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Resveratrol inhibits obesity-associated adipose tissue dysfunction and tumor growth in a mouse model of postmenopausal claudin-low breast cancer

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Abstract

Adipose tissue dysregulation, a hallmark of obesity, contributes to a chronic state of low-grade inflammation and is associated with increased risk and progression of several breast cancer subtypes, including claudin-low breast tumors. Unfortunately, mechanistic targets for breaking the links between obesity-associated adipose tissue dysfunction, inflammation, and claudin-low breast cancer growth have not been elucidated. Ovariectomized female C57BL/6 mice were randomized ($n = 15/\text{group}$) to receive a control diet, a diet-induced obesity (DIO) diet, or a DIO + resveratrol (0.5% wt/wt) diet. Mice consumed these diets ad libitum throughout study and after 6 weeks were orthotopically injected with M-Wnt murine mammary tumor cells, a model of estrogen receptor (ER)-negative claudin-low breast cancer. Compared with controls, DIO mice displayed adipose dysregulation and metabolic perturbations including increased mammary adipocyte size, cyclooxygenase-2 (COX-2) expression, inflammatory eicosanoid levels, macrophage infiltration, and prevalence of crown-like structures (CLS). DIO mice (relative to controls) also had increased

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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systemic inflammatory cytokines and decreased adipocyte expression of peroxisome proliferator-activated receptor gamma (PPAR γ) and other adipogenesis-regulating genes. Supplementing the DIO diet with resveratrol prevented obesity-associated increases in mammary tumor growth, mammary adipocyte hypertrophy, COX-2 expression, macrophage infiltration, CLS prevalence, and serum cytokines. Resveratrol also offset the obesity-associated downregulation of adipocyte PPAR γ and other adipogenesis genes in DIO mice. Our findings suggest that resveratrol may inhibit obesity-associated inflammation and claudin-low breast cancer growth by inhibiting adipocyte hypertrophy and associated adipose tissue dysregulation that typically accompanies obesity.

Keywords

diet-induced obesity; mammary tumor; resveratrol

1 | INTRODUCTION

The prevalence of obesity, an established risk and prognostic factor for several subtypes of breast cancer, has increased dramatically in the US and globally over the past 35 years.¹ Obese, relative to normoweight, postmenopausal women with estrogen receptor (ER)-negative breast cancer are more likely to have poorer response to treatment and higher rates of recurrence.² The ER-negative claudin-low subtype of breast cancer, which is energy balance-responsive in humans and mice and often confers a poor prognosis due to a current lack of targeted therapies.^{3,4} While obesity-associated inflammation is implicated in the generally poor response to therapy and increase in mortality in obese women compared with their lean counterparts, the mechanism by which inflammation promotes ER-negative claudin-low breast cancer growth is unknown.^{5,6}

Chronic positive energy balance in obesity results in the remodeling of the adipose microenvironment and results in hypertrophic adipocytes. Studies of C57BL/6 mice have established strong correlations between fat pad mass and mean adipocyte size, a measure of hypertrophy.⁷ Furthermore, with increasing adipocyte size the adipocyte secretome is enriched in pro-inflammatory adipokines and cytokines, including tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6).⁸ Adipocyte size also positively correlates with macrophage infiltration in the adipose tissue as well as systemic insulin resistance.^{9,10} TNF- α is a potent inhibitor of adipogenesis and can prevent preadipocytes from differentiating into mature adipocytes. Partially transdifferentiated preadipocytes can possess a macrophage-like phenotype and secrete cytokines similar to macrophages.¹¹ Isakson et al harvested preadipocytes from obese and non-obese adipose tissue biopsies and found that the number of preadipocytes that differentiated into adipose cells was negatively correlated with both BMI and adipocyte cell size of the donors.¹²

The crosstalk between hypertrophied adipocytes and macrophages promote and sustain a pro-inflammatory environment in adipose tissue, manifested by an influx of immune cells and metabolic dysfunction. Macrophages that have infiltrated into adipose tissue propagate the recruitment of additional macrophages by secreting chemoattractants, including

monocyte chemoattractant protein-1 (MCP-1). Macrophages also induce cyclooxygenase-2 (COX-2) expression resulting in high levels of arachidonic acid metabolites such as prostaglandins that disrupt fatty acid homeostasis by inhibiting adipogenesis. Prostaglandin (PG) F₂ alpha (PGF_{2α}) and PGE₂ inhibit peroxisome proliferator-activated receptor gamma (PPARγ),¹³ a transcription factor regulating adipocyte differentiation and maintenance of insulin sensitivity. A higher ratio of PPARγ to COX-2, as achieved by COX-2 inhibition via non-steroidal anti-inflammatory drugs (NSAIDs) or PPARγ-agonists such as thiazolidinediones (TZD), decreases macrophage infiltration and improves insulin sensitivity as manifested by adipocyte glucose uptake.^{14–16} Although not well studied in the context of cancer, pharmacologic strategies that have been successful in resolving obesity-associated inflammation in diabetes were dependent on restoring adipogenesis and adipocyte turnover.^{17,18}

Resveratrol is a natural polyphenolic compound produced in several species of plants in response to pathogens.¹⁹ Resveratrol supplementation increases PPARγ expression in vivo in the adipose tissue of obese mice and in vitro in cultured macrophages and cancer cells.^{20–22} Furthermore, resveratrol has demonstrated potential as a cancer chemopreventive agent due to its established ability to attenuate the pro-inflammatory effects of obesity.^{23,24} Specifically, resveratrol decreases TNF-α production,²⁵ nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation²⁶ and COX-2 activity,^{27,28} targets critical to perpetuating the cancer-promoting effects of adipose dysfunction in obesity.

The present study evaluated the ability of resveratrol to offset the effects of obesity on adipose tissue dysfunction, inflammation, and claudin-low breast cancer growth. We and others have demonstrated success in disrupting the obesity-cancer link in a variety of cancer types by targeting inflammation with omega-3 fatty acids, NSAIDs or selective COX-2 inhibitors.^{29–31} Here we hypothesized that resveratrol, via its ability to prevent adipose tissue remodeling and inhibit pro-inflammatory cytokine production, would protect against the protumorigenic effects of obesity in our murine model of claudin-low breast cancer.

