JPET Fast Forward. Published on March 22, 2018 as DOI: 10.1124/jpet.117.246256
This article has not been copyedited and formatted. The final version may differ from this version.

JPET#246256

Title Page

Indomethacin enhanced brown fat activity

Authors:

Lei Hao, Jamie Kearns, Sheyenne Scott, Dayong Wu, Sean D. Kodani, Christophe Morisseau, Bruce D. Hammock, Xiaocun Sun, Ling Zhao, Shu Wang.

Author affiliations:

Department of Nutritional Sciences, Texas Tech University, Lubbock, Texas, United States of America (L. H., S. S., S. W.); Department of Nutrition (J. K., L. Z.) and Research Computing Support (X. S.), The University of Tennessee, Knoxville, Tennessee, United States of America; Nutrition Immunology Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, Massachusetts, United States of America (D. W.); Department of Entomology and Nematology, and UC Davis Comprehensive Cancer Center, University of California, Davis, Davis, California, United States of America (S. D. K., C. M., B. D. H.)

JPET Fast Forward. Published on March 22, 2018 as DOI: 10.1124/jpet.117.246256
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title Page

Running title: Indomethacin enhanced brown fat activity

*Correspondences:

*Shu Wang, Department of Nutritional Sciences, Texas Tech University, Box 41270, Lubbock, TX 79409-1270, US, Email: shu.wang@ttu.edu, Telephone number: (806) 834-4050, Fax number: (806) 742-2926.

*Ling Zhao, 1215 W. Cumberland Ave, Room 229 Jessie Harris Building, Knoxville, TN 37996-1920, Email: ling.zhao@utk.edu, Telephone number: (865) 974-1833, Fax number: (865) 974-3491.

Number of text pages: 36

Number of tables: 2

Number of figures: 5

Number of references: 36

Number of words in the Abstract: 199

Number of words in the Introduction: 746

Number of words in the Discussion: 1094

Abbreviations: Acadm, acyl-Coenzyme A dehydrogenase; ATGL, adipocyte triglyceride lipase; BAT, brown adipose tissue; C/EBPs, CCAAT/enhancer-binding proteins; Cidea, cell death-

inducing DFFA-like effector A; COX, cyclooxygenases; COX4b, cytochrome c oxidase subunit IV isoform 2; COXII, cytochrome c oxidase subunit 2; eWAT, epididymal WAT; CT, computed tomography; FDA, US Food and Drug Administration; HDL-C, high-density lipoprotein cholesterol; HSL, hormone-sensitive lipase; iBAT interscapular BAT; iWAT, inguinal WAT; LDL-C, low-density lipoprotein cholesterol; NEFA, non-esterified fatty acids; NE, norepinephrine; NSAID, non-steroidal anti-inflammatory drug; PET, positron emission tomography; PGC-1α, PPAR-γ coactivator 1-alpha; Plin1, perilipin 1; Plin2, perilipin 2; PPARγ, peroxisome proliferator-activated receptor gamma; Prdm16, PR domain containing 16; SNS, sympathetic nervous system; TC, total cholesterol; TG, triglycerides; Tfam, mitochondrial transcription factor A; UCP1, uncoupling protein 1; VLDL-C, very-low-density lipoprotein cholesterol; WAT, white adipose tissue.

Section assignment: Drug Discovery and Translational Medicine

Abstract

Indomethacin, a non-steroidal anti-inflammatory drug, has been shown to induce white adipocyte differentiation; however, its roles in brown adipocyte differentiation, and activation in brown adipose tissue (BAT) and obesity are unknown. To address this issue, we treated mouse pre-brown cells with different doses of indomethacin, and delivered indomethacin to interscapular BAT (iBAT) of obese mice using implanted osmotic pumps. Indomethacin dose-dependently increased brown adipocyte differentiation, and upregulated both mRNA and protein expression of uncoupling protein 1 (UCP1) and peroxisome proliferator-activated receptor (PPAR) gamma (γ) coactivator 1-alpha. The mechanistic study showed that indomethacin significantly activated the reporter driven by PPAR response element, indicating that indomethacin may work as a PPARγ agonist in this cell line. Consistently, indomethacin significantly decreased iBAT mass and fasting blood glucose levels in the high-fat diet induced obese (DIO) mice. Histological analysis showed that brown adipocytes of indomethacin-treated mice contained smaller lipid droplets compared to the control mice, suggesting that indomethacin alleviated the "whitening" of BAT induced by the high-fat diet. Moreover, indomethacin significantly increased UCP1 mRNA expression in the iBAT. Taken together, this study indicates that indomethacin can promote mouse brown adipocyte differentiation, and might increase brown fat and glucose oxidation capacity in DIO mice.

Introduction

In recent decades, obesity has become a major public health problem in developed and even developing countries (Ng et al., 2014). More than two-third of U.S. adults are considered either overweight or obese (Flegal et al., 2012). Obesity is positively associated with a number of complications including type-2 diabetes, cardiovascular disease, gallbladder disease, osteoarthritis, and even some types of cancers (Jensen et al., 2014). Imbalance between energy intake and expenditure is thought to contribute to obesity (Spiegelman and Flier, 2001). Adipose tissue can be classified into white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is the major site for energy storage, and BAT is the major site for thermogenic energy expenditure by metabolizing glucose and fatty acids to produce heat. Historically, it was believed that BAT only exists in human neonates. Newly developed technologies, especially positron emission tomography (PET) in combination with computed tomography (CT), have demonstrated that adult humans do have BAT (Cypess et al., 2009; Van et al., 2009; Virtanen et al., 2009).

Despite extensive research, few drugs have been approved for treating obesity by the US Food and Drug Administration (FDA) (Narayanaswami and Dwoskin, 2016). Currently approved drugs target energy intake either by suppression of appetite (phentermine) or by interfering with nutrient absorption (orlistat). However, these drugs have severe cardiovascular and/or psychiatric side effects, and obesity relapse may occur when stopping the drugs (Kakkar and Dahiya, 2015). Emerging evidence on the role of functional BAT in increasing energy expenditure in human adults opens a new direction for anti-obesity therapy.

Indomethacin was discovered in 1963 (Hart and Boardman, 1963) and approved as a non-steroidal anti-inflammatory drug (NSAID) by the US FDA in 1965. Indomethacin's anti-inflammatory

effect is mainly mediated by inhibiting cyclooxygenases (COX), the key enzymes catalyzing the production of prostaglandins from arachidonic acid (Nalamachu and Wortmann, 2014). The adipogenic effect of indomethacin was first documented by Williams and Polakis, who found that addition of insulin and indomethacin significantly induced ATP-citrate lyase activity and biosynthesis of triglycerides during 3T3-L1 adipocyte differentiation (Williams and Polakis, 1977). Some researchers speculated that the adipogenic effects of indomethacin could be partly explained by the inhibited production of prostaglandins (Chen and London, 1981). Later, one study demonstrated that ob 17 cells treated with a combination of insulin and indomethacin differentiated into adipocytes more rapidly than the cells treated with insulin alone (Verrando et al., 1981). Other investigators (Ye and Serrero, 1998; Negrel et al., 1989) further confirmed the effect of indomethacin in promoting adipogenic differentiation. The mechanism for this effect of indomethacin was not revealed until when Lehmann et al. reported that indomethacin induced C3H10T1/2 adipocyte differentiation by binding and activating PPARγ (Lehmann et al., 1997). It is well recognized that the differentiation of both white and brown adipocytes from adipose stem cells is regulated by transcriptional factors that include CCAAT/enhancer-binding proteins (C/EBPs) and PPARγ (Tontonoz et al., 1994; Wu et al., 1999). PPARγ is a critical and irreplaceable transcriptional factor in regulation of UCP1 expression and brown adipocyte differentiation (Koppen and Kalkhoven, 2010). PPARγ agonists, such as rosiglitazone, are capable of inducing brown preadipocyte differentiation into mature brown adipocytes (Hwang et al., 2011). However, the effects and underlying mechanisms of indomethacin on brown adipocyte differentiation have not been investigated.

