ORIGINAL ARTICLE

Supplemental estrogen and caloric restriction reduce obesity-induced periprostatic white adipose inflammation in mice

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Abstract

Obesity is associated with an increased incidence of high-grade prostate cancer (PC) and worse prognosis for PC patients. Recently, we showed in men that obesity-related periprostatic white adipose tissue (WAT) inflammation, characterized by macrophages surrounding dead or dying adipocytes forming crown-like structures, was associated with high-grade PC. Possibly, interventions that suppress periprostatic WAT inflammation will improve outcomes for men with PC. Here, we tested the hypothesis that supplemental 17 β -estradiol (E2) could decrease periprostatic WAT inflammation in obese male mice. Mice were fed a high-fat diet to induce periprostatic WAT inflammation before being treated with supplemental E2. E2 supplementation suppressed caloric intake, induced weight loss, decreased periprostatic WAT inflammation and downregulated the expression of genes linked to inflammation including *Cd68*, *Mcp1* and *Tnf*. Similar to the effects of E2 supplementation, treatment with diethylstilbestrol, a synthetic estrogen, also suppressed caloric intake and reduced periprostatic WAT inflammation. To determine whether the observed effects of supplemental estrogen could be reproduced by caloric restriction (CR) alone, obese mice were put on a 30% CR diet. Like estrogen treatment, CR was effective in reducing body weight, periprostatic WAT inflammation and the expression of pro-inflammatory genes. Transcriptomic analyses of periprostatic fat showed that obesity was associated with enrichment in inflammatory response pathways, which were normalized by both supplemental E2 and CR. Taken together, these findings strengthen the rationale for future efforts to determine whether either CR or supplemental estrogen will decrease periprostatic WAT inflammation and thereby improve outcomes for men with PC.

Introduction

Obesity is associated with the development of high-grade prostate cancer (PC) and worse prognosis for men diagnosed with PC (1–4). A hallmark of obesity is chronic white adipose tissue (WAT) inflammation, characterized by dead or dying adipocytes

surrounded by macrophages forming crown-like structures (CLS) (5). The prostate is covered by a thin connective tissue capsule. Beyond the capsule, the prostate is surrounded by visceral WAT. We previously showed that periprostatic WAT inflammation

Received: December 9, 2018; Revised: April 13, 2019; Accepted: May 7, 2019

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Abbreviations

CLS	crown-like structure
CR	caloric restriction
DES	diethylstilbestrol
HFD	high-fat diet
IPA	Ingenuity Pathway Analysis
LFD	low-fat diet
NK	natural killer
PC	prostate cancer
WAT	white adipose tissue

occurs in approximately 50% of PC patients and is positively associated with high-grade PC, suggesting a potential contributory role (6). Periprostatic WAT inflammation was associated with adipocyte hypertrophy, insulin resistance and reduced levels of adiponectin, an anti-inflammatory adipokine (6). Both insulin resistance and low levels of adiponectin have been linked to the pathogenesis of PC (6–8). Therefore, pharmacological or behavioral interventions that decrease obesity-related periprostatic WAT inflammation may be useful for improving the prognosis of PC patients. Recently, we developed a mouse model of obesity-related periprostatic WAT inflammation that provides an opportunity to test potential anti-inflammatory interventions (9).

Supplemental estrogen is known to suppress appetite and to possess anti-inflammatory properties (10–12). Previously, we showed that 17 β -estradiol (E2) reversed obesity-induced WAT inflammation in the mammary glands of female mice (13). Because estrogens have been used to treat men with advanced PC (14,15), the first aim of this study was to test the hypothesis that supplemental estrogen will attenuate periprostatic WAT inflammation in obese male mice. Whether lifestyle interventions such as weight loss can improve the prognosis of obese men with PC is an important unanswered question. Hence, the second goal of this study was to determine whether caloric restriction (CR) can attenuate periprostatic adipose inflammation and normalize-related changes in gene expression. Here, we demonstrate that obesity-induced inflammation in periprostatic fat, including high CLS density and elevated levels of pro-inflammatory mediators, is attenuated by supplemental E2 in association with reduced caloric intake and weight loss. Moreover, CR alone was sufficient to decrease obesity-induced periprostatic WAT inflammation and elevated levels of pro-inflammatory mediators. Treatment of obese mice with E2 or CR led to a normalization of the transcriptomic profile of periprostatic WAT. Collectively, our data highlight the utility of E2 supplementation and CR as effective strategies to reduce obesity-induced periprostatic WAT inflammation and strengthen the rationale for future translational studies in PC patients.

