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Jonas Laget, Claire Vigor, Agathe Nouvel, Amandine Rocher, Jérémy Leroy, et al.. Reduced production of isoprostanes by peri-pancreatic adipose tissue from Zucker fa/fa rats as a new mechanism for β -cell compensation in insulin resistance and obesity. *Free Radical Biology and Medicine*, 2022, 182, pp.160-170. 10.1016/j.freeradbiomed.2022.02.013 . hal-03599233

HAL Id: hal-03599233

<https://hal.science/hal-03599233>

Submitted on 11 Mar 2022

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Reduced production of isoprostanes by the peri-pancreatic adipose tissue from Zucker fa/fa rats as a new mechanism for β -cell compensation in insulin resistance and obesity

Jonas Laget^{a,b}, Claire Vigor^c, Agathe Nouvel^a, Amandine Rocher^c, Jérémy Leroy^a, Laura Jeanson^a, Guillaume Reversat^c, Camille Oger^c, Jean-Marie Galano^c, Thierry Durand^c, , Sylvie Péraldi-Roux^{a,c}, Jacqueline Azay-Milhau^a, Anne-Dominique Lajoix^{a,*}

^aBiocommunication in Cardio-Metabolism (BC2M), University Montpellier, France

^bRD-Néphrologie, Montpellier, France

^c

Institut des Biomolécules Max Mousseron (IBMM)
Pôle Chimie Balard Recherche, University Montpellier, CNRS, ENSCM, France

* Corresponding author

Email address: anne-dominique.lajoix@umontpellier.fr

Keywords: Type 2 diabetes, obesity, adipose tissue, isoprostanooids, inflammation

Abbreviations

eWAT	epididymal white adipose tissue
GPX-1	glutathione peroxidase 1
GPX-4	glutathione peroxidase 4
HTRF	Homogeneous time resolved fluorescence
iBOP	[1S-[1 α ,2 α (Z),3 β (1E,3S*),4 α]]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid
IL-1 β	interleukin-1 β
IL-10	interleukin-10
IL-18	interleukin-18
MGL1	Galactose-Type C-Type Lectin1
MCP-1	monocyte chemoattractant protein-1
PDX-1	pancreas/duodenum homeobox protein 1
PLA2	phospholipase A2
pWAT	pancreatic white adipose tissue
SQ29548	[1S-[1 α ,2 α (Z),3 α ,4 α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid
TLR2	Toll-like receptor 2
T2D	Type 2 diabetes

Highlights

- pWAT but not epididymal white adipose tissue (eWAT) of obese animals releases less amounts of isoprostanoids compared to lean animals.
- Induction of antioxidant defenses and reduced activity of some PLA2s in obese animals can account for reduced production of isoprostanoids in pWAT.
- 15-F_{2t}-isoprostane epimers decreased glucose-induced insulin secretion in rat islets, through binding to the thromboxane A₂ receptor and reduction of cAMP signaling.
- The modulation of isoprostanoids production in adipose tissue can locally improve β -cell compensation during early stages of diabetes.
- Understanding isoprostanoids homeostasis linked to WAT adaptation in obesity could lead to new therapeutic strategies in type 2 diabetes.

Abstract

During early stages of type 2 diabetes, called prediabetes, pancreatic β -cells compensate for insulin resistance through increased insulin secretion in order to maintain normoglycemia. Obesity leads to the development of ectopic fat deposits, among which peri-pancreatic white adipose tissue (pWAT) that can communicate with β -cells through soluble mediators. We thus investigated whether pWAT produced oxygenated lipids, namely isoprostanes and neuroprostanes and whether they can influence β -cell function in obesity. In the Zucker *fa/fa* rat model, pWAT and epididymal white adipose tissue (eWAT) displayed different inflammatory profiles. In obese rats, pWAT, but not eWAT, released less amounts of 5-F_{2t}-isoprostanes, 15-F_{2t}-isoprostanes, 4-F_{4t}-neuroprostanes and 10-F_{4t}-neuroprostane compared to lean animals. These differences could be explained by a greater induction of antioxidant defenses enzymes such as SOD-1, SOD-2, and catalase in pWAT of obese animals than in eWAT. In addition, sPLA2 IIA, involved in the release of isoprostanooids from cellular membranes, was found decreased in pWAT of obese animals, but not in eWAT, and may also account for the reduced release of oxidized lipids by this tissue. At a functional level, 15-F_{2t}-isoprostane epimers, but not 5-F_{2t}-isoprostanes, were able to decrease glucose-induced insulin secretion in pancreatic islets from Wistar rats. This effect appeared to be through activation of the thromboxane A₂ receptor and reduction of cAMP signaling in pancreatic islets. In conclusion, through the removal of an inhibitory tone exerted by isoprostanes, we have evidenced, for the first time, a new mechanism allowing β -cells to compensate for insulin resistance in obesity and involving a biocommunication between adipose tissue and β -cells.