2 | MATERIALS AND METHODS

2.1 | In vivo studies

All animal studies and procedures were approved and monitored by the University of Texas Institutional Animal Care and Use Committee. As summarized in Figure 1A, 8-week-old ovariectomized female C57BL/6 mice were randomized ($n = 15$ per group, based on sample size calculations) to receive one of the following three dietary regimens: (i) control diet (catalog # D12450B; 10% kcal from fat); (ii) diet-induced obesity (DIO) regimen (catalog # D12492; 60% kcal from fat), or DIO regimen supplemented with resveratrol (5 g resveratrol/kg DIO diet). Diets were purchased from Research Diets, Inc. (New Brunswick, NJ) and stored at 4°C in sealed bags. Mice were fed ad libitum and food was replaced biweekly to prevent oxidation. After 5 weeks on diet mice underwent a retro-orbital bleed for serum collection. One week later, mice received an orthotopic injection of 25 000 M-Wnt murine mammary tumor cells. These cells were clonally derived from the Wnt-1 transgenic mouse model of mammary cancer and display transcriptional profiles that closely align with human claudin-low breast tumors.⁴ Transplanted tumor growth was monitored for 5 weeks,

and tumors were measured weekly with calipers. At study endpoint (week 11), mice were euthanized by CO₂ inhalation followed by cervical dislocation. Tumors and mammary fat pad were excised and divided in portions to be formalin fixed or flash frozen in liquid nitrogen and stored at -80°C until further analysis. Ex vivo tumor volume was calculated using the ellipsoid formula $1/6\pi \times D_1 \times D_2 \times D_3$ where D₁, D₂, and D₃ indicate three distinct diameter measurements in millimeters.

2.2 | Quantitative magnetic resonance analysis

Body composition was measured on all mice at end of study by quantitative magnetic resonance (qMR) (Echo Medical Systems, Houston, TX). Measurements included lean mass, fat mass, and total water mass. Percent body fat was calculated by dividing fat mass by total body weight.

2.3 | Serum hormone, adipokine, and cytokine measurement

After 5 weeks on diet, blood was collected from mice fasted 4-6 h by retro-orbital bleed, allowed to clot for 30 m at room temperature, and serum was collected and flash frozen for subsequent analyses. Insulin and leptin were measured using mouse adipokine LINCOplex® Multiplex Assays (Millipore, Inc., Billerica, MA). Insulin-like growth factor 1 (IGF-1) concentrations were measured using a Millipore Milliplex Rat/Mouse IGF-1 Single Plex Assay. Adiponectin concentrations were measured using a Milliplex Mouse Adiponectin Single Plex (Millipore, Inc.). Serum concentrations of mouse cytokines, including IL-1β, IL-6, IL-10, IL-17A, interferon-γ (IFN-γ), TNF-α, and MCP-1, were measured using a Bio-Plex Pro™ Mouse Cytokine 7-Plex Assay (Bio-Rad, Inc., Hercules, CA). All assays were completed and analyzed on a Bio-Rad Bio-Plex 200 analysis system (Bio-Rad, Inc., Hercules, CA).

2.4 | Histopathology and immunohistochemical staining

Paraffin-embedded mammary and tumor tissue were cut into 4 μm thick sections for either hematoxylin and eosin (H&E) staining or immunohistochemical analysis. Slides were deparaffinized and processed as previously described.³² Mammary tissues were stained with the following primary antibodies (source, dilution, and incubation conditions presented parenthetically): COX-2 (Cayman, Ann Arbor, MI catalog #160126, 1:500, 1 h at room temperature (RT)) and PPARγ (Abcam, Cambridge, MA #ab59256, 1:250, overnight at 4°C). Tumor tissues were stained with the following primary antibodies: Macrophage marker (RM0029-11H3), a pan-macrophage antibody (Santa Cruz, Dallas, TX sc-101447, 1:100, overnight at 4°C) and F4/80 (Abcam #ab6640, 1:200, 2 h at RT). Slides were incubated with Dako EnVision™ labeled polymer for 30 m at RT, followed by incubation with Dako diaminobenzidine to develop the antibody stain then by a hematoxylin counterstain to visualize nuclei. Slides were scanned and digitized using the Aperio CS2 Digital Pathology Scanner (Leica Biosystems, Wetzlar, Germany). Quantification was performed using Aperio Digital Pathology platform using individually developed scoring algorithms.²⁹

2.5 | Analysis of mammary and intratumoral adipocyte size

H&E staining on sections from mammary fat pad tissue ($n = 12$ -15 mice/group) and tumor tissue ($n = 10$ /group) was processed, scanned and imaged at 40 $\times$ magnification. Representative areas ($n = 3$ /mouse) were viewed at 8.8 $\times$ magnification (300 μ m) and the size of mammary fat pad adipocytes (calculated as two-dimensional area) was quantified using ImageJ Version 1.51e (National Institute of Health, Bethesda, Maryland). An adipocyte tool macro (MRI Adipocyte Tools.txt) was downloaded from (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Adipocytes_Tool) and imported into ImageJ. The area values from the representative three digital images yielded between 345 and 1991 measurements for each mouse, which were then compiled to calculate the 10th, 50th, and 90th percentile of adipocyte area. Resultant percentiles were then averaged by group.

2.6 | Quantitative RT-PCR

Total RNA was extracted from flash-frozen mammary fat pad samples using TRI-Reagent (Sigma-Aldrich, St.Louis, MO) according to manufacturer's instructions. RNA concentration was spectrophotometrically determined using a nanodrop (Thermo Scientific, Logan, UT) and quality was confirmed using an Agilent 2100 Bioanalyzer (Santa Clara, CA). RNA was reverse transcribed with Multiscribe RT (Applied Biosystems, Carlsbad, CA). Resulting cDNA from tissue samples were assayed in triplicate for PCR using Taqman® Gene Expression Assays for IL-6, serum amyloid A 3 (Saa3), MCP-1, c-x-c motif chemokine ligand 12 (CXCL12), granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), TNF- α , macrophage migration inhibitory factor (MIF), EGF-like module receptor 1 (Emr1), and PPAR γ (Applied Biosystems). PCR reactions were completed using a ViiA™ 7 Real time PCR system (Applied Biosciences, Foster City, CA). Gene expression data were normalized to the housekeeping gene β -actin and analyzed using the delta delta C_T cycle threshold method.

2.7 | Crown-like structure analysis

The formation of crown-like structures (CLS) was assessed from H&E staining of 4 μ m thick sections prepared from formalin-fixed paraffin-embedded mammary fat pad tissue. Briefly, the total number of CLS were counted in a blinded fashion. CLS density measures were achieved by dividing CLS by the total slide area used for analysis using ImageScope Viewing Software Version 12.0 (Leica Biosystems, Wetzlar, Germany). Values expressed as CLS/area of tissue in cm².