Materials and methods

Materials

RNeasy Mini Kits were purchased from Qiagen. Murine Leukemia virus reverse transcriptase, RNase inhibitor, oligo (dT)₁₆ and Fast SYBR green PCR master mix were obtained from Applied Biosystems. Real-time PCR (RT-PCR) primers were synthesized by Sigma-Genosys. 17 β -estradiol (E2), diethylstilbestrol (DES), and placebo pellets were purchased from Innovative Research of America.

Animal models

Diet-induced obesity

Male C57BL/6J mice were obtained from The Jackson Laboratory and fed low-fat diet (LFD, 10 kcal% fat, 12450Bj; Research Diets) or high-fat diet (HFD, 60 kcal% fat, D12492i) beginning at 6 weeks of age. The mice were continued on these diets for a total of 12 weeks before being killed at 18 weeks of age.

Estrogen treatment

Male C57BL/6J mice were fed LFD or HFD for 12 weeks as described previously. After 12 weeks of feeding, the HFD-fed mice were divided into two groups. One group of HFD-fed mice received a 0.25 mg E2 pellet (60-day continuous release). A second group of HFD-fed mice received a placebo pellet. Pellets were subcutaneously implanted using a 10 gauge trocar. All mice continued on their respective diets for an additional 8 weeks until killed at 26 weeks of age. In the case of the DES study, the study design was similar except a 0.025 mg DES pellet was used. The weights of the mice and calories consumed were monitored throughout the experiment. To measure caloric consumption, amount of diet consumed by each mouse over 24 h was determined by measuring the starting weight of the diet in the cage hopper minus the final weight after 24 h, divided by number of mice in each cage (i.e. weight of diet consumed/mouse/day). Measurements were taken over three 24 h periods each week and averaged to determine daily consumption each week. The known energy density of each diet (LFD: 3.82 kcal/g, HFD: 5.21 kcal/g) was used to convert weight of diet consumed to calories consumed.

Caloric restriction

Male C57BL/6J mice were fed LFD or HFD for 12 weeks as described in the diet-induced obesity study. The LFD group was fed *ad libitum* for an additional 7 weeks until killed. The HFD-fed mice were divided into two groups. One group was fed *ad libitum* for an additional 7 weeks until killed, whereas the other group was switched to a 30% CR feeding regimen for 7 weeks until killed. Each day for 7 weeks the CR group received 70% of the amount consumed by the HFD group that was fed *ad libitum*.

Following killing in all studies, periprostatic WAT was snap-frozen in liquid nitrogen and stored at –80°C for molecular analyses or formalin fixed for histological analyses. All animal studies were approved by the institutional animal care and use committee at Weill Cornell Medicine (New York, NY).

Quantitative RT-PCR

Total RNA was isolated from periprostatic WAT using the RNeasy Mini Kit. RNA was reverse transcribed to complementary DNA using murine leukemia virus reverse transcriptase and oligo (dT)₁₆ primer. RT-PCR was performed using 2 $\times$ Fast SYBR Green PCR Master Mix on a 7500 Fast Real-Time PCR System (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous normalization control. Relative fold-induction was determined using the ddC_t (relative quantification) analysis protocol.

Light microscopy

Four-micron-thick sections were cut from formalin-fixed, paraffin-embedded periprostatic WAT followed by staining with hematoxylin and eosin. The total number of CLS per section was quantified by a pathologist (D.G.) and the area of periprostatic WAT present on each slide was determined using National Institutes of Health Image J. Inflammation was quantified as number of CLS/cm² of periprostatic fat tissue.

