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EFFECT OF MELATONIN ON OBESITY, LOW-GRADE INFLAMMATION AND OXIDATIVE STRESS IN DIABETIC FATTY (ZDF) RATS

DOCTORADO EN BIOMEDICINA

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D. AHMAD AGIL, profesor de Farmacología de la Facultad de Medicina de la Universidad de Granada, certifica que: Dña. Aroa Jiménez Aranda, Licenciada en Bioquímica, ha realizado bajo mi dirección en el Instituto de Neurociencias, Centro de Investigación Biomédica, Parque Tecnológico de Ciencias de la Salud, y Departamento de Farmacología, Facultad de Medicina, Universidad de Granada, el presente trabajo de investigación, titulado: **"EFFECT OF MELATONIN ON OBESITY, LOW-GRADE INFLAMMATION AND OXIDATIVE STRESS IN DIABETIC FATTY (ZDF) RATS"**, que ha sido objeto de su Tesis Doctoral, reuniendo las condiciones necesarias para optar al grado de Doctor.

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ABREVIATURAS

5-HT: 5-hydroxytryptamine

5-HETE: 5-hydroxyeicosatetraenoic acid

AFMK: N1-acetyl-N2-formyl-5-methoxykynuramine

AMK: N1-acetyl-5-methoxykynuramine

BAT: brown adipose tissue

BMI: body mass index

CAT: catalase

C/EBP: Ccaat-enhancer-binding proteins

COX-2: cyclooxygenase 2

CRP: C-reactive protein

CS: citrate synthase

CV: cardiovascular

DNA: deoxyribonucleic acid

ER: endoplasmic reticulum

ETC: electron transport chain

FFA: free fatty acids

FGF21: fibroblast growth factor 21

GPCR: G protein-coupled receptor

GPx: glutathione peroxidase

GSH: glutathione

H_2O_2: hydrogen peroxide

Hb: hemoglobin

HDL: high-density lipoprotein

HOMA: homeostatic model assessment

HRP: horseradish peroxidase

H&E: hematoxylin-eosin

IKK: I kappa B kinase

IL-6: interleukin 6

iWAT: inguinal white adipose tissue

LDL: low-density lipoprotein

LPO: lipid peroxidation

MCP1: monocyte chemoattractant protein

MDA: malondialdehyde

MS: metabolic syndrome

MT: melatonin

NF-κβ: nuclear factor

NLR: NOD-like receptor

NO: nitric oxide

NOS: nitric oxide synthase

O^{2-}: superoxide anion

OH^-: hydroxyl radical

$ONOO^-$: peroxynitrite anion

PAI-1: plasminogen activator inhibitor

PDI: protein disulfide isomerase

PKA: protein kinase A

PPARγ: peroxisome proliferator-activated receptor

PRDM16: zinc finger-containing protein PR domain containing 16

PTP: permeability transition pore

RBP4: resistin and retinol binding protein

RNS: radical nitrogen species

ROR/RZR: retinoid orphan/retinoid Z

ROS: radical oxygen species

SAT: subcutaneous adipose tissue

SOD: superoxide dismutase

T2DM: type 2 diabetes mellitus

TEM: transmission electron microscopy

TLR: toll like receptor

TNFα: tumor necrosis factor

UCP: uncoupling protein

UPR: unfolded protein response

VAT: visceral adipose tissue

WAT: white adipose tissue

ZDF: Zucker diabetic fatty

ZL: Zucker lean

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RESUMEN

Resumen

El síndrome metabólico es un conjunto de factores de riesgo de enfermedad cardiovascular y diabetes mellitus tipo 2 que incluye la obesidad. Para producirse el síndrome metabólico, deben combinarse al menos tres de los siguientes parámetros: presión arterial ≥ 135/85 mmHg, HDL < 40 mg / dL para los hombres y < 50 mg / dL para las mujeres, glucosa en ayunas > 100 mg / dL, triglicéridos > 150 mg / dl y obesidad abdominal (circunferencia de la cintura ≥ 102 cm en varones y ≥ 88 en mujeres). Actualmente, supone un problema de salud pública a nivel mundial. Esto provoca una alta morbilidad y mortalidad, así como una enorme carga económica. La búsqueda de fármacos seguros y eficaces que pueden mejorar esta situación ha resultado infructuosa.

Varios estudios sugieren que la melatonina, una molécula natural con una alta seguridad toxicológica y de bajo costo, podría ayudar a mejorar la situación. Pero la base científica de sus beneficios sobre la obesidad y sus complicaciones metabólicas, son poco conocidos. Esta neurohormona es producida por la glándula pineal durante la noche, y también a nivel local en muchos otros tejidos. Además de regular los ritmos circadianos, la melatonina y varios de sus metabolitos son eficaces antioxidantes y agentes antiinflamatorios.

Numerosos estudios han demostrado que la administración de melatonina en roedores reduce el exceso de peso asociado a diferentes condiciones experimentales o deficiencias fisiológicas (como el envejecimiento). Nuestro grupo ha demostrado que la melatonina mejora el sobrepeso y la dislipidemia en ratas ZDF jóvenes. También reduce la ganancia de peso corporal sin afectar a la ingesta de alimentos. La melatonina puede ejercer su efecto antihiperglucémico ya sea por mejora de la acción de la insulina, por mejora de la secreción de insulina, o ambos. Nuestro grupo también mostró que la melatonina oral se acompaña de una disminución de hiperinsulinemia, que se refleja en la mejora de la resistencia a la insulina, como se evidencia por un índice HOMA - IR inferior.

Por otra parte, el tejido adiposo ha mostrado un papel principal en el desarrollo de la enfermedad metabólica, y la diferencia entre los adipocitos blancos, pardos y beige se ha convertido en una clave importante para la investigación. La disfunción mitocondrial y de retículo endoplásmico vista en el síndrome metabólico se correlaciona con la inflamación crónica, que conecta el desequilibrio con el tejido adiposo.

Por todo ello, este trabajo relaciona el papel de la melatonina en el síndrome metabólico centrándose en sus efectos en la obesidad, resistencia a la insulina y la inflamación.

El objetivo inicial fue evaluar la influencia de la melatonina en la inflamación de bajo grado y en los depósitos de tejido adiposo, tanto a nivel local como a nivel mitocondrial. Esto incluye los mecanismos moleculares por los que la melatonina mejora el sobrepeso en un modelo animal de la obesidad y la diabetes, la rata ZDF, con el fin de explorar su aplicación potencial en el tratamiento de la obesidad humana.

Se analizaron en primer lugar varios marcadores plasmáticos de inflamación de bajo grado, como son la IL-6, el TNF-α y la CRP, así como el estrés oxidativo a nivel plasmático mediante un análisis de la peroxidación lipídica (MDA). Estos primeros resultados mostraron un efecto positivo ejercido a nivel de la inflamación y el estrés oxidativo crónico, sin afectar negativamente a la situación fisiológica.

En la siguiente fase de nuestro estudio, mediante la observación de los depósitos de tejido adiposo en ratas obesas (y concretamente la zona inguinal), se encontró que sólo aquellas ratas tratadas con melatonina tenían un tipo de tejido adiposo diferente, el beige. Se realizó un estudio profundo a nivel morfológico y una comparación con los tejidos obtenidos a partir del mismo depósito en el control y los animales tratados. La segunda evidencia llamativa fue reportada por la microscopía óptica (tinción H&E), que nos permitió identificar estos grumos de tejido adiposo beige dentro de zonas típicas de tejido adiposo blanco. El análisis detallado de cada almohadilla de grasa de forma individual, junto con los recientes hallazgos de otros grupos, indicaba que estábamos ante un depósito de grasa especial, con características similares al tejido adiposo pardo clásico. Por ello, llevamos a cabo una detallada comparación con el tejido adiposo blanco en el que se encontraba, para caracterizarlo a nivel bioenergético y funcional. Con ello, pudimos comprobar que el tejido adiposo beige tiene la capacidad de aumentar su actividad termogénica y su capacidad mitocondrial. Además, la melatonina resultó ser altamente efectiva estimulando estas actividades.

En conclusión, el tratamiento oral con melatonina atenúa la inflamación de bajo grado y el estrés oxidativo, presente en un modelo experimental de diabetes tipo 2, la rata ZDF. Esto contribuye a apoyar el concepto de que la melatonina puede ser útil para el tratamiento de la diabetes tipo 2 y el síndrome metabólico. Además, la melatonina se comporta como un inductor del "browning" con propiedades termogénicas.

Estas observaciones, junto a su alta seguridad farmacológica, hacen de la melatonina una herramienta potencialmente útil para una terapia independiente o complementaria para la obesidad. Por ello, los resultados presentados en esta tesis representan el comienzo de una nueva estrategia de lucha contra la obesidad y la diabetes mellitus tipo 2, donde la melatonina es la columna vertebral de todas las mejoras observadas.

INTRODUCTION

1. Background

Current lifestyle, characterized by caloric abundance, reduced physical activity, and other manners, increased the incidence of obesity, propelling it to an alarming worldwide expansion. This situation led to a breakthrough in research of obesity and its associated diseases, and one of them is metabolic syndrome [1]. The metabolic syndrome is a cluster of risk factors for cardiovascular disease, including abdominal obesity, and proinflammatory states [2, 3]. There are several sets of defining criteria for metabolic syndrome, being the one provided by the ATPIII mostly used worldwide, the combination of at least three of the following parameters: blood pressure ≥135/85 mmHg, HDL<40 mg/dL for men and <50 mg/dL for women, impaired fasting glucose (>100 mg/dL), triglycerides>150 mg/dL and abdominal obesity (waist circumference ≥ 102 cm in males and ≥ 88 in women). Since different definitions are actually being used, it is difficult to compare the prevalence between countries [4]. According to various sources, metabolic syndrome in the United States affects at least 30% of the population, and the prevalence increases with age [5].

There has been also an increased interest on the adipose tissue, which has evolved from being a simple deposit of fat to being considered as a major orchestrator of the metabolic syndrome pathophysiology [6]. Obesity causes a metabolic problem when adipose tissue fails to meet the increased demands of fat storage, so that the excess of fat, that cannot be efficiently and safely stored in it, accumulates in other non-adipose organs, causing lipotoxicity and metabolic complications [7]. The excessive accumulation of adipose tissue is a major risk factor for metabolic disease, and beyond the continual growth, the adipocyte begins exhibiting hypertrophy and associated mechanical stress, compositional changes of lipids and other nutrients, hypoxia, disruption of mitochondrial function, production of reactive oxygen species, apoptotic signaling, increased fatty acid release, altered adipokine signaling, inflammation, and endoplasmic reticulum stress [8]. Although the molecular mechanisms of the metabolic syndrome are unknown, the inflammation is emerging as an important factor to understand the disorders of the metabolic syndrome, due to its relevance at the level of the adipose tissue [9]. The possible dysfunction of the endoplasmic reticulum related to this inflammation is also discussed as a new option for the study of the metabolic syndrome and its risk factors. Additionally to the endoplasmic reticulum, many researchers point to the mitochondrial dysfunction linked to the metabolic syndrome [2].

