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ORIINAL ARTICLE

Inflammation Biomarker Response to Oral 2-Hydroxybenzylamine (2-HOBA) Acetate in Healthy Humans

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Abstract— Inflammation is associated with the formation of reactive oxygen species (ROS) and the formation of lipid-derived compounds, such as isolevuglandins (IsoLGs), malondialdehyde, 4-hydroxy-nonenal, and 4-oxo-nonenal. The most reactive of these are the IsoLGs, which form covalent adducts with lysine residues and other cellular primary amines leading to changes in protein function, immunogenicity, and epigenetic alterations and have been shown to contribute to a number of inflammatory diseases. 2-Hydroxybenzylamine (2-HOBA) is a natural compound found in buckwheat seeds and reacts with all IsoLG adducts preventing adduct formation with proteins and DNA. Therefore, 2-HOBA is well positioned as an agent for the prevention of inflammatory-prone diseases. In this study, we examined the potential beneficial effects of 2-HOBA on oxidative stress and inflammatory biomarkers in two cohorts of healthy younger and older adults. We utilized the Olink® targeted inflammation panel before and after an oral 15-day treatment regimen with 2-HOBA. We found significant relative changes in the plasma concentration of 15 immune proteins that may reflect the *in vivo* immune targets of 2-HOBA. Treatment of 2-HOBA resulted in significant increased levels of CCL19, IL-12β, IL-20Rα, and TNFβ, whereas levels of TWEAK significantly decreased. Ingenuity Pathway Analysis identified canonical pathways regulated by the differentially secreted cytokines, chemokines, and growth factors upon 2-HOBA treatment and further points to biofunctions related to the recruitment, attraction, and movement of different immune cell types. In conclusion, 2-HOBA significantly altered the protein biomarkers CCL19, IL-12β, IL-20Rα, TNFβ, and TWEAK, and these may be responsible for the protective effects of 2-HOBA against reactive electrophiles, such as IsoLGs, commonly expressed in conditions of excessive oxidative stress. 2-HOBA has a role as a IsoLG scavenger to proactively improve immune health in a variety of conditions.

KEY WORDS: electrophiles; reactive aldehyde; oxidative damage; humans; 2-hydroxybenzylamine.

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INTRODUCTION

Globally, chronic inflammatory diseases are a major health concern and are recognized as the most significant cause of death in the world today, with more than 50% of all deaths attributable to inflammation-related illnesses: ischemic heart disease, stroke, cancer, diabetes mellitus, chronic kidney disease, non-alcoholic fatty liver disease (NAFLD), and autoimmune and neurodegenerative conditions [1–3]. This health burden has put the spotlight on determining the underlying mechanisms for the pathologic progression of the inflammatory pathways to affecting morbidity and mortality [2, 4, 5].

Inflammation is characterized by the activation of immune and non-immune cells and is critical to protection from various infections such as viruses, bacteria, other pathogens, and toxins. Normally, inflammation is self-limiting, and its resolution is the first step in the repair and regeneration of injured tissues [6–8]. Failure of inflammation resolution can lead to the progression of several diseases including cardiovascular, metabolic (*e.g.*, diabetes, metabolic syndrome), chronic pulmonary, and musculoskeletal, kidney, or neurodegenerative diseases [3].

Both acute and chronic inflammation are associated with the formation of reactive oxygen species (ROS) and the formation of lipid-derived compounds, such as alkanes, aldehydes, furans, and most importantly highly reactive compounds termed dicarbonyl electrophiles [9]. This latter group includes isolevuglandins (IsoLGs) [10], malondialdehyde (MDA) [11], and methylglyoxal (MGO) [12]. The most reactive of these are IsoLGs which form covalent bonds with amine macromolecules present in nucleic acid bases [13, 14] and with lysine residues [15] forming irreversible covalent adducts, which trigger DNA damage, denature proteins, and lead to changes in cell signaling [16, 17] and epigenetic alterations [18]. These IsoLG-macromolecule adducts appear to be essential in the pathophysiology of inflammatory diseases [19] and lead to enhanced risks of the development of increased morbidity and mortality associated with chronic diseases [20–22].