1. Introduction

Type 2 diabetes (T2D) is preceded by a silent phase, called prediabetes, a state of intermediate hyperglycemia, characterized by either an impaired fasting glucose or an impaired glucose tolerance [1]. During this phase, pancreatic β -cells compensate for insulin resistance through an increased capacity to secrete insulin, although evidence suggests that insulin hypersecretion could be the primary defect leading to insulin resistance in some cases (for review see [2]).

The mechanisms underlying insulin hypersecretion are still under investigation. In rodent models, such as the Zucker *fa/fa* rat and the high fat diet-fed mouse, pancreatic islets display an increased β -cell mass [3,4] and hypertrophy [5], although this phenomenon appears to be less pronounced in humans [6]. It was also shown that β -cells exhibited an increased sensitivity to glucose in rats and humans [7, 8]. In addition, an enhanced β -cell function is also observed, involving numerous functional adaptations such as increased glucose utilization and oxidation [9] and an improved fatty acid esterification/lipolysis process [10]. Except glucose, the driving cause for insulin hypersecretion is unknown.

During obesity, the limited expandability of the subcutaneous adipose tissue leads to the accumulation of visceral and ectopic fat deposition, associated with macrophage infiltration, and subsequent insulin resistance. In addition to energy storage, adipose tissue secretes numerous factors, among which adipokines, chemokines, and free fatty acids (FFAs) with autocrine, paracrine and endocrine functions [11]. Among ectopic fat deposits, the peri-pancreatic white adipose tissue (pWAT) establishes close-contact with pancreatic islets, allowing a local biocommunication that could influence β -cell function [12, 13, 14]. Indeed, pWAT-secreted factors were able to modulate β -cell proliferation in a rat model of obesity and insulin resistance [12, 13].

In the present study, we used the Zucker *fa/fa* rat, as a relevant model of insulin hypersecretion and obesity, associated with pWAT deposition. As potential pWAT-secreted factors, we focused on isoprostanoids (isoprostanes and neuroprostanes), which are lipid-derivatives produced from the non-enzymatic peroxidation of arachidonic and docosahexaenoic acids, respectively, present in phospholipid bilayer of membranes [15]. These oxygenated lipids are considered as *in vivo* markers of oxidative stress, and related to several pathologies, like metabolic disease [15]. Moreover, isoprostanoids are recognized as cellular mediators involved in several biological functions [15], mainly through their binding to the thromboxane A2 receptor [16]. Our aim was thus to investigate whether isoprostanoids released by the pWAT could be new modulators of β -cell function, able to influence its ability to compensate for insulin resistance in obesity.

2. Methods

2.1. Animals

Male Zucker *fa/fa* and *fa/+* rats, Wistar Han rats were purchased from Charles River Laboratories (L'Arbresle, France). Animals were housed (3-4 per cage) in our conventional animal facility (n°D34-172-25) under conditions of constant temperature (20-22°C), humidity (45-50%) and a standard dark cycle (20.00-08.00 hours) with free access to food (SAFE A04, Safe, Rosenberg, Germany) and water. The experiments were performed in accordance with the "Guide for the care and use of laboratory animals" (8th edition, 2011). The protocol was approved by the ethical committee of our University.

2.2. Tissue sampling and islet isolation

Rats (9-10 weeks old) were anesthetized with 4% isoflurane, killed by decapitation. Blood was sampled and centrifuged at 2000 g for 10 min (4°C) for plasma storage at -80°C. Adipose tissues were removed, rinsed in PBS and stored at -80°C, or used for further experiments. Pancreatic islets were isolated using collagenase digestion (Roche, Basel, Switzerland). They were separated from exocrine tissue and collected after centrifugation on a Ficoll density gradient.

2.3. Insulin secretion and cAMP assay

Isolated islets were cultured for 24h in RPMI1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 1.25 µg/ml amphotericin B (Sigma-Aldrich, Saint-Quentin Fallavier, France). Then, islets were incubated for 24h in the same medium containing synthetic 15- or 5-F_{2t}-isoprostanes [17,18], iBOP ([1S-[1α,2α(Z),3β(1E,3S*),4α]]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-

oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; Cayman Chemicals, Ann Harbor, MI, USA), SQ29548 ([1S-[1 α ,2 α (Z),3 α ,4 α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; Cayman Chemicals) or conditioned medium (1/3 dilution). After 1h preincubation in KRB buffer, insulin secretion was then stimulated by 11.2 mmol/l glucose for 1 h at 37°C. Each experimental condition was replicated at least 10 times. Insulin concentration in the culture supernatants was finally measured by HTRF using the insulin high range assay kit (Cisbio, Codolet, France).

For cAMP assay, 100 pancreatic islets were cultured for 24 hours in the presence or not of forskolin (Sigma-Aldrich) and isoprostanoids. Then, intracellular cAMP concentration was determined using the cyclic AMP Select ELISA kit (Cayman Chemicals) according to the manufacturer's recommendation. cAMP concentration was normalized according to DNA concentration and expressed as % over forskolin alone.

