Characterization of 3(3,4-dihydroxy-phenyl) propionic acid as a novel microbiome-derived epigenetic modifier in attenuation of immune inflammatory response in human monocytes

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Abstract

Recent studies suggest that microbiome derived 3(3,4-dihydroxy-phenyl) propionic acid (DHCA) attenuates IL-6 cytokine production through downregulation of the epigenetic modifier DNA Methyltransferase 1 (DNMT1) expression and inhibition of DNA methylation at the 5’—C—phosphate—G—3′ (CpG)-rich IL6 sequence introns 1 and 3 in a mouse model of depression. In this study, we extended the investigation of DHCA epigenetic mechanisms in IL-6 expression in human peripheral blood mononuclear cells (PBMC). Using Lucia Luciferase reporter gene system we identified CpG-rich sequences in which of methylation is influenced by DHCA similar to what observed in response to treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine. Correlation study showed that DNA methylation at select CpG motifs in the IL-6 promoter correlates with IL-6 gene expression. Our study suggests that DHCA is effective in reducing IL-6 expression in human PBMCs, in part, by regulation of methylation in the IL-6 promoter region.

Keywords
IL-6; inflammation; epigenetics; polyphenols; PBMC; 3(3,4-dihydroxy-phenyl) propionic acid (DHCA)

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CONFLICT OF INTEREST STATEMENT
The authors declare no conflicts of interest.

1AZA-DC = 5’-Aza-2’-deoxycytidine; BDPP = bioactive dietary polyphenol preparation; CpG = 5’-C-phosphate-G-3′; DHCA = 3(3,4-dihydroxy-phenyl) propionic acid; DNMT1 = DNA Methyltransferase 1; HPRT = Hypoxanthine phosphoribosyltransferase; IL-6 = Interleukin 6; MDD = Major depressive disorder; PBMC = Peripheral blood mononuclear cells
1.0 Introduction

Major depressive disorder (MDD) is a serious psychiatric disorder that is known to cause and exacerbate a multitude of pathologies including Atherosclerosis, Hypertension, Vessel occlusion, Dyslipidemia, Anxiety and Ventricular arrhythmias\(^1\). Additionally, neuroimaging studies have implicated MDD in the reduction of brain volume in certain areas of the striatum, amygdala, thalamus and the prefrontal cortex\(^2\). Currently, there are 241 million incidences of MDD in the world and it is the third leading cause of disability globally\(^3,4\). Despite its prevalence, effective treatments remain elusive\(^5,6\). Therefore, there is a need for the development of novel therapeutics. MDD is highly complex involving biological, psychological and social factors as such, it’s underlying disease mechanism still remains unidentified\(^7\). MDD has been associated with a chronic, low-grade inflammatory response which is thought to induce functional abnormalities in the brain that then lead to depression\(^8,9\). This inflammatory response is led by pro-inflammatory cytokines such as peripheral interleukin 6 (IL-6). Twenty-four studies have shown that increased IL-6 is associated with MDD\(^10\). Peripherally introduced IL-6 can alter brain function indicating that IL-6 is able to cross the bold-brain barrier\(^11\). Further evidence advocates that depression associated behavior is increased by the intracranial infusion of IL-6\(^12\). Taken together, there is strong evidence that suggests that pro-inflammatory cytokines such as IL-6 play a major role in MDD pathogenesis and should be explored as a target for novel therapeutic strategies to combat MDD.

While the role of inflammation in MDD has been known for quite some time, current antidepressant medications do not specifically treat MDD associated inflammation. Polyphenols have been found to suppress IL-6 and other pro-inflammatory cytokines\(^13\). Our group has identified a bioactive dietary polyphenol preparation (BDPP), comprised of Concord grape juice, grape seed extract and trans-resveratrol, that target a multitude of mechanisms associated with multiple neurological disorders\(^14–18\). We previously isolated a phytochemical, \(d3(3,4\text{-dihydroxy-phenyl})\) propionic acid (DHCA), derived from BDPP that had been microbiome metabolized, post-absorption. We found that DHCA attenuated IL-6 production via downregulation of DNMT1 expression and inhibition of DNA methylation at the CpG-rich IL-6 sequence introns 1 and 3 in a mouse model of depression\(^19\). In this study, we extended the investigation to humans. This study provides preclinical evidence that supports the DHCA mediated targeting of the MDD associated inflammation agent, pro-inflammatory cytokine IL-6, through epigenetic modification as a novel intervention for depression. The safety and efficacy of DHCA demonstrates that it can directly translate into human clinical studies for the treatment of MDD either alone or in combination with currently available antidepressants\(^19\).