2-Hydroxybenzylamine (2-HOBA) is a compound found in buckwheat seeds [23]. It is a gluten-free foodstuff that originated thousands of years ago in Southeast Asia and is currently cultivated worldwide. 2-HOBA has been shown to react with IsoLGs at a rate 2.5–3 orders of magnitude faster than IsoLGs react with lysine, thus preventing adduct formation with macromolecules [24, 25] such as proteins and DNA. We previously showed evidence in several *in vitro* and *in vivo* studies in mice, rats, and rabbits that 2-HOBA is not toxic or mutagenic [26–29]. Effectiveness of 2-HOBA has also been established in several preclinical models of oxidative damage including hypertension [21], atherosclerosis [30], systemic lupus erythematosus [31], pulmonary hypertension [32], gastric cancer [33], and Alzheimer's disease [20]. In addition, we have established its safety in two Phase I human clinical trials [34, 35]. Therefore, 2-HOBA is well positioned as a chemopreventive agent for the prevention of inflammatory-prone diseases. In this study, we examined the potential beneficial effects of 2-HOBA on oxidative stress and inflammatory biomarkers in two cohorts of healthy younger and older adults.

MATERIALS AND METHODS

Reagents

2-Hydroxybenzylamine acetate (CAS 1206675–01-5; 2-HOBA) was manufactured by TSI Group Ltd. (Shanghai, China). Hard gel capsules (Capsugel, Jiangsu, China) containing 250 mg of 2-HOBA acetate (corresponding to 168 mg 2-HOBA) were prepared by TSI Group Ltd. *In vitro* pharmacology [28] and first-in-human studies reporting safety, tolerability, and *in vivo* pharmacokinetics of 2-HOBA were recently described [34, 35].

Subjects and Sample Collection

The study was registered at Clinicaltrials.gov (multiple dose studies—NCT03555682 and NCT03556319). The Vanderbilt University Institutional Review Board approved all study protocols and study documents. Informed consent was obtained from all subjects before voluntary study participation. Two cohorts of participants were studied at the Vanderbilt University Medical Center (VUMC) Clinical Research Center (CRC) (Table 1). The first cohort contained healthy volunteers ($n = 7$ male and 10 non-pregnant female) between the ages of 18 and 59 years. These data were collected between August 2018 and May 2019; full details of these subjects were previously reported [35]. One volunteer was lost to follow-up. The second older adult cohort consisted of $n = 10$ men and $n = 6$ post-menopausal women between 60 and 75 years of age. These data were collected between March 2019 and July 2021; the delay in

Table 1 Subject Demographics

Variable	Younger cohort	Older cohort	Mann–Whitney test
N	17	16	
Age (yrs.)[a]	33.8 ± 12.6	66.1 ± 4.3	< 0.0001
Sex (male/female)	7/10	6/10	ns
Weight (kg)	69.2 ± 11.1	90.1 ± 18.7	< 0.001
Height (cm)	170.3 ± 11.1	168.6 ± 10.8	ns
BMI (kg/m^2)	23.8 ± 2.2	31.4 ± 5.6	< 0.00001
Race (*n*)			
Black/African American	3	2	
White	14	12	
Asian	0	1	
American Indian	0	1	
Ethnicity (*n*)			
Hispanic/Latino	2	1	
Not Hispanic/Latino	14	15	
Not reported	1	0	

[a]Mean ± SD

recruitment was adapted mid-study due to the COVID-19 pandemic. Two volunteers were lost to follow-up because of a COVID-19 shutdown.

Study Design and Sample Collection

The study was a double-blind, randomized, placebo-controlled, multiple dose escalation designed to assess the effects of 2-HOBA acetate on plasma inflammatory biomarkers in two separate cohorts. Nine participants were enrolled per dose level, including 6 randomized to 2-HOBA and 3 randomized to placebo in each cohort as previously described [34]. Randomization was performed by the study statistician; a computer-generated randomization sequence using the stratified permuted block randomization, with blocks of size 3, was used to assign participants at a 1:2 ratio to placebo or 2-HOBA for dose level. The Vanderbilt University Hospital Pharmacy provided all treatments, and all study staff were blinded to treatment assignments. The 2-HOBA acetate dose levels were 500 and 750 mg; these levels correspond to 336 and 504 mg 2-HOBA, respectively. 2-HOBA acetate was provided in 250-mg capsules; the placebo was identical in appearance and physical properties but contained no 2-HOBA acetate. These doses and dose frequency (every 8 h) were designed to achieve peak plasma levels at steady state that corresponded to the peak plasma levels observed at the two highest doses as we previously reported [34]. Plasma samples for biomarker analyses were collected at baseline day 1 and after 14 days of HOBA acetate administration on day 15. The samples on day 15 were taken 120 min after the last 2-HOBA acetate dose.