2.4. Conditioned-medium preparation

Minced adipose tissues were cultured for 24 h in RPMI 1640 containing 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (5 ml of medium/g of tissue). Conditioned medium was finally collected, filtered through a 0.22 μ m sterile filter and stored at -80°C. For isoprostanoids analysis, butylated hydroxytoluene (40 μ g/ml; Sigma) was added to conditioned media before freezing, to prevent further oxidation.

2.5. Isoprostanoids quantification

Isoprostanoids were extracted from conditioned medium according to the protocol described by Lee et al. [19]. Briefly, after addition of internal standards into samples, they were purified using solid phase extraction anion-exchange cartridge separation (Waters, Milford, MA, USA) : interferences removal with first 2 mL of NH₃ (2% (v/v)), then 2 mL of a mixture of formic acid (20 mM):MeOH (70:30, v/v) and finally with 2 mL of hexane. The interest

compounds were eluted with 2 mL of a hexane:ethanol:acetic acid (70:29.4:0.6, v/v/v) mixture. Elution solvent was evaporated under a nitrogen stream and concentrated residue was then solubilized. Concentration of 7 isoprostanoids was determined by liquid chromatography-mass spectrometry, as recently described [20]. Concerning tissue isoprostanoids, lipid fraction was recovered with Folch extraction after homogenization of adipose tissue. Then, an alkaline hydrolysis was performed allowing quantification of total isoprostanoids (esterified and free forms). This last step was also applied for extraction and determination of plasmatic isoprostanoids. All LC-MS analyses were carried out using an Eksigent® MicroLC 200 Plus (Eksigent Technologies, CA, USA) combined with an AB SCIEX QTRAP 5500 (Sciex Applied Biosystems, ON, Canada). Each measurement was performed in duplicate.

2.6. RNA isolation and quantitative RT-PCR

Total RNA extraction was performed on RNAlater-preserved (Qiagen, Venlo, Netherlands) pWAT, eWAT or isolated islet using RNA Now reagent (Biogentex, League City, TX, USA). RNA concentrations, quality and purity were evaluated using a Nanodrop One (Thermo Scientific, Waltham, MA, USA) and agarose gel electrophoresis. First strand cDNA was synthesized from 500 ng RNA using Omniscript Reverse Transcription kit (Qiagen) and oligo(dT) primers. Then, quantitative PCR (qPCR) was performed using 0.2 ng cDNA, LightCycler 480 SYBR Green I Master mix (Roche), and 500 nM specific primers (Table 1) in 10µl reaction volume, on a LightCycler 96 system (Roche). Each experimental point was done in triplicate. Data were analyzed with the LightCycler 96 software 1.1.

2.7. Immunohistochemistry and immunofluorescence

Adipose tissue samples were fixed in 10% formalin for at least 24h. After dehydration and embedding in paraffin, sections of 5µm were cut using microtome. Immunoreactivity was

revealed by an anti-CD68 antibody (BioRad Laboratories, Hercules, CA, USA) and Vectastain ABC kit (Vector Labs, Burlingame, USA). A negative control was performed in the absence of the primary antibody. Images were acquired using an Eclipse TE300 inverted microscope (Nikon) equipped with a camera.

Immunofluorescence was performed on dispersed cells from pancreatic islets, on a poly-L-lysine (Sigma) coated Lab-Tek System. After 3 days of culture, cells were immunostained overnight with anti- thromboxane A2 receptor (Abcam, Cambridge, UK), anti-insulin (Sigma-Aldrich), anti-glucagon (Sigma-Aldrich), or anti-somatostatin (ThermoFischer Scientific) antibodies. Fluorescence was observed with Zeiss (Oberkochen, Germany) LSM 780 confocal microscope using the Montpellier RIO Imaging platform and analyzed using Omero Software.

2.8. Western blotting

Pancreatic islets or INS-1 cells were homogenized in 20 mM Tris lysis buffer pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 0.1% SDS and a cocktail of protease inhibitors. Proteins (30 µg) were separated on a 10% SDS-polyacrylamide gel. Incubation with anti-thromboxane A2 receptor (Abcam) or anti-β-actin (Sigma-Aldrich) antibodies was performed overnight. Signals were acquired with a Vilbert Lourmat imager and quantified by Image J analysis software. Each experiment shown is representative of 3 independent experiments.

2.9. Statistical analysis

Experimental values represent the mean of n experiments and are plotted on the graphs as means ± SEM. Statistical analysis was performed with GraphPad Prism 5 software using Student's t test or one-way ANOVA when applicable.

3. Results

3.1. Macrophages infiltration and polarization in pWAT

At mRNA level, we observed an overexpression of the macrophage marker *Cd68* in pWAT of Zucker *fa/fa* versus *fa/+* rats (+79%; n=6; P<0.05) (Fig. 1A). Macrophages infiltration was confirmed by immunohistochemistry in pWAT with an anti-CD68 antibody (P<0.05) (Fig. 1B). In addition, we found that monocyte chemoattractant protein-1 (MCP-1 or C-C motif chemokine 2 (*Ccl2*)) was 5.3 times more expressed in pWAT from obese rats, as compared to lean animals (n=6, P<0.001) (Fig. 1C). At the opposite, RANTES (*Ccl5*) expression, found increased in white adipose tissue of diabetic rats [21], was reduced by 2.6-fold in pWAT from *fa/fa* rats (P<0.05) (Fig. 1C).

