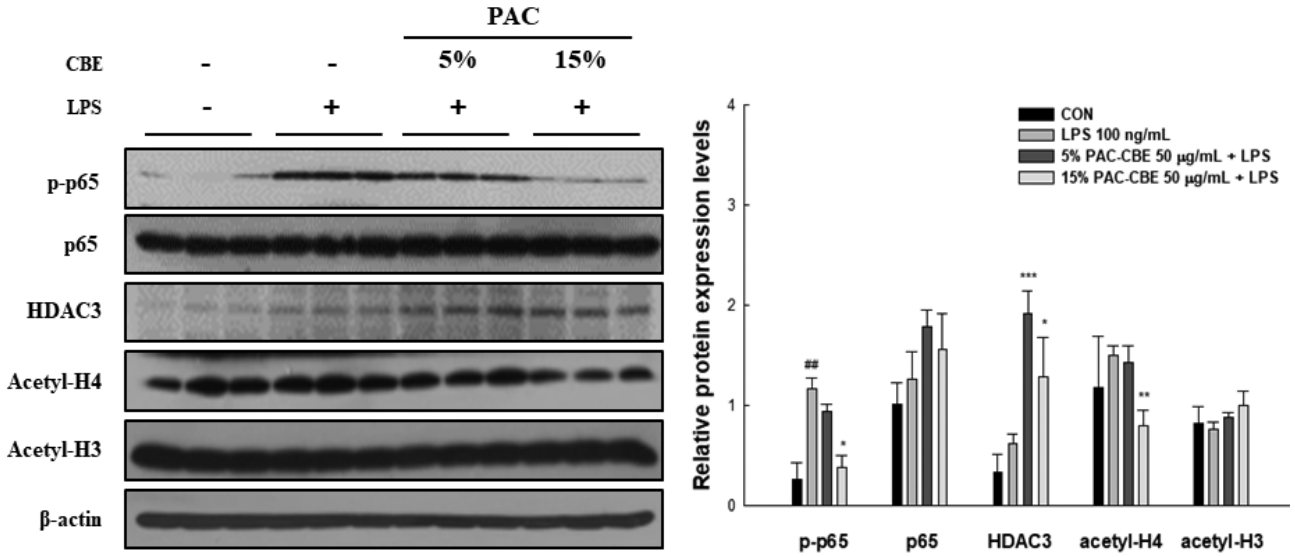

Figure 3. CBE inhibits NF-κB p65 phosphorylation and histone H4 acetylation while increasing HDAC3 expression in LPS-stimulated RAW264.7 macrophages. After preincubation with CBE for 1 hour, cells were co-treated with LPS for 1 hour, and proteins were obtained and analyzed with western blotting. Bands were quantified by normalizing to β-actin. n = 3 of independent experiments, bars represent mean and S.D. # P<0.05, ## P<0.01, ### P<0.001 as compared to control (unstimulated cells), * P<0.05, ** P<0.01, *** P<0.001 as compared to only LPS-stimulated cell

3.3. CBE Suppresses Phosphorylation of the NF-κB p65 and Acetylation on Histone H4 in RAW 264.7 Macrophages Stimulated with LPS

We next determined whether CBE suppresses the phosphorylation of NF-κB p65 and/or histone acetylation to downregulate the expression of pro-inflammatory mediators in LPS-stimulated macrophages (Figure 3). LPS significantly increased the phosphorylation of p65 without changing the protein expression of total p65 in RAW264.7 macrophages. On the other hand, CBE with both 5% and 15% PAC decreased the phosphorylation of p65 without affecting total p65 protein expression, but statistically significant difference was observed from 15% PAC-CBE. Moreover, while no differences in the levels of acyl-H3 and acyl-H4 as well as HDAC3 protein expression were observed between LPS-stimulated and unstimulated cells, both 5% and 15% PAC-CBEs significantly increased the protein expression levels of HDAC3 compared to LPS-stimulated RAW 264.7 macrophages. Further, treatment of 15% PAC-CBE showed a significant reduction in the acetylation levels of histone H4 protein, but not H3 protein.