Plasma Proteomic Biomarkers

A large panel with 92 inflammatory proteins (Olink®, Boston, MA) [38] was used to investigate potential biomarkers of inflammation in the pre- and post-2-HOBA supplementation periods. Plasma levels of proteins were analyzed by the Proximity Extension Assay (PEA) technique as described [39]. Data outputs were reported as normalized protein expression (NPX) values, Olink Proteomics' arbitrary unit on log2 scale.

Ingenuity Pathway Analysis

The fold change between the control and the 2-HOBA treatment groups was used as an input for the Ingenuity Pathway Analysis (IPA) (Qiagen, USA). The differential expression of cytokines, chemokines, and growth factors was examined via a core analysis in IPA

to deduce differentially regulated canonical pathways, upstream regulators, diseases and biofunctions, and novel gene networks based on the Fisher exact test (p-value cut off at 0.05). The comparison analysis module in IPA was used to deduce the differentially regulated canonical pathways and diseases and biofunctions in the experimental groups compared to the control. The heatmaps of differentially regulated canonical pathways and diseases and biofunctions related to the connective tissue disorders were generated using the IPA.

Statistics

GraphPad Prism 9.5 (San Diego, CA, USA) was used for data analysis. Plasma protein biomarkers using log2-NPX expression were analyzed by a 2×2 ANOVA including time and treatment as main effects. Pearson correlation was used to evaluate the daily 2-HOBA dose and inflammatory protein levels in younger and older adults. Milligram per kilogram body weight dosing was correlated with log2-transformed changes. Cohort demographics were compared using a Mann–Whitney test of significance. p-values < 0.05 were considered statistically significant.

RESULTS

Plasma samples from thirty-three subjects (Table 1) enrolled in a double-blind, randomized, placebo-controlled, multiple dose (500 mg or 750 mg) study of daily oral 2-HOBA acetate were profiled for biomarkers of systemic inflammation. Immune-protein profiles at baseline on day 0 and on day 15 taken 120 min after the last dose of 2-HOBA are shown in Supplemental Table 1. Fifteen markers, namely C–C motif chemokine 19 (CCL19), CUB domain–containing protein 1 (CDCP1), C-X-C motif chemokine 9 (CXCL9), Delta- and Notch-like EGFR receptor (DNER), eotaxin 1 (CCL11), fibroblast growth factor 5 (FGF-5), fibroblast growth factor 21 (FGF-21), interferon gamma (IFN-γ), interleukin 10 Rβ (IL-10Rβ), interleukin 12β (IL-12β), interleukin 20Rα (IL-20Rα), monocyte chemotactic protein 1 (MCP-1), stem cell factor (SCF), tumor necrosis factor beta (TNFβ, also known as lymphotoxin alpha), and tumor necrosis factor ligand superfamily, member 12 (TWEAK), showed significant dose by time interactions ($p \leq 0.05$).

Our previously published data [35] suggest the fixed, repeated, oral 2-HOBA dosing at 500 mg and 750 mg demonstrated similar systemic 2-HOBA exposure profiles; we next examined the dichotomous effects of oral 2-HOBA *versus* placebo on the temporal abundance changes in several immune proteins (Fig. 1). The levels of four biomarkers, namely CCL19, IL-12β, IL-20Rα, and TNFβ, were significantly increased after 2-HOBA but not with placebo treatment, whereas levels of TWEAK significantly decreased after 2-HOBA but not with placebo treatment. Only one biomarker, SCF, increased after control, but not 2-HOBA treatment (data not shown). Multiple comparison testing for CCL11, CDCP1, CXCL9, DNER, FDF-G, FGF-21, INF-γ, IL-10Rβ, and MCP-1 showed no significant differences in the levels of these immune biomarkers over the 15-day 2-HOBA treatment period (data not shown).

The effects of 2-HOBA dosing expressed per unit of body weight on immune biomarker abundance were further analyzed using regression analysis (Supplemental Fig. 1). Nine proteins, namely CCL11, CCL19, DNER, FGF-5, IL10-Rβ, MCP-1, SCF, TNFβ, and TWEAK, trended higher; four proteins, namely CXCL9, FGF21, IL-20Rα, and IFN-γ, trended lower; and 2 proteins (CDCP1 and IL-12Rβ) remained unchanged with increased 2-HOBA dosing; however, none of these responses was significant.