Since metabolic syndrome and its associated disorders are becoming a major global health problem, the aim of this research is to present evidence on the mechanisms underlying these risk factors, and the connection between the impaired tissues, the low-grade inflammation and the mitochondrial dysfunction. Since pineal disturbances could influence the pathogenesis and the phenotype variations of the metabolic syndrome [10], the use of melatonin to prevent such adverse outcomes should also be considered.

2. Adipose tissue in the metabolic syndrome

The adipose tissue has an important role in maintaining the energy balance and lipid homeostasis. Classically, there are two types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT). The different WAT depots have specific roles based on their level of lipolysis and the rate of triglycerides storage. WAT is distributed throughout the body in the form of two major divisions, subcutaneous or inguinal adipose tissue (iWAT). In this iWAT appears a new adipose tissue, which was recently described as beige adipose tissue, and visceral adipose tissue (VAT or classical WAT) (Figure 1) [(11)].

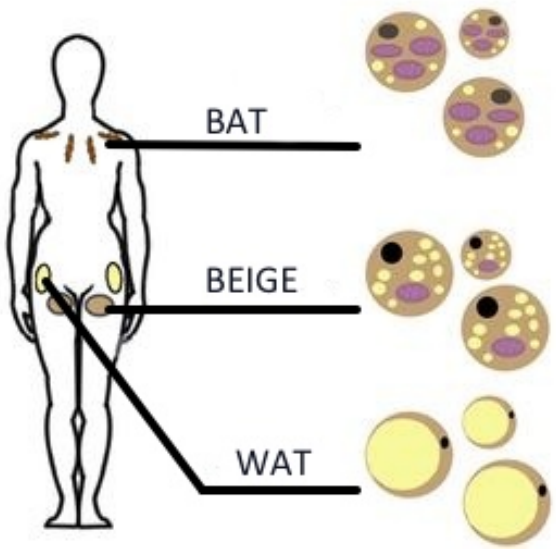


Figure 1: Human location of adipose depots.

A recent study has expanded the characterization of these depots, including six different fat groups [(12)]. The expandability of these depots, particularly in the central obesity, determines the onset of metabolic syndrome. Meanwhile VAT increases the risk of obesity-related disease, beige iWAT, especially located to the lower-body parts, protects from lipotoxic effects [(13)]. Due to its high susceptibility to inflammation and higher lipolytic activity, VAT can induce detrimental metabolic effects such as insulin resistance, diabetes mellitus, hypertension and dyslipidemia. In contrast, beige iWAT benefits the metabolic profile by improving glucose homeostasis, and its expansion does not imply metabolic risk [(9)]. Obesity is characterized by a huge increase in white fat, with hyperplasic and hypertrophic adipocytes [(14)], that exhibits a lower density of insulin receptors, which facilitates the initiation of the proinflammatory state [(15)].

While WAT store energy, BAT has a thermogenic function generating body heat as a defense against hypothermia and obesity. This tissue, mainly located in the interscapular area, metabolizes fatty acids to produce heat instead of ATP. It was traditionally believed that BAT is only functional

in neonatal mammals, rodents and hibernators, but recent studies have shown the presence of substantial depots of brown adipose tissue in adult humans [16, 17].

Adaptive thermogenesis is an energy dissipating process in response to cold exposure and energy intake that contributes to energy expenditure [18, 19], and its activation represents a crucial defense against obesity. The intensity of this process is highly variable among individuals. Since low energy expenditure is a risk factor for obesity, unraveling the underlying mechanisms of adaptive thermogenesis can be beneficial in the struggle against metabolic syndrome. All these differences between adipose depots are shown in Figure 2.

The adipose tissue is an endocrine and auto-paracrine organ that releases a large variety of cytokines involved in metabolic homeostasis the development of chronic diseases, due to its direct or indirect effect on insulin resistance, inflammation and endothelial dysfunction. The alterations that influence the expression and secretion of these biomarkers in different tissues are responsible of the modification in the pro-inflammatory status.

ADIPOSE DEPOTS	BAT	Beige (in iWAT)	WAT	Reference
Lipid content	Multilocular droplets	Multilocular droplets	Single large droplet	*11*
Vascularity	Highly vascularized	Highly vascularized	Normal	*18*
Mitochondria	Abundant +++	Abundant +	Few	*46*
Origin	Myf-5 positive cells	Myf-5 negative cells	Myf-5 negative cells	*46*
UCP	UCP1 +++	UCP1 +	UCP1 -	*13, 54*
Functions	- Non-shivering thermogenesis - Burn triglycerides for heat production - Energy expenditure - Insulin sensitivity	- Non-shivering thermogenesis - Burn triglycerides for heat production - Energy expenditure - Insulin sensitivity	- Store energy - Detrimental metabolic effects	*46*

Figure 2: Adipose tissue depots in humans.

2.1. Brown adipose tissue

Brown fat acts in the thermogenic response and in energy balance. In humans, the role of brown adipose tissue is still controversial. The last emerging studies for understanding obesity show that functional and substantial amounts of brown adipose tissue (BAT) persist in adult humans. Until recently, it was thought that the activation of brown depots can induce a weight loss and its metabolic rate can be regulated by physiological or pharmacological manipulations. However, we now know that is the beige adipose tissue (the brown-like adipocytes) the one with this capacity. If these processes can be applied to humans, BAT could be a novel target for new drug development to treat metabolic syndrome [20-23]. Indeed, the appearance of brown-like adipocytes within white adipose tissue depots is associated with improved metabolic phenotypes [24]. These brown fat-like cells are known as beige or "brite" cells and can be found in the inguinal subcutaneous adipose tissue and sometimes in epididymal adipose tissue [25].

Brown adipose tissue has different functions, all related to its exclusive localization in mammals. In a cold atmosphere the sympathetic nervous system activates the hypothalamus, leads to secretion of norepinephrine in BAT. The huge innervation of BAT allows quick stimulation of adipocyte membrane adrenergic receptor, resulting in activation of G protein through PKA (protein kinase A), cAMP-dependent actives lipases, which generates free fatty acids from triglycerides activating cytochrome c oxidase into mitochondria. Due to these free fatty acids generation, the process ends with the production of H^+ concentration gradient by the electron transport chain, and in the presence of UCP1, thermogenesis will occur.

Activating the production of brown adipocytes has many positive aspects to combat the effects of metabolic syndrome, in contrast to the negative effects that white adipose tissue can exert in the development of this cluster of risk factors.

2.1.1. Molecular signature of BAT

Many studies have identified the cascade of adipogenic factors involved in WAT and BAT, a gene expression profiling with a vast array of common adipogenic markers, including both, fat store and fat mobility markers [26]. Some of these adipogenic markers are the transcriptional factors, like peroxisome proliferator-activated receptor γ (PPARγ), which is responsible for the expression and central adipogenic activation [27]. The PPARγ and the Ccaat-enhancer-binding proteins (C/EBP) are the most notable transcriptional factors.

PPARγ is indispensable for brown and white adipocyte formation. Its function is to induce the expression of target genes related with lipid and glucose metabolism, including mitochondrial biogenesis and secretion of adipokines [28]. Is a nuclear factor, which plays a key role in lipid and

glucose homeostasis. White adipose tissue's main role is the synthesis and buildup of lipids from fatty acids and triglycerides, while brown adipose tissue functions as a thermogenic organ. In both cases PPARγ is involved, and this is consistent with the fact that PPARγ is the main subtype of PPAR expressed in adipose tissue [29]. This factor is sufficient to induce adipocyte differentiation, and it also regulates energy homeostasis by stimulation of the expression of uncoupling mitochondrial proteins (UCP1, UCP2 and UCP3).

There are several nuclear factors associated with the formation of brown fat cells. The most notable of these factors has been PGC-1α[30], until the recent discovery of PRDM16 (zinc finger–containing protein PR domain containing 16). PGC-1α, considered as a co-activator for PPARγ, is highly expressed in BAT compared with WAT, and is responsible for regulating mitochondrial biogenesis and thermogenesis [31]. It is also needed to promote UCP1 transcription in brown adipocytes [32]. One of the most relevant and best-studied PGC-1α functions is the role it plays in the process of adaptive thermogenesis, where it acts as a link between nuclear receptors and the specific transcriptional program of adaptive thermogenesis. It has been observed that the expression of PGC-1α is highly induced in response to cold in BAT, in the newly identified as "beige" adipocytes and in skeletal muscle. This induction takes place through the adrenergic pathway in BAT [23, 33]. Its induction in muscle by exercise may also stimulate the beneficial effects of this activity [34].

Recent discoveries have brought to light the possibility of future investigations, related to this transcriptional co-activator [35-38]. One of them indicates that there are some factors secreted from muscle under control of this co-activator that might increase whole body energy expenditure, including a hormone, irisin, which is regulated by PGC1-α, secreted from muscle into blood and activates thermogenic function in adipose tissues. The importance of subcutaneous fat depots is reflected in these results, and shows the need to deeply analyze this tissue. The possible white adipose tissue "browning" could provide a new perspective for treating the obesity and type 2 diabetes mellitus, included in the metabolic syndrome [38].

The PGC1-α induction is also associated with an endocrine hormone, fibroblast growth factor 21 (FGF21), a cold-induced secreted protein that increases the appearance of brown-like cells in white adipose tissue [25]. The beneficial effect of this hormone on glucose metabolism and body weight control supports the interest to use it as a potential treatment of metabolic syndrome. Nevertheless, the function of FGF21 must be studied in more detail.

PRDM16 is a zinc finger transcriptional factor, with two points of zinc finger DNA-binding domain [39]. Its main function in brown adipocyte is protein-protein bindings' production. It is believed that PRDM16 acts as a mediator of PGC-1α and PPARγ[40]. Opposite to PPAR, PRDM16 is a determinant but not essential [22] for brown differentiation. Exogenous PRDM16 in white preadipocytes leads to increment of PPAR expression, which in turns, coactivate PGC-1 and UCP1 [41]. Furthermore, specific markers of white fat cells are inhibited by PRDM16. Brown preadipocytes

with PRDM16 suppressed will express skeletal muscle cells phenotype [39].

In conclusion, PRDM16 inhibits WAT and skeletal muscle differentiation, and these studies identify PRDM16 as a master regulator of brown adipocyte differentiation.

2.2. Beige adipose tissue

In addition to classical BAT depots, other brown-fat-like cells are present in the subcutaneous white adipose tissue in animals and also in humans [42]. These cells, with many of the morphological and functional characteristics of classical brown adipocytes, are known as beige or brite (brown-in-white) adipocytes [24, 43, 44]. Brown and white adipocytes were thought to derive from the same precursor cell, but recent studies have shown that brown adipocytes arise from a distinct population of progenitors [31]. The specific progenitors give rise to both brown adipocytes and myocytes, but fail to produce white fat cells. The PRDM16 works as a key molecular switch, determining the development of brown adipocytes due to the expression of the myoblast marker Myf-5. Classical brown adipocytes that reside in the interscapular and perirenal regions develop during the prenatal stage from Myf-5 positive myoblast precursors. However, beige adipocytes are sporadically found as a copious cluster in the WAT of adult animals that have been chronically exposed to browning agents [42].