Although a number of studies have investigated adipogenic effects of indomethacin *in vitro*, only a few studies reported the effects of indomethacin on obesity *in vivo*. One study showed that

indomethacin supplemented in food (16 mg/kg diet) prevented high fat diet-induced obesity (DIO) in C57BL/6 mice, but failed to prevent hyperglycemia and impaired glucose tolerance (Fjaere et al., 2014). Interestingly, the same group in an earlier study reported that *in vitro* treatment with indomethacin (1 μM) significantly inhibited UCP1 expression in primary inguinal adipocytes and dietary indomethacin supplementation (16 mg/kg diet) increased weight gain and prevented high fat diet-induced UCP1 expression in inguinal WAT (iWAT) in male Sv129 mice (Madsen et al., 2010). While the discrepancy between the two *in vivo* studies might be related to different strains of mice used, it is also possible that indomethacin could enhance differentiation of both white and brown adipocytes *in vivo*. To further clarify this issue, we first investigated the effect of indomethacin on brown adipocyte differentiation and its underlying mechanism *in vitro*. We further conducted a pilot animal study to deliver indomethacin to interscapular BAT (iBAT) of DIO mice to maximize brown differentiation while minimizing white differentiation, leading to desirable outcomes in treating obesity and its associated metabolic disorders

Materials and Methods

Chemicals and reagents

For cell culture work, indomethacin, insulin, triiodothyronine, rosiglitazone, and dimethyl sulfoxide (DMSO) were from Sigma (St. Louis, MO). Primary antibody for UCP1 was from Sigma Aldrich (St. Louis, MO), for PGC-1α was from Millipore (Temecula, CA); ERK1/2 and horseradish peroxidase-conjugated goat anti-rabbit were from Cell Signaling Technology (Danvers, MA). For animal study, Kolliphor® HS 15 was a gift from BASF (Florham Park, NJ), and indomethacin was purchased from Cayman Chemical Company (Ann Arbor, MI).

Cell culture and treatment

The murine brown fat cell line is a gift from Dr. Johannes Klein (University of Lubeck, Lubeck, Germany), who has generated the cell line from iBAT of newborn C57BL/6 mice. Brown preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA) at 37°C humidified atmosphere of 5% CO_2 in air, until they reached confluence (designated as day 0). The cells were differentiated by incubation in the differentiation media containing DMEM supplemented with 20% FBS, 1 nM triiodothyronine, and 20 nM insulin and media were changed every other day until day 5. To study the effects of indomethacin on brown adipocytes, indomethacin (2, 5, 10, 20, 50 μM) or the vehicle control (DMSO) was added at D0 and replaced with each change of the media during the differentiation process.

Western blot analysis

Total cell lysates were prepared using 1X lysis buffer (Cell Signaling, Danvers, MA), and protein concentrations were determined by the BCA assay kit (Thermo Scientific, Waltham, MA). Total cell lysates were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked in 20 mM Tris·HCl, 137 mM NaCl, and 0.1% Tween 20 (pH 7.4) containing 5% nonfat milk. The membrane was immunoblotted with primary antibodies at 4°C overnight followed by secondary antibody conjugated with horseradish peroxidase for 1 h. The membrane was exposed on X-ray film after developed by ECL Western blot detection reagents (Pierce, Rockford, IL). The signal was quantified by densitometry using a ChemiDocXRS+ imaging system with an ImageLab^{TM} software (Bio-Rad).

Quantitative real-time PCR analysis

Total RNA was prepared using a TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Total RNA abundance was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was carried out using the High Capacity cDNA Reverse Transcription kit (Thermo Scientific, Pittsburgh, PA) according to the manufacturer's instructions. The mRNA expression of target genes and the housekeeping gene 36B4 was measured quantitatively using Power Up SYBR master mix (Applied Biosystems, Austin, TX). PCR reactions were run in a 96-well format using an ABI 7300HT instrument. Cycle conditions were 50°C 2 min, 95°C 10 min and then 40 cycles of 95°C for 15 s, 60°C for 1 min. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, which normalizes against 36B4. All primer sequences are listed in Table 1.

Total DNA was prepared using the TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions, and DNA concentrations were quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). COX II, a mitochondrial Cytochrome C Oxidase subunit 2**,** and 18S rRNA control genes were measured quantitatively using PowerUp^{TM} SYBR master mix (Applied Biosystems, Austin TX). PCR reactions were run in a 96-well format using an ABI 7300HT instrument. Cycle conditions were 50°C 2 min, 95°C 10 min and then 40 cycles of 95°C for 15 s, 60°C for 1 min. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, which normalizes against 18s rRNA.

Reporter gene assays

Brown preadipocytes seeded on 48-well plates were transiently transfected with PPAR Response Element (PPRE)-Luc reporter (Addgene plasmid #1015) and β-galactosidase expression plasmid with Lipofectamine 2000 transfection reagent and Plus reagent (Thermo Fisher Scientific, Carlsbad, CA). After 24 h of transfection, the cells were treated with indomethacin or the vehicle control for 18 h. The cell lysate was prepared and reporter luciferase and β-galactosidase activities were measured using a GloMax Luminometer (Promega, Madison, WI). Relative luciferase activities were normalized by β-galactosidase activities.

Animal study

Six-week-old male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine) were housed in an animal facility with a 12-h light and dark cycle. After 1-week of acclimation, mice were fed a high fat diet (45% kcal from fat, D12451) (Research Diets, New Brunswick, NJ) for 9 weeks, then

JPET Fast Forward. Published on March 22, 2018 as DOI: 10.1124/jpet.117.246256
This article has not been copyedited and formatted. The final version may differ from this version.

the mice were weighed and randomly assigned to either the control or the indomethacin group (3 mice/group). Indomethacin (10 mg/mL) dissolved in saline containing 18.75% Kolliphor® HS15 (wt%) was put into three ALZET® mini-osmotic pumps (model 2006) (DURECT Corporation, Cupertino, CA), which were reported to have a delivery rate of 0.15 µL/h. Additional three pumps delivered only saline and the same concentration of Kolliphor® HS15. We connected a piece of silicon tubing at the end of each pump, and sutured the tubing on the mouse back muscle to ensure that the opening of the tubing was on the iBAT of the mouse during pump implantation. Mice had free access to water and food, and their body weight and food intake were recorded weekly. After 4 weeks, mice were euthanized by CO_2 asphyxiation. Blood samples were collected in EDTA-coated tubes. Plasma was obtained by blood centrifugation at 2,000 x g at 4°C for 20 min. Organs and tissues were immediately dissected, weighed, and flash-frozen in liquid nitrogen. A portion of WAT and iBAT was fixed with 10% PBS buffered formalin for histological analysis. The animal protocol was approved by the Institutional Animal Care & Use Committee of Texas Tech University (TTU) (protocol number: 16028).

Plasma lipid profile

Plasma concentrations of triglycerides (TG), total cholesterol (TC), and high-density lipoprotein-cholesterol (HDL-C) and very low-density lipoprotein-cholesterol (VLDL-C) were measured using the AU400 clinical chemistry analyzer with enzymatic reagents (Beckman Coulter, Inc., Brea, CA) by following the AU400 procedural manual. Low-density lipoprotein-cholesterol (LDL-C) concentrations were calculated using the Friedewald formula. Plasma concentrations of non-esterified fatty acids (NEFA) were measured using a NEFA quantification kit (ab65341) from abcam (Cambridge, MA).