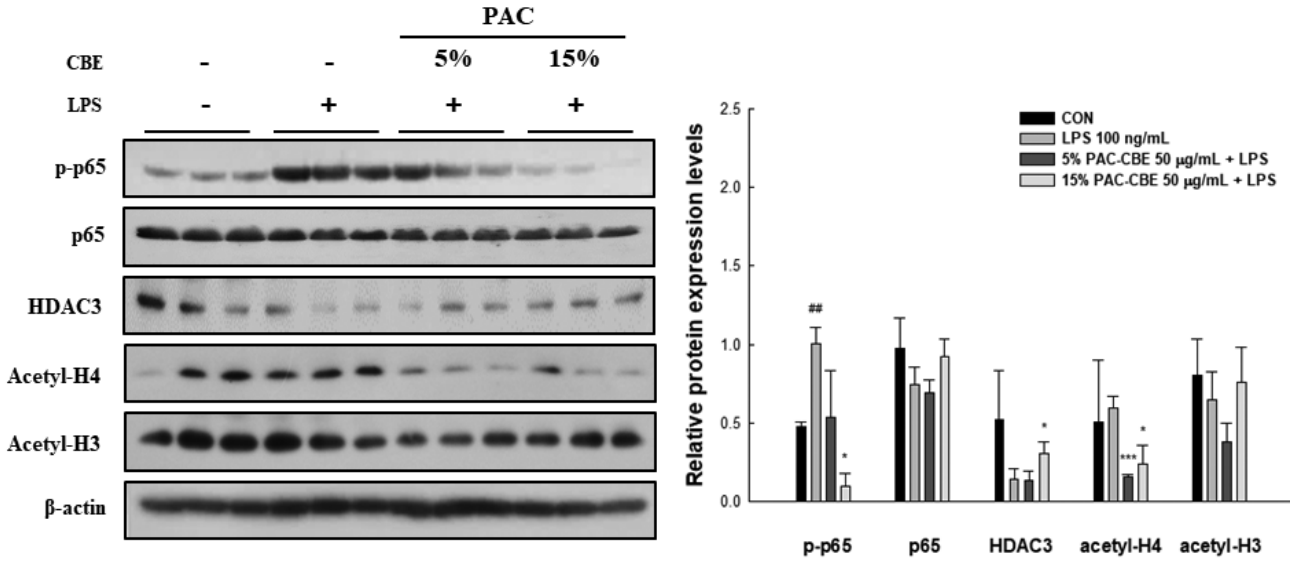

Figure 4. CBE suppresses p65 phosphorylation and histone H4 acetylation but increases HDAC3 expression in LPS-stimulated mouse BMDMs. After preincubation with CBEs for 1 hour, cells were co-treated with LPS for 3 hours, and proteins were obtained and analyzed with western blotting. Bands were quantified by normalizing to β-actin. n = 3 of male mice, bars represent mean and S.D. # P<0.05, ## P<0.01, ### P<0.001 as compared to control (unstimulated cells), * P<0.05, ** P<0.01, *** P<0.001 as compared to only LPS-stimulated cell

3.4. CBE Targets p65 Phosphorylation and Histone H4 Acetylation in Primary Mouse BMDMs Stimulated with LPS

To confirm that the anti-inflammatory effect of CBE was not limited to RAW 264.7 macrophage cell line, PAC-dependent effects on NF-κB p65, acyl-H3 and acyl-H4, and HDAC 3 protein abundance were carried out using mouse BMDMs. Similar to the observations in RAW 264.7 macrophages, we found that treatment of CBE with either 5% or 15% PAC greatly reduced the phosphorylation of p65 in LPS-stimulated BMDMs without altering p65 protein expression levels (Figure 4). The statistical significance on the inhibition of p65 phosphorylation was observed in the cells treated with 15% PAC-CBE. No differences in the acetylation levels of histone H3 and H4 and HDAC3 protein abundance between LPS-treated and unstimulated cells were observed. However, we found that CBE with either 5% or 15% PAC significantly decreased the acetylation levels of histone H4, but not H3, when compared to LPS-treated BMDMs, consistent with the observations made in RAW264.7 macrophages (Figure 3). Moreover, HDAC3 protein expression levels were significantly increased by 15% PAC-CBE treatment, suggesting that CBE inhibits histone acetylation via HDAC3 expression.