The browning process, described as an induction of brown adipocytes in WAT depots or the activation of thermogenic programs in WAT, led to further studies in adipose tissue [38, 42, 43, 47]. Brown fat-like cells can emerge in most white fat depots. These cells can be found in the inguinal adipose tissue after its induction, and are characterized for being UCP1-positive cells that emerge from a non Myf-5 lineage [42]. They have a gene expression pattern distinct from either white or brown fat, with their own markers (CD137, Cited1 or TBX1). Therefore, despite a common ability to undergo thermogenesis, brown and beige cells have many distinguishing characteristics and should be considered as different cells (Figure 3).

Recent data suggest that the previously described depots in human BAT are of the beige type and raise the question of whether humans lack classical brown adipocytes altogether [45]. This finding supports the hypothesis that the BAT present at birth is different from the thermogenic adipose tissue observed in human adults [48].

One of the most promising characteristics of these brown-like adipocytes is its induction with adrenergic stimuli [43] or exposure to the cold [42]. Regardless of whether these adipose depots have a major role in body weight reduction, there is no question that expanding the activity of brown fat, beige fat or both in mice through genetic manipulation, drugs or transplantation suppresses metabolic disease [46].

2.2.1. Beige adipose tissue activation

The activities of brown and beige fat cells reduce metabolic disease, including obesity. Therefore, its activation or manipulation provides a promising therapeutic target for metabolic syndrome [46].

There has been controversy regarding the origin of beige adipocytes, since these cells could originate from proliferation and de novo differentiation of a specific pool of precursor cells contained in WAT depots, or from trans differentiation from pre-existing white adipocytes [49].

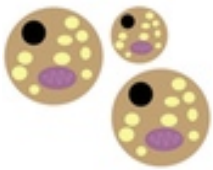
THERMOGENIC ADIPOSE DEPOTS	Brown adipocytes	Beige adipocytes	References
Location in human	Neck Interscapular (Newborns)	Supraclavicular	*46, 47*
Location in mice	Interscapular Cervical Perirenal	Inguinal (Subcutaneous fat)	*46*
Markers	Zic1 Lhx8 Eva1 Pdk4	Cd137 Tbx1 Cited1	*43, 46*
Development	Non-recruitable Disappears with age	Recruitable	*43, 46-48*
Adipocyte structure			---

Figure 3: Differences between brown and beige adipocytes [46].

Following cAMP stimulation, beige cells generated adipocytes with high UCP1 and mitochondrial respiration capacity in vitro [48]. Under physiological conditions, the exposure of rodents such as experimental mice to cold augments UCP1-dependent nonshivering thermogenesis pathways in subcutaneous WAT (a beige phenotype) via activation of the sympathetic system [49]. Angiogenesis is simultaneously activated, resulting in increased vascular density. Cold is a classic activator of beige adipocyte development and function, and the tendency of WAT depots to undergo beiging is highly correlated with their density of sympathetic nerve fibers [46].

Interestingly, browning of WAT can also be induced by exercise, which selectively drives WAT browning through irisin, the exercise-induced myokine [38]. β-adrenergic drugs [50]and other

pharmacological agents, such as prostaglandins, can also induce the beige adipocytes appearance [51].

There are many agents known to favor the acquisition of beige adipocytes and many others that stimulate the activity of BAT [149], but only FGF21 has been proved to act in both ways. However, our main hypothesis is that melatonin could promote these actions (Figure 4).

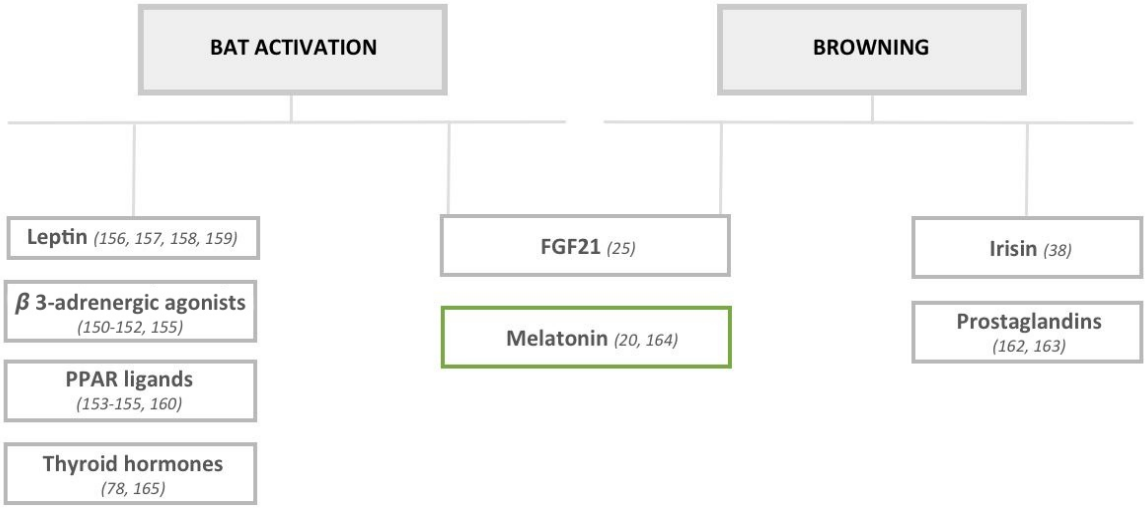


Figure 4: Pharmacological agents and endogenous signals causing BAT activation and browning [149].

Moreover, as we show in our last results [164], the iWAT browning is activated by chronic melatonin treatment. This hypothesis could demonstrate that beige adipose tissue is a better therapeutic tool than BAT, since BAT disappears with age and is a non- recruitable tissue.

In conclusion, although further studies are needed, cold-induced activation of oxidative metabolism in adipose tissue [49] should be deeply analyzed, as well as physical activity and neuropathy, comparing both melatonin-treated and untreated individuals.

2.3. Role of UCP

The most frequently studied mechanism for the adaptive thermogenesis is mitochondrial uncoupling in brown adipocytes. This uncoupling process is executed by uncoupling protein 1, a unique inner membrane protein for BAT and beige depots. UCP1 causes a reflux of protons into the mitochondrial matrix, by passing the ATP synthase. Instead of using the energy stored in the proton gradient to produce ATP, which is the intermediate form of energy in the organism, heat is dissipated due to what is called proton leakage [52]. In conclusion, UCPs are responsible for consuming energy and producing heat.

Mitochondrial biogenesis and increased synthesis of the UCP1 are hallmarks of the thermogenic recruitment process. UCP1 is considered the main marker for the identification of BAT because it is the most specific protein in the brown adipose tissue [53].

These mitochondrial proteins exert a protective role, due to its relation with the proton motive force, and the mitochondrial uncoupling proteins are concerned with the energy expenditure and the control of reactive oxygen species (ROS) homeostasis. Lipotoxicity has been associated with the development of insulin resistance, type 2 diabetes and other pathologies linked to the metabolic syndrome. In particular, the accumulation of reactive lipid peroxides inside the mitochondrial matrix may impair mitochondrial function. The activity of the UCPs may help protecting against mitochondrial damage, because uncoupling reduces reactive oxygen species production and the UCPs may also facilitate the export of fatty acid anions and their peroxides away from the mitochondria [19]. UCP3 has been proposed as an anionic fatty acid exporter to prevent accumulation of free fatty acids inside the mitochondria. Accumulation of anionic free fatty acids resulted in mitochondrial dysfunction, the phenomenon called mitochondrial lipotoxicity [54, 55].

On the other hand, the main outcome of UCP2 activity in the pancreatic β cell is an impairment of glucose-stimulated insulin secretion, which depends on ATP-dependent process, while uncoupling lowers ATP production during glucose metabolism. UCP2 has been found to have an enormous potential in metabolic processes. It is expressed ubiquitously and it is more difficult to control its function. This uncoupling protein is upregulated by cytokines.

Thus, different purported roles of the UCPs have implications for the development of the metabolic syndrome. In addition, PGC1, originally linked to adaptive thermogenesis, is an important determinant of the oxidative capacity of tissues such as skeletal muscle and white adipose tissue.

2.4. Mitochondria as selective intracellular targets

The mitochondria are indirectly selective victims of excessive lipid storage in the adipocyte and in peripheral fat-storing tissues. The impaired functioning and the structural damage of these organelles run in parallel, and have undesirable reciprocal effects [56]. Damaging factors related to insulin-resistance and chronic inflammation such as hypoxia, oxidative stress and mechanical stress due to hypertrophy, cumulatively result in organelle dysfunction, particularly in mitochondria [57].

Mitochondria are organelles controlling the life and death of the cell. They participate in key metabolic reactions, synthesize ATP, and regulate a number of signaling cascades [165].

Mitochondria are the most important organelle of brown adipose tissue, and they have a central role in energy metabolism. They are the main cellular sites of ATP generation through the process of oxidative phosphorylation. Mitochondria are also a site of steroid biosynthesis, participate in regulation of cellular calcium homeostasis and are involved in the production of free radicals [59]. It is

essential for the adipose tissue to have well-functioning mitochondria, as many evidences show a relation between reduced mitochondrial function and altered biogenesis in the etiology of obesity, insulin resistance and type 2 diabetes mellitus [13].

The mitochondrial function and the respiratory chain have an important role in those studies. The respiratory chain consists of five distinct complexes that are bound to the mitochondrial inner membrane. During the passage of electrons, a proton translocation occurs at complex I, III and IV. The general mechanistic principle of oxidative phosphorylation explaining the coupling between mitochondrial respiration and ATP synthesis is referred to as the chemiosmotic theory [58].

It is now well recognized that several mammalian mitochondrial electron-transporting enzymes, components of the inner mitochondrial membrane and their functionality can be altered in the metabolic syndrome. Therefore, a better characterization of mitochondrial parameters is essential for understanding the relationship between mitochondrial dysfunction and disease development [59, 60]. Since each tissue has unique characteristics, researchers found it useful to compare mitochondrial parameters in all tissues involved in the metabolic syndrome.

Investigations of mitochondrial oxidative phosphorylation have been mainly carried out in isolated mitochondria, where the experimental conditions can be precisely set [61]. There are several parameters that can provide information about the disease extent. Since it has been shown that complex III (ubiquinol:cytochrome c oxidoreductase) and complex I (NADH:ubiquinone oxidoreductase) are the main respiratory chain components where ROS are produced [62], these complexes have been studied by many researchers[62, 166, 167]. In addition to these assays, the calcium retention or the expression of mitochondrial proteins and transcriptional factors allowed us to understand the relationship between the mitochondrial damage and the metabolic syndrome.

3. Chronic inflammation in the metabolic syndrome

Inflammation is a physiological response of the organism to harmful stimuli. The response usually conducts to reestablish homeostasis, and to coordinate the action of many cell types and mediators, whose intervention depends on the nature of the initial stimulus and resulting responses thereafter [63]. The inflammatory state that accompanies the metabolic syndrome shows a quite peculiar presentation, as no massive tissue injury seems to take place. Furthermore, the inflammatory activity is not so large and it is often called "low-grade" chronic inflammation. The *Hotamisligil* group [64] has attempted to name this inflammatory state as "metaflammation", meaning metabolically triggered inflammation.