H&E staining

Tissues were fixed with 10% PBS-buffered formalin for 48 h. Hematoxylin and eosin stained was performed by the Department of Pathology of TTU Health Sciences Center. Briefly, the paraffin sections of adipose tissues (5 μm) were dewaxed with xylene and rehydrated with alcohol. The samples were washed with water to remove any reagent residue, incubated with Gill's Hematoxylin II for 4 min, washed several times using water. The samples were counterstained with eosin for 1 min. Then the samples were dehydrated through absolute alcohol for 3 times. Finally, samples were cleaned with xylene and mounted with xylene based mounting medium.

Body composition

Body composition was measured at week 0, 2 and 4 using an EchoMRI^{TM} Body Composition Analyzer (EchoMRI LLC, Houston, TX) according to the manufacturer's instructions at the TTU animal facility.

Insulin tolerance test (ITT)

After fasting mice for 4 h on week 4, blood glucose concentrations were measured by tail snip using OneTouch Ultra2 glucometer (Milpitas, CA) and blood glucose test strips. Insulin (0.75 U/kg body weight) was given to mice via intraperitoneal injection, and blood glucose concentrations were measured using the above method at 15, 30, 45, 60 and 120 min of post injection.

Statistical analysis

All data presented in text, table, and figures are mean ± SEM. Measurements were performed in at least three independent experiments. Statistical analysis was performed using Prism 5

(GraphPad Software, Inco., San Diego, CA). One-way analysis of variance (ANOVA) with repeated measures followed by multiple comparisons test (Student-Newman-Keuls method) was performed to determine the differences of group means between the treatment groups in the cell culture study. Student's t-test was performed to compare two group means in the animal study. The level of significance was set at $P < 0.05$.

JPET Fast Forward. Published on March 22, 2018 as DOI: 10.1124/jpet.117.246256
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Indomethacin stimulated differentiation of mouse brown adipocytes

We examined brown preadipocyte differentiation in the presence of increasing doses of indomethacin. Indomethacin dose-dependently increased brown adipocyte differentiation, as revealed by Oil Red O (ORO) stained cell morphology (Fig. 1A). Quantitation of lipid accumulation by ORO absorbance indicated that compared to DMSO control, 20 and 50 μM of indomethacin significantly increased cellular lipid accumulation by 28% and 50%, respectively (Fig. 1B).

Real time PCR was performed to measure mRNA expression of brown adipocyte related genes in response to indomethacin in brown adipocytes. Brown adipocyte selective markers, both mRNA (Fig. 1C and D) and protein levels (Fig. 1E) of UCP1 and PPARγ coactivator 1-alpha (PGC-1α), were upregulated in a dose-dependent manner by indomethacin at 5, 10, and 20 μM (P<0.001). Adipocyte differentiation markers, PPARγ and PR domain containing 16 (Prdm16) were also induced by indomethacin in a dose-dependent manner at 5-20 μM (Fig. 2A and B), whereas nuclear respiratory factor 1 (Nrf-1) expression was significantly increased by indomethacin only at the high concentration (50 μM) (P<0.001) (Fig. 2C). Furthermore, the DNA content of cytochrome c oxidase subunit 2 (COXII) gene, one of the mitochondrial biogenesis markers, was significantly increased when cells were treated with 20 or 50 μM of indomethacin (Fig. 2D).

Induction of PPRE-driven luciferase activity by indomethacin

The PPRE-driven luciferase activities were evaluated in mouse preadipocytes after transient transfection of PPRE-Luc cells were treated with indomethacin at the concentrations indicated. The

data showed that indomethacin dose-dependently activated PPRE-Luc reporter, reaching significance at 20 μM in mouse preadipocytes (Fig. 2E), indicating that indomethacin may work as a PPARγ agonist in this cell line.

Metabolic effects of local delivery of indomethacin to iBAT in obese mice

We next investigated the metabolic effects of local delivery of indomethacin to iBAT using ALZET® mini-osmotic pumps in DIO mice *in vivo*. The indomethacin treatment did not change food intake and body weight. However, as compared to the control mice, indomethacin-treated mice had a 34% reduction in the percentage of epididymal WAT (eWAT) mass (P=0.14), resulting in a 32% reduction in the percentage of body fat mass (P=0.23) (Table 2). Additionally, indomethacin significantly decreased the percentage of iBAT mass by 13% compared with the control (P=0.03). Fasting blood glucose concentrations were 19% lower in indomethacin-treated mice (185 ± 13.58 mg/dl) compared to the control mice (229 ± 6.84 mg/dl) (P=0.02) although insulin tolerance tests showed no differences between the two groups (data not shown). Fasting blood concentrations of VLDL-C, HDL-C and NEFA were similar between the control and indomethacin groups. Indomethacin group had numerically lower fasting blood concentrations of TG, TC and LDL-C compared to the control group, but the differences did not reach statistical significance, possibly due to the small sample size together with large variability in response.

Indomethacin alleviated brown fat whitening and increased expression of genes involved in brown fat activation in obese mice

Since indomethacin was directly delivered to iBAT through ALZET osmotic mini-pumps, we specifically examined the effects of indomethacin on brown adipocyte morphology and expression of iBAT marker genes. H&E staining of iBAT cells in the control mice had enlarged lipid droplets and enhanced lipid deposition, giving the appearance of "whitening" of iBAT (Fig. 3A). By contrast, the whitening phenotype was not observed in iBAT cells in indomethacin-treated mice. As compared to the control, indomethacin significantly increased mRNA expression of UCP1, perilipin 1(Plin1) and perilipin 2 (Plin2) (Fig. 3B). There was no significant differences in other brown markers including Prdm16, PGC-1α, PPARγ, mitochondrial transcription factor A (Tfam), and Nrf-1, in fatty acid β-oxidation genes including acyl-Coenzyme A dehydrogenase (Acadm) and cytochrome c oxidase subunit IV isoform 2 (COX4b), and in lipolysis genes including adipocyte triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) between the indomethacin-treated and control mice (Fig. 3B).

To evaluate browning of WAT, H&E staining and real time PCR were performed in both eWAT and iWAT (Fig. 4 and 5). The H&E staining revealed similar morphology in both eWAT and iWAT in the indomethacin-treated mice (Fig. 4A and Fig. 5A) compared to the control mice. The expression of UCP1, PGC-1α, PPARγ, and Prdm16 was 80% higher in the eWAT isolated from the indomethacin-treated mice compared to the control mice, but this difference did not reach statistical significance (Fig. 4B). However, we observed that lipid utilization genes, Plin1 and Plin2 were significantly higher in eWAT isolated from the indomethacin-treated mice compared to the control mice ($P<0.05$) (Fig. 4B). Unexpectedly, expression of browning related markers in iWAT tended to be lower in the indomethacin-treated mice compared to the control mice (Fig. 5B). We also measured the expression of genes related to fatty acid oxidation, and lipid utilization in iWAT, but found no significant difference between the treatment and control groups (Fig. 5B).

Discussion

The effects of indomethacin on brown adipocyte differentiation and activities have not been investigated. Our current study demonstrated that indomethacin stimulated mouse brown adipocyte differentiation by enhancing lipid accumulation and gene expression of brown adipocyte specific markers. We further showed that indomethacin activated the PPAR responsive reporter in mouse brown preadipocytes *in vitro*. In our pilot animal study, we validated that local delivery of indomethacin to iBAT increased expression of genes involved in thermogenesis in iBAT and improved hyperglycemia found in DIO mice.