2.8 | Eicosanoid analysis

Eicosanoids in the mammary adipose tissues were extracted and analyzed by liquid chromatography/tandem mass spectroscopy (LC/MS/MS) using the modified method described previously.³³ LC/MS/MS analyses were performed using an Agilent 6460 Triple Quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with an Agilent 1200 binary pump high-performance liquid chromatography (HPLC) system as described previously.³⁴

2.9 | Adipogenesis PCR array

RNA was extracted from the mammary fat pad of control, DIO, and DIO + resveratrol mice ($n = 6/\text{group}$, randomly selected) as described above. RNA was reverse transcribed using a cDNA conversion kit according to the manufacturer's instructions. The cDNA was plated on the real-time Mouse Adipogenesis PCR Array (Qiagen, Hilden, Germany, RT² Profiler PCR Array catalog # PAMM-049Z) in combination with RT² SYBR® Green qPCR Mastermix (catalog # 330529). C_T values were uploaded to the Qiagen data analysis web portal at <http://www.qiagen.com/geneglobe> and fold change/regulation was calculated using the delta delta C_T method.

2.10 | Tissue resveratrol analysis

One milliliter of an extraction solvent consisting of 20% 0.1 M Sodium Acetate buffer (pH 3.8) and 80% methanol was added to each tissue sample designated for analysis. Piceatannol (Sigma) was added to the samples as an internal standard to a final concentration of 0.5 µg/mL. The sample was disrupted using a sonic dismembrator for 1 min, then centrifuged at 10 000g for 10 min. An aliquot of 600 µL was taken from the supernatant and dried using a Speed-Vac system. The samples were then resuspended in 100 µL of the mobile phase which consisted of 25% methanol, 10% acetonitrile, and 1% acetic acid in distilled water and aliquots were injected into a Waters Acquity UPLC H-Class (Waters Corporation, Milford, MA). Analysis was accomplished using the peak area of the fluorescence response of resveratrol and piceatannol, with the lower limit of detection at 5 ng/mL (0.005 µg/mL). The results are reported as an average of two technical replicates.

2.11 | Statistical analyses

A priori sample size calculations were completed to determine the required number of mice/treatment group ($n = 15$) to provide >90% power to detect at least 25% differences in the primary study outcome, which is mean tumor volume between treatment groups.³⁵ Values are presented as group mean ± standard deviation (s.d.). For all tests, GraphPad Prism software was used (GraphPad Software Inc., La Jolla, CA), and $P < 0.05$ was considered statistically significant. Differences between groups in weekly body weight and calorie intake were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Differences between groups in body fat percentage, serum hormones, adipokines, and cytokines were analyzed by one-way ANOVA followed by Tukey's post hoc test. Differences between groups in adipocyte size, gene expression, CLS, immunohistochemical staining, tumor volume, and tumor adipocyte area were analyzed by one-way ANOVA followed by Tukey's post hoc test. Differences in gene expression detected using the Adipogenesis Array were calculated by Student's *t*-test using Qiagen data analysis web portal.

3 | RESULTS

3.1 | Resveratrol supplementation does not impact the establishment of diet-induced obesity

The DIO (alone or with resveratrol supplementation) and control regimens effectively generated obese and normoweight phenotypes, respectively (Figures 1B and 1C). Significant differences in body weight were observed after 3 weeks on diet and continued throughout the remainder of study between control mice and DIO mice. No statistically significant differences in body weight or calorie intake were detected between DIO and DIO + resveratrol mice throughout study. Average body weight is shown prior to tumor implantation for the first 6 weeks on diet (Figure 1B). DIO + resveratrol mice consumed on average 390.5 ± 40.1 – 675.6 ± 27.4 mg resveratrol/body weight (kg) per day (Supplementary Table S1). Percent body fat, obtained at end of study, was significantly lower in control mice ($30.1 \pm 3.6\%$) versus both DIO ($49.3 \pm 3.0\%$; $P < 0.0001$) and DIO + resveratrol mice ($46.2 \pm 4.7\%$; $P < 0.0001$), with no statistical differences between DIO and DIO + resveratrol mice (Figure 1C).

3.2 | Resveratrol supplementation protects against serum metabolic and inflammatory perturbations in DIO mice

As shown in Table 1, DIO mice had significantly higher serum levels of insulin, IGF-1, and leptin compared with control mice ($P < 0.01$ for each analyte). DIO + resveratrol mice displayed intermediate levels of insulin and IGF-1 that were not significantly different from DIO or control mice. Leptin was significantly increased in both DIO and DIO + resveratrol mice compared with control mice (DIO vs control $P < 0.0001$; DIO + resveratrol vs control $P < 0.0001$). No differences in serum adiponectin levels were detected between groups, but control mice on average had a significantly lower leptin to adiponectin ratio compared with DIO and DIO + resveratrol mice (Table 1).

Furthermore, DIO mice, relative to control mice, had significantly increased serum levels of IFN- γ , IL-1 β , IL-6, MCP-1, and TNF- α ($P < 0.05$ for each analyte). DIO + resveratrol mice displayed significantly lower serum IL-1 β and IL-6 compared with DIO mice ($P < 0.05$ for each analyte), with levels not statistically different than controls. DIO + resveratrol mice had an intermediate level of IFN- γ , MCP-1, and TNF- α , which were not significantly different from DIO or control mice. No significant differences were observed between groups in serum IL-10 and IL-17A (Table 1).

3.3 | Resveratrol prevents adipocyte hypertrophy in the mammary fat pad of DIO mice

We measured the two-dimensional area of each adipocyte using stained H&E sections of mammary fat pad tissue from 12 to 15 mice/ group (Figure 2A). The average 10th percentile of adipocyte area for DIO mice ($1614 \pm 628 \mu\text{m}^2$) was significantly greater than the average 10th percentile of control ($337 \pm 133 \mu\text{m}^2$) and DIO + resveratrol mice ($399 \pm 197 \mu\text{m}^2$; Figures 2B and 2C). The average median (50th percentile) adipocyte area for the DIO group ($4891 \pm 1097 \mu\text{m}^2$) was significantly greater compared with control ($1334 \pm 494 \mu\text{m}^2$) and DIO + resveratrol ($2081 \pm 540 \mu\text{m}^2$). However, DIO + resveratrol mice had a median adipocyte area that was also significantly greater than controls (Figures 2B and 2D). A

similar trend was also seen in the average 90th percentile of adipocyte area, with the largest values in DIO mice ($8940 \pm 1560 \mu\text{m}^2$), intermediate values in DIO + resveratrol mice ($5415 \pm 579 \mu\text{m}^2$), and the lowest values in controls ($3311 \pm 1004 \mu\text{m}^2$; Figures 2B and 2E). All statistical differences were at $P < 0.0001$. These findings on diet-dependent effects on adipocyte area provide additional support that the DIO mice possessed hypertrophied adipocytes, and supplementation of the DIO diet with resveratrol inhibited the pathogenic increase in adipocyte size.