2.0 Materials and Methods

2.1 Human blood collection and isolation of Peripheral blood mononuclear cells (PBMCs)

Blood from six healthy, age-matched male donors (age between 25 to 30) following informed consent (IRB #11–00866) was collected in heparin-coated tubes and PBMCs were isolated using Ficoll-Paque (GE Healthcare, Sweden). Briefly, blood samples were diluted with phosphate-buffered saline (PBS) and carefully laid over Ficoll-Paque and centrifuged
400g for 30 minutes at room temperature in a swinging bucket rotor without brake. The mononuclear cell layer at the interphase was carefully isolated and washed once with PBS at 300g for 10 minutes. The cell pellet was washed once more with PBS at 200g for 10 minutes to remove platelets.

2.2 PBMC stimulation by lipopolysaccharide (LPS)

Human PBMCs were seeded at 2x10⁶ cells per well in 6-well plate in culture medium RPMI-1640 (Sigma) supplemented with 20% horse serum, 10% FBS, 2.05 mM L-glutamine, 25 mM Hepes and 100U/ml penicillin/streptomycin. PBMCs were treated with a vehicle (PBS) or DHCA (final concentration 50μM) for 16 hours before stimulated with 100 ng/ml LPS for 24 hours. The cell pellets were washed once with ice-cold PBS and snap frozen for DNA and RNA.

2.3 Gene expression analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed using Superscript III® (Invitrogen). Gene expression was measured in 4 replicates by quantitative RT-PCR using Maxima SYBR Green master mix (Fermentas) in ABI Prism 7900HT. Mouse hypoxanthine phosphoribosyltransferase (HPRT) expression level was used as an internal control. Data were normalized using the 2⁻ΔΔCt method as previously described[20,21]. Levels of target gene mRNAs were expressed relative to those in control mice and plotted in GraphPad Prism.

2.4 DNA extraction

For human PBMCs, DNA and RNA were simultaneously extracted using the Zymo Duet DNA/RNA kit (Zymo, Irvine, CA) according to the manufacturer’s instructions. The DNA concentration was measured with Qubit fluorometer (Life technologies) and stored at −20°C until further processing.

2.5 Bisulfite-sequencing PCR

For BSP assays, 500 ng of genomic DNA from human PBMCs was bisulfite-treated with the Epitect Bisulfite kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Primers specific to 3 regions of the IL-6 promoter were then used to amplify bisulfite-converted DNA as described previously[22] and amplicons were PCR purified with Diffinity Rapid Tips (Diffinity Genomics, West Chester, PA). Purified DNA amplicons were then Sanger sequenced and data were analyzed using 4Peaks chromatogram software by taking the ratio of G/(G/A) peaks at target CpG sites to measure the bisulfite conversion rate and therefore methylation status (6 sites included). IL-6 BSP Primer sequences: For1: 5’-TATTATTTTGAGGGAAGGGTTTTT- 3’; Rev1: 5’-TACTTCCCCACCTACCATAACTCT- 3’; For2: 5’-TTTTTTAAGTGAGTTGAAGTGTGAT- 3’; Rev2: 5’-AAAAAAATTAAAACTAACATAAC- 3’; For3: 5’-CAAAAGTTGTGAGTTAATTAAAG- 3’; Rev3: 5’-TCATAACTAAACTCCCTAAAAAA- 3’.
2.6 Cloning and expression of CpG rich sequence from human IL-6 promoter