IPA core analysis was performed to understand the various canonical pathways regulated by the differentially secreted cytokines, chemokines, and growth factors upon 2-HOBA treatment (Fig. 2). A listing of the top 10 most enriched canonical pathways which changed with 2-HOBA treatment is shown in Fig. 2A. All mapped canonical pathways, p-values, and Z-scores are listed in Supplemental Table 2. Five enriched canonical pathways were identified by applying a $-\log(p\text{-value}) > 2$ threshold. The "pathogen induced cytokine storm signaling pathway" was the highest-ranking signaling pathway with a $-\log$ (p-value) of 21.5. Taking Z-score > 2 as the threshold of significant activation, we found significant activation of the "differential regulation of cytokines by IL-17α and IL-17F" (Z-score $= 2.449$) and "role of hypercytokinemia in the pathogenesis of influenza" (Z-score $= 2.236$).

A histogram of 10 biofunctions mediated by 2-HOBA is shown in Fig. 2B. The top categories ranked in accordance with their $-\log$ (p-value) are shown. The complete disease and function classifications are provided in Supplemental Table 3. Biofunctions related to the recruitment, attraction, and movement of different immune cell types including phagocytes, lymphocytes, myeloid cells, antigen-presenting cells, and dendritic cells were predominant

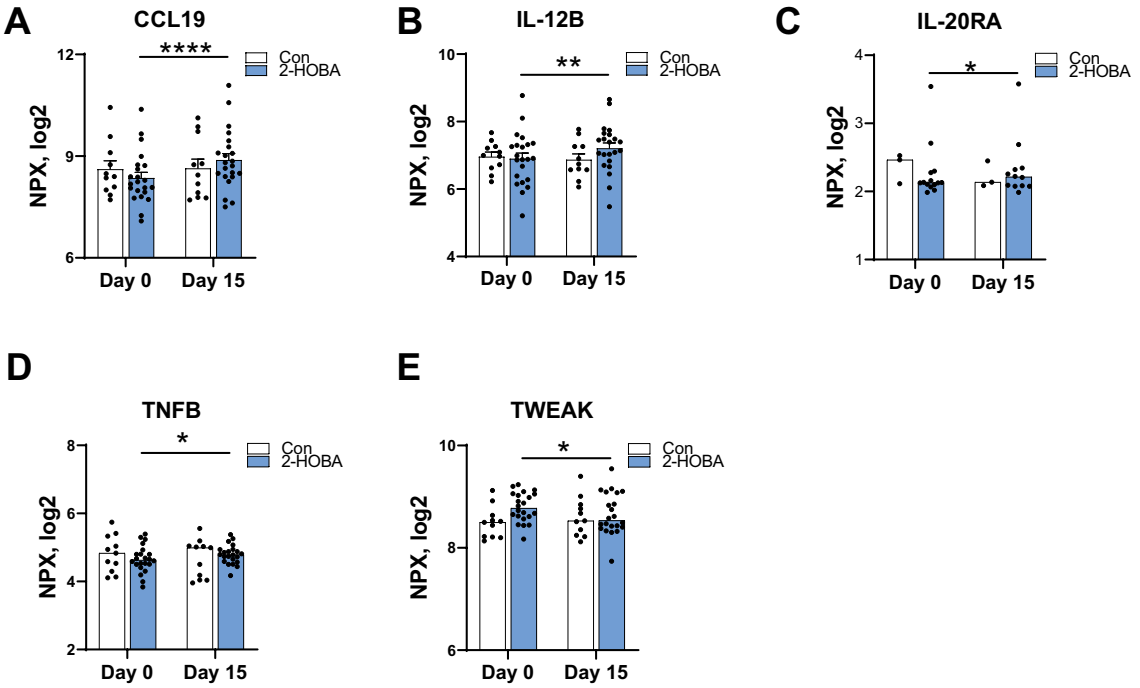

Fig. 1 Differential abundance of plasma inflammatory markers after oral 2-HOBA. Plasma concentrations of **A** C–C motif chemokine 19 (CCL 19), **B** interleukin 12 receptor subunit β (IL-12β), **C** interleukin 20 receptor subunit α (IL-20Rα), **D** tumor necrosis factor β (TNFβ; lymphotoxin alpha), and **E** tumor necrosis factor ligand superfamily, member 12 (TWEAK).

when mapped by the 2-HOBA biomarker dataset. Noteworthy were other biofunctions including "polarization of blood cells" [−log (*p*-value) = 8.08 e − 16, *Z*-score = 1.915], inflammation of liver [−log (*p*-value) = 1.46 e − 15, *Z*-score = 1.307], and "mobilization of calcium 2 + " [−log (*p*-value) = 1.47 e − 14, *Z*-score = 1.691].