3.4 | Resveratrol protects against inflammatory and macrophage gene signaling in DIO mice

We measured gene expression in the mammary fat pad of mice for mediators of inflammation and genes implicated in macrophage recruitment, proliferation, and secretory products of activated macrophages. DIO mice had significantly increased gene expression of the pro-inflammatory mediators IL-6 compared with control and DIO + resveratrol mice (DIO vs control $P < 0.01$; DIO vs DIO + resveratrol $P < 0.05$) and Saa3 (DIO vs control $P < 0.01$; DIO vs DIO + resveratrol $P < 0.01$; Figure 3A). DIO mice displayed increased gene expression of MCP-1 ($P < 0.05$) compared with DIO + resveratrol, but not control mice. CXCL12 expression was increased in DIO mice compared with both control ($P < 0.01$) and DIO + resveratrol ($P < 0.01$; Figure 3B). DIO mice, relative to control mice and DIO + resveratrol mice, also displayed increased expression of GM-CSF (DIO vs control $P < 0.01$; DIO vs DIO + resveratrol $P < 0.01$) and M-CSF (DIO vs control $P < 0.05$; DIO vs DIO + resveratrol $P < 0.01$; Figure 3C), which promote macrophage proliferation. Gene expression of TNF- α and MIF, which are both predominantly secreted by macrophages in the adipose tissue, were significantly increased in DIO mice compared with control mice ($P < 0.01$; $P < 0.05$, respectively) and DIO + resveratrol mice ($P < 0.01$; $P < 0.01$, respectively; Figure 3D).

3.5 | Resveratrol blunts mammary gland crown-like structure formation and COX-2 protein expression in DIO mice

To extend our findings from mammary gene expression profiles suggesting resveratrol supplementation modulated macrophage activity, we measured the number of CLS in the mammary fat pads of 14-15 mice/group. CLS are indicative of macrophages surrounding dead or dying adipocytes. The density of CLS (expressed as CLS/area of tissue in cm^2) in the mammary fat pad was significantly increased in DIO mice ($15.5 \pm 5.6 \text{ CLS}/\text{cm}^2$) relative to control ($9.6 \pm 4.0 \text{ CLS}/\text{cm}^2$; $P < 0.01$) and DIO + resveratrol mice ($11.2 \pm 3.5 \text{ CLS}/\text{cm}^2$; $P < 0.05$; Figures 4A and 4B). Gene expression of Emr1, which is specific to macrophages, was also increased in DIO mice compared to both control ($P < 0.05$) and DIO + resveratrol ($P < 0.05$; Figure 4C).

We also quantified COX-2 protein expression using immunohistochemistry analysis on formalin-fixed, paraffin-embedded tissue from the mammary fat pads of mice fed the control, DIO, and DIO + resveratrol diets. DIO mice displayed approximately a two-fold increase in COX-2 protein expression relative to control ($P < 0.0001$) and DIO + resveratrol mice ($P < 0.0001$; Figures 4A and 4D).

3.6 | Resveratrol modulates mammary tissue levels of eicosanoids and other inflammatory lipid derivatives in DIO mice

To test if modulation of COX-2 expression in the mammary fat pad resulted in corresponding effects in levels of downstream metabolites, we analyzed the mammary fat pad from four randomly selected mice/ group for levels of inflammatory lipid derivatives. DIO mice had significantly increased levels of arachidonic acid (AA) pro-inflammatory mediators $\text{PGF}_{2\alpha}$ ($P < 0.05$ vs control; $P < 0.05$ vs DIO + resveratrol), PGE_2 ($P < 0.05$ vs control; $P < 0.05$ vs DIO + resveratrol), 13,14-dihydro-15-keto- PGE_2 , as well as linoleic acid derived 13-hydrox-yoctadecadienoic acid (13-HODE), compared with DIO + resveratrol mice. Relative to DIO mice, control mice had significantly decreased $\text{PGF}_{2\alpha}$ and 13-HODE but intermediate levels of PGE_2 and 13,14-dihydro-15-keto- PGE_2 which was not significantly different from DIO or DIO + resveratrol mice. No substantial differences between the diet treatment groups were observed in the anti-inflammatory intermediates PGE_1 and PGE_3 or the pro-inflammatory intermediates 6ke-to $\text{PGF}_{1\alpha}$, PGD_2 , thromboxane B2 (TXB_2), 5-hydroxyeicosatetraenoic acid (5-HETE), 12-HETE and 15-HETE (Table 2).

3.7 | Resveratrol maintains mammary tissue PPAR γ expression in DIO mice

COX-2 metabolites $\text{PGF}_{2\alpha}$ and PGE_2 are known to inhibit PPAR γ , a key regulator of adipogenesis,¹³ and these interactions could explain the differences in adipocyte size observed between DIO and DIO + resveratrol mice despite both groups having equivalent body fat percentage. Accordingly, we performed immunohistochemistry analysis on paraffin-embedded mammary tissue for protein levels of PPAR γ . Relative to DIO mice, control and DIO + resveratrol mice had significantly increased staining of positive nuclei for PPAR γ in the mammary fat pad (DIO vs control $P < 0.0001$; DIO vs DIO + resveratrol $P < 0.0001$; Figures 5A and 5B). Furthermore, DIO mice had significantly decreased PPAR γ gene expression compared with DIO + resveratrol mice ($P < 0.01$); control mice displayed intermediate gene expression, not statistically different from DIO or DIO + resveratrol mice (Figure 5C).