Indomethacin stimulated mouse brown adipocyte differentiation via activating PPARγ, which is consistent with a previous study showing that indomethacin can bind to and activate PPARγ (Lehmann et al., 1997). In addition, increased ratio of COX II to 18S rRNA DNA content (COX II/18S) in these cells suggests that mitochondrial biogenesis was enhanced by indomethacin. It has been proposed that browning effects of PPARγ agonist may be due to its ability to induce expression of PGC-1α (Hondares et al., 2006; Wilson-Fritch et al., 2003). In agreement with this, our *in vitro* study demonstrated that indomethacin increased both mRNA and protein expression of PGC-1α, a master regulator of mitochondrial biogenesis (Jornayvaz and Shulman, 2010).

To extend our *in vitro* findings into mice *in vivo*, we further performed a pilot study to deliver indomethacin directly to the iBAT of DIO mice. We found that indomethacin significantly decreased iBAT mass and induced UCP1 mRNA expression in iBAT. The expression of genes regulating lipid utilization, such as PLIN1 and 2, in iBAT were also increased by indomethacin. These data indicated that iBAT fat burning activity might be increased after local delivery of indomethacin. The results of the animal study demonstrated that delivery of indomethacin to the

iBAT alleviated its whitening induced by the high fat diet. One possible explanation is that indomethacin alleviated iBAT whitening via enhancing UCP1-mediated thermogenesis and fat utilization as our data showed that both UCP1 and lipid droplet proteins, such as Plin 1 and Plin 2, were significantly increased in the iBAT of indomethacin-treated mice. It is believed that lipid droplet proteins, including Plin family and several others, play an important role in fat utilization in brown adipocytes (Townsend and Tseng, 2014). Local delivery of indomethacin appears to differentially affect white fat depending on the depot, as the expression of browning markers was increased in eWAT but decreased in iWAT by the indomethacin treatment in this study. At this point, this depot-related difference in white fat browning in response to indomethacin is not well understood. Studies have shown that more beige adipocytes were formed in eWAT than in iWAT in response to some drugs/nutrients, such as β3-agonist CL316,243 (Warner et al., 2016) and retinaldehyde (Kiefer et al., 2012). We speculate that the concentrations of indomethacin reached in eWAT and iWAT may be different, resulting in different responses in indomethacin-targeted signaling events. For example, Lehmann et al. suggested that indomethacin at low concentrations may block COX activity but without activating PPARγ; however, at high concentrations (micromolar range) it may function as both COX inhibitor and PPARγ agonist (Lehmann et al., 1997). Therefore, it is possible that indomethacin, depending on its concentrations in particular tissues, would differentially affect the signaling molecules generated from both COX and PPARγ pathways in the affected locations. Since COX-2 is required for recruitment of beige cells in WAT (Vegiopoulos et al., 2010), inhibition of COX-2 by indomethacin may lead to suppression of UCP1, which may well explain the lower expression of those browning markers in iWAT measured in our indomethacin-treated mice. Of note, a recent study showed that subcutaneous injection of

indomethacin (2.5 mg/kg of body weight) suppressed cold-induced UCP1 gene expression in iWAT of C57BL/6 mice (Madsen et al., 2010).

We also found that indomethacin significantly decreased fasting blood glucose concentrations. In contrast to our results, Fiaere et al. reported that indomethacin treatment (16 mg/kg of diet) prevented a high fat/high sucrose (HF/HS)-induced obesity but deteriorated glucose intolerance in C57BL/6J mice (Fjaere et al., 2014). The different results could be explained by differences in the study design, such as the timing of indomethacin administration, diets, indomethacin doses, and delivery routes between two studies. It is worth pointing out that increased concentrations of plasma glucose by oral delivery of indomethacin found in Fjaere et al's study was thought to be due to increased liver gluconeogenesis and impaired HF/HS-induced glucose-stimulated insulin secretion (Fjaere et al., 2014), which is the possible consequence of systemic side effects of indomethacin. The decreased fasting blood glucose concentrations can be explained by the fact that local delivery of indomethacin might only induce the differentiation of BAT, not WAT. Activated BAT can take up and burn more circulating glucose (Townsend and Tseng, 2014). Emerging evidence suggests that BAT might protect against obesity and type-2 diabetes through BAT-derived endocrine factors (i.e., batokines) that act in a paracrine or autocrine manner (Townsend and Tseng, 2012). Therefore, indomethacin may stimulate the release of batokines, such as FGF21, interleukin-6 (IL-6), and neuregulin 4 (Villarroya et al., 2013; Villarroya et al., 2017), which might in turn contribute to activation of iBAT and lowered blood glucose concentrations. Future studies are needed to examine effects of indomethacin on the release of batokines.

We recognize that the relatively small sample size is a limitation in this study. However, our animal pilot study does support the findings from our *in vitro* studies showing that indomethacin promoted

brown adipocyte differentiation and enhanced gene expression of brown adipocyte specific markers in the iBAT. More animal studies are required to confirm our findings and to investigate additional underlying mechanisms of indomethacin in regulating brown adipocyte differentiation and activity. Another limitation is the DIO mouse model. Mice and humans differ significantly in term of morphometry, physiology and life history (Lai et al., 2014). For example, as mice are significantly smaller than humans, their basal metabolic rate is seven times greater than that of humans (Demetrius, 2005). Nevertheless, these DIO mice are believed to be the best choice for investigating novel anti-obesity drugs, because they better mimic human obesity than most of the genetically modified models (Lutz & Woods, 2012).

In conclusion, the present studies demonstrated that indomethacin, a commonly used NSAID, promoted mouse brown adipocyte differentiation *in vitro*. Local delivery of indomethacin to iBAT improved hyperglycemia, and increased brown fat thermogenesis capacity in DIO mice. Our studies highlighted the advantages of local or targeted BAT delivery of indomethacin to maximize its beneficial effects on promoting brown adipogenesis and to minimize its toxic side effects often associated with systemic delivery to WAT (increased white adipogenesis) and the liver (increased gluconeogenesis).

JPET Fast Forward. Published on March 22, 2018 as DOI: 10.1124/jpet.117.246256
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

The authors thank Yujiao Zu, Jie Liu and Md Shahjalal Khan for their help in the animal study.

JPET Fast Forward. Published on March 22, 2018 as DOI: 10.1124/jpet.117.246256
This article has not been copyedited and formatted. The final version may differ from this version.

Authorship Contributions

Participated in research design: Hao, Kearns, Zhao, Wang.

Conducted experiments: Hao, Kearns, Zhao, Wang, Scott, Wu.

Performed data analysis: Hao, Kearns, Sun, Zhao, Wang, Scott, Wu.

Wrote or contributed to the writing of the manuscript: Hao, Kearns, Zhao, Wang, Scott, Wu, Kodani, Morisseau, Hammock.

References

Chen JJ and London IM (1981) Hemin enhances the differentiation of mouse 3T3 cells to adipocytes. *Cell* **26**:117-122.

Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng YH, Doria A, Kolodny GM and Kahn CR (2009) Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* **360**:1509-1517.

Demetrius L (2005) Of mice and men. When it comes to studying ageing and the means to slow it down, mice are not just small humans. *EMBO reports* **6 Spec No**:S39-44.

Fjaere E, Aune UL, Roen K, Keenan AH, Ma T, Borkowski K, Kristensen DM, Novotny GW, Mandrup-Poulsen T, Hudson BD, Milligan G, Xi Y, Newman JW, Haj FG, Liaset B, Kristiansen K and Madsen L (2014) Indomethacin treatment prevents high fat diet-induced obesity and insulin resistance but not glucose intolerance in C57BL/6J mice. *J Biol Chem* **289**:16032-16045.

Flegal KM, Carroll MD, Kit BK and Ogden CL (2012) Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. *Jama* **307**:491-497.