DISCUSSION

Oxidative stress is a major mechanism underlying natural aging and is elevated with the preclinical and pathogenic development and subsequent progression of several chronic diseases including Alzheimer's disease, heart disease, rheumatoid arthritis, and cancer. Reactive oxygen species (ROS) are generated during normal respiration but increase with inflammation, mitochondrial dysfunction, and metabolic stress [36, 37] contributing to the progression of chronic diseases. ROS-catalyzed lipid peroxidation generates a number of reactive lipid aldehydes such as MDA, HNE, and ONE, with IsoLGs being the most reactive and which rapidly adduct to lysine and other cellular primary amines, leading to changes in protein function, cross-linking, and immunogenicity. Previous studies have documented the formation of IsoLGs in dendritic cells, monocytes, leukocytes, and T cells from multiple mouse models of hypertension [38–40] and proteinuric kidney injury [41] and in humans with resistant hypertension [42], lupus erythematosus [42], and cardiac dysfunction [43].

The use of several antioxidants and antioxidant enzymes has been proposed as targets to prevent the formation of free radicals and ROS [44]. Supplementation with moderate to high doses of vitamins C and E has shown equivocal results in humans and has failed to demonstrate any disease reduction [45, 46]. The failures could be related to the necessity of using remarkably high doses of these antioxidants, which are often limited by associated toxicity [47]. We and others have shown that 2-HOBA, a compound found in buckwheat seeds, is a potent scavenger of IsoLGs [24, 25] and consequently quells immune cell activation and reduces proinflammatory cytokine formation [38, 42, 48]. The mechanism of