3.8 | Resveratrol protects against obesity-induced dysregulated expression of adipogenesis-related genes in DIO mice

To further characterize the effects of maintained PPAR γ expression in DIO + resveratrol mice, we performed a quantitative real-time Adipogenesis PCR array on mammary fat pad tissue for genes involved in preadipocyte commitment, differentiation and adipocyte maturation. Differential expression data (Figure 6 and Supplementary Table S2) demonstrate that DIO mice display patterns of decreased gene expression of targets across the spectrum of adipogenesis compared with control and DIO + resveratrol mice. Furthermore, DIO + resveratrol mice displayed a unique increase in expression of genes implicated in the beiging of adipocytes, relative to both control and DIO mice.

Generally, DIO mice, relative to controls, had significantly decreased expression of factors promoting stem cell differentiation to preadipocytes, including adipogenin (Adig), fibroblast growth factor 2 (Fgf2), and nuclear receptor subfamily 0 group B member (Nr0b2). DIO + resveratrol mice generally had intermediate expression levels of these preadipocyte differentiation factors that did not significantly differ from either control or DIO mice.

Genes that facilitate adipogenesis, including the master regulators PPAR γ and CCAAT/enhancer binding protein α (Cebpa), were similarly expressed in control and DIO + resveratrol mice; both groups displayed expression levels of these adipogenesis regulators that were generally higher than in DIO mice. Furthermore, downstream targets of PPAR γ , including insulin receptor substrate 2 (Irs2), fatty acid binding protein 4 (Fabp4) and lipoprotein lipase (Lpl) that are known to facilitate a mature adipocyte phenotype, were decreased in DIO mice compared with control and DIO + resveratrol mice.

The expression of the transcriptional regulator PRD1-BF1-RIZ1 homologous domain containing 16 (Prdm16) and Forkhead Box C2 (Foxc2), which both promote the beiging of adipose tissue, was significantly increased in DIO + resveratrol mice compared with both control and DIO mice. The expression of uncoupling protein 1 (Ucp1), a specific marker of adipose browning, was decreased in DIO mice compared with control and DIO + resveratrol mice (Figure 6 and Supplementary Table S2).

3.9 | Resveratrol protects against enhanced M-Wnt murine mammary tumor size, infiltrating adipocyte size, and tumor associated macrophages in DIO mice

To characterize the effects of obesity with and without adipose tissue dysfunction (the latter achieved in DIO + resveratrol mice) on mammary tumor growth, we orthotopically injected mice with M-Wnt mammary tumor cells, which model claudin-low breast cancer.⁴ DIO mice had significantly increased mean *ex vivo* tumor volume ($364.5 \pm 98.1 \text{ mm}^3$) compared with DIO + resveratrol mice ($224.8 \pm 113.0 \text{ mm}^3$; $P < 0.05$) and control mice ($159.8 \pm 115.1 \text{ mm}^3$; $P < 0.01$; Figure 7A). Similarly, the *ex vivo* tumor weight was significantly greater in DIO mice ($0.50 \pm 0.13 \text{ g}$) compared to DIO + resveratrol ($0.35 \pm 0.14 \text{ g}$; $P < 0.05$) and control ($0.26 \pm 0.16 \text{ g}$; $P < 0.001$; Figure 7B). Furthermore, both measurements of tumor size in DIO + resveratrol mice did not statistically differ from the corresponding measurement in control mice (Figure 7A and 7B). We confirmed the presence of resveratrol in tumor tissue by HPLC (Supplementary Table S3). H&E staining of 10 randomly selected tumors/group revealed a markedly increased accumulation of adipocytes within the tumor tissue of DIO mice compared with control and DIO + resveratrol mice. We measured intratumoral adipocyte size via two-dimensional area analysis of each adipocyte present in the tumor. The median adipocyte area for DIO mice was statistically significantly higher than control ($P < 0.01$) and DIO + resveratrol mice ($P < 0.05$; Figures 7B and 7E).

We also quantified the presence of intratumoral macrophages by utilizing immunohistochemistry on sections of tumor tissue with two different markers: (i) Macrophage marker RM0029-11H3 ($n = 3$ randomly selected tumors/group), a pan-macrophage marker; and (ii) F4/80 ($n = 5-7$ randomly selected tumors/group), a stain more specific to tumor-associated macrophages.^{36,37} Tumors from DIO mice displayed nearly a three-fold increase in positive staining for both Macrophage marker (RM0029-11H3) compared with control ($P < 0.01$) and DIO + resveratrol mice ($P < 0.01$; Figures 7C and 7E). Similarly, DIO tumors had significantly increased positive staining for F4/80 compared with control ($P < 0.01$) and DIO + resveratrol mice ($P < 0.01$; Figures 7D and 7E). The tumors from DIO + resveratrol mice did not statistically differ from control tumors in their positivity for Macrophage marker and F4/80 (Figures 7D and 7E).

4 | DISCUSSION

We report that resveratrol supplementation prevents obesity-induced adipose tissue dysfunction, chiefly adipocyte hypertrophy, and reduces tumor growth in obese mice orthotopically transplanted with an aggressive claudin-low subtype of breast cancer. The anticancer effects of resveratrol in DIO mice were associated with: (1) smaller mammary adipocyte size; (2) lower expression of genes (normalized to control levels) involved in promoting inflammation, macrophage recruitment, and signaling; (3) decreased mammary CLS; (4) lower mammary expression of COX-2 and pro-inflammatory eicosanoids; (5) altered systemic cytokines (particularly IL-1 β and IL-6); and (6) maintenance of mammary expression of pro-adipogenic genes, notably PPAR γ . A summary of these key findings is shown in Figure 8.

This study contributes vital knowledge about the impact of resveratrol supplementation on obesity-associated metabolic dysfunction and mammary tumor progression. Our findings are in agreement with and expand upon several other studies demonstrating that resveratrol supplementation in the context of a DIO diet exerts multifactorial effects, including favorable modulation of systemic measures of insulin sensitivity and adipose tissue inflammation to levels comparable to lean, metabolically healthy counterparts.^{25,38,39} Furthermore, the anticancer benefits we observed on claudin-low mammary tumor growth are consistent with findings investigating the effects of resveratrol in lung, skin, ovarian, colon, and prostate cancer studies.^{40–45} The observation that resveratrol exerts potent anticancer effects in obese mice supports the hypothesis suggested by several reports that resveratrol primarily exerts beneficial effects in the face of metabolic perturbations or inflammation (as occurs with obesity). For example, Kang et al showed that resveratrol improved insulin signaling only in the face of an insulin resistant state in vitro and in vivo.⁴⁶ Carreras et al demonstrated that resveratrol supplementation in male mice reduced visceral white adipose tissue macrophages under conditions of intermittent hypoxia in a mouse model of sleep apnea, but not under normal conditions in wild-type mice.⁴⁷ One apparent exception to the notion that resveratrol exerts anticancer effects primarily in the context of a pro-inflammatory state is from a DMBA-induced mammary carcinogenesis study in which resveratrol enhanced cell-mediated immune response and apoptosis and reduced epithelial carcinogenesis in lean mice.⁴⁸ However, even though the mice in the Yusuf et al study were lean, they still may have had inflammatory or metabolic perturbations, as topical DMBA treatment is well known to increase cutaneous oxidative stress and inflammation regardless of body weight or adiposity.^{28,42,48}