Concerning macrophage polarization, we observed an increased expression of both CD11c (*Itgax*) (+5.15-fold, n=6, P<0.05) and Toll-like receptor 2 (*Tlr2*) (+8.7-fold, n=6, P<0.05) in pWAT from *fa/fa* rats (Fig. 1D), suggesting the local accumulation of proinflammatory M1 macrophages. Interestingly, we also detected an overexpression of Macrophage Galactose-Type Lectin1 (*Mgl1*) (+2.4-fold; n=6; P<0.05) and Transglutaminase 2 (*Tgm2*) (+3.9-fold; n=6; P<0.05) in pWAT from *fa/fa* rats, suggesting a rise of the M2 macrophage phenotype (Fig. 1E). Overall, our results suggest that pro- and anti-inflammatory processes occur in pWAT of obese *fa/fa* rats, as compared to lean animals. This was confirmed by the cytokine profile of pWAT showing an increased expression of the pro-inflammatory cytokine TNF- α (*Tnfa*) (+16.6-fold; n=5, P<0.01) and the regulatory cytokine TGF- β (*Tgfb*) (+3.1-fold, P<0.01) in obese animals, while interleukin-1 β (IL-1 β , *Il1b*), interleukin-10 (IL-10, *Il10*), and interleukin-18 (IL-18, *Il18*) remained unchanged (Fig. 2A).

For comparison with pWAT, we found an increased expression of *Cd68* in eWAT from obese rats versus lean animals (+2.1-fold, n=6, P<0.05) (Fig. 3A). The increased infiltration of

macrophages was associated with an enhanced expression of the chemokine MCP-1 in obese rats (+3.9-fold *versus* lean ones; n=6; P<0.01), while RANTES remained unchanged (Fig. 3B). Regarding M1 macrophage polarization, CD11c has been found enhanced in obese rats (+5.6-fold, n=6; P<0.05) and *Tlr2* was not significantly modified (P=0.09) (Fig. 3C). However, M2 markers, remained unchanged (n=6) (Fig. 3D). The cytokine profile of eWAT showed an enhancement of the pro-inflammatory cytokine TNF- α (+2.2-fold; n=8; P<0.05), IL-1 β (+1.7-fold; n=9; P<0.01) and IL-18 (+1.9-fold; P<0.001), and of the regulatory cytokine TGF- β (+1.7-fold; P<0.001) in obese animals, while IL-10 was not modified (Fig. 2B).

3.2. Release of isoprostanoids by pWAT

In conditioned medium obtained from pWAT, four main species of F_{2t}-IsoP and F_{4t}-NeuroP could be detected and quantified (Fig. 4A). Surprisingly, concentration of 5-F_{2t}-IsoPs (including the R/S isomers) was found diminished from 127.1 \pm 18.2 in *fa/+* rats (n=5) to 33.1 \pm 4.1 ng/ml in *fa/fa* rats (-3.8-fold, n=15, P<0.01). 15-F_{2t}-IsoPs (as R/S isomers) were less abundant than 5-F_{2t}-IsoPs (by 2.8 to 3.7-fold) and also reduced from 45.8 \pm 7.5 in *fa/+* rats to 9 \pm 1.3 ng/ml in *fa/fa* rats (-5.1-fold, P<0.01). 4-F_{4t}-NeuroPs, which were less abundant than F_{2t}-IsoPs (by 5 to 30-fold), were similarly decreased from 4.1 \pm 0.55 in *fa/+* rats to 1.8 \pm 0.33 ng/ml in *fa/fa* (-2.3-fold, P<0.01). Finally, 10-F_{4t}-NeuroP, the less abundant species, was reduced from 0.373 \pm 0.55 in *fa/+* to 0.123 \pm 0,023 ng/ml in *fa/fa* rats (-3-fold, P<0.01). Interestingly, in whole pWAT (containing both free and membrane-bound lipid derivatives cleaved during the process), less 5-F_{2t}-IsoPs and 15-F_{2t}-IsoPs were recovered from pWAT of obese rats as compared to lean animals (respectively -2.5-fold, P<0.01 and -2.1-fold, n=7, P<0.01) (Fig. 4B). Concerning 4-F_{4t}-NeuroPs, an average concentration of 0.295 ng/g tissue (n=7) could be detected in pWAT from *fa/+* rats, whereas it remained undetectable in pWAT from *fa/fa* rats. 10-F_{4t}-NeuroP could not be measured, due to sensitivity limitation of the assay.