The adipose tissue has the ability to secrete factors such as adipokines, leptin, tumoral necrosis factor (TNFα), interleukin-6 (IL-6), plasminogen activator inhibitor (PAI-1), adiponectin, resistin and retinol binding protein (RBP4). Most of these factors are related to inflammatory state and

metabolic processes associated with many risk factors involved in the pathogenesis of the metabolic syndrome (type 2 diabetes, obesity, hypertension, atherosclerosis and fatty liver), and could be the link between adiposity and its complications, justifying the chronically elevated inflammation in metabolic syndrome. In addition to the changes in concentrations of adipokines in the chronic inflammatory process, also the concentrations of other inflammatory markers such as C-reactive protein and markers of endothelial dysfunction as the selectins and adhesion molecules, all are augmented to relevant levels to increase the risk of cardiovascular disease [15].

The metabolic syndrome inflammation is a noninfectious, chronic, systemic and low-grade inflammation. It is also defined by a vasodilatation, vascular permeability, the presence of inflammatory cells, and a manifestation of an increased oxidative stress due to the insulin resistance and endothelial dysfunction that occurred in the metabolic syndrome [65]. The exact mechanisms for releasing these factors remain unclear, but in the last decade a new concept has been reached, which indicates that an expanded fat mass leads to inflammation. Several theories have been proposed to link adiposity with inflammation, and the hypothesis includes adipocyte necrosis which triggers the recruitment of macrophages [66], endoplasmic reticulum stress leading to an insulin resistance and a ROS production, expanded fat mass with higher levels of proinflammatory cytokines [67] and free fatty acids-mediated activation of Toll-like receptor (TLR)[68]. The recruitment and activation of macrophages promoted by CD8 T-cells found by Nishimura *et al*. [69] may support the fact that CD8 T-cells play an essential role in the initiation and propagation of adipose inflammation.

Inflammatory pathways are upregulated in obese adipose tissue, leading to increased expression of downstream cytokines, such as TNF-α, some interleukins (IL-6, IL-8), PAI-1, tissue factor, monocyte chemoattractant protein (MCP-1), and C-reactive protein (CRP) representing an extremely relevant group in adipobiology [70]. Recently, the cytokine interleukin-1β (IL-1β) has also emerged as a prominent instigator of the proinflammatory response in obesity [71]. Since inhibition of chronic inflammation is a critical component in the strategies for prevention and treatment of metabolic syndrome, a greater knowledge of these pathways must be desirable to understand the disease and, therefore, to clarify the metabolic syndrome pathophysiology.

The inflammatory response is commonly triggered as a consequence of endoplasmic reticulum (ER) stress. The continued cell damage leads to a progressive destruction in which the immune system initiates ineffective attempts to repair this damage. This situation is characteristic of chronic inflammation [72]. In this sense, the role of the nucleotic-binding and oligomerization domain, NOD-like receptor (NLR) family in the inflammatory response is becoming clear [73]. Some NLR family members, termed inflammasomes, serve as platforms for caspase-1 activation and subsequent proteolytic maturation of the IL-1β and IL-18. These cytokines are synthesized as latent cytosolic precursors that require caspase-1 processing secretion [74] (Figure 5).

This NLRP3 inflammasome is a cytosolic protein complex, and among various NLR family members, the NLRP3 inflammasome has drawn the most attention, due to its association with numerous inflammatory diseases. It can sense the presence of endogenous danger signals that are associated with cellular damage or stress. Recent studies suggest that the NLRP3 inflammasome responds to ER stress, thus providing mechanistic insight to the link between ER stress and chronic inflammatory diseases [75], by using different factors that are capable to induce the ER stress and activate the NLRP3 inflammasome.

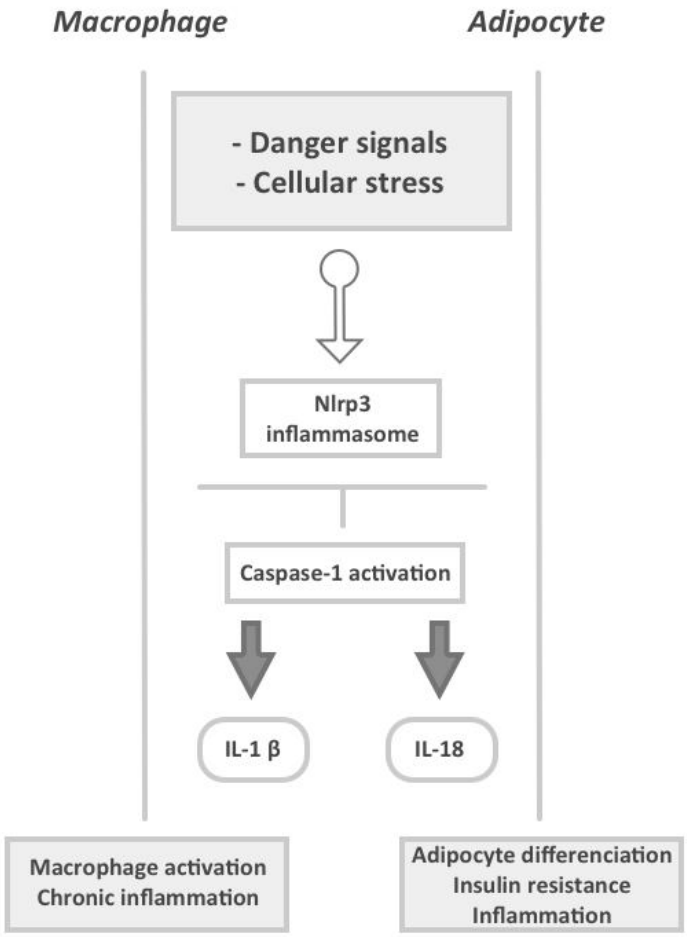


Figure 5: Consequences of Nlrp3 inflammasome activation in macrophage and adipocytes [74].

The activation of the NLRP3 inflammasome may be triggered by a wide variety of signals such as components of necrotic cells or damaged tissues, or noxious exogenous substances. The mitochondria have a central role, monitoring the activity of the mitochondrion and acting as an integrator of danger signals. This mechanism results in an elevated production of ROS, which enhances the activation of NLRP3 inflammasome. Consequently, mitochondrial dysfunction drives NLRP3 inflammasome activation, and establishes a link between mitochondria and chronic inflammation [73]. According to these findings, IL-1β has a pathogenic role in the development of

obesity and type 2 diabetes, working on adipose tissue and pancreatic islets, activation of this inflammasome results in inflammation and insulin resistance [74].

Although clinical evidence is currently lacking, inhibition of this pathway may represent an alternative therapeutic target to limit pathological complications associated with obesity and type 2 diabetes [22]. Considering that the dynamics of ER and mitochondria interaction has a central role in NLRP3 inflammasome activation [75], targeting ER stress and mitochondria may represent an enormous therapeutic goal to combat chronic inflammation.

3.1. Adipose tissue inflammation

Recent studies showed that adipose tissue plays a major role in production of cytokines like IL-1, IL-6 and TNF-α**,** which stimulates the production of C-reactive protein from the liver and this is the origin of metabolic syndrome [76]. TNF-α stimulates cellular kinase complex known as I Kappa B Kinase (IKK), which activates nuclear factor (NF)-κβ, a transcription factor that, in turn, drives the production of proinflammatory cytokines.

Many important findings in obesity-associated inflammation are made in epididymal fat, including identification of TNF-α expression and macrophage infiltration in adipose tissue [77]. The excessive gain of adipose tissue causes a dysfunction at many levels, and cellular inflammation is emerging as a central mediator of this dysfunction [64]. The metabolic overload causes an impact on the adipose tissue, leading to an organelle stress. This metabolic stress to which adipose tissue is subjected in obesity results in organelle dysfunction, particularly in mitochondria and the endoplasmic reticulum, contributing to inflammation and related pathological outcome. In addition, problems of hyperplasic adipocytes to achieve homeostasis represent another factor that promotes adipocyte hypertrophy and inflammatory response. These adipocytes attract and activate macrophages due to the rupture caused of the hypertrophic ones. The ROS production, the activation of kinases that potentiate the transcription of inflammatory genes and the inflammatory cytokines production contribute to the metabolic dysregulation [78] (Figure 6).

Besides these processes, systemic blood circulation provides fuels for proper WAT function as well as transport capabilities for adipokines. Recently, great attention has been focused on adipose circulation and its potential interplay with the micro vascular endothelium. In general, visceral fats have less blood flow than the subcutaneous fats, and this difference may be responsible for the high level of inflammation and lipolysis in the visceral fat since the lack of blood flow can induce hypoxic response and chronic inflammation [79]. Also blood supply could be the key for the higher expression of inflammatory cytokines and macrophage infiltration seen at visceral fat depots relative to the subcutaneous fat.