Multiphoton microscopy

Multiphoton microscopy imaging was carried out using an upright Olympus Fluoview FV1000MPE imaging system (Olympus America, Center Valley, PA), with a Mai Tai DeepSee laser, tunable between 690 and 1040 nm, as the excitation source (Spectra Physics, Santa Clara, CA). The periprostatic fat specimen was fixed and labeled with BODIPY FL to label fat, and 4',6-diamidino-2-phenylindole to label nuclei. It was placed at the center of a glass-bottomed 35-mm dish, hydrated with phosphate-buffered saline and overlaid with a coverslip, on which another drop of phosphate-buffered saline was placed to achieve water immersion. The specimen was imaged using a 25 $\times$ /1.05NA water immersion objective, using a two-photon excitation wavelength of 780 nm, and a scanner zoom factor of 3. The 4',6-diamidino-2-phenylindole signal was collected using an emission filter with a bandpass of 420–460 nm, and the BODIPY FL signal was collected using a filter with a bandpass of 495–540 nm. The signals in individual channels were collected as separate grayscale images, color-coded and merged in Fiji/ImageJ (National Institutes of Health) to produce the final image. Minor adjustments of brightness and color balance were carried out using Adobe Photoshop CC 2015. Scale bar: 20 μ m.

Adipocyte diameter

Adipocyte diameter was quantified using the Linear Dimension tool in Canvas 11 (ACS Systems of America) as described previously (16).

Transcriptome analysis of periprostatic WAT

Total RNA from periprostatic fat was isolated using the RNeasy Mini Kit. Estrogen treatment study: sequencing libraries were constructed following the Illumina TrueSeq Stranded Total RNA Library preparation protocol with ribosomal RNA depletion. Next-generation sequencing was performed with paired-end 50 bp using the Illumina HiSeq 4000 platform (Weill Cornell Medicine Genomics Resources Core Laboratory). Raw sequenced reads were aligned to the Mouse reference genome (version mm10 from UCSC) using STAR (version 2.4.2) aligner. Aligned reads were quantified against the reference annotation (mm10 from UCSC) to obtain FPKM (Fragments per Kilobase per Million) and raw counts using Cufflinks (version 2.2.1) and HTSeq, respectively. Diet-induced Obesity Study and Caloric Restriction Study: Samples were labeled and hybridized to oligonucleotide microarrays (GeneChip Mouse Gene 2.0 ST, Affymetrix) according to standard protocols at the Weill Cornell Medicine Genomics Resources Core Facility. Raw image data were background corrected, normalized and summarized into probeset expression values using the Robust Multi-chip Average algorithm within GeneSpring 12.6 Software (Agilent Technologies). Log2-transformed expression values for within-array replicate probes were condensed by their average. Differential gene expression analyses were carried out using limma package in R 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria). Genes with false discovery rate less than 0.05 and fold change more than 2 in between-group comparisons were considered significantly differentially expressed. Ingenuity Pathway Analysis (IPA, Qiagen) was used to identify the diseases and functions enriched with the top differentially expressed genes associated with HFD-induced obesity, E2 supplementation and CR. All transcriptomics data were deposited onto GEO and given the accession numbers: GSE123348, GSE123355, GSE123357.

xCell profiling of immune cell types and abundance

Abundance estimation of immune cell types, including macrophages, dendritic cells, T cells and natural killer (NK) cells, in periprostatic WAT, was performed using the computational tool xCell (17). Normalized bulk RNAseq expression data (FPKM) and normalized microarray expression were used as input data. Before running xCell, mouse gene names were converted into their respective human orthologous gene names, using the Mouse Genome Informatics database (18), for adaptation to xCell. The cell type abundance enrichment scores were then visualized across all study groups followed by statistical calculations using the Wilcoxon test.

Statistical analysis

In these studies, mouse body weights over time and consumption over time were examined and summarized graphically. End points of interest include mouse body weights at killing and various measurements including number of CLS/cm², average adipocyte diameter and relative messenger RNA expression level in periprostatic WAT. To examine differences in the end points across multiple groups, analysis of variance or the non-parametric Kruskal–Wallis test was used where appropriate. Student's t-test or the non-parametric Wilcoxon rank-sum test was used to examine differences in end points between two independent groups where appropriate. All statistical tests were two sided and P-value of less than 0.05 was considered statistically significant.