Hart FD and Boardman PL (1963) INDOMETHACIN: A NEW NON-STEROID ANTI-INFLAMMATORY AGENT. *Br Med J* **2**:965-970.

Hondares E, Mora O, Yubero P, Rodriguez de la Concepcion M, Iglesias R, Giralt M and Villarroya F (2006) Thiazolidinediones and rexinoids induce peroxisome proliferator-activated receptor-coactivator (PGC)-1alpha gene transcription: an autoregulatory loop controls PGC-1alpha expression in adipocytes via peroxisome proliferator-activated receptor-gamma coactivation. *Endocrinology* **147**:2829-2838.

Hwang HH, Moon PG, Lee JE, Kim JG, Lee W, Ryu SH and Baek MC (2011) Identification of the target proteins of rosiglitazone in 3T3-L1 adipocytes through proteomic analysis of cytosolic and secreted proteins. *Mol Cells* **31**:239-246.

Jensen MD, Ryan DH, Apovian CM, Ard JD, Comuzzie AG, Donato KA, Hu FB, Hubbard VS, Jakicic JM, Kushner RF, Loria CM, Millen BE, Nonas CA, Pi-Sunyer FX, Stevens J, Stevens VJ, Wadden TA, Wolfe BM, Yanovski SZ, Jordan HS, Kendall KA, Lux LJ, Mentor-Marcel R, Morgan LC, Trisolini MG, Wnek J, Anderson JL, Halperin JL, Albert NM, Bozkurt B, Brindis RG, Curtis LH, DeMets D, Hochman JS, Kovacs RJ, Ohman EM, Pressler SJ, Sellke FW, Shen WK, Smith SC, Jr. and Tomaselli GF (2014) 2013 AHA/ACC/TOS guideline for the management of overweight and obesity in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and The Obesity Society. *Circulation* **129**:S102-138.

Jornayvaz FR and Shulman GI (2010) Regulation of mitochondrial biogenesis. *Essays Biochem* **47**:69-84.

Kakkar AK and Dahiya N (2015) Drug treatment of obesity: current status and future prospects. *Eur J Intern Med* **26**:89-94.

Kiefer FW, Vernochet C, O'Brien P, Spoerl S, Brown JD, Nallamshetty S, Zeyda M, Stulnig TM, Cohen DE, Kahn CR and Plutzky J (2012) Retinaldehyde dehydrogenase 1 regulates a thermogenic program in white adipose tissue. *Nat Med* **18**:918-925.

Koppen A and Kalkhoven E (2010) Brown vs white adipocytes: the PPARgamma coregulator story. *FEBS Lett* **584**:3250-3259.

Lai M, Chandrasekera PC and Barnard ND (2014) You are what you eat, or are you? The challenges of translating high-fat-fed rodents to human obesity and diabetes. *Nutrition & diabetes* **4**:e135.

Lehmann JM, Lenhard JM, Oliver BB, Ringold GM and Kliewer SA (1997) Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J Biol Chem* **272**:3406-3410.

Lutz TA and Woods SC (2012) Overview of animal models of obesity. *Current protocols in pharmacology* **Chapter 5**:Unit5.61.

Madsen L, Pedersen LM, Lillefosse HH, Fjaere E, Bronstad I, Hao Q, Petersen RK, Hallenborg P, Ma T, De Matteis R, Araujo P, Mercader J, Bonet ML, Hansen JB, Cannon B, Nedergaard J, Wang J, Cinti S, Voshol P, Doskeland SO and Kristiansen K (2010) UCP1 induction during recruitment of brown adipocytes in white adipose tissue is dependent on cyclooxygenase activity. *PLoS One* **5**:e11391.

Nalamachu S and Wortmann R (2014) Role of indomethacin in acute pain and inflammation management: a review of the literature. *Postgrad Med* **126**:92-97.

Narayanaswami V and Dwoskin LP (2016) Obesity: Current and potential pharmacotherapeutics and targets. *Pharmacol Ther*.

Negrel R, Gaillard D and Ailhaud G (1989) Prostacyclin as a potent effector of adipose-cell differentiation. *Biochem J* **257**:399-405.

Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, Mullany EC, Biryukov S, Abbafati C, Abera SF, Abraham JP, Abu-Rmeileh NM, Achoki T, AlBuhairan FS, Alemu ZA, Alfonso R, Ali MK, Ali R, Guzman NA, Ammar W, Anwari P, Banerjee A, Barquera S, Basu S, Bennett DA, Bhutta Z, Blore J, Cabral N, Nonato IC, Chang JC,

Chowdhury R, Courville KJ, Criqui MH, Cundiff DK, Dabhadkar KC, Dandona L, Davis A, Dayama A, Dharmaratne SD, Ding EL, Durrani AM, Esteghamati A, Farzadfar F, Fay DF, Feigin VL, Flaxman A, Forouzanfar MH, Goto A, Green MA, Gupta R, Hafezi-Nejad N, Hankey GJ, Harewood HC, Havmoeller R, Hay S, Hernandez L, Husseini A, Idrisov BT, Ikeda N, Islami F, Jahangir E, Jassal SK, Jee SH, Jeffreys M, Jonas JB, Kabagambe EK, Khalifa SE, Kengne AP, Khader YS, Khang YH, Kim D, Kimokoti RW, Kinge JM, Kokubo Y, Kosen S, Kwan G, Lai T, Leinsalu M, Li Y, Liang X, Liu S, Logroscino G, Lotufo PA, Lu Y, Ma J, Mainoo NK, Mensah GA, Merriman TR, Mokdad AH, Moschandreas J, Naghavi M, Naheed A, Nand D, Narayan KM, Nelson EL, Neuhouser ML, Nisar MI, Ohkubo T, Oti SO, Pedroza A, et al. (2014) Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* **384**:766-781.

Spiegelman BM and Flier JS (2001) Obesity and the regulation of energy balance. *Cell* **104**:531-543.

Tontonoz P, Hu E and Spiegelman BM (1994) Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell* **79**:1147-1156.

Townsend K and Tseng YH (2012) Brown adipose tissue: Recent insights into development, metabolic function and therapeutic potential. *Adipocyte* **1**:13-24.

Townsend KL and Tseng YH (2014) Brown fat fuel utilization and thermogenesis. *Trends Endocrinol Metab* **25**:168-177.

van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, Schrauwen P and Teule GJ (2009) Cold-activated brown adipose tissue in healthy men. *N Engl J Med* **360**:1500-1508.

Vegiopoulos A, Muller-Decker K, Strzoda D, Schmitt I, Chichelnitskiy E, Ostertag A, Berriel Diaz M, Rozman J, Hrabe de Angelis M, Nusing RM, Meyer CW, Wahli W, Klingenspor M and Herzig S (2010) Cyclooxygenase-2 controls energy homeostasis in mice by de novo recruitment of brown adipocytes. *Science* **328**:1158-1161.

Verrando P, Negrel R, Grimaldi P, Murphy M and Ailhaud G (1981) Differentiation of ob 17 preadipocytes to adipocytes. Triggering effects of clofenapate and indomethacin. *Biochim Biophys Acta* **663**:255-265.

Villarroya F, Cereijo R, Villarroya J and Giralt M (2017) Brown adipose tissue as a secretory organ. *Nat Rev Endocrinol* **13**:26-35.

Villarroya J, Cereijo R and Villarroya F (2013) An endocrine role for brown adipose tissue? *Am J Physiol Endocrinol Metab* **305**:E567-572.

Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, Taittonen M, Laine J, Savisto NJ, Enerback S and Nuutila P (2009) Functional brown adipose tissue in healthy adults. *N Engl J Med* **360**:1518-1525.