CpG-rich sequences from human IL-6 promoter P-1165/−965, P-697/−394 and P-366/−95 were cloned into the pCpGgree-basic Lucia, a Lucia® reporter plasmid without a promoter and devoid of CpG dinucleotides (InvivoGen) using In-Fusion Cloning Kit (Clontech Laboratories). Plasmids were prepared in E.coli GT115 cells and sequences were verified by Sanger DNA sequencing (GenScript). Plasmids were then expressed in N2A neuroblastoma cells using lipofectamine 2000 (ThermoFisher) according to the manufacturer’s instruction. pCpGfree-basic plasmids contain a multiple cloning site (MCS) upstream of the mSEAP or Lucia, a secreted luciferase, reporter gene was used to assess the expression vector CpG-free for promoter CpG methylation study pCpGfree-basic are reporter plasmids completely devoid of CpG dinucleotides. These plasmids lack the entire promoter region (compared to pCpGfree plasmids). Expression of reporter in cells transfected with this plasmid depends on the insertion of a functional promoter or enhancer/promoter cassette upstream from the reporter gene. Thus, pCpGfree-basic plasmids allow to study the effect of CpG methylation on a promoter, alone or combined with enhancer elements. Following overnight recovery, cells were subjected to 5 μM of methylation inhibitor 5′-Aza-2′-deoxycytidine (AZA-DC) treatment. Twenty-four hours later, 10 μl of medium was incubated with 50 μl of QUANTI-Luc luciferase substrate for luciferase activity assessment.

3.0 Results

3.1 Role of DHCA in modulating LPS-mediated IL-6 expression in human PBMCs

PBMCs from six healthy male subjects aged between 25 and 30 were isolated and treated with vehicle control (CTRL) or DHCA (50 μM) for 16 hours, according to our previously established protocols. PBMCs then were stimulated with LPS for 24 hours and cells were collected, and RNA was isolated for IL-6 gene expression analysis. We found that all 6 subjects had increased expression of IL-6 variants 1 and 2 following 24 hours LPS stimulation (Figs. 1A and 1B). Consistent with our previous observation conducted in rodent, DHCA treatment significantly reduced IL-6 variant 1 expression in all 6 healthy subjects and reduced IL-6 variant 2 expression in 5 subjects (Figs. 1A and 1B).

3.2 Role of LPS and DHCA in IL-6 promoter CpG methylation

Based on evidence that DNA methylation at a single CpG in the IL-6 promoter region effectively alters IL-6 gene expression, we next tested whether LPS or DHCA can modulate IL-6 gene expression through methylation mechanisms. Genomic DNA isolated from the human PBMCs was treated with bisulfate followed by bisulfate sequencing to determine methylation status at CpG dinucleotides. There are 19 CpG dinucleotides along the IL-6 promoter. Consistent with previous work, IL-6 promoter region CpG methylation of the 7 motifs between −1099 bp and −1001 bp had much higher levels of methylation in all samples (Fig. 2). Levels of methylation in the rest of the motifs were much lower (Fig. 2). Due to the large variability between individuals, we did not find any significant differences in methylation status in any of the motifs when comparing the three groups (Fig. 2).
3.3 Correlation of CpG methylation and IL-6 gene expression

To further dissect the relationship between promoter region CpG methylation and IL-6 expression, we conducted the correlation studies of methylation of individual CpG methylation and IL-6 gene expression. For IL-6 variant 1, we found that methylation of motif 16 was positively correlated with IL-6 RNA variant 1 expression (Supplementary Fig. 1). Examination of IL-6 variant 2 found that methylation of motif 11 was negatively correlated with IL-6 RNA variant 2 expression while methylations of motif 16 and 17 were positively correlated with IL-6 RNA variant 2 expression (Supplementary Fig. 2).

To test whether methylation of the CpG motif identified had functional relevance to IL-6 expression, we employed the CpG-free luciferase reporter system. We cloned the ~200 bp CpG rich DNA segment from the human IL-6 promoter region (P-1165/−965) that contains the first seven CpG motifs (Figs. 2 and 3) into the promoter-less CpG-free reporter construct and transfected the construct into cultured N2a cells. We found the CpG-rich IL-6 promoter segment presents no inherent promoter activity; no differences in luciferase activity were observed between cultured cells transfected with the IL-6 promoter-reporter luciferase construct and the control reporter (Fig. 4A–B), suggesting the CpG motifs in P-1165/−965 does not play any role in the promoter activity of IL-6. Next, we cloned the ~300 bp CpG rich DNA segment from the human IL-6 promoter region (P-697/−394) that contains the CpG motifs 7–14 (Figs. 2 and 3) into the promoter-less CpG-free reporter construct and transfected the construct into cultured N2a cells. We found that the luciferase activity was significantly lower than the control reporter, suggesting that methylation may reduce the promoter activity (Fig. 4B). This is consistent with our correlation study that methylation of CpG motif 11 negatively correlated with IL-6 expression (Supplementary Fig 1).