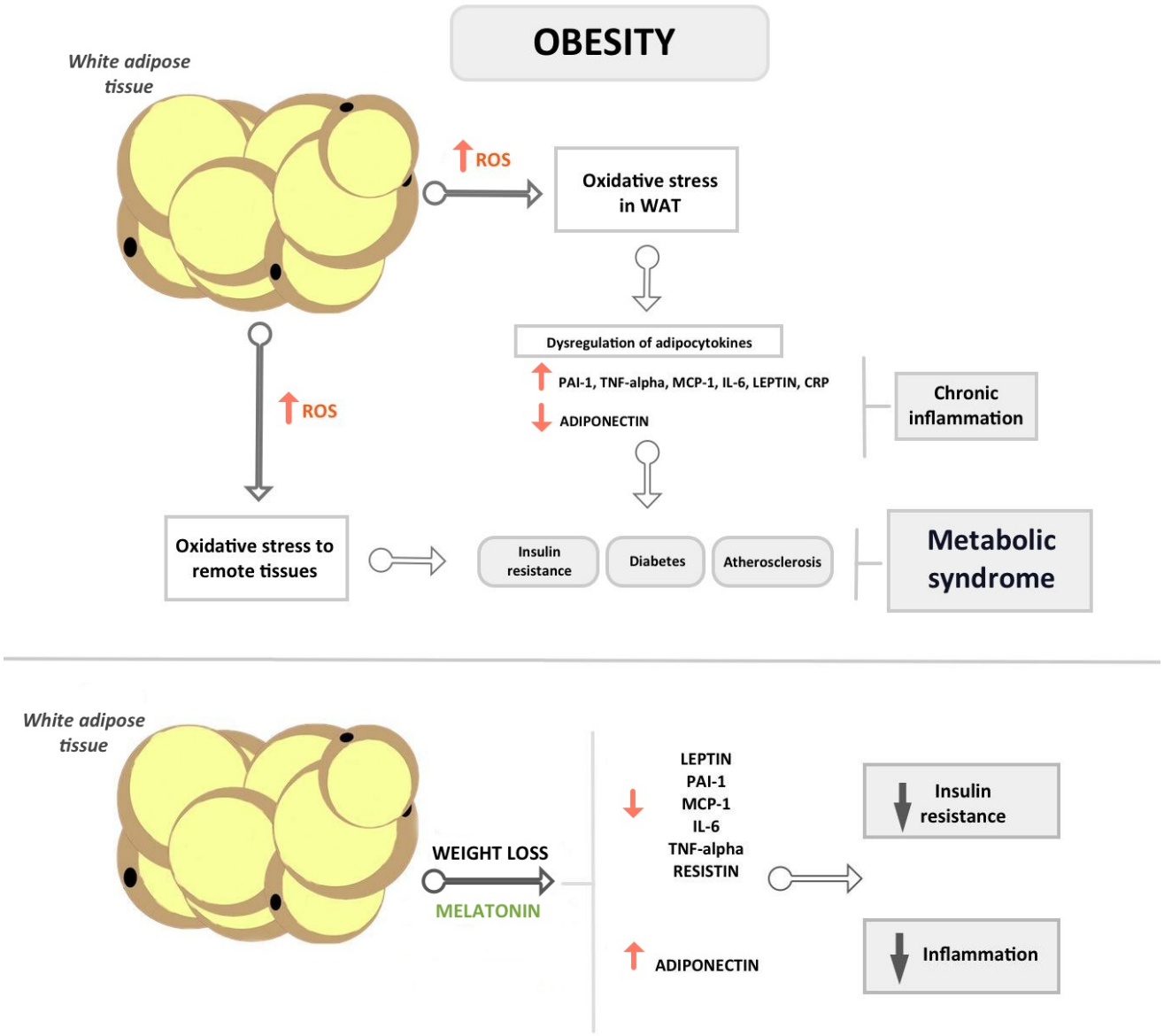


Figure 6: In obesity, adipose tissue is exposed to stress, which triggers the inflammatory response. The impairment could be counteracted by weight loss (or melatonin [122]).

Some investigators have suggested that adipose tissue inflammation may constitute an important mechanism of insulin resistance, establishing a link between increased adipokine expressions, macrophage infiltration in adipose tissue and insulin resistance [80, 81]. Recent data support the proposal that obesity increases cardiovascular risk through its effects on insulin resistance and endothelial function. Adipokines appear to serve as the cellular link mediating both the metabolic syndrome of insulin resistance and the endothelial dysfunction present in the obese state. Adipokine levels may correlate closely with adiposity, with increasing levels in subjects with higher body mass index (BMI) values [82].

In addition to adipose tissue, recent studies suggest that skeletal muscle may also be a source of low-grade inflammation, particularly in inactive and overweight individuals [83]. Thus, the study of other tissues may provide more information to clarify the pathophysiology associated with the chronic inflammation, and therefore with the metabolic syndrome.

3.2. Main adipokines in chronic inflammation

The proinflammatory factors are produced or regulated by adipose tissue, which acts as an endocrine and secretory organ. Several findings have shown that metabolic dysfunction may partly result from an imbalance in the expression of pro- and anti-inflammatory adipokines, thereby contributing to the development of obesity-linked complications. Accordingly, the concept that adipokines function as regulators of body homeostasis has received widespread attention from the research community [84].

The number of identified cytokines secreted by adipocytes has continued to grow over the years, and these discovered factors appear to modulate both metabolic and immune pathways [7]. Many of them promote vascular damage and the development of endothelial dysfunction. However, adipose tissue also produces adipokines that exerts protective effects on vascular function.

Figure 7 shows the most representative adipokines for the metabolic syndrome studies and its functions.

ADIPOKINES	Primary source	Function	References
Leptin	Adipocytes	↓Appetite ↑Insulin sensitivity ↑Fatty acid oxidation	*15, 23, 87*
Resistin	Peripheral blood mononuclear cells, adipocytes	Promotes insulin resistance Inflammation	*15, 84*
TNFα	Stromal vascular fraction cells, adipocytes	↓GLUT-4 receptor ↑Lipolysis ↓Insulin sensitivity	*88-90*
IL-6	Adipocytes, stromal vascular fraction cells, liver, muscle	↑Energy expenditure ↓Insulin sensitivity ↓Appetite	*88, 91*
Adiponectin	Adipocytes	Anti-inflammatory ↑Insulin sensitivity ↑Fatty acid oxidation	*15, 92*
CRP	Liver	↑ PAI-1 expression and activity in endothelial cells	*76, 82, 93*

Figure 7: Sources and functions of main adipokines. Adapted from Ouchi et. al[84]

4. Free radicals and stress

Free radicals can be defined as highly reactive and unstable molecules containing one or more unpaired electrons in atomic or molecular orbitals [96]. They become stable by obtaining electrons from biological nearby non-radical macromolecules, mainly nucleic acids, proteins, carbohydrates and lipids [85]. This electron stealing causes a cascade of chain reactions resulting in cell injury, inhibiting their normal function, triggering apoptosis and necrosis [168, 169]. The free radicals generated as a result of aerobic metabolism can be formed in multiple cell compartments, being the mitochondria the main former of these species [86].

There are many types of free radicals in living systems, but we focus here on the oxygen and nitrogen radicals. In healthy individuals, reactive oxygen species (ROS), reactive nitrogen species (RNS), and antioxidants coexist in a prooxidant-antioxidant delicate balance that leads to redox homeostasis [96].

4.1. Oxidative stress

Oxidative stress can be defined as an imbalance between antioxidant and pro-oxidant species, potentially triggering cellular damage [57].

Most of the O_2 taken up by mammalian cells is processed in the mitochondria and reduced to water via mitochondrial complex IV. Since the effectiveness of the electrons flow through the electron transport chain (ETC) is not complete, it can generate partially reduced oxygen molecules, forming ROS [94]. The most toxic oxygen-derived metabolites are the superoxide anion (O^{2-}), hydrogen peroxide (H^2O^2) and hydroxyl radical (OH^-) [75]. The superoxide radical can react with other compounds such as nitric oxide (NO) resulting in the peroxynitrite anion ($ONOO^-$), nitrogen highly reactive species which can cause irreversible inhibition of mitochondrial respiration, induced mitochondrial swelling and therefore opening permeability transition pore (PTP), and finally apoptosis [83]. The hydroxyl radical is one of the most cytotoxic ROS. This radical is capable of capturing a hydrogen atom from the unsaturated, particularly abundant in the cell membrane, generating a loss of the biological function of the cell membrane fatty acids. This process is known as lipid peroxidation (LPO).

However, ROS are continuously formed in the cell and they are indispensable for many physiological processes [95]. There is a difference between oxidative stress and oxidative damage; the first is defined as the disturbance of the balance that should exist between free radicals and antioxidant molecules, and the second refers to the failure of antioxidant systems to repair a damage caused by ROS, either by a deficiency in antioxidant levels or exacerbated by an increase in free radicals [96].

To counteract the oxidative activities, we have the antioxidants, defined as any molecule able to delay or entirely prevent the oxidation of a given substrate. There are some endogenous antioxidants, those that are synthesized by the body itself: the enzyme superoxide dismutase (SOD), responsible for eliminating the radical O^{2-} [97], the catalase (CAT) and the glutathione peroxidase (GPx).

Under normal physiological conditions, the pro-oxidant - antioxidant balance allows the crucial maintenance of the physiological functions and protects against cell damage. This balance can be affected by exposure to many physiological, pathological or pharmacological agents [98].

4.2. Nitrosative stress

Reactive nitrogen species are derived from nitric oxide (NO), synthesized by a set of three nitric oxide synthases (NOS) that catalyze the oxygen and NADPH-dependent oxidation of L-arginine to L-citrulline and NO [178].

NO is a diffusible free radical signaling molecule, mediator of many physiological processes including vasodilation, inflammation, thrombosis, immunity and neurotransmission [177]. Moreover, is a physiological regulator of diverse functions in several tissues including cardiovascular, neuromuscular, neurological, gastrointestinal and renal [15]. Inhibitors of nitric oxide synthase reduce NO production and prevent the decrease in insulin secretion caused by free fatty acids [170].

NO itself possesses limited toxicity, but may reversibly suppress mitochondrial respiration and ATP synthesis if produced in excess. The majority of the pathological consequences of nitrosative stress are mediated through the reaction of NO with metals or the intermediate formation off RNS, which impart many types of modifications in proteins and other biologically active molecules [179]. Furthermore, by uncontrolled or excessive NO production, nitrosative stress may develop, and combined with oxidative stress can result in peroxinitrite production, a potent oxidant that can cause DNA fragmentation and lipid peroxidation [177].

According to these alterations, several organs and tissues with high-energy dependence may then be damaged, and the result could be an overall inflammatory situation, hyperinsulinemia and obesity.

4.3. Mitochondrial dysfunction and ROS/RNS in the metabolic syndrome

Mitochondrial dysfunction is a common feature of most of the diseases related to ROS/RNS excess. Up to five percent of all oxygen molecules entering the mitochondrial ETC can acquire an electron to form the superoxide free radical, which can be converted to various powerful ROS/RNS. Once formed, these ROS/RNS react indiscriminately with proteins, membrane lipids, nucleic acids

or DNA, causing, among other things, mitochondrial dysfunction. This will cause the production of yet more ROS/RNS, leading to a self-sustaining vicious cycle [99].

Biochemical analysis of the presence of mitochondrial alterations have revealed the existence of ETC defects, impairment of ATP production, oxidative stress, and high sensitivity to PTP in different degree [86].

In metabolic syndrome and associated diseases, oxidants are overproduced [100]. Fatty acids are particularly sensitive to ROS/RNS oxidation. It has been demonstrated that excess free fatty acids induce nitrosative damage in pancreatic islets and promote a dysfunction associated with type 2 diabetes [96]. Increasing oxidative stress in adipose tissue causes dysregulated production of adipocytokines, and also leads to increased oxidative stress in blood, affecting different organs. It has been proposed that increased ROS/RNS in accumulated fat is an early initiator and one of the important underlying causes of obesity. Hence, the redox state in adipose tissue is a potentially useful target in new therapies against metabolic syndrome [101].

Inhuman pathologies, the main factor triggering an overproduction of mitochondrial ROS is the so-called Warburg effect: when there is enough glucose and/or the cell is proliferating, the glycolytic pathway is rather used to generate ATP, inhibiting the pyruvate entry into the mitochondria [173]. Numerous studies have shown that in patients with metabolic dysfunction or diabetes, the body is subjected globally to the Warburg effect, and a general increase in glycolytic metabolism and suppression of mitochondrial oxidative activity is promoted [174].

It has also been observed that this situation is associated with increased mitochondrial ROS production and the onset of oxidative stress situations. Hence, excessive production of ROS, associated with metabolic dysfunction is a consequence of detoxification mechanisms and protection against ROS, that are suppressed when mitochondrial metabolic activity is inhibited, being PGC-1α responsible for the co-regulation of mitochondrial activity and protection systems against ROS [175, 176].