Warner A, Kjellstedt A, Carreras A, Bottcher G, Peng XR, Seale P, Oakes N and Linden D (2016) Activation of beta3-adrenoceptors increases in vivo free fatty acid uptake and utilization in brown but not white fat depots in high-fat-fed rats. *Am J Physiol Endocrinol Metab* **311**:E901-e910.

Williams IH and Polakis SE (1977) Differentiation of 3T3-L1 fibroblasts to adipocytes. The effect of indomethacin, prostaglandin E1 and cyclic AMP on the process of differentiation. *Biochem Biophys Res Commun* **77**:175-186.

Wilson-Fritch L, Burkart A, Bell G, Mendelson K, Leszyk J, Nicoloro S, Czech M and Corvera S (2003) Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. *Mol Cell Biol* **23**:1085-1094.

Wu Z, Puigserver P and Spiegelman BM (1999) Transcriptional activation of adipogenesis. *Curr Opin Cell Biol* **11**:689-694.

Ye H and Serrero G (1998) Stimulation of adipose differentiation related protein (ADRP) expression by ibuprofen and indomethacin in adipocyte precursors and in adipocytes. *Biochem J* **330 (Pt 2)**:803-809.

Footnotes

This work was supported by the National Center for Complementary & Integrative Health (Grants R15AT007013 and R15AT008733); the National Institute of Environmental Health Sciences Superfund Research Program (Grants P42 ES0044699 and R01 ES002710); National Institute of Diabetes and Digestive and Kidney Diseases (Grant R01 DK103616); the College of Human Sciences, Texas Tech University, Lubbock, TX; and the University of Tennessee, Knoxville, TN.

L. H. and J. K. contributed equally to this work.

JPET Fast Forward. Published on March 22, 2018 as DOI: 10.1124/jpet.117.246256
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Fig. 1 Indomethacin dose-dependently induced brown adipocyte differentiation. (A) Oil Red O stained cell morphology at day 5; (B) Lipid accumulation measured by Oil Red O absorbance; (C) UCP1 gene expression; (D) PGC-1α gene expression; (E) Western blotting of UCP1 and PGC-1α. Data = mean ± SEM (n=3). ***, $P<0.001$; **, $P<0.01$;*, $P<0.05$ compared to the DMSO group.

Fig. 2 Indomethacin dose-dependently increased brown adipocyte related markers. (A-C) Gene expression of brown fat selected markers, negative control cells were set at fold 1; (D) Gene expression of mitochondrially encoded cytochrome c oxidase II; (E) Luciferase activity of PPAR reporter. Data = mean ± SEM (n=3). ***, $P<0.001$; **, $P<0.01$;*, $P<0.05$ compared to the DMSO group.

Fig. 3 Indomethacin alleviated BAT whitening and increased thermogenesis related markers in DIO mice.(A) Representative H&E staining images of iBAT (B) Relative mRNA levels of BAT markers, genes involved in fatty acid oxidation, and lipid utilization in iBAT. Data= mean ± SEM (n=3). *, $P<0.05$ compared to the control group.

Fig. 4 Indomethacin slightly increased browning markers in eWAT. (A) Representative H&E staining images of eWAT. (B) Real -time PCR analysis of the expression of BAT markers, genes involved in fatty acid oxidation, and lipid utilization in eWAT. Data= mean ± SEM (n=3). *, $P<0.05$ compared to the control group.

Fig. 5 Indomethacin slightly inhibited browning markers in iWAT. (A) Representative H&E staining images of iWAT. (B) Gene expression analysis of BAT markers, fatty acid oxidation, and lipid utilization in iWAT. Data= mean ± SEM (n=3). *, $P<0.05$ compared to the control group.

JPET Fast Forward. Published on March 22, 2018 as DOI: 10.1124/jpet.117.246256
This article has not been copyedited and formatted. The final version may differ from this version.

Tables

Table 1. Primer sequences for both mouse pre-brown cells and C57BL6 mice

JPET Fast Forward. Published on March 22, 2018 as DOI: 10.1124/jpet.117.246256
This article has not been copyedited and formatted. The final version may differ from this version.

Gene name	**Primer sequences**	
36B4	Forward	GCGACATACTCAAGCAGGAGCA
	Reverse	AGTGGTAACCGCTCAGGTGTTG
UCP1	Forward	GCTTTGCCTCACTCAGGATTGG
	Reverse	CCAATGAACACTGCCACACCTC
PGC-1α	Forward	GAATCAAGCCACTACAGACACCG
	Reverse	CATCCCTCTTGAGCCTTTCGTG
PPARγ	Forward	GTACTGTCGGTTTCAGAAGTGCC
	Reverse	ATCTCCGCCAACAGCTTCTCCT
Prdm16	Forward	ATCCACAGCACGGTGAAGCCAT
	Reverse	ACATCTGCCCACAGTCCTTGCA
Tfam	Forward	GAGGCAAAGGATGATTCGGCTC
	Reverse	CGAATCCTATCATCTTTAGCAAGC
Nrf-1	Forward	GGCAACAGTAGCCACATTGGCT
	Reverse	GTCTGGATGGTCATTTCACCGC
ATGL	Forward	TTGTCCTGCCCCACTAAGAG
	Reverse	ACTCAGAGAACCCCGTGAAG
HSL	Forward	AGACACTTCGCTGTTCCTCA
	Reverse	CTTCAGCCTCTTCCTGGGAA
CPT1	Forward	GGACTCCGCTCGCTCATT
	Reverse	GAGATCGATGCCATCAGGGG
Acadm	Forward	TCAAGATCGCAATGGGTGCT
	Reverse	GCTCCACTAGCAGCTTTCCA

JPET Fast Forward. Published on March 22, 2018 as DOI: 10.1124/jpet.117.246256
This article has not been copyedited and formatted. The final version may differ from this version.

Plin1	Forward	TATGCCCAGAGAGAAGCCTG
	Reverse	AATCTGCCCACGAGAAAGGA
Plin2	Forward	AGCTCCAGACTACATGCCAG
	Reverse	AGCTTTGACCTCAGACTGCT
Cox4b	Forward	GGGCAGCTCTGGATAGTTCC
	Reverse	TCAACGTAGGGGGTCATCCT
Cox II	Forward	GCTCTCCCCTCTCTACGCAT
	Reverse	AGCAGTCGTAGTTCACCAGG
18S	Forward	GATTCTCCGTTACCCGTCAA
	Reverse	CGTGCTGACGACAGATCACT

JPET Fast Forward. Published on March 22, 2018 as DOI: 10.1124/jpet.117.246256
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. Metabolic parameters of mice in both control and indomethacin-treated groups

JPET Fast Forward. Published on March 22, 2018 as DOI: 10.1124/jpet.117.246256
This article has not been copyedited and formatted. The final version may differ from this version.

	Control	Indomethacin	*p* value
Body weight (g)	30.73±1.57	31.30±2.04	0.41
Body composition			
% fat mass	19.62±3.05	13.32±3.07	0.23
% lean mass	79.51±3.19	83.47±2.88	0.20
% BAT of BW	0.32±0.01	0.28±0.16	0.03
% rWAT of BW	0.95±0.17	0.81±0.25	0.34
% eWAT of BW	3.41±0.64	2.25±0.66	0.14
% iWAT of BW	1.24±0.38	1.29±0.09	0.44
Food intake (g/day)	2.91±0.16	3.02±0.18	0.34
Fasting glucose (mg/dl)	229.30±6.84	185.00±13.58	0.02
Blood lipid profile			
TG (mg/dl)	95.00±5.00	85.00±2.89	0.07
TC (mg/dl)	160.00±32.79	133.30±11.67	0.24
LDL-C (mg/dl)	63.33±28.48	41.67±7.27	0.25
VLDL-C (mg/dl)	18.33±1.67	16.67±1.67	0.26

HDL-C (mg/dl)	77.50±5.35	74.67±4.51	0.35
NEFA (nm/ml)	13.97±0.49	13.88±1.18	0.47

Note: All data are expressed as mean ± SEM (n=3) and analyzed by student t tests.