Using the same strategy, we cloned the ~270 bp CpG rich DNA segment from the human IL-6 promoter region (P-366/−95) that contains the CpG motifs 15–19 (Fig. 2) into the promoter-less CpG-free reporter construct and transfected the construct into cultured N2a cells. We found that the luciferase activity was significantly higher than the control reporter, suggesting the sequence may contain promoter activity and methylation may increase the expression of IL-6 (Fig. 4B).

To confirm whether methylation plays a role in the promoter activity in plasmid constructs containing P-697/−394 sequence and P-366/−95 sequence. We expressed the two constructs and control plasmid in the N2a cells. Twelve hours after transfection, we added AZA-DC, a DNA methylation inhibitor or DHCA into the cells. We found that neither AZA-DC nor DHCA significantly changes the luciferase activities (Fig. 4C) confirming the lack of methylation in the pCpG free-basic plasmid. We found that AZA-DC and DHCA treatment increased the luciferase activity in plasmid containing P-697/−394 sequence, however, it did not reach statistical significance (One-way ANOVA, P=0.075, Fig. 4D). Treatment of cells expressing plasmid containing P-366/−95 sequence with AZA-DC or DHCA significantly reduced the expression of the reporter gene (One-way ANOVA, P<0.0001, Fig. 4E), suggesting that the methylation of the CpG motif in the sequence can influence the expression. Interestingly, we also found significant differences in the expression of the reporter gene between the AZA-DC treated cells and the DHCA cells, suggesting DHCA
may influence the expression through methylation-dependent and methylation-independent mechanisms.

4.0 Discussion

Antidepressants are commonly prescribed to treat MDD. However, the treatment protocols are influenced by clinical practice and personal preferences rather than by a personalized medicine approach that targets the underlying cause. Unfortunately, despite the number of different antidepressants currently available, only a third of patients respond to treatment, and up to half of them relapse in less than a year. Inflammatory cytokines play a major role in MDD pathogenesis. Multiple studies have demonstrated that the exogenous administration of inflammatory cytokines induced the development of MDD symptoms. Therefore, this pathway is of interest for the development of novel therapeutics. While various inflammatory cytokines have been found to influence MDD, increased levels of IL-6 have been consistently identified to be implicated with MDD associated inflammation. MDD associated upregulation of IL-6 is influenced by monocytes. Monocytes regulate the innate and adaptive immune inflammatory responses. Circulating monocytes are divided into three major subsets based on the expression of the LPS receptor CD14 and the FcγRIII low-affinity IgG receptor CD16. Once activated by LPS, monocytes secrete cytokines, such as IL-6 resulting in inflammation and its associated pathogenesis.

Given that current antidepressant treatments have relatively low response rates coupled with the variety of adverse events there is a critical need for novel therapeutics to target the underlying pathologies of MDD. Phytochemicals have been of medicinal interest because of their strong antioxidant, antimicrobial, anti-inflammatory, and anti-cancer properties. Natural sources such as BDPP have been used for their medicinal properties throughout history. BDPP has been used successfully in the past to attenuate cognitive dysfunction in various animal models. Previous work by our laboratory identified the ability of DHCA to attenuate the release of pro-inflammatory cytokines, including IL-1β, IL-12, and IL-6, in vivo. We specifically identified epigenetic mechanisms by which the DHCA attenuates the release of IL-6 in response to stress. Here we have validated the use of DHCA, a polyphenol, derived from gut metabolized BDPP to lower IL-6 expression in human PBMCs upon LPS stimulation in part, through epigenetic mechanisms.

Polyphenols are bioactive compounds found in fruits and vegetables that influence their color, flavor, and medicinal properties. Polyphenols have been demonstrated to target many inflammatory components and lower inflammation through various mechanisms. Polyphenols regulate immunity by interfering with the regulation of immune cells, synthesis of pro-inflammatory cytokines, and gene expression. They are known to lower the production of reactive oxygen species by scavenging radicals and chelating metal ions.