Inflammation reprograms the cellular composition of adipose tissue, including an influx of macrophages and other immune cells that secrete factors to propagate additional macrophage recruitment. TNF- α is produced by macrophages and is the predominant cytokine contributor to insulin resistance in the adipose tissue. TNF- α inhibits adipogenesis, reduces the expression of genes in mature adipocytes that enable an adequate insulin response, and directly interferes with insulin-induced glucose metabolism by downregulating glucose transporter 4 (GLUT-4) as well as the activity of Irs-1.^{49,50} In our study, we demonstrated that resveratrol supplementation decreased serum TNF- α as well as mammary TNF- α gene expression. Our findings are consistent with Kang et al which

showed that resveratrol inhibited TNF- α release from macrophages and adipocytes after exposure to conditioned media from macrophages treated with resveratrol.⁵¹ Adipose tissue inflammation, including high levels of TNF- α , drives adipose-derived stem cells toward an endothelial cell or fibroblast fate rather than differentiation into a mature adipocyte.⁵² The anti-inflammatory activity of resveratrol, including the decreased TNF- α expression and prevention of macrophage infiltration into the adipose tissue, protects against inhibited adipogenesis observed in obesity. Our findings of pro-adipogenic effects of resveratrol are consistent with the findings of Hu et al, who examined the in vitro effects of resveratrol supplementation in 3T3-L1 preadipocytes at varying doses and found that resveratrol treatment at physiologically relevant levels (1 and 10 μ M) enhanced 3T3-L1 adipocyte differentiation while doses above 10 μ M resveratrol induced cell death.⁵³ Other groups have shown that deleterious levels of TNF- α , prevalent in obesity, can stimulate the dedifferentiation of adipocytes, which delipidate and take on a fibroblast-like phenotype and lack the ability to respond to insulin and store triglyceride.⁵⁴ The apparent dose-dependent effects on preadipocyte fate is supported by several reports showing that resveratrol at high concentrations inhibits PPAR γ expression, adipocyte differentiation, and adipocyte viability in vitro.^{55–57} In addition to impacting preadipocyte fate, resveratrol has also been shown to inhibit breast cancer stem cell proliferation by suppressing the Wnt/ β -Catenin signaling pathway, which is involved in tumor growth and metastasis and can also regulate adipogenesis.⁵⁸

We report here that supplementation of resveratrol in the diet (0.5% wt/wt) resulted in an average intake/kg body weight/day of 500.4 ± 100.1 mg/kg/day, which is in the upper half of the range of doses shown to have anticancer effects in multiple preclinical cancer studies.^{38,43,59} We found that this pharmacologic level of resveratrol supplementation in DIO + resveratrol mice relative to DIO mice did not impact body weight or body fat percentage, but did impart considerable protection against obesity-associated inflammation. Preventing adipocyte hypertrophy and the associated shift in pro-inflammatory cytokines (including TNF- α and IL-6) produced by hypertrophied adipocytes may explain why DIO + resveratrol mice, relative to DIO mice, had lower gene expression of cytokines associated with recruitment of macrophages, as well as lower COX-2 expression. Notably, our adipogenesis array in the mammary fat pad revealed that resveratrol offset the obesity-induced decrease of pro-adipogenic gene signaling (Figure 6), with a shift in gene expression in DIO + resveratrol towards that observed in control rather than DIO mice. Konings et al reported that resveratrol supplementation (at 150 mg/day, ~ 1.5 mg/kg body weight/day) for 30 days in obese men significantly decreased abdominal subcutaneous adipocyte size.⁶⁰ Similar to our findings, Konings et al also completed a microarray analysis, which indicated a downregulation in Wnt signaling. Additionally, Jimenez-Gomez et al observed that rhesus monkeys on a high-fat, high-sugar diet supplemented with resveratrol (80 mg/day in year one; 480 mg/day in year 2) displayed an increase in the number of small adipocytes in visceral white adipose tissue and a significant decrease in mean adipocyte size compared to rhesus monkeys on the high-fat, high-sugar diet.³⁸

From our adipogenesis array, we observed that DIO + resveratrol mice displayed Pgc1 α and Fabp4 expression levels that were not statistically different from control mice, while DIO mice had significantly lower expression of both genes. Pgc1 α and Fabp4, which facilitate

adipose beiging and fatty acid uptake, respectively, are downstream targets of PPAR γ .⁶¹ Prevention of the aberrant decrease in gene expression of adipogenesis-related and beiging-related genes observed in DIO mice may also contribute importantly to the protective effects of resveratrol against tumor growth in DIO + resveratrol mice. Enrichment of beige/brown adipose tissue relative to white adipose tissue could change the adipose secretome and limit lipids and other nutrients available to the tumor. Additional evidence that resveratrol enhances beige adipocyte development, comes from Zou et al, who found white adipose tissue browning in male mice that were the offspring of obese female mice supplemented with resveratrol in the diet (0.2% wt/wt) during pregnancy.⁶² Resveratrol also induced adipose browning in rats when added to an obesogenic diet.⁶³

There were several limitations to our study. First, we attempted to measure levels of resveratrol in the serum of fasted mice, but had few samples pass the threshold of detection, which was likely due to resveratrol being largely cleared from the blood in the mice analyzed. Thus, we are unable to report the serum levels that our dose achieved. However, we were successful in measuring resveratrol levels in tumors samples and found a range from 157.7 nmol/g to 661.5 nmol/g (Supplementary Table S3). This is comparable to the levels in the murine prostate reported by Narayanan et al that range from 175.2 nmol/g to 416.2 nmol/g.⁴⁵ To our knowledge tissue resveratrol concentrations in human tumors following resveratrol supplementation have not been widely reported so we unfortunately cannot compare the levels observed in our preclinical study to human studies. Another limitation of our study is the lack of a normal weight control group supplemented with resveratrol, which could have more clearly delineated the effects (if any) of resveratrol in the absence of obesity. Finally, we observed no effect of resveratrol on the gene expression of the sirtuin pathway in our study (data not shown). Sirt-1 is reportedly associated with the anticancer effects of resveratrol in vitro, although the effects of resveratrol on the sirtuin pathway are likely dose- and tissue-dependent.⁶⁴