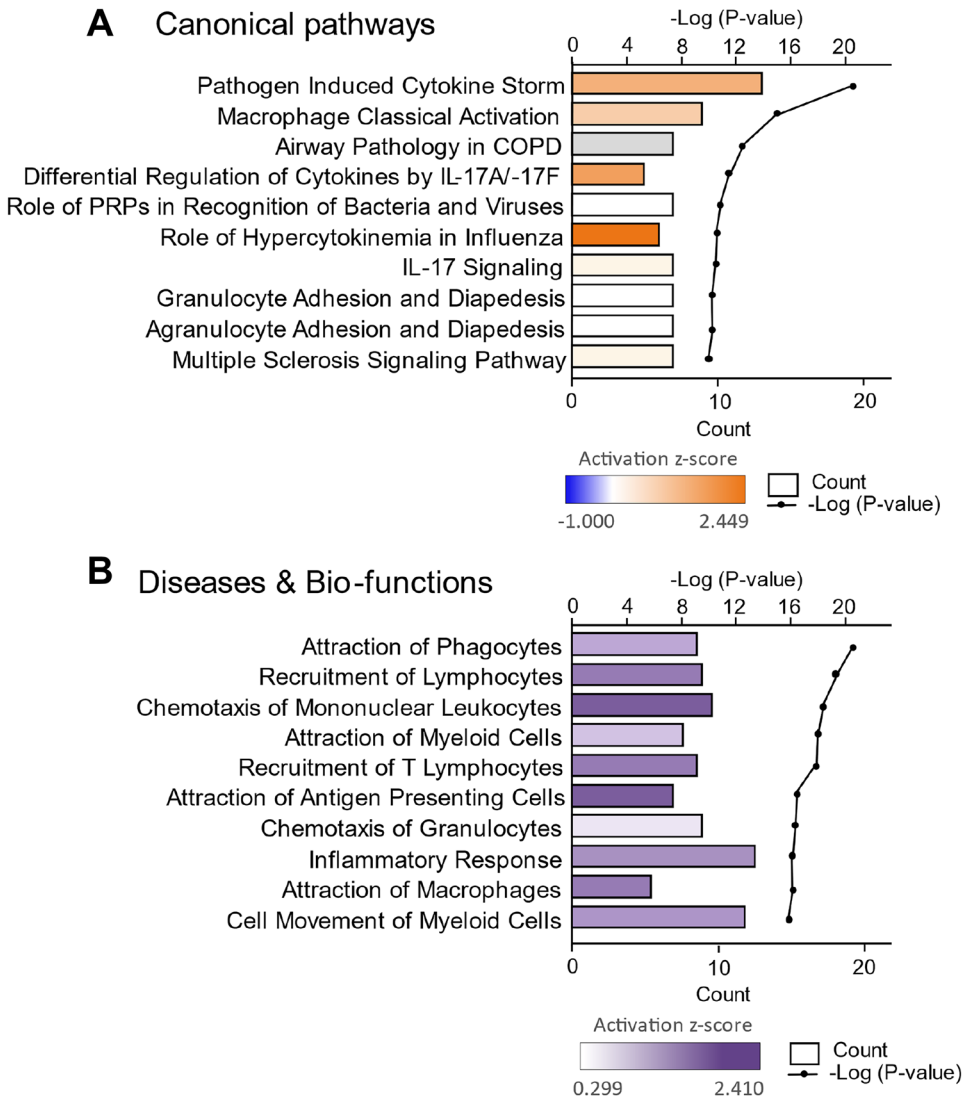

Fig. 2 Ingenuity pathway core analyses of the differentially regulated cytokines, chemokines, and growth factors after 2-HOBA treatment. **A** Histogram of the top 10 canonical pathways. **B** Histogram of the 10 top diseases and biofunctions. The top *y*-axis represents the − log (*p*-value), and the bottom *y*-axis represents the number of genes counted of each of the representative canonical pathways.

IsoLGs' formation has been reviewed by others [19, 49]; however, the mechanism by which they elicit an inflammatory response is not fully understood and may differ depending on the disease pathologies. Data demonstrate that IsoLGs and IsoLG-protein adducts directly impact biological activity through covalent modification of proteins affecting their biological activity [50]. This contributes to the development of a variety of diseases through multiple mechanisms, including protein accumulation, cell death, inflammation, mitochondrial dysfunction, and endoplasmic reticulum stress. Low-dose exposure of IsoLGs, at concentrations as low as 500 pM, caused proinflammatory effect through ERK, JNK, and NF-kB activation, resulting in increased expression of proinflammatory cytokines and the ensuing hepatic stellate cell activation [51], and accumulation of IsoLG-protein adducts in dendritic cells increased the production of proinflammatory cytokines IL-1β, IL-6, and IL-23 and induction of T cell proliferation and production of TNF-α, IFN-γ, and IL-17A which contribute to hypertension [38, 52, 53]. Treatment

with 2-HOBA prevented vascular inflammation and prevented DC and T cell activation [39].

We previously showed that 2-HOBA exhibits favorable safety, tolerability, and pharmacokinetic profiles [34, 35]. In this study, we extend such observations and show that the use of 2-HOBA in two cohorts of healthy adult humans, young and elderly, appears to favorably alter a panel of inflammation protein biomarkers, specifically, CCL19, IL-12B, IL-20RA, TNFB, and TWEAK, and these may be responsible for the protective effects of 2-HOBA against reactive electrophiles, such as IsoLGs, commonly expressed in conditions of excessive oxidative stress.

In this study, the use of oral supplementation of 2-HOBA resulted in a significant increase in CCL19, also known as EBI1 ligand chemokine (ELC), a small cytokine in the CC chemokine family that binds to the chemokine receptor CCR7. CCL19 is expressed abundantly in the thymus and lymph nodes, with moderate levels in the trachea and colon and low levels in the stomach, small intestine, lung, kidney, and spleen [54]. CCR7 is expressed on dendritic cells (DC) and distinct T and B cell subpopulations where it regulates DC and T cell interactions, calcium mobilization, and chemotaxis [55]. CCL19 has been shown to inhibit CRC angiogenesis by promoting miR-206, thus inhibiting Met/ERK/Elk-1/HIF-1α/VEGF-A pathway [56]. We have recently shown that 2-HOBA reduced the degrees of gastritis and inflammation in *H. pylori*–infected mice with gastric cancer [33] and reduced the development of gastric dysplasia and carcinoma, and concomitantly, reduced DNA damage. In addition, 2-HOBA reduced gene expression of innate effectors IL-1β, TNF-α, and NOS2, and the chemokine CXCL1, the Th17 cytokine IL-17, and the prototype Th1 cytokine IFN-γ were also significantly decreased. We and our collaborators also showed that the use of scavengers, including 2-HOBA, reduced the development of colitis-associated carcinoma [57, 58].