Fig. 1

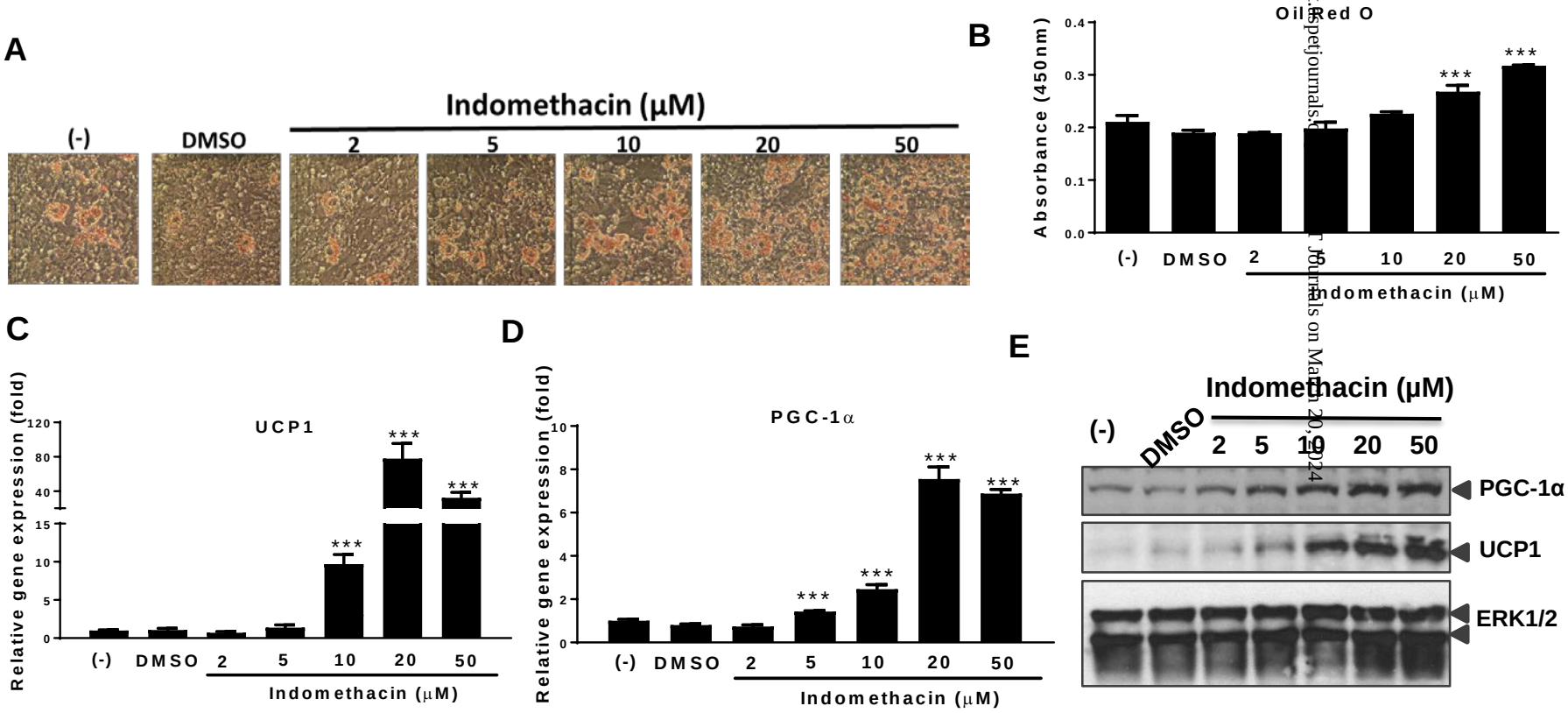

Fig. 2

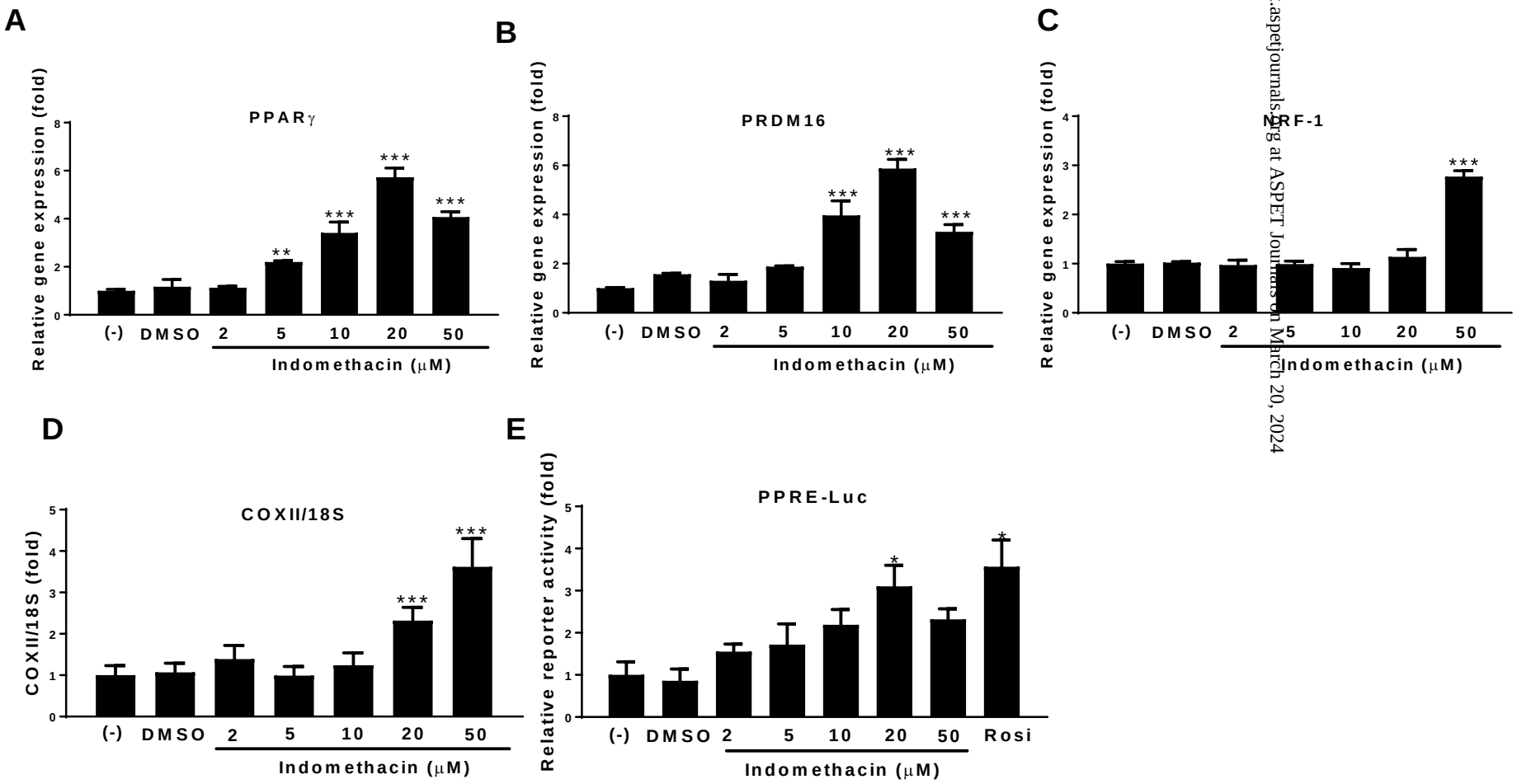