Polyphenols inhibit the activity of cyclooxygenase, lipoxygenase, and nitric oxide synthase. Analogs of the polyphenol resveratrol inhibit Signal transducer and activator of transcription 3 (STAT3) which is involved with macrophage activation and the expression of IL-6 by inhibiting IKK and IκBα phosphorylation. Polyphenols also inhibit inflammation.
by disrupting signaling pathways such as, NF-κB signaling pathway, MAPK signaling pathway, and the arachidonic acid signaling pathway\textsuperscript{13}. In this study, we have demonstrated the efficacy of the polyphenol, DHCA to lower LPS induced upregulation of IL-6 gene expression in human PBMCs. We identified that the DNA methylation at CpG motifs, 11, 16, and 17 in the IL-6 promoter correlates with IL-6 gene expression. Further investigation of the IL-6 promoter region using pCpG free Lucia system identified the P-366/–95 CpG-rich sequence that has some promoter activity. DHCA induced inhibition of methylation of the P-366/–95 CpG-rich sequence significantly attenuated expression of the reporter gene. This suggests that DHCA is also effective in reducing IL-6 expression in human PBMCs, by regulation of methylation in the IL-6 promoter region. We found DHCA functions similar to and in certain cases better than the DNA methylation inhibitor AZA-DC.

Previous work by our lab has shown DHCA lowers expression of other inflammatory cytokines including IL-1β and IL-12 in vivo, both of which have been reported to be elevated in MDD subjects. However, it remains unclear whether DHCA mediated modulation of these cytokines is via the same epigenetic mechanisms as seen with IL-6\textsuperscript{19}. Polyphenols have been known to lower DNA methylation by inhibiting DNA methyltransferases and histone acetyltransferases\textsuperscript{39,40}. DHCA may use these same mechanisms to inhibit DNA methylation at the P-366/–95 CpG-rich sequence of IL-6. DHCA treated cells significantly lowered the expression of the reporter gene containing the P-366/–95 CpG-rich sequence when compared to AZA-DC treated cells suggesting that DHCA also acts via methylation-independent mechanisms mentioned previously, however, further investigation is required. In our previous work, we demonstrated that the use of DHCA in combination with the polyphenol malvidin-3′-O-glucoside (Mal-gluc) conferred resilience against the development of depression-like phenotypes in both mice models and human PMBCs\textsuperscript{19,41}. While further study is required to identify how DHCA influences IL-6 expression through methylation-dependent and methylation-independent mechanisms, here we have demonstrated the efficacy and basic mechanism by which DHCA lowers IL-6 expression. Furthermore, DHCA does not interfere with monoaminergic systems that are targeted by classical antidepressants and can, therefore, be developed as a novel therapeutic agent that can be a viable element in combination therapies targeting multiple mechanisms of MDD\textsuperscript{19}.

Collectively the present study suggests that DHCA can immediately translate into human clinical studies for the treatment of MDD either alone or in combination with currently available antidepressants.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

• 3(3,4-dihydroxy-phenyl) propionic acid (DHCA) has been previously shown to reduce IL-6 cytokine production in models of depression in vivo

• DHCA downregulates Il-6 through epigenetic mechanisms in human peripheral blood mononuclear cells (PBMC) by promoting methylation of CpG motifs, similarly to 5-aza-2’-deoxycytidine

• DNA methylation at select CpG motifs in the IL-6 promoter correlates with IL-6 gene expression

• DHCA is effective in reducing IL-6 expression in human PBMCs, in part, by regulation of methylation in the IL-6 promoter region
Fig. 1. Role of DHCA on LPS-induced IL-6 expression.
The effect of LPS and DHCA on IL 6 gene expression in PBMCs isolated from six age-matched, male healthy donors. The numbers expressed as percentage of individual controls.
Fig. 2. Role DHCA on methylation of CpG dinucleotides of IL-6 promoter.
DNA methylation status of the nineteen CpG dinucleotides in healthy human PBMC samples treated with vehicle control, LPS or LPS and DHCA. Data are shown as mean ± SEM, N=6, in triplicate.
Fig. 3. Human IL-6 promoter region and CpG motifs.

The nineteen CpG dinucleotides in the IL-6 promoter region are highlighted in red. Sequences P-1165/−965, P-697/−394, P-366/−95 enriched in CpG motifs that were cloned into the CpG-free Lucia plasmid for gene expression are highlighted in yellow.
Fig. 4. Examination of methylation of GpC-enriched motifs in IL-6 promoter region and the role of HDAC.

(A) Lucia CpG-free basic plasmid. (B) Luciferase activity in N2a cells transfected with CpG-free basic plasmids with CpG enriched sequences from IL-6 promoter P-1165/-965, P-697/-394 and P-366/-95. (C-E) Luciferase activity in the presence of vehicle, AZA and DHCA following in cells transfected with control and plasmid containing P-697/-394 and P-366/-95 sequences. Data are shown as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001. n=3–5 per assays in duplicate.