In contrast, in conditioned medium from eWAT, no significant difference in isoprostanoid levels could be observed in obese *versus* lean rats (n=6) (Fig. 4C). In addition, eWAT released less isoprostanoids than pWAT (up to 10-fold between the two adipose tissues). Such discrepancies may not be related to differences in adipocyte number and size, as the total protein content/g of adipose tissue was not significantly modified between eWAT and pWAT: 1.57 ± 0.1 mg/ml *versus* 1.38 ± 0.08 mg/ml for *fa/+* rats and 1.05 ± 0.03 mg/ml *versus* 0.97 ± 0.03 mg/ml for *fa/fa* rats. This data thus confirmed that adipocytes from obese animals were similarly hypertrophic in pWAT and eWAT.

For comparison, all four series of oxygenated lipids were up to 100-fold less abundant in plasma than in conditioned medium of pWAT, with a concentration of 5-F_{2t}-IsoPs > 15-F_{2t}-IsoPs > 4-F_{4t}-NeuroPs > 10-F_{4t}-NeuroP (Fig. 4D). F_{2t}-IsoPs as well as F_{4t}-NeuroPs were found significantly higher in plasma of obese rats (n=10) *versus* lean animals (n=6) (P<0.05) (Fig. 4D), with analytes being 2.5 to 3 times more concentrated in the plasma of obese animals, as previously described [22].

3.3. Antioxidant defenses in pWAT

Antioxidant enzymes are known to protect cells against intracellular oxidative stress [23] and can limit isoprostanoids formation. Gene expression of [Cu-Zn]- and Mn-dependent superoxide dismutases (SOD1 and SOD2, respectively) was enhanced by 52% for *Sod1* (n=6; P<0.05) and 70% for *Sod2* (n=6; P<0.01), respectively, in obese *versus* lean animals (Fig. 5A). Moreover, catalase (*Cat*), glutathione peroxidase 1 (GPX-1, *Gpx1*), and 4 (GPX-4, *Gpx4*) were also found overexpressed by 2.5, 1.8 and 2.1-fold, respectively, in the pWAT from *fa/fa* rats (n=6; P<0.05) (Fig. 5A).

In eWAT from *fa/fa* rats, expression of *Sod1*, *Sod2*, and *Gpx1* were significantly increased (respectively, 28%, 49%, 52%; n=6; P<0.01), whereas *Gpx4* and catalase remained unchanged compared to lean animals (Fig. 5B).

When comparing both types of adipose tissue, we observed that eWAT displayed higher mRNA expression of antioxidant enzymes than pWAT, except for *Sod1*. For example, *Sod2* is approximately 7 times more expressed in eWAT than in pWAT of lean and obese animals. This observation is in line with the lower isoprostanoids level we found in eWAT conditioned medium as compared to pWAT (see Fig. 4C). In addition when comparing *fa/fa* and *fa/+* rats, overexpression of *Sod1*, *Sod2*, and most importantly catalase was greater in pWAT than in eWAT.

3.4. Phospholipases A2 expression in pWAT

As phospholipases A2 (PLA2s) are known to be involved in the release of isoprostanoids from cellular membranes [24], we selected PLA2s with potential involvement in isoprostanoids release from adipose tissue [25, 26, 27].

In pWAT, we found sPLA2 IIA (*Pla2g2*) being the most strongly expressed enzyme, compared to other PLA2s studied (Fig. 5C). Overall, PLA2s appeared to be enhanced in pWAT of obese rats, reaching significance for sPLA2 V (*Pla5g5*) (+12.4-fold; n=6; P<0.05) and Lp-PLA2 (*Pla5g7*) (+2.9 fold; P< 0.01). In contrast, the predominant sPLA2 IIA enzyme was significantly reduced in the pWAT of obese animals (-2.1-fold; P<0.05).

Expression of PLA2s in eWAT displayed a very different profile as for pWAT. The sPLA2 IIA enzyme is less expressed and reached the same level as cPLA2 (*Pla2g4*), and iPLA2 γ (*Pnpla8*), the latter being more expressed than in pWAT (Fig. 5D). There were no significant changes in PLA2s expression between lean and obese animals in eWAT, except for Lp-PLA2, which was strongly increased in obese rats (+5.4-fold; n=6; P<0.01).

3.5. Effect of conditioned medium of pWAT on glucose-induced insulin secretion of pancreatic islets

After 24 hours culture with conditioned medium from pWAT of *fa/+* rats, we observed a significant decrease in insulin secretion induced by 11.2 mmol/l glucose (-24.4%; n=9; P<0.001) (Fig. 6A). However, conditioned medium from *fa/fa* rats was unable to modify glucose-induced insulin secretion. For comparison, culture of islets with conditioned medium from both eWAT of *fa/+* and *fa/fa* rats reduced insulin secretion (n=4; P<0.05) (Fig. 6B). Differences between pWAT and eWAT effects could be related to changes in released isoprostanooids with a potential effect on insulin secretion.