Oral intake of 2-HOBA also resulted in a small, yet significant increases in two interleukins, IL-12β and IL20Rα. IL-12β is a subunit of interleukin 12, a cytokine that acts on T and natural killer (NK) cells and has a broad array of biological activities. It has been shown to be important in sustaining a sufficient number of memory/effector-Th1 cells to mediate long-term protection to intracellular pathogens [59]. IL-20Rα comprises part of the IL20Rα/IL20Rβ dimer, a receptor for IL-19, IL-20, and IL-24 [60]. We also noted small increases in TNFβ, a protein in humans encoded by the LTA gene encoding lymphotoxin alpha [61]. TNFβ has considerable influence on the maintenance of the immune system [62] and is involved in the regulation of cell survival, proliferation, differentiation, and apoptosis [63]. Furthermore, TNFβ plays an important role in innate immune regulation, and its presence has been shown to prevent tumor growth [64] and cancerous cell lines. 2-HOBA resulted in a significant decrease in only one immune biomarker, TWEAK (TNF-related weak inducer of apoptosis), a tumor necrosis factor ligand superfamily member 12. TWEAK is expressed in leukocytes and can promote the proliferation and migration of endothelial cells [65, 66] and induce apoptosis. When activated chronically after injury, TWEAK promotes inflammation, fibrosis, and angiogenesis [66].

Limitations of the present study include the modest sample size in a cohort with predominantly European ancestry without known underlying chronic disease and with no clinical evidence of inflammation and/or oxidative stress. Hence, the findings may not be applicable to individuals across the diversity spectrum [67]. Furthermore, with the sample size, a number of anti-inflammatory cytokines [59], such as IL-10, did not reach statistical significance in this trial. Lastly, the IPA was limited by only including the inflammation protein biomarkers, and the scope of the analysis could have been improved with the inclusion of additional classes of proteins.

A number of preclinical animal models have demonstrated a link between IsoLG and IsoLG-adduct formation in the development of a variety of pathologies, including cardiovascular diseases obesity and diabetes, cancer, and neurodegeneration, as well as aging. The use of 2-HOBA, which has been shown to be highly tolerable and relatively no toxicity, efficiently eliminates the toxic effects of IsoLGs and should be considered a potential proactive tool for the improvement of immune health associated with oxidative stress. This is supported by the data from this manuscript showing that oral consumption of 2-HOBA, a naturally produced food product, for 2 weeks in healthy volunteers, resulted in significant changes in 15 immune proteins using the Olink® targeted platform. IPA identified changes to canonical pathways that differentially regulate secreted cytokines, chemokines, and growth factors. Given that 2-HOBA has a proven high safety margin, then its use as a nutritional supplement in healthy individuals has a high likelihood of favorably changing the biofunctions related to the recruitment, attraction, and movement of different immune cell types.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1007/s10753-023-01801-w.

AUTHOR CONTRIBUTION

All authors contributed to the conception and design of the study. Material preparation, data collection, and analysis were performed by C.R.F. and J.A.R. The first draft of the manuscript was co-written by C.R.F., N.N.A., and J.A.R., and all authors commented on previous versions of the manuscript. All authors read and approved the final version of the manuscript.

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AVAILABILITY OF DATA AND MATERIALS

The datasets generated and/or analyzed during the current study are available from the corresponding authors on reasonable request.

DECLARATIONS

Ethics Approval The study protocol was approved by the Vanderbilt University Institutional Review Board.

Consent to Participate All participants provided written informed consent before participating in the study.

Consent for Publication Not applicable.

Competing Interests The authors declare the following financial interests/personal relationships which may be considered potential competing interests: John A. Rathmacher, John C. Fuller, Jr., and Charles R. Flynn report financial support was provided by National Institute on Aging. John A. Rathmacher reports financial support was provided by the National Heart, Lung, and Blood Institute. John Rathmacher is employed by MTI Biotech Inc. and is named as an inventor on a number of 2-hydroxybenzylamine patents. MTI Biotech Inc. has a licensing agreement with Vanderbilt University for the future use of electrophile scavenger drugs, including the 2-hydroxybenzylamine. Charles R. Flynn is employed by Vanderbilt University Medical Center and is named as an inventor on a 2-hydroxybenzylamine patent. John C. Fuller, Jr., and Naji N. Abumrad are employed by Metabolic Technologies, LLC. Naji N. Abumrad is the CEO of MTI BioTech Inc.

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