Given the global prevalence of obesity, the emerging association between obesity and the development and progression of several breast cancer subtypes, and the current lack of targeted therapies for claudin-low and additional subtypes of triple-negative breast cancer, our findings have several important implications. Our preclinical data are the first to establish that resveratrol supplementation prevents obesity-associated increases in claudin-low mammary tumor growth, mammary adipocyte hypertrophy, COX-2 expression, macrophage infiltration, adipose CLS enrichment, and serum cytokines. Resveratrol supplementation also ameliorates downregulation of PPAR γ and other adipogenesis genes in the mammary tissue of DIO mice. We conclude that preventing obesity-associated adipose tissue dysregulation can be achieved with resveratrol supplementation, which may represent an effective intervention strategy for breaking the link between obesity and triple-negative breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Resveratrol supplementation does not impact the establishment of diet induced obesity (A) Study design. B, The DIO and control regimens effectively generated obese and normal weight phenotypes, with significant differences in body weight, beginning after three weeks on diet (DIO vs control, $P < 0.0001$; DIO + resveratrol vs control $P < 0.05$; DIO vs DIO + resveratrol *NS*). After 6 weeks on diet and when mammary tumor cells were injected, DIO and DIO + resveratrol had significantly greater body weights than control mice (DIO vs control, $P < 0.0001$; DIO + resveratrol vs control $P < 0.0001$; DIO vs DIO + resveratrol *NS*). C, Percent body fat, obtained at end of study, was significantly lower in control mice vs both DIO ($P < 0.0001$) and DIO + resveratrol ($P < 0.0001$), with no differences between DIO and DIO + resveratrol mice. Data presented as mean $\pm$ s.d. Differences in significance denoted by different letters (a,b), *NS* = not significant

FIGURE 2.

Resveratrol prevents adipocyte hypertrophy in the mammary fat pad of DIO mice. A, Representative image of H&E stained sections of the mammary fat pads from control, DIO and DIO + resveratrol mice. B, Histogram distribution (10th, 50th, and 90th percentiles) of the adipocyte areas of mammary fat pad sections from control, DIO and DIO + resveratrol. Red dashed lines indicate the median of each percentile. DIO mice had significantly higher adipocyte area for the (C) 10th percentile compared to both control and DIO + resveratrol (DIO vs control $P < 0.0001$; DIO vs DIO + resveratrol $P < 0.0001$; control vs DIO + resveratrol NS). DIO mice had significantly higher adipocyte area for the (D) 50th percentile compared to control and DIO + resveratrol (DIO vs control $P < 0.0001$; DIO vs DIO + resveratrol $P < 0.0001$). However, DIO + resveratrol mice also had significantly higher adipocyte area for the 50th percentile compared to control ($P < 0.05$). Similarly, DIO mice had significantly higher adipocyte area for the 90th percentile compared to control and DIO + resveratrol (DIO vs control $P < 0.0001$; DIO vs DIO + resveratrol $P < 0.0001$). Additionally, DIO + resveratrol mice also had significantly higher adipocyte area for the 90th percentile compared to control ($P < 0.0001$). Data presented as mean $\pm$ s.d Differences in significance denoted by different letters (a,b), NS = not significant

FIGURE 3.

Resveratrol protects against inflammatory and macrophage gene signaling in DIO mice. DIO mice had significantly increased gene expression of the pro-inflammatory mediators (A) IL-6 and Saa3 relative to both control and DIO + resveratrol mice. DIO mice also had significantly increased gene expression of (B) macrophage signaling molecules MCP-1 and CXCL12 compared to DIO + resveratrol mice. DIO mice had significantly increased expression of (C) GM-CSF and M-CSF, which promote macrophage proliferation, compared to both control and DIO + resveratrol mice. Gene expression of secretory products of activated macrophages, including (D) TNF- α and MIF were significantly increased in DIO mice compared to both control and DIO + resveratrol mice. Data presented as mean $\pm$ s.d Differences in significance denoted by different letters (a,b), *NS* = not significant.

FIGURE 4.

Resveratrol blunts mammary gland crown-like structure formation and COX-2 protein expression in DIO mice. A, Representative images of CLS and COX-2 protein expression in the mammary fat pads of control, DIO and DIO + resveratrol mice ($n = 14-15/\text{group}$). B, The prevalence of CLS (expressed as CLS/area of tissue in cm²) in the mammary fat pads was significantly increased in DIO mice relative to control and DIO + resveratrol mice (DIO vs control $P < 0.01$; DIO vs DIO + resveratrol $P < 0.05$; control vs DIO + resveratrol NS). C, Gene expression of Emr1, a macrophage-specific marker, was significantly increased in DIO mice compared with control and DIO + resveratrol mice. D, COX-2 protein expression was significantly increased in DIO mice compared with both control and DIO + resveratrol mice (DIO vs control $P < 0.0001$; DIO vs DIO + resveratrol $P < 0.0001$; control vs DIO + resveratrol NS). Data presented as mean $\pm$ s.d. Differences in significance denoted by different letters (a,b), NS = not significant

FIGURE 5.

Resveratrol maintains mammary tissue PPAR γ expression in DIO mice. A, Representative image and (B) quantitation of immunohistochemically stained sections from control, DIO, and DIO + resveratrol mice for PPAR γ protein expression. Protein levels of PPAR γ was significantly decreased in DIO mice compared to both control and DIO + resveratrol mice (DIO vs control $P < 0.0001$; DIO vs DIO + resveratrol $P < 0.0001$; control vs DIO + resveratrol NS). Similarly, DIO mice had significantly decreased (C) PPAR γ gene expression compared to DIO + resveratrol mice, however control mice displayed intermediate gene expression, (DIO vs control NS ; DIO vs DIO + resveratrol $P < 0.01$; control vs DIO + resveratrol NS). Data presented as mean $\pm$ s.d. Differences in significance denoted by different letters (a,b), NS = not significant

FIGURE 6.