All these facts indicate that mitochondrial reactive species are involved in both the pathogenesis and long-term complications of diabetes. In individuals with type 2 diabetes, the high nutrient flux and consequent ROS production appear to mediate loss of β-cell function. In insulin-sensitive tissues, including liver, muscle and heart, high fatty acid flux leads to oxidative damage. At the same time, non-insulin-sensitive tissues, including the eye, kidney, nervous system and vasculature, are exposed to both high circulating glucose and fatty acid levels and, consequently, ROS-induced diabetic complications [172].

Both inflammation and oxidative stress are closely linked via positive feedback mechanisms and occur at local systemic levels, but mainly in visceral adipose tissue, liver, muscle pancreatic β-cell and the arterial wall. Inflammation is accompanied by an excessive release of TNF-α, IL-6 and free fatty acids, and contribute to hyperglycemia and dyslipidemia.

Finally, endoplasmic reticulum stress and ROS/RNS also contribute to the systemic damage, leading to a vicious circle, which promotes blood hypertension (Figure 8). However, according to our studies [108, 109, 122] this situation could be reverted.

The combination of oxidative and ER stress, together with autophagy insufficiency and inflammation, may contribute to β-cell death or dysfunction in type 2 diabetes [171]. Hence, elucidating the cellular mechanisms of stress, inflammation and cell death has contributed toward our understanding of these processes, which may lead to new therapeutic agents for treating type 2 diabetes.

In conclusion, to ameliorate or prevent these obesity-related metabolic and cardiovascular complications, pharmacological treatment should aim to modulate this pro-inflammatory and pro-oxidant scenario.

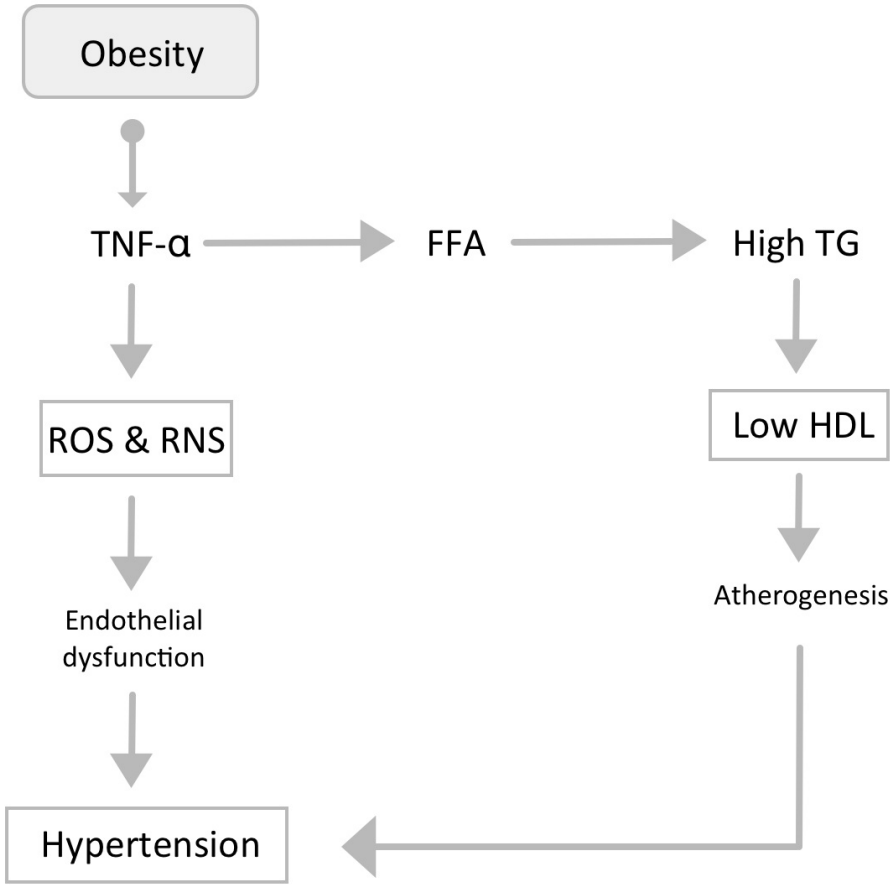


Figure 8: Features involved in the impairment of metabolic syndrome. Adapted from Gutiérrez-Salmeán et al [180].

5. Therapeutic approach

To understand the pathogenesis of metabolic syndrome and the damage of the adipose tissue, the therapeutic strategies and the diagnosis should be based on avoidance of mitochondrial toxins and protecting mitochondria from further damage, as well as avoiding chronic low-grade inflammation.

Metabolic syndrome is a disorder strongly associated with obesity and a sedentary lifestyle. Excess body weight is the most modifiable risk factor of all that involve this syndrome. Hence, the weight reduction is a key target to treat the metabolic abnormalities, but a healthy diet and increased physical activity are not enough. Besides the control of obesity, the therapy must control the insulin resistance and all the individual components of the metabolic syndrome [42].

Weight loss induced by currently available anti-obesity drugs is only modest [102]. Whilst it is attractive to consider pharmacological therapy in order to control these disorders, the generation of effective and safely new treatments is the main target in therapies to reduce the metabolic syndrome risk factors. Current pharmacological treatments include metformin, sibutramine, orlistat and thiazolidinedione [103]. To date, many researchers are focusing on the study of melatonin and its clinical approach for treatment of various human diseases.

Some recent data suggest the positive effects of melatonin as an antioxidant and antiinflammatory therapy in neonates, cancer, neurodegenerative diseases, ageing, and others [104, 105].

In conclusion, in obesity, changes in brown-fat-like activity contribute to the overall impairment of thermogenesis, while its activity was reduced in obese and elder subjects [106]. A better understanding of adipogenesis and a detailed knowledge of different adipose tissue depots may provide new therapeutic strategies. Moreover, the possibility to direct adipogenesis using cold, catecholamines, melatonin or maybe irisin, is another subject needs further consideration.

5.1. Protecting role of melatonin

Nutritional and pharmacological strategies for the prevention and treatment of the metabolic syndrome aimed at potentiating fat oxidation and thermogenesis.

Over the past years, there has been increasing scientific interest in the so-called antioxidant hypothesis. Tryptophan and serotonin, which are melatonin precursors, are present in many organisms at the early stages of cell phylogeny, suggesting the presence of melatonin [107]. These metabolites are antioxidants, and several experiments indicate that melatonin may be a potential key to combat the physiopathological consequences of metabolic diseases [108, 109].

Melatonin is a hormone that is a metabolite of the neurotransmitter serotonin (5-hydroxytryptamine, or 5-HT), which in turn has been converted from the amino acid tryptophan. It occurs mainly in the pineal gland, and participates in a variety of cellular processes,

neuroendocrine and neurophysiological. Melatonin has a short half-life of less than an hour and remains in the bloodstream for only 20-90 minutes [43], due to its high lipophilicity and partial hydrophilicity, which allow the molecule to cross cell membranes readily and distribute extensively in the body, easily reaching subcellular compartments including mitochondria, where it seems to accumulate in high concentrations [110, 115].

One of the most striking features about the pineal biosynthesis of melatonin is its variability along the 24-hour cycle, and precise response to changes in ambient lighting. Melatonin is produced under the influence of the suprachiasmatic nucleus of the hypothalamus, which receives information from the retina about the daily patterns of light and darkness. Due to this role, melatonin is an endogenous substance involved in the regulation of biorhythms, according to which the body organizes biochemical strategies designed to maintain the correct balance of their biological and psychological functions, in relation to rhythmic variations in the surrounding environment. It is secreted by the pineal gland during the dark photoperiod, as a biochemical messenger announcing the night, prepares mental and physical functions for night period [104]. Melatonin is involved in sleep/wakefulness, in the rhythms of cell division and regulating the immune system. Exogenous administration are used in the correction of sleep/wakefulness, insomnia characterized by difficulty in falling asleep, but also as a natural antioxidant and free radical scavenger, immunostimulant and has antiobese effect [13, 20, 108, 109] and other actions that enhance the efficacy and reduce the toxicity of many drugs. The uses of melatonin are varied, and there is much potential for further clinical and diagnostic use of the agent [44]. The most relevant actions related with the melatonin activity are showed in Figure 9 [111].

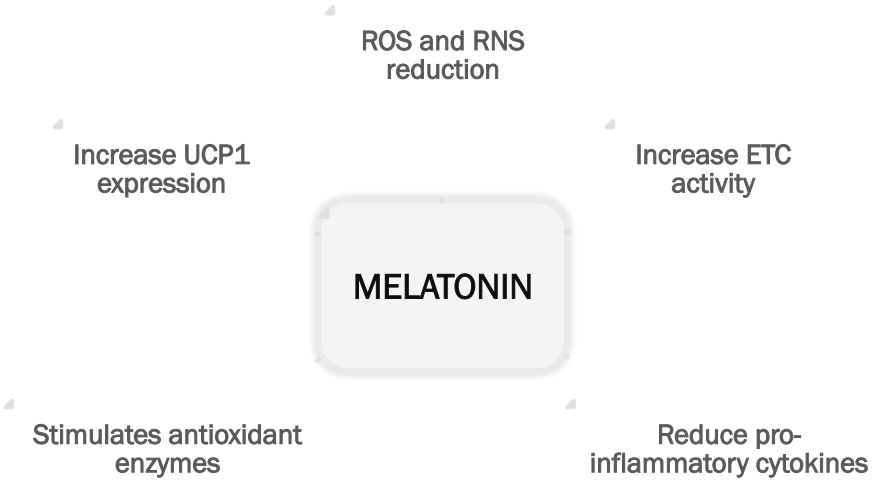


Figure 9: Melatonin actions include scavenging oxygen and nitrogen free radicals, stimulation of antioxidative enzymes, and beneficial actions at the mitochondrial level [111].

5.2. Melatonin actions on mitochondria

Many researchers have shown [104, 108, 109], melatonin modulates or attenuates several of the undesired processes associated with metabolic disorders. The designation of the mitochondria as a powerhouse of disease [112] is related with its increasingly recognized role in multiple disorders. The involvement of mitochondria in many dysfunctions exceeds by far their participation in apoptosis. The impairment seen at mitochondrion frequently starts with partial blockades of electron flux through the electron transport chain, leading to electron leakage. At this point, melatonin has been found to reduce the mitochondrial production of ROS and RNS, protecting against oxidative stress [113, 114, 115]. Besides its damage to several essential cell constituents, the ROS overload activates a cascade of stress-sensitive pathways, hindering the insulin-receptor signaling and the insulin action, leading to a possible inhibition of insulin secretion [116]. A melatonin treatment may diminish these alterations, since some studies have shown an antidiabetic effect accompanied by other concerted metabolic benefits [108, 109, 117].

In conclusion, melatonin has two effects on mitochondria. As an antioxidant, melatonin can directly scavenge ROS produced during the normal metabolism of mitochondria and indirectly promote the activity of the antioxidant enzymes. On the other hand, melatonin increases the activities and the expression of complexes I and IV of the electron transport chain, restoring their activities and promoting an increased ATP production [118]. Related to its brownish effect at brown adipose tissue, the role of melatonin is its ability to increase thermogenesis [119]. As previously mentioned, the mitochondrial protein UCP1 is responsible for this mechanism, and the improvement of this process could be due to the action of melatonin at mitochondrial level, both activating the UCP1 expression and restoring the damaged mitochondria hindered during the metabolic disorders.