4. Discussion

Obesity-mediated chronic inflammation has been linked to increased incidence of and deaths from metabolic diseases such as T2DM and CVD [3]. The recruitment and the resultant activation of BMDMs in tissues are recognized as a key cause of obesity-induced inflammation [2,4]. Moreover, liposome-mediated deletion of visceral adipose macrophages significantly suppressed the expression of genes for lipogenesis and pro-inflammatory cytokines and concomitantly improved levels of serum lipids, glucose and insulin in diet-induced obese mice [5]. A body of evidence has also shown the anti-inflammatory effects of cranberries in obese individuals and animals [15,16,18]. Furthermore, in vitro studies using RAW264.7, U937, and THP-1 suggest that extracts from cranberries inhibit the production of pro-inflammatory cytokines by reducing the transcriptional activity of NF-κB. However, little has been appreciated regarding the underlying mechanisms by which cranberry extracts inhibits NF-κB transcriptional activity and the relevance of potential mechanisms in BMDMs. Here we found the anti-inflammatory effects of CBE on RAW 264.7 macrophages and primary mouse BMDMs, which are the major source of tissue macrophages and inflammation in obesity [2,25]. We also found that CBE significantly decreased LPS-induced upregulation of pro-inflammatory genes such as Tnfα, Mcp-1 and Il-6 by suppressing the NF-κB p65 phosphorylation in both RAW 264.7 macrophage cell line and mouse BMDMs. Consistent with our data, it was reported that cranberry extracts significantly decreased the secretion of pro-inflammatory cytokines such as IL-6 and TNFα from human peripheral mononuclear cells and inhibited NF-κB transactivation in human T lymphocytes [20]. Furthermore, it has been demonstrated that PACs isolated from cranberries dose-dependently suppressed the release of pro-inflammatory cytokines and inhibited NF-κB p65 transactivation in oral epithelial cells [26]. Therefore, these comparable and consistent observations support the anti-inflammatory properties of cranberries, indicating that cranberry-originated PAC targets NF-κB p65-mediated inflammatory responses in immune cells, including macrophages as supported by the current findings.

To the best of our knowledge, this is the first study that CBE increased the protein expression level of HDAC3 with a concomitant decrease in the acetylation level of histone H4, but not H3, in both RAW 264.7 macrophage cell line and mouse BMDMs. In quiescent state, it has been shown that the promoter regions of inflammatory genes are restrained by histone deacetylation, and HDAC3 is identified as a key regulator of the histone deacetylation in mouse BMDMs [11]. Upon activation, phosphorylated p65 subunit, which forms a heterodimer with p50 accompanies histone acetylation and binds to its element to induce the expression of pro-inflammatory genes [11,12]. In line with this, it was reported that trichostatin A (TSA), an inhibitor of HDAC, significantly promoted LPS-induced inflammatory responses in mouse BMDMs [27]. Given the epigenetic regulation of NF-κB p65 signaling, our findings may indicate that PAC-rich CBE increases the expression levels of HDAC3, resulting in reduced the acetylation of histone H4, which in turn prevent NF-κB p65-mediated inflammatory responses in tissue macrophages. The differential regulation of CBE-mediated histone acetylation on histone H3 and H4 may indicate that, as previously reported [28], the level of acetylation of histone H3 may not be as rapid as that of histone H4 in response to inflammatory stimuli. This is also consistent with the observation made by others [29], where LPS-induced inflammatory response in RAW264.7 macrophages was inhibited by veratric acid with concomitantly increased deacetylation of histone H4, but not H3. Therefore, the epigenetic regulation of HDAC3 by PAC-rich CBE promotes HDAC3 expression that may serve as an additional potential molecular mechanism for suppressing the NF-κB p65-dependent inflammatory responses in macrophages. When interpreting these findings, one should consider a limited bioavailability of the extracts as the cranberry PAC bioavailability has been reported approximately between 6 and 12 % in humans [23,30]. The CBE-PAC samples used in this study are crude extracts, some of which may be limited in the intestinal absorption. Therefore, further work needs to be done to identify primary bioactive compounds in cranberry PACs and their metabolites and also to study whether such bioactives would exert anti- inflammatory effects by modulating of the NF-κB p65 phosphorylation and histone acetylation in macrophages.

In conclusion, our current study demonstrated that PAC-rich CBE inhibits LPS-induced inflammatory responses in RAW264.7 macrophages and mouse BMDMs. Potential mechanisms are suggested to be through the suppression of the NF-κB p65 phosphorylation and acetylation levels of histone H4 with a concomitant increase in HDAC3 protein expression in

both cell types. Therefore, these findings warrant further investigations in preclinical and/or clinical in vivo models.

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Conflicts of Interest Statement

The authors declare that there is no conflicting interest in this manuscript.

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