3.6. Effects of synthetic isoprostanooids on glucose-induced insulin secretion of pancreatic islets

No effect on insulin secretion could be observed with the most abundant isoprostanooid, 5-F_{2t}-IsoP or its epimer, either at a low (10 nmol/l) or a higher (10 μmol/l) concentration (n=3) (Fig. 6C). At the opposite, an inhibition of glucose-induced insulin secretion was obtained when islets were cultured with 10 nmol/l or 10 μmol/l of 15-F_{2t}-IsoP (respectively, -30%, P<0.05 and -43%, P<0.001; n=3) (Fig. 6D). Its epimer, 15-*epi*-15-F_{2t}-IsoP, similarly reduced insulin secretion by 32 and 35% at respectively 10 nmol/l or 10 μmol/l (P<0.01) (Fig. 6d). Overall, our results showed that some isoprostanooids can negatively regulate glucose-induced insulin secretion in rat islets.

3.7. Isoprostanooids target thromboxane A2 receptor for the modulation of insulin secretion

We first investigated whether thromboxane A2 receptor was expressed in pancreatic β-cells. In rat islets and in the β-cell line, INS-1 cells, we found the presence of thromboxane A2 receptor mRNA (Fig. 7A). We were also able to evidence the protein production, by Western blotting (Fig. 7B). Using immunofluorescence, we confirmed that thromboxane A2 receptor immunoreactivity was localized in β-cells with a punctuated staining in the cytoplasm and a

stronger staining in the nucleus, as previously described in oligodendrocytes [28] [Fig. 7C). Concerning α -cells, a faint staining was observed in the cytoplasm and a more pronounced one, in the nucleus (Fig. 7D). In δ -cells, thromboxane A2 receptor staining was weak in the cytoplasm, stronger in the nucleus and in part colocalised with somatostatin (Fig. 7E). In addition, in islets from *fa/fa* rats, the receptor was slightly overexpressed (+45%, $P<0.05$) as compared to islet from lean animals (Fig. 7F).

To confirm the involvement of thromboxane A2 receptor, we used the potent thromboxane A2 analog, IBOP ($EC_{50} = 0.34$ nmol/l) [29], and the receptor antagonist SQ29548 ($IC_{50} = 12$ nmol/l) [30]. Interestingly, IBOP induced a reduction of insulin secretion stimulated by 11.2 mmol/l glucose, with a maximal effect at 10 nmol/l (-37%, $P<0.001$, $n=3$) (Fig. 8A). In addition, the receptor antagonist SQ29548 (at 10 μ M) partially reversed the inhibitory effects of 15- F_{2t} -IsoP (used at 10 μ M) on glucose-stimulated insulin secretion (Fig. 8B). Both observations allowed us to assume that isoprostanoids modulate insulin release through binding to thromboxane A2 receptor. Binding of the two 15- F_{2t} -IsoPs to thromboxane A2 receptor did not modify mRNA expression of insulin (*Ins1*) or pancreas/duodenum homeobox protein 1 (PDX-1, *Pdx1*) gene in islets (Fig. 8C). As thromboxane A2 receptor can induce cAMP-dependent signaling [31], we stimulated intracellular cAMP production in islets with forskolin (10 μ M) during 24 hours. 15- F_{2t} -IsoPs and IBOP, but not 5- F_{2t} -IsoPs (at 10 μ M) significantly decreased forskolin-stimulated cAMP levels ($P<0.05$ and $P<0.01$, respectively) (Fig. 8D).

Discussion

Peri-pancreatic white adipose tissue is an ectopic tissue that expands during obesity and establishes close contact with pancreatic islets. From previous studies, we and others have found that pWAT secrete proteic factors able to modulate β -cell proliferation and/or function in rat model of obesity [12, 13, 14] or type 2 diabetes [32].

In obese Zucker *fa/fa* rats, pWAT displays an increased macrophage infiltration, as we recently found in pWAT from prediabetic ZDF rats [32]. Macrophage recruitment is also enhanced in eWAT of *fa/fa* rats, similarly to what was observed in mouse models of obesity [33]. However, the inflammatory profile of the two adipose tissues appears clearly different. In eWAT, a predominance of M1 phenotype and proinflammatory cytokines can be detected, which is consistent with other studies done in diet-induced obese (DIO) mice [34]. The regulatory cytokine, TGF- β , is also found enhanced, but this factor can be secreted by adipocyte precursors to enhance their self-proliferation [35]. pWAT contains macrophages of both M1 and M2 phenotype and a mixed cytokine profile. This data suggests that M1 macrophages are recruited, in parallel with the maintenance of a pool of M2 macrophages, allowing a balance between pro- and anti-inflammatory processes in this adipose tissue. Alternatively, this can reflect the presence of macrophages of mixed phenotype with M1/M2 markers, as previously described in adipose tissue from DIO mice [36] or obese subjects [37]. Moreover, in the Zucker rat model, both pWAT and eWAT are able to produce isoprostanooids as oxygenated lipids derived from phospholipids of bilayer membranes. Surprisingly, we demonstrate that pWAT of obese animals release less isoprostanes and neuroprostanes than lean animals, whereas they remain unchanged in eWAT secretions from both animals. At the opposite, isoprostanooids are found increased in plasma of obese animals, as previously described [22]. Even if the source of plasma isoprostanooids remains unclear, expansion of

visceral adipose tissue in *fa/fa* rats could partially account for this plasmatic increase. Indeed, in humans, the volume of visceral adipose tissue is highly associated with a rise in urinary 15-F_{2t}-IsoPs levels, as compared to subcutaneous adipose tissue [38]. However, the concentrations of isoprostanoids in plasma are 100-fold less abundant than in conditioned media, and this amounts are probably insufficient for a direct effect of circulating isoprostanoids on target tissues.