Results

Diet-induced obesity is associated with periprostatic fat inflammation and significant alterations in gene expression

To determine whether obesity caused periprostatic WAT inflammation as defined by the presence of CLS, male mice were fed a LFD or HFD. Body weight and periprostatic WAT inflammation were determined at killing. After feeding these diets for 12 weeks, the HFD-fed mice weighed approximately 40%

more than the mice that received the LFD (Figure 1A). In association with the increase in weight, HFD-fed mice developed periprostatic WAT inflammation (Figure 1B and C). In parallel to the increase in body weight and periprostatic WAT inflammation, microarray analysis showed significant alteration in transcriptomic profile of periprostatic WAT from obese mice compared with lean controls. Compared with LFD-fed control mice, 299 genes were significantly upregulated and 112 genes significantly downregulated in association with HFD-induced obesity. The top 50 genes differentially regulated by HFD in periprostatic WAT are shown in Figure 1D and Supplementary Table 1, available at Carcinogenesis Online. Next, IPA was used to determine how these gene expression changes related to diseases and functions. A significant impact on the 'inflammatory response' was predicted by IPA in response to the HFD with a total of 82 molecules involved in this function altered (Figure 1E, Supplementary Table 2, available at Carcinogenesis Online). Our findings reveal a pro-inflammatory signature in periprostatic WAT in association with obesity.

Supplemental E2 reverses obesity-induced periprostatic fat inflammation in association with decreased calorie consumption and weight loss

Here, we tested whether E2 could reverse obesity-induced periprostatic WAT inflammation. Mice were fed HFD for 12 weeks to induce obesity and periprostatic WAT inflammation (Figure 2A). The HFD-fed mice were then randomized to receive a subcutaneous placebo pellet or a continuous release pellet containing E2 for 8 weeks while continuing on HFD. In parallel, a group of mice was fed LFD for the duration of the study to serve as lean controls. As shown in Figure 2B, mice given a placebo pellet continued to gain weight on HFD whereas E2 supplementation led to a significant reduction in body weight. Consistent with the decrease in body weight, caloric consumption was suppressed following E2 treatment compared with the placebo group (Figure 2C). A number of studies have shown that periprostatic fat thickness is a negative prognostic marker for PC (19–21). Therefore, we measured periprostatic WAT wet weight at killing. Periprostatic WAT from mice in the HFD + placebo group weighed significantly more than periprostatic WAT from mice in the LFD group (Figure 2D). Supplemental E2 attenuated the elevation in periprostatic WAT weight with a strong correlation seen with body weight (Figure 2D–E). Because adipocyte size correlates with both body weight and WAT inflammation (5,16,22), we determined whether supplemental E2 led to a change in adipocyte size. E2 supplementation led to a significant decrease in periprostatic adipocyte diameter compared with mice given placebo (Figure 2F). Consistent with the reduction in adipocyte diameter, histological analysis of periprostatic WAT demonstrated that E2 supplementation significantly reduced the severity of periprostatic WAT inflammation (Figure 2G and H).

HFD-induced alterations in periprostatic fat transcriptome are normalized by supplemental E2

Having found that obesity leads to significant alterations in periprostatic WAT transcriptome, we next determined whether E2 supplementation could prevent these changes. RNA-seq analysis was conducted on periprostatic WAT isolated from mice in the LFD, HFD + placebo and HFD + E2 groups. A gene expression heatmap was generated consisting of the top 50 genes differentially regulated by HFD-induced obesity and E2 supplementation. As shown in Figure 3A, E2 supplementation in obese mice shifted the gene expression profile toward a pattern of expression closely resembling the LFD group. Fold change of the top 50