Resveratrol protects against obesity-induced dysregulated expression of genes involved in adipogenesis in DIO mice. Quantitative real-time Adipogenesis PCR array on mammary fat pad tissue for genes involved in preadipocyte commitment, differentiation, adipocyte maturation, and adipocyte beiging. Heatmap representation of log₂ fold change ($\log_2$ (experimental group) – $\log_2$ (average of all groups)) of control, DIO and DIO + resveratrol mice ($n = 4-5$ /group). Black indicates a log₂ fold change of zero, representing a lack of change between the experimental group and the the average of all groups. Increasing brightness of green indicates an increase in expression ($\log_2$ fold change > 0). Increasing brightness of red indicates a lower level of the expression in the experimental group in comparison to the average of all groups ($\log_2$ fold change < 0).

FIGURE 7.

Resveratrol protects against enhanced M-Wnt murine mammary tumor size, infiltrating adipocyte size, and tumor associated macrophages in DIO mice. A, DIO mice had significantly increased ex vivo tumor volume compared to DIO + resveratrol ($P < 0.01$) and control ($P < 0.0001$). Similarly, the ex vivo tumor weight (B) was significantly greater in DIO mice compared to DIO + resveratrol ($P < 0.05$) and control ($P < 0.001$). C, The median area of intratumoral adipocytes in tumors from DIO mice was significantly increased compared to both control ($P < 0.001$) and DIO + resveratrol ($P < 0.0001$). D, DIO mice had significantly higher Macrophage marker (RM0029-11H3) compared with control ($P < 0.01$) and DIO + resveratrol mice ($P < 0.01$). The abundance of (E) F4/80 positive staining was significantly higher in DIO mice compared with control ($P < 0.01$) and DIO + resveratrol mice ($P < 0.01$). F, Representative images displayed. Data presented as mean $\pm$ s.d. Differences in significance denoted by different letters (a,b)

FIGURE 8.

Proposed mechanism of the anticancer effects of resveratrol in DIO mice. Highlighted aspects of the proposed mechanism of the anticancer effects of resveratrol. DIO results in abnormal adipocyte expansion, which leads to upregulated expression of pro-inflammatory mediators, enhanced macrophage signaling, increased prevalence of CLS, increased COX-2 expression, and decreased expression of pro-adipogenic genes such as PPAR_γ. This adipocyte reprogramming is associated with larger tumors, larger intratumoral adipocytes and a high abundance of tumor-associated macrophages. Supplementation of DIO mice with resveratrol attenuates many of the pro-cancer effects of DIO

TABLE 1

Serum hormones, adipokines, and cytokines

	Control	DIO	DIO + resveratrol
Serum hormones and adipokines (ng/mL)			
Insulin	0.3 ± 0.2 ^a	1.0 ± 0.8 ^b	0.5 ± 0.2 ^{a,b}
IGF-1	250.9 ± 85.3 ^a	406.6 ± 102.8 ^b	323.4 ± 71.3 ^{a,b}
Leptin	1.2 ± 0.5 ^a	7.6 ± 2.7 ^b	6.2 ± 2.6 ^b
Adiponectin	6.2 ± 3.1 ^a	4.8 ± 2.2 ^a	4.1 ± 2.3 ^a
Leptin: Adiponectin	0.5 ± 0.9 ^a	1.9 ± 1.5 ^b	2.0 ± 1.4 ^b
Serum cytokines (pg/mL)			
IFN- γ	12.4 ± 5.5 ^a	25.4 ± 7.3 ^b	17.5 ± 6.7 ^{a,b}
IL-1 β	140.9 ± 62.9 ^a	225.6 ± 58.4 ^b	125.6 ± 50.0 ^a
IL-6	10.0 ± 2.8 ^a	16.3 ± 2.0 ^b	11.1 ± 4.2 ^a
IL-10	51.4 ± 21.5 ^a	75.8 ± 31.8 ^a	51.3 ± 13.8 ^a
IL-17A	143.1 ± 11.1 ^a	148.6 ± 30.4 ^a	154.3 ± 20.6 ^a
MCP-1	174.5 ± 70.3 ^a	311.0 ± 52.3 ^b	220.1 ± 61.4 ^{a,b}
TNF- α	542.2 ± 161.4 ^a	870.4 ± 152.5 ^b	666 ± 265.6 ^{a,b}

IGF-1, insulin like growth factor-1; IFN- γ , interferon-gamma; IL-1 β , interleukin-1 beta; IL-6, interleukin-6; IL-10, interleukin-10; IL-17A, interleukin-17A; MCP-1, monocyte chemoattractant protein-1; TNF- α , tumor necrosis factor-alpha.

Statistical significance among groups denoted by different letters (a,b).

TABLE 2

Inflammatory lipid derivatives in mouse mammary tissue

Metabolite	Control	DIO	DIO + resverat
5-HETE	1.5 ± 0.8 ^a	2.3 ± 1.4 ^a	1.2 ± 0.5 ^a
12-HETE	62.6 ± 40.1 ^a	34.5 ± 31.3 ^a	24.1 ± 8.1 ^a
15-HETE	5.2 ± 3.3 ^a	4.9 ± 3.2 ^a	5.0 ± 1.4 ^a
13-HODE	69.9 ± 19.7 ^a	328.4 ± 124.7 ^b	98.8 ± 79.8 ^a
PGD ₂	43.6 ± 19.0 ^a	110.8 ± 69.5 ^a	52.0 ± 34.6 ^a
PGE ₁	3.3 ± 1.7 ^a	3.8 ± 2.0 ^a	1.9 ± 1.3 ^a
PGE ₂	27.2 ± 13.4 ^{a,b}	54.4 ± 12.0 ^a	16.0 ± 7.8 ^b
PGF _{2α}	7.4 ± 3.5 ^a	15.0 ± 4.3 ^b	8.1 ± 2.3 ^a
PGE ₃	0.3 ± 0.1 ^a	0.4 ± 0.2 ^a	0.9 ± 0.8 ^a
13PGE ₂	1.6 ± 0.6 ^{a,b}	2.4 ± 0.8 ^a	0.8 ± 0.2 ^b
6ketoPGF _{1α}	32.3 ± 7.9 ^a	35.5 ± 35.0 ^a	13.7 ± 8.2 ^a
TXB ₂	9.8 ± 5.4 ^a	11.4 ± 7.9 ^a	8.2 ± 6.2 ^a

HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin; TX, thromboxane. Statistical significance among groups denoted by different letters (a,b).