It is difficult to predict if all these findings could be extrapolated to the human condition. However, if the mitochondrial dysfunction observed in rat models also occurs under human conditions, this may explain part of the mechanisms of metabolic syndrome and possibly the dysfunction of patients. It would be of interest to investigate the complete mechanism by which melatonin improves these metabolic disorders, as well as its role restoring the mitochondrial function. Further studies are needed to identify the mechanisms by which melatonin increases the activity of mitochondrial elements such as UCP, and applied that to adipose tissue.

5.3. Melatonin and inflammation

The pharmacological strategies to confront the chronic inflammation must maintain the physiological inflammatory response, since many synthetic anti-inflammatory agents generate considerable side effects [120]. From this point of view, current researches are focusing on the use of

endogenous molecules as possible supplements for this response. According to this, melatonin is an attractive option as it may act as a regulator of the inflammatory cell compartment, with a potent antioxidant potential able to reduce the oxidative environment of chronic inflammation and to regulate leukocyte function and number [121]. As recently shown for the first time, chronic oral melatonin administration is able to improve circulating biomarkers of systemic low-grade inflammation in young ZDF rats [122]. Moreover, several in vitro studies indicate that melatonin may have a potential use as a pharmaceutical agent [123-129].

Oxidative stress plays an important role at the initial phase of inflammation, activating signaling pathways and leading to different actions, such as release of the inflammatory mediators and regulation of leukocyte recruitment and maturation [130]. Chronic inflammation is also characterized by chronic ROS production; therefore an antioxidant action is desirable in anti-inflammatory therapies, to reduce the burden of inflammatory mediators at the inflammatory site [120]. For this reason, melatonin plays a protective role, due to its antioxidant power (scavenging free radicals) and its ability in activating antioxidant defenses (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase [126]). In order to know how the melatonin exerts this action, in a recently published study performed by our research group we studied the antioxidant activity of melatonin in diabetes in relation to the regulation and levels of plasma Cu, Zn, Fe, Mn and Se in Zucker diabetic fatty (ZDF) and lean (ZL) rats [133]. In this study, we found a relationship between their plasma concentrations and the increased antioxidant enzymes (SOD and CAT) induced by melatonin treatment [134, 135-141]. Future studies would be necessary to discover the exact mechanism through which this indolamine regulates plasma levels of antioxidant minerals like by measuring their tissue levels, and therefore those present in BAT and WAT in inflammation processes characteristic of metabolic syndrome before and after melatonin administration. Probably, these future studies would help to better understanding of how after melatonin supplementation in animal and human studies the enhancement of antioxidant enzymes (SOD, GSH-Px, catalase) is established [142-148].

On the other hand, the important concept in the inflammation involves the differential pro- and antiinflammatory roles of melatonin, regulating and counteracting inflammation simultaneously. Since melatonin could reduce oxidative tissue injuries, facilitates the promotion of inflammatory response and stimulates pro-inflammatory mediators such as arachidonic acid and 5-HETE [106], it could then avoid complications of chronic inflammation.

5.4. Melatonin as thermogenic tool

Alterations in nonshivering thermogenesis are presently discussed as being both potentially causative of and able to counteract obesity [19]. Nonshivering thermogenesis is fully due to brown

adipose tissue activity, and adaptation corresponds to the recruitment of the tissue. However, the role of beige adipose tissue should be kept in mind. Diet-induced thermogenesis is probably also due to brown-like activity.

Mounting evidence suggests that melatonin might increase energy expenditure by activating nonshivering thermogenesis in rodent BAT. Since Heldmaier and Hoffman firstly reported that melatonin stimulates growth of BAT in Djungarian hamsters [181], many subsequent studies confirm this observation in the same [182, 183] and other species of rodents [184-189]. In addition, several of these works demonstrated that melatonin also increases BAT activity.

To our knowledge the role of melatonin as a WAT browning inducer has not previously been explored. Our recent work demonstrates that oral melatonin administration may induce white fat browning [164] by multiple mechanisms. Firstly, by acting on MT1 receptors on the suprachiasmatic nucleus neurons, melatonin can activate sympathetic drive to inguinal fat and to other tissues, leading to noradrenaline release. Alternatively, melatonin may act directly on noradrenergic terminals where it releases the catecholamine. In addition, by acting through one or more of its receptors, i.e., either membrane G-protein-coupled receptors (MT1/MT2) or via nuclear retinoid orphan/retinoid Z receptors (ROR/RZR), the indoleamine may directly promote differentiation of precursors of beige adipocytes or trans-differentiate white adipocytes. Regardless of the origin of melatonin-induced new brown-like adipocytes in WAT, melatonin may also stimulate the thermogenic capacity of these cells by multiple mechanisms, as it is suggested from studies in BAT [20].

In conclusion, chronic melatonin treatment in rats behaves as a white fat browning inducer with thermogenic properties. This may be one of the mechanisms that underlies the anti-obesity effect of melatonin and thereby explains its metabolic benefits, i.e., anti-diabetic and lipid-lowering properties.

These observations together with its high pharmacological safety profile make melatonin a potentially useful tool as anti-obesity therapy.

As concluding remarks, we can summarize all the positive effects of melatonin regulating the imbalance associated with metabolic syndrome (Figure 10). Consequently, the good results obtained in recent years marks this hormone as a promising tool that should be taken into account in future research.

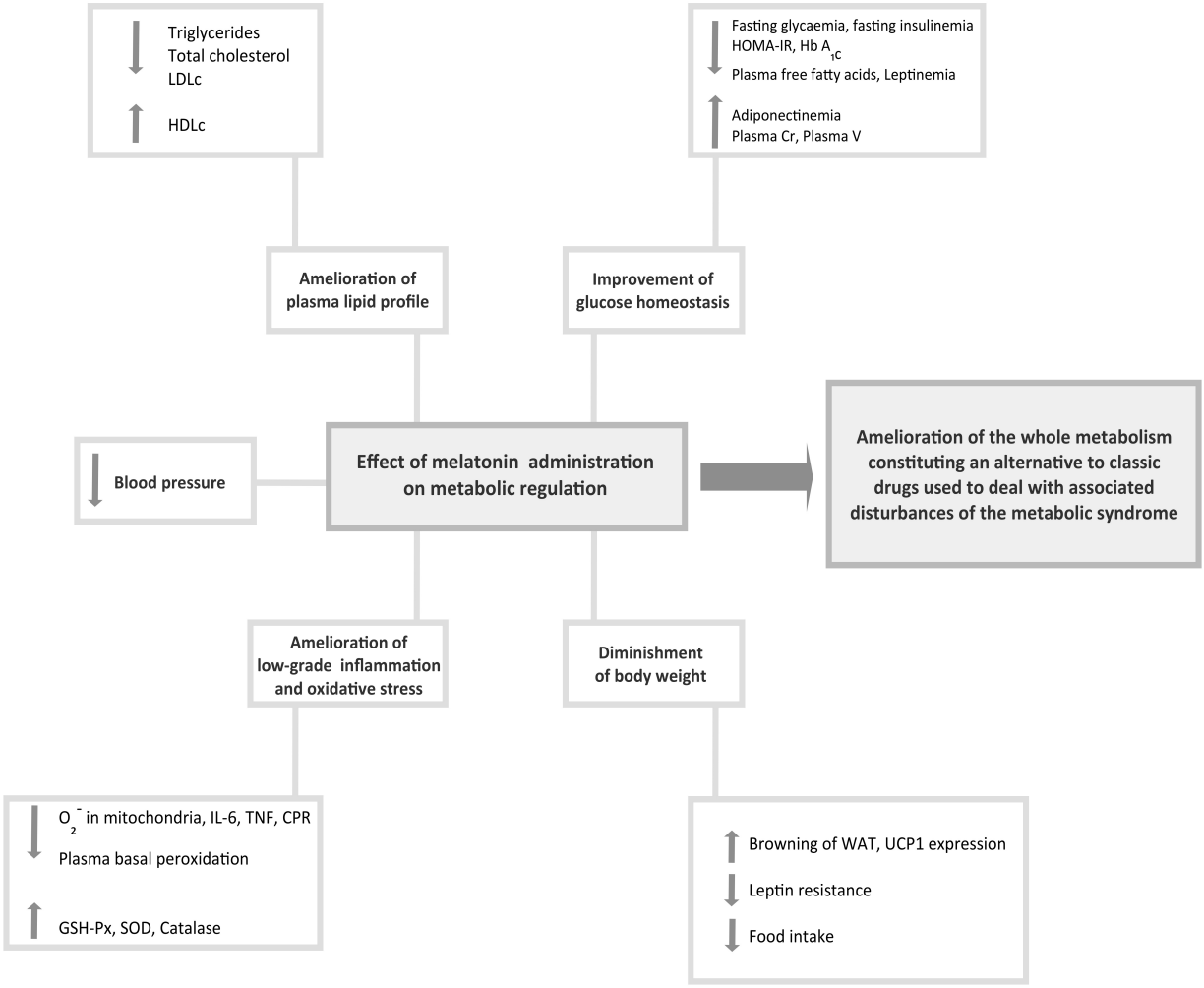


Figure 10: Effect of melatonin administration on metabolic regulation.

6. Future perspectives

First of all, we must consider the main conclusion of this study. As melatonin acts specifically to protect mitochondria electron leakage and failure of the respiratory chain, melatonin may be useful as adjunctive therapy in the metabolic syndrome to reduce insulin resistance, dyslipidemia and overweight in obese people. Therefore, any research that aims to extend the study of mitochondrial alterations in tissues affected by metabolic syndrome would provide more and better information in this area. We can include here the study of fatty liver, the apoptosis induction or structural analysis of mitochondria by electron microscopy (TEM).

Over the past decade, it has become clear that obesity is associated with cellular stress signaling (including mitochondrial and ER stress) and inflammatory pathways. ER is responsible for the synthesis, maturation, and trafficking of a wide range of proteins, facilitating the transport and

release of correctly folded proteins. The lumen of the ER contains molecular chaperones and folding enzymes including Grp78 (BiP), Grp94, protein disulfide isomerase (PDI), calnexin, and calreticulin. As the major site for proper protein folder, only correctly folded proteins are exported to the Golgi organelle, while incompletely folded proteins are retained in the ER [126]. If unfolded proteins appear in the lumen, a cellular adaptation program named the unfolded protein response (UPR) is triggered to increase the folding and degradation capacity of the ER [191]. The UPR is activated in obese, insulin-resistant tissues in experimental models, and is more prominent in the adipose tissue [190]. Several evidences indicate that ER stress is induced in obesity due to an augmented demand for protein synthesis under nutrient excess and by saturated free fatty acids, which activate UPR and causes ER stress [191].

Whether the UPR is the inductor of the inflammation or the contrary, it remains unclear. Furthermore, the induction may be dependent upon ROS [192]. Given all these assumptions, a better understanding of the metabolic syndrome may establish a relationship between the occurrence of altered functions and the development of disease.