Fig. 3

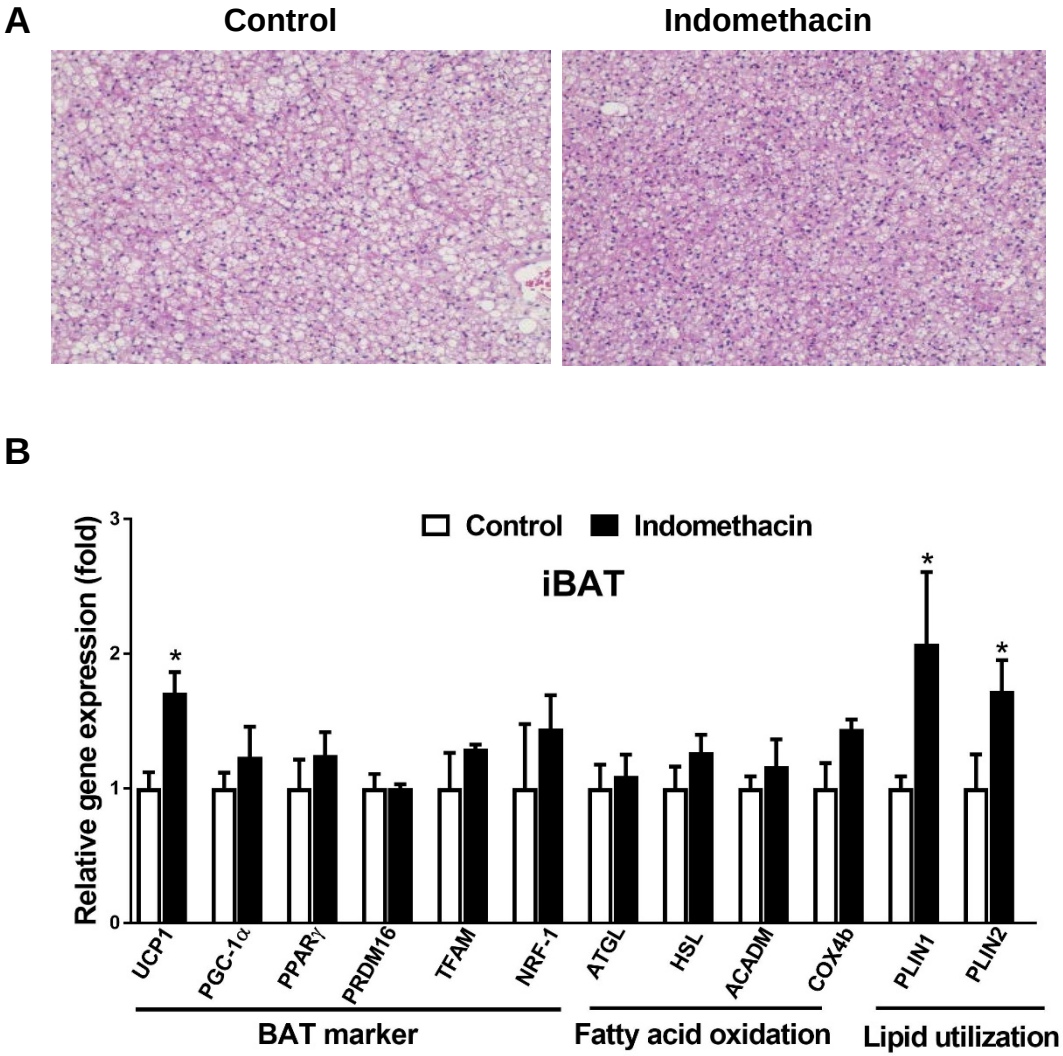

Fig. 4

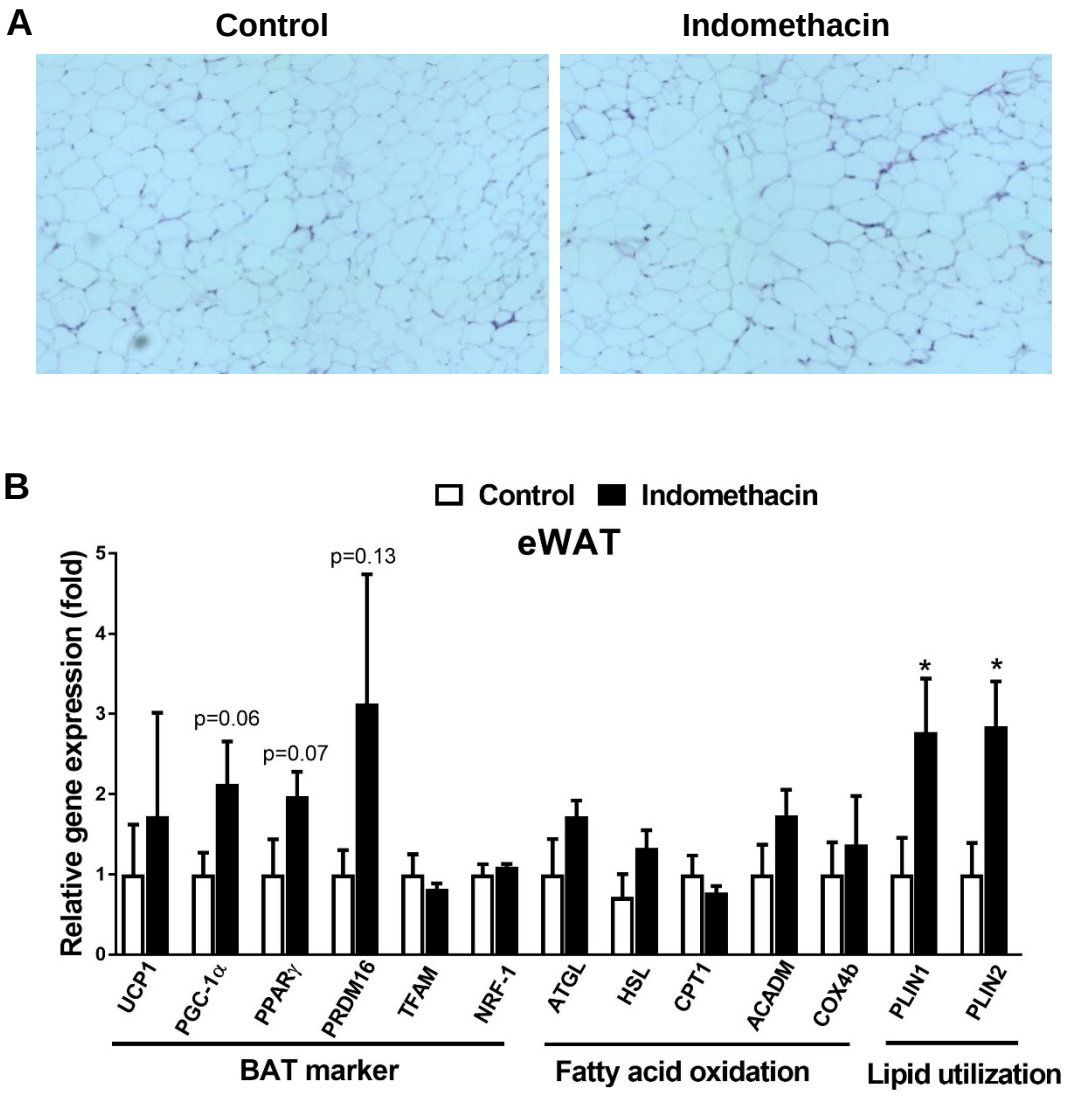

Fig. 5