The generation of reactive oxygen species is controlled by cellular enzymatic and non-enzymatic antioxidant defenses, such as [Cu-Zn]- and Mn-dependent superoxide dismutases, glutathione peroxidases 1 and 4, and catalase. In pWAT, the five antioxidant enzymes are strongly overexpressed in *fa/fa* versus *fa/+* rats, showing the stimulation of antioxidant defenses in this tissue. In eWAT, induction of these enzymes in obese animals is less pronounced than in pWAT and no induction of catalase expression (this study) and activity [23] is observed. This data suggests an intensification of defenses mechanisms in ectopic pWAT of obese animals, partly accounting for the reduced production and release of isoprostanoids. Interestingly, mice overexpressing *Sod1* and *Sod2* are protected against high fat diet induced glucose intolerance [39]. In addition, overexpression of catalase and *Sod1* in mouse adipocytes improves insulin sensitivity through a greater adipose tissue expansion and protection against ectopic lipid deposits [40].

PLA2 enzymes are responsible for isoprostanoids release from membrane phospholipids. Among the six PLA2s tested, sPLA2 IIA displays the highest expression in adipose tissue and is found downregulated in pWAT of obese animals. This suggests that sPLA2 IIA could be involved in the reduced release of isoprostanoids we observed, all the more that a higher expression of this enzyme was recently correlated with an increase in free F₂-isoprostanes in preeclamptic placenta [41]. In contrast, sPLA2 IIA level remains unchanged in eWAT in *fa/fa* rats, which contrasts with an overexpression found in adipose tissue from high fat diet fed mice [42] and rats [43]. In addition, in this latter model, a selective inhibitor of sPLA2 IIA

reduced the production of prostaglandin E2 (another oxygenated lipid from arachidonic acid) and improved glucose tolerance [43].

At a more functional level, we showed that conditioned media from pWAT of *fa/+* rats diminishes glucose-induced insulin secretion, whereas the one issued from *fa/fa* rats has no effect. Such an observation suggests that pWAT secretome exerts an inhibitory tone on insulin secretion, this effect being alleviated in obese animals. This observation is in line with the surgical removal of pWAT in HFD fed mice that induces higher basal and glucose-stimulated insulin levels, even though the effects of the tissue removal are probably multifactorial [14]. We can correlate this effect to changes into release of isoprostanoids by pWAT, as we showed that the ability of this tissue to produce/release isoprostanoids is strongly reduced in obese rats.

Most importantly, we observe, for the first time, that both 15-F_{2t}-IsoP epimers are able to reduce glucose-induced insulin secretion in rat islets, whereas the other series, the 5-F_{2t}-IsoPs remained inactive. Concentrations of isoprostanoids we applied on islets are in the range of those measured in conditioned medium of *fa/+* rats (around 10⁻⁷ M) and what was used in other studies [44]. These results are consistent with the effect of 15-F_{2t}-IsoPs on contractile activity of microvasculature, which is not observed with 5-F_{2t}-IsoPs [45]. Therefore, inhibition of insulin release we observed with conditioned media from pWAT of lean rats may be related to an inhibitory tone of isoprostanoids, which is alleviated in obese animals. This suggests that, *in vivo*, β-cell exposure to isoprostanoids released by pWAT could be locally high and exerts negative modulation on insulin secretion. Other ectopic adipose tissues were also found able to modulate functions of neighboring cells. For example, perivascular adipose tissue has a vasorelaxant effect on vessels, a function altered in obesity [46].

Investigating isoprostanoids mechanisms of action, we observed, for the first time, that thromboxane A2 receptor is expressed in rat pancreatic β-cells. Importantly, the receptor agonist, IBOP [29], decreased glucose-induced insulin secretion and the receptor antagonist,

SQ 29548 [30], reversed 15-F_{2t}-IsoPs' effects, confirming that oxygenated lipids influence β -cells activity via binding to this receptor. As coupling mechanisms, thromboxane A2 receptor activation is known to induce Ca²⁺ mobilization via Gq or RhoA kinase activation via G_{12/13} [16] or cAMP-dependent signaling [31]. In islets, we found that 15-F_{2t}-IsoPs and IBOP induced a decrease in intracellular cAMP stimulated by forskolin that can be related to the inhibitory effects of the compounds on insulin secretion, as previously described in platelets [31].

In conclusion, we have evidenced a novel mechanism involved in β -cell compensation in insulin resistance. Metabolic and inflammatory adaptation of pWAT in obesity appears to be responsible for a reduced production of isoprostanoids leading to an improved insulin response of β -cells necessary for the maintenance of glucose tolerance. Better understanding of isoprostanoids homeostasis linked to WAT adaptation in obesity could lead to new therapeutic strategies in type 2 diabetes.