Figure 1. High-fat diet-induced obesity is associated with periprosthetic WAT inflammation in mice. (A) Body weight at killing of male mice fed a 10 kcal% LFD or 60 kcal% HFD for 12 weeks ($n = 7/\text{gp}$). (B) Representative image of a CLS in periprosthetic fat after 12 weeks of HFD feeding shown by hematoxylin and eosin staining (left) and two-photon microscopy (right, green = BODIPY FL labeling of adipocytes, blue = 4',6-diamidino-2-phenylindole labeling of nuclei). (C) Periprosthetic white adipose inflammation defined as CLS/cm² was quantified at the time of killing ($n = 7/\text{gp}$). (D) Microarray RNA profiling was conducted on periprosthetic fat from mice fed LFD and HFD for 12 weeks ($n = 4\text{--}5/\text{gp}$). Heat map of the top 50 differentially expressed genes is shown with false discovery rate less than 0.05 and absolute log₂ (fold change) more than 1, ranked by absolute log₂ (fold change). (E) Top 5 'Diseases and Biological Functions' determined by IPA to be enriched by HFD feeding are shown and ranked by the -log₁₀(P-value) from the Fisher's Exact test across all the observations. For panels A and C, mean $\pm$ SD (error bars) are shown, *** $P < 0.01$, **** $P < 0.001$.

genes shown in the heatmap is listed in [Supplementary Table 3](#), available at [Carcinogenesis Online](#). IPA was used to determine the effect of E2 supplementation on HFD-induced activation of the inflammatory response in periprosthetic WAT. As expected, significant activation of the inflammatory response was identified in the HFD + placebo group compared with LFD controls with 179 molecules involved in this function altered and an activation z-score of +5.6. E2 supplementation significantly inhibited

activation of the inflammatory response compared with placebo with 154 molecules altered and an activation z-score of -3.7. The diseases/functions under the umbrella of the 'inflammatory response' that are enriched by HFD and significantly reversed by E2 supplementation are shown in [Figure 3B](#).

Quantitative RT-PCR was used to confirm that E2 downregulated the expression of several genes known to be associated with inflamed adipose tissue ([Figure 3C](#)).

Figure 2. Estrogen suppresses inflammation in periprostacic white adipose tissue in mice fed a high-fat diet. (A) Study schema. At 6 weeks of age, C57BL/6J male mice were fed either LFD or HFD. After 12 weeks of feeding, the HFD mice were randomized to receive either a placebo pellet ($n = 16$) or 0.25 mg continuous release E2 pellet ($n = 15$) for 8 additional weeks before killing. The LFD-fed mice were killed after 20 weeks of feeding ($n = 16$). (B) Body weights of mice in different treatment groups. (C) Caloric consumption was monitored in mice following E2 pellet implantation until killed. (D) Periprostacic fat (PPF) wet weight was measured at killing. (E) PPF wet weight was correlated with bodyweight at kill. (F) Mean PPF adipocyte diameter ($n = 6/\text{gp}$). (G) Representative images of PPF from each treatment group is shown with CLS indicated by arrows. (H) Periprostacic white adipose inflammation was quantified as CLS/cm² ($n = 6\text{--}12/\text{gp}$). Mean $\pm$ SD (error bars) are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ comparing HFD + E2 with HFD + placebo.

Consistent with the CLS findings, levels of the macrophage marker *Cd68* are elevated in the HFD + placebo group and significantly attenuated by E2 supplementation. Similarly, expression of monocyte chemoattractant protein-1 (*Mcp1*), which plays a significant role in macrophage recruitment, and *Tnf*, a pro-inflammatory mediator, were both elevated in the HFD + placebo group and significantly decreased by E2 supplementation (*Mcp1*) or decreased with a trend toward significance (*Tnf*).

DES suppresses periprostacic fat inflammation in obese mice

In the clinical setting, a synthetic, non-steroidal form of estrogen called DES has been used to treat PC. To extend on our E2 findings, obese mice were treated with subcutaneous DES pellets to determine its effects on periprostacic WAT inflammation (Figure 4A). Like E2, DES-induced significant weight loss compared with placebo in association with reduced caloric consumption and a significant attenuation of periprostacic WAT inflammation (CLS/cm²; Figure 4B–E).

CR attenuates obesity-induced periprostacic WAT inflammation

Because both supplemental E2 and DES suppressed periprostacic WAT inflammation in association with reduced caloric intake and weight loss, we next investigated whether CR alone would suppress inflammation. To this end, we fed male mice HFD for 12 weeks to induce obesity and periprostacic WAT inflammation. The mice were then randomized either to continue HFD feeding *ad libitum* or to a 30% CR diet for an additional 7 weeks (Figure 5A). In parallel, a group of mice was fed LFD for the duration of the study to serve as lean controls. Consistent with the effects of E2 treatment, CR led to significant decreases in body weight, adipocyte diameter, CLS/cm² in periprostacic WAT and pro-inflammatory gene expression (Figure 5B–F).