Data availability

The data used to support the findings of this study are included within the article.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgments

ADL was supported by a funding from the technology transfer acceleration company SATT AxLR. J.L. received travel grants from the European Association for the Study of Diabetes (EASD) and Société Francophone du Diabète (SFD).

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Figure legends

Fig. 1. Inflammatory profile of pWAT from Zucker *fa/fa* versus *fa/+* rats. A. Relative quantification of *Cd68* by RT-qPCR in obese versus lean rats (n=6). B. Immunohistochemistry staining of CD68 and quantification of macrophage infiltration/mm² in sections of pWAT from obese versus lean rats. Ctr- is a negative control performed in absence of primary antibody. Magnification x20. Relative quantification of (C) chemokines, (D) M1 macrophage markers, and (e) M2 macrophage markers by RT-qPCR in obese versus lean rats (n=6). *, P< 0.05; ***, P< 0.001.

Fig. 2. Cytokine profile of pWAT and eWAT from Zucker *fa/fa* versus *fa/+* rats. A. Relative quantification of cytokines of pWAT by RT-qPCR in obese versus lean rats (n=5). B. Relative quantification of cytokines of eWAT by RT-qPCR in obese versus lean rats (n=8-9). *, P<0.05; **, P< 0.01; ***, P< 0.001.

Fig. 3. Inflammatory profile of eWAT from Zucker *fa/fa* versus *fa/+* rats. A. Relative quantification of *Cd68* by RT-qPCR in obese versus lean rats (n=6). Relative quantification of (B) chemokines, (C) M1 macrophage markers, and (D) M2 macrophage markers by RT-qPCR in obese versus lean rats (n=6). *, P< 0.05; **, P< 0.01.

Fig. 4. Production and release of isoprostanoids in pWAT and eWAT from Zucker *fa/fa* versus *fa/+* rats. A. Quantification of isoprostanoids in conditioned medium from pWAT of obese (n=15) versus lean (n=5) rats. B. Evaluation of intracellular isoprostanes after chemical hydrolysis from pWAT of obese (n=15) versus lean (n=5) rats. C. Quantification of isoprostanoids in conditioned medium from eWAT of obese versus lean rats (n=6).

Measurement of isoprostanoids in plasma of obese (n=10) versus lean (n=6) rats. IsoP, isoprostanes, NeuroP, neuroprostanes. *, P< 0.05; **, P< 0.01.

Fig. 5. Analysis of expression profile of antioxidant defenses enzymes and PLA2s by RT-qPCR in pWAT and eWAT. Relative quantification of (A) antioxidant enzymes and (C) PLA2s in pWAT of obese versus lean rats (n=6). Relative quantification of (B) antioxidant enzymes and (D) *PLA2s* in eWAT of obese versus lean rats (n=6). *, P< 0.05; **, P< 0.01.

Fig. 6. Effects of F_{2t}-isoprostanes on glucose-stimulated insulin release in isolated islets from Wistar rats. Effects of conditioned medium from (A) pWAT (n=9) and (B) eWAT (n=3) of obese versus lean rats on insulin secretion induced by 11.2 mmol/l glucose. C. Insulin secretion induced by 11.2 mmol/l glucose in the presence or not of 5-F_{2t}-IsoP and 5-*epi*²-5-F_{2t}-IsoP (n=3). D. Insulin secretion induced by 11.2 mmol/l glucose in the presence or not of 15-F_{2t}-IsoP and 15-*epi*-15-F_{2t}-IsoP (n=3). *, P<0.05; **, P< 0.01; ***, P< 0.001.

Fig. 7. Involvement of thromboxane A₂ receptor in modulation of insulin secretion. A. Analysis of thromboxane A₂ receptor expression by RT-qPCR (A) and Western blotting (B) in rat pancreatic islets and INS-1 cells. Cellular localization of thromboxane A₂ receptor (TRX) by immunofluorescence in (C) β-cells (insulin (INS) staining), (D) α-cells (glucagon (GLUC) staining), and (E) δ-cells (somatostatin (SOMATO) staining). (F) Western blotting analysis of thromboxane A₂ receptor expression in islets from Zucker *fa/fa* and *fa/+* rats. *, P<0.05.

Fig. 8. Effects of the thromboxane A₂ receptor agonist and antagonist glucose-stimulated insulin release in isolated islets from Wistar rats. A. Insulin secretion induced by 11.2 mmol/l glucose in the presence or not of the receptor agonist, IBOP. B. Insulin secretion induced by

11.2 mmol/l glucose in the presence or not of the receptor antagonist, SQ 29548. C. Effects of isoprostanoids on insulin (*Ins1*) and *Pdx-1* gene expression measured by RT-qPCR. D. Effects of 15-F_{2t}-IsoP, 5-F_{2t}-IsoP, and IBOP on intracellular cAMP concentration. t, 0.05<t<0.1*, P<0.05; ***, P<0.001.