Both CR and E2 normalize obesity-induced periprostacic WAT transcriptome changes

Transcriptomic analysis of periprostacic WAT from mice in the CR study shows that mice fed HFD *ad libitum* displayed

Figure 3. Obesity-induced alterations in periprostic fat transcriptome are normalized by supplemental E2. (A) Heatmap of the top 50 genes differentially regulated by HFD-induced obesity and normalized with E2 treatment as identified by RNA-seq analysis of periprostic fat ($n = 5-6/\text{gp}$). False discovery rate was set at less than 0.05 and absolute log₂(fold change) more than 1. Genes are ranked by absolute log₂ (fold change). (B) Diseases and functions related to the 'Inflammatory Response' as identified by IPA are ranked by P-value with the colors in the heat map indicating their activation z-score. The first column represents the effect of HFD feeding, comparing the HFD+Placebo (HFD) group with the LFD group. The second column represents the effects of E2 treatment, comparing the HFD + E2 (E2) group with HFD + placebo (Placebo) group. (C) Relative expression of *Cd68*, *Mcp1* and *Tnf* in periprostic fat was determined by quantitative real-time PCR. Mean $\pm$ SD (error bars) are shown. * $P < 0.05$, *** $P < 0.001$ comparing HFD + E2 with HFD + placebo.

significantly altered transcriptome compared with LFD fed mice (Figure 6A). However, HFD mice treated with CR had a gene expression profile distinct from the HFD *ad libitum* mice and more similar to LFD-fed mice (Figure 6A). Fold change of the top 50 genes differentially regulated by HFD and reversed by CR is shown in Supplemental Table 4, available at Carcinogenesis Online. As we found with E2 treatment, CR also inhibited HFD-induced enrichment in the inflammatory response. IPA predicted this function to be activated in periprostic WAT from mice fed HFD *ad libitum* (+3.2 activation z-score, 77 molecules altered) whereas it was decreased with CR (-2.4 activation z-score, 42 molecules altered). The diseases/functions under the umbrella of the 'inflammatory response' that are enriched by HFD and significantly reversed by CR are shown in Figure 6B. Comparing the overall effects on periprostic WAT gene expression, we found a large number of genes significantly altered by HFD-induced obesity to be similarly normalized by both E2 and CR treatment (Figure 6C). Furthermore, IPA's Upstream Regulator analysis identified inhibition of the same pro-inflammatory genes (*Tgfb1*, *Tnf*, *Ifng*, *Il-6*, and *Il-1 β*) as regulators of the gene expression changes associated with both E2 and CR treatment (Supplemental Table 5, available at Carcinogenesis Online).

Several recent studies have demonstrated an association between obesity-induced alterations in immune cell function/number and adipose inflammation, tumor growth and response to immune therapy (23-26). Therefore, we used the computational screening tool xCell to determine whether the abundance of various immune cells was altered in periprostic WAT of obese mice and normalized by E2 or CR treatment. HFD was associated with significantly higher abundance of macrophages and dendritic cells that were reduced by both E2 and CR treatment (Supplementary Figure 1, available at Carcinogenesis Online). No differences were observed in T cell (CD4+ and CD8+) or NK cell abundance in association with HFD or with either E2 or CR treatment (Supplementary Figure 1, available at Carcinogenesis Online).

Discussion

Obese men are at increased risk of developing high-grade PC that is associated with worse prognosis. A hallmark of obesity is chronic subclinical WAT inflammation, defined by the presence of CLS. Previously, we reported that periprostic WAT inflammation occurs in approximately 50% of PC patients in association with high-grade PC (6). This finding was consistent with

Figure 4. Diethylstilbestrol attenuates periprostatic white adipose tissue inflammation in obese mice. (A) Study schema. At 6 weeks of age, C57BL/6J male mice were fed either LFD or HFD. After 12 weeks of feeding, the HFD mice were randomized to receive either a placebo pellet ($n = 10$) or 0.025 mg continuous release DES pellet ($n = 13$) for 8 additional weeks before killing. The LFD-fed mice were killed after 20 weeks of feeding ($n = 10$). (B) Body weights of mice in different treatment groups. (C) Caloric consumption was monitored in mice following E2 pellet implantation until killed. (D) Representative images of periprostatic fat from each treatment group are shown with CLS indicated by arrows. (E) Periprostatic white adipose inflammation was quantified as CLS/cm². Mean $\pm$ SD (error bars) are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ comparing HFD + DES with HFD + placebo.

previous reports by our group and others demonstrating that WAT inflammation is an independent predictor of poor prognosis for other cancers (27–29). Given the link between WAT inflammation and cancer, the main objective of this preclinical study was to identify strategies to decrease periprostatic WAT inflammation as the basis for future translational studies. In a mouse model of obesity-related periprostatic WAT inflammation, we report significant anti-inflammatory effects of both supplemental estrogens and CR.

Estrogens possess anti-inflammatory effects and have been used to treat men with PC (13–15,30). Here, treatment with E2 led to reduced calorie consumption and weight loss with associated decreases in adipocyte size, periprostatic WAT inflammation and levels of pro-inflammatory mediators. Although these effects of exogenous estrogen on periprostatic fat were previously unknown, the findings are consistent with the known biological effects of estrogen. Supplemental estrogen has been reported to suppress appetite via direct effects on the hypothalamus, protect against adipocyte hypertrophy and reduce adipose inflammation in other fat depots in mice (12,13,31–33). The reduction in weight may be due to both reduced appetite and increased metabolism (11,34,35). The observed anti-inflammatory effects of E2 in periprostatic fat could be a consequence of its ability to stimulate weight loss. Alternatively, estrogen is also known to possess anti-inflammatory activity. For example, estrogen can block the induction of pro-inflammatory pathways in macrophages (36). Activation of nuclear factor-kappaB, a pro-inflammatory transcription factor, can be blocked by estrogen (37). This is potentially relevant because CLS are comprised of macrophages and nuclear factor-kappaB is known to be activated in inflamed WAT in both mice and humans (16,38–40).

Notably, DES, a form of estrogen given to patients with PC, was also effective at suppressing calorie consumption, inducing weight loss and reducing periprostatic WAT inflammation.

Currently, it is uncommon for estrogens to be used to treat PC, at least in part, due to side effects like cardiovascular toxicity associated with oral administration (41). However, new forms of estrogen delivery may be safer. Indeed, a large randomized phase III clinical trial called the Prostate Adenocarcinoma TransCutaneous Hormones (PATCH) trial is underway in the United Kingdom testing the safety and efficacy of transdermal E2 versus another PC treatment, luteinizing hormone-releasing hormone agonist. Initial reports from this study indicate that this route of E2 administration is safe with no cardiovascular events reported and better overall quality of life compared with patients randomized to luteinizing hormone-releasing hormone agonist (42,43). Our study provides further support for the potential benefits of E2 treatment in PC in the setting of obesity.

Ideally, E2 could also be used in the prevention setting to reduce periprostatic WAT inflammation and potentially decrease the risk of developing PC. A limitation of this approach is the requirement for a biopsy of periprostatic WAT for pathological assessment of inflammation. New strategies are needed to non-invasively detect WAT inflammation with the goal of identifying the subset of men who might benefit from the anti-inflammatory effects of estrogens. Also limiting the use of E2/DES in the prevention setting are the cardiovascular side effects associated with high dose DES (5 mg) traditionally used for the management of PC. A number of studies have found that a low dose of DES (1 mg) is also effective in the treatment of PC without the cardiovascular toxicity associated with high doses (15,44,45). Possibly, either low dose DES or non-oral route of delivery (e.g.

Figure 5. Caloric restriction suppresses inflammation in periprostatic white adipose tissue in mice fed a high-fat diet. (A) Study schema. At 6 weeks of age, C57BL/6J male mice were fed either LFD or HFD. After 12 weeks of feeding, the HFD mice were randomized to either continue receiving HFD *ad libitum* ($n = 15$) or to a 30% CR feeding regimen ($n = 15$) for 7 additional weeks before killing. The LFD-fed mice were killed after 19 weeks of feeding ($n = 16$). (B) Body weights of mice in different treatment groups over time after initiation of CR. (C) Average periprostatic fat (PPF) adipocyte diameter ($n = 8$ /gp). (D) Representative images of PPF from each treatment group are shown with CLS indicated by arrows. (E) Periprostatic white adipose inflammation was quantified as CLS/cm² ($n = 9$ –10/gp). (F) Relative expression of *Cd68*, *Mcp1* and *Tnf* in periprostatic fat was determined by quantitative real-time PCR. Mean $\pm$ SD (error bars) are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, comparing HFD + CR with HFD.

transdermal, pending results of the PATCH trial) may be effective strategies for reducing the cardiovascular effects of DES and allowing for its safe use in men with periprostatic WAT inflammation.

Because both E2 and DES suppressed caloric intake in association with reducing periprostatic WAT inflammation, we also tested whether CR would be sufficient to phenocopy the protective effect of estrogens on periprostatic WAT inflammation. Analogous to treatment with estrogens, CR led to significant weight loss, decreased adipocyte size, reduced severity of WAT inflammation and normalized the expression of a large number of genes and pathways associated with inflammation. These data indicate that CR, a behavioral intervention, may be an effective strategy to decrease periprostatic WAT inflammation in the approximately 50% of men with PC. Moreover, it is possible that combining estrogens with CR will be more effective than either treatment alone.

Several recent studies have suggested that obesity is a determinant of both immune cell function and response to checkpoint inhibitors in patients with cancer (23–26,46). Immune cell profiling by xCell suggested that the abundance of macrophages and dendritic cells in periprostatic WAT increased in response to

HFD, effects that were attenuated by CR or treatment with E2. In contrast, neither the abundance of T cells (CD8+, CD4+) nor NK cells appeared to be affected. It is possible that even though the abundance of NK and T cells was not changed, immune cell activity could have been affected. In addition, gene expression data from whole periprostatic WAT, and not an enriched population of immune cells, were used in the xCell analysis to infer immune cell types. This may explain the very low overall abundance of T cells and NK cells detected, which are probably too low to observe differences between groups. Given the potential limitations of a computational screening approach using bulk RNA, other approaches including flow cytometry or single cell sequencing could be used to better define the potential changes in immune cell populations in periprostatic WAT in response to HFD, E2 and CR.

Collectively, we demonstrate the efficacy of estrogens in attenuating obesity-induced periprostatic WAT inflammation in mice. Similar anti-inflammatory effects are achieved with CR, presenting a behavioral intervention that can be utilized as a risk-reduction strategy without the side effects of estrogen treatment. Whether such interventions attenuate periprostatic WAT inflammation in men or inhibit the development or progression of PC warrants further investigation.

Figure 6. Caloric restriction and E2 normalize obesity-induced periprostatic WAT transcriptome changes. **(A)** Heatmap of the top 50 genes differentially regulated by HFD-induced obesity and normalized by CR treatment as identified by microarray analysis of periprostatic fat ($n = 5-6/\text{gp}$). False discovery rate was set at less than 0.05 and absolute log₂ (fold change) more than 1. Genes are ranked by absolute log₂ (fold change). **(B)** Diseases and functions related to the 'Inflammatory Response' as identified by IPA are ranked by P -value with the colors in the heatmap indicating their activation z-score. The first column represents the effect of HFD feeding, comparing the HFD *ad libitum* group with the LFD group. The second column represents the effects of CR treatment, comparing the HFD + CR group with HFD *ad libitum* group. **(C)** The numbers of unique and shared genes significantly upregulated by HFD feeding (top panel) or downregulated by HFD feeding (bottom panel) and normalized by both CR and E2 treatment are shown in the Venn diagrams.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

Funding

Tokyo Association for Clinical Surgery (to T.I. and M.M.); Patricia and William Kleh (to A.J.D.); National Institutes of Health/ National Cancer Institute (P50 CA092629 to A.J.D.).

Conflict of Interest: None declared.

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