Unfortunately, the melatonin action on endoplasmic reticulum remains unclear. There are not many studies about this relationship, but the effects of melatonin on inflammation suggest that its effect on the endoplasmic reticulum would be positive and would enhance its activity. A recent study [193] has proved a delay on the endoplasmic reticulum stress-induced apoptosis correlated with the melatonin supplementation. Therefore, the age-related decrease on the endogenous melatonin production is directly linked to oxidative stress, and thus the integrity of other cellular structures, as well as mitochondria, are involved in this process. Although these results were obtained in leukocytes, it would be interesting to analyze the effect of melatonin on both brown and white adipocytes endoplasmic reticulum.

In conclusion, the study of melatonin effects at chaperones levels and ER stress could be a great target to further understand the pathogenesis associated with the metabolic syndrome. We should also keep in mind that MT2 receptor is a G protein coupled receptor, which is known to regulate circadian and seasonal rhythms. Structural alterations provoked by mutations or genetic variations in the gene sequence of G protein-coupled receptors (GPCRs) may lead to abnormal function of the receptor molecule. Frequently, this leads to disease. While some mutations lead to changes in domains involved in agonist binding, receptor activation, or coupling to effectors, others may cause misfolding and lead to retention/degradation of the protein molecule by the quality control system of the cell. Therefore, the development of pharmacological chaperone that stabilize mutant proteins of the melatonin pathway or compounds that enhance their defective activity could also be a future strategy.

HYPOTHESIS AND OBJECTIVES

1. HYPOTHESIS

A dual pathogenesis (insulin resistance and impaired insulin secretion) characterizes obesity-related T2DM. Chronic low-grade inflammation and oxidative stress constitute pathogenic background substrates for obesity-related insulin resistance and development of T2DM and its micro- and macro- vascular complications. Both inflammation and oxidative stress are closely linked via positive feedback mechanisms and occur at local and systemic levels, including the adipose tissue.

Chronic oral melatonin administration in Zucker diabetic fatty rats has an antidiabesogenic effect by increasing the basal energy expenditure through different mechanisms, including the increased mitochondrial biogenesis and function (thermogenesis) of brown and beige adipose tissue. Moreover, the mitochondrial dysfunction seen at metabolic syndrome correlates with the chronic inflammation, connecting the imbalance with the adipose tissue.

Since adipose tissue has shown a main role in the metabolic disease development, and the difference between both white and brown adipocytes has become an important key for research, it is necessary to evaluate the variety of dysfunctions that contribute to the disease, where the mitochondrial impairment has a central role.

To date, few data have been published which examined melatonin's protective effect toward obesity-related metabolic complications. Therefore, a positive effect of melatonin on systemic low-grade inflammation and oxidative stress would be desirable.

Also, if chronic oral melatonin were able to influence adipose tissue function, it would contribute to improve obesity and diabetic-related metabolic derangements.

The melatonin effect at adipose tissues remains unclear, but its ability to increase thermogenesis indicate that melatonin and brown adipose tissue are closely related, and that melatonin could be a safe and effective way to increase thermogenesis. Thereby, as the beige adipose tissue has only been characterized molecularly and genetically but not bioenergetically, the browning studies would provide great novelty to this field.

2. OBJECTIVES

The main objective was to investigate the molecular mechanisms whereby melatonin improves obesity and diabetes in the Zucker diabetic fatty (ZDF) rat. The expected outcome of our study is a better understanding of melatonin's role in obesity and type 2 diabetes mellitus developments, and its

relation to human chronobiology. We further hope to provide new therapeutic perspectives for its treatment.

2.1. Specific aims

2.1.1. Study the melatonin effect on low-grade inflammation and oxidative stress in young Zucker diabetic fatty rats

2.1.2. Study the melatonin potential as browning inducer in white adipose tissue in Zucker diabetic fatty rats

2.1.3. Study the melatonin effect improving the mitochondrial function in inguinal white adipose tissue of Zucker diabetic fatty rats

METHODS

1. Sample size and experimental group

Male ZDF rats (fa/fa) and male lean littermates (ZL, fa/-) were obtained at the age of 5 weeks. Animals were maintained on a special diet Purina 5008 rat chow (protein 23%, fat 6.5%, carbohydrates 58.5%, fiber 4%, and ash 8%) and housed in a 12-h dark/light cycle controlled room, in a climate-controlled room at 28-30ºC and 30-40% relative humidity.

In the first week after arrival, the animals were acclimated to room conditions, and water intake was recorded. Food was placed in the grid-cage cover. The leftover food was calculated and added to subsequent feeds. Grid-cages were refilled, and food consumption was calculated three times weekly.

The sample size was 30 for low-grade inflammation studies and 8 for browning and mitochondrial determinations.

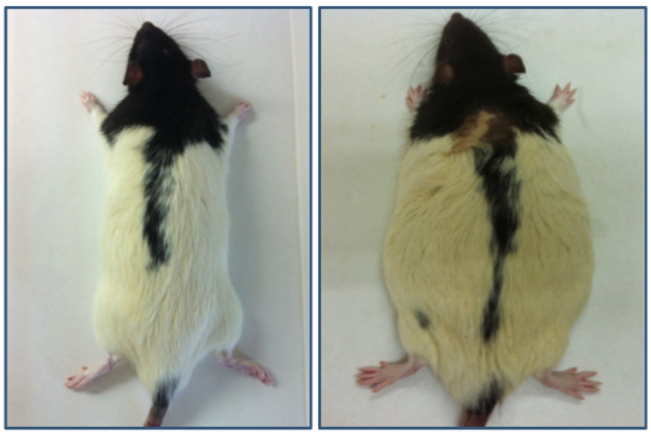


Figure 11: Zucker lean (left) and Zucker diabetic fatty rat (right).

2. Melatonin treatment

Both ZL and ZDF rats were subdivided into two groups: animals treated for 6 weeks with melatonin in drinking water (melatonin-treated, M-ZDF and M-ZL) and a control group (C-ZDF and C-ZL).

Melatonin was dissolved in a minimum volume of absolute ethanol and diluted in the drinking water to yield a dose of 10 mg/kg/day, with a final concentration of 0.066% (w/v) ethanol. Fresh melatonin was prepared twice a week, and the dose was adjusted to the body weight throughout the study period. Water bottles were covered with aluminum foil to protect from light.

3.1. Acute cold challenge and thermal images

Acute cold exposure was performed between 09-11 AM, for a 5-min, by placing the rat on a hot/cold plate analgesia meter pre-cooled to 4ºC (Panlab SLU, Barcelona) and located in a contiguous room at 20-24 ºC. This device is based on a 16.5 x 16.5 cm metal plate, which can be cooled to -3ºC or heated to 65 ºC. An electronic thermostat maintains the plate's temperature and a front panel digital thermometer displays the current plate temperature. The plate was surrounded by transparent plastic walls, which maintain inside temperature around 10 ºC.

Thermal images of inguinal regions were taken with a thermal imaging camera (FLIR B425, FLIR Systems AB, Danderyd, Sweden) with a range limit of -20ºC to 120ºC. Perpendicularly distanced (20 cm) photos were taken before (at thermoneutral temperature) and immediately after cold challenge.

3.2. Locomotor activity

The locomotor activity was evaluated in the open field by counting the squares crossed in 5 min with a four-paw criterion and total rears. The test was performed at night, 3 h after light off, in a four-sided 100x100x50 cm chamber, and the floor of the open field was divided into 25 squares (20 cm). Rats were placed in the middle of the open field, and each rat was given 5 min to explore. After this period, the number of rears and lines crossed were used as a measure of locomotor activity [242].

Figure 12: Open field test for locomotor activity.

3.3. Tissue collection

After the six-week treatment period, animals were anesthetized with sodium thiobarbital (thiopental) and killed. Inguinal subcutaneous fat pads, both white and beige areas were visually inspected and collected by surgical excision.

3.4. Mitochondrial studies

3.4.1. Mitochondrial isolation

Adipose tissue samples (~0.3 mg) were excised from white and beige regions of inguinal fat pad. Adipocyte mitochondria were isolated from these depots by serial centrifugation. Tissues were removed, excised, washed with cold saline, and homogenized in isolation medium (10 mm Tris, 250 mm sucrose, 0.5 mm Na_2EDTA, and 1 g/L free fatty acid BSA, pH 7.4, 4ºC) with a Teflon pestle.

The homogenate was centrifuged at 1,000 g for 10 min at 4ºC and the supernatant was centrifuged again at 15,000 g for 20 min at 4ºC. The resultant pellet was resuspended in 1 mL of isolation medium without BSA, and an aliquot was frozen for protein measurement. The remaining mitochondrial suspension was centrifuged at 15,000 g for 20 min at 4ºC and resuspended in 1 mL of respiration buffer (20 mm HEPES, 0.5 mm EGTA, 3 mm $MgCl_2$, 20 mm taurine, 10 mm KH_2PO_4, 200 mm sucrose, and 1 g/L fatty acid free BSA). The mitochondrial suspension was kept on ice for 10–15 min before starting the experiments to permit the rearrangement of the membranes. To prepare submitochondrial particles, mitochondrial pellets were frozen and thawed twice, sonicated, and suspended in the corresponding medium. The protein concentration was 0.2-0.4 mg protein/mL for each assay.

3.4.2. Mitochondrial respirometry

Oxygen consumption was measured with a high-resolution oxygraph (Oroboros Instruments), consisting in a two-chamber respirometer with a peltier thermostat and electromagnetic stirrers. Analysis of respiration was performed in 2 mL of respiration medium at 30ºC. The medium was previously equilibrated in each chamber with air at 30ºC and stirred at 750 rpm until a stable signal at air saturation was obtained. A final concentration of 0.2–0.3 mg/mL fresh proteins of mitochondria in the respiratory buffer was used for the experiments. Mitochondria were used to measure bioenergetic parameters. The mitochondria were suspended in incubation medium supplemented with glutamate (5mM)/malate (2.5mM) or with succinate (5mM) in the presence of

rotenone as energizing substrates, Jo2 was recorded at 30ºC in a constantly stirred oxygraph vessel after successive additions of 1mM ADP (state 3), 0.75 mM oligomycin (state 4), and 75 mM DNP (uncoupling). The respiratory control index (RCR) (the ratio of state 3 to state 4), and the ADP:O ratio (the ratio between the amount of ADP added to the respiratory medium and the oxygen consumed during state 3) were also calculated.

3.4.3. Mitochondrial enzyme activity

Citrate synthase activity was measured spectrophotometrically at 412 nm as the increased absorbance produced by the appearance of TNB since the rate-limiting reaction catalyzed by citrate synthase is coupled to this product. DTNB (0.2 mM), Triton X-100 (0.1 mM) and acetyl CoA (0.1 mM) diluted in Tris HCl (100 mM), were used. The assay was initiated by the addition of 20 mM oxalacetate.

Complex IV activity (nmol oxidized cytochrome c/min/mg prot) was determined in a medium containing 75 mM potassium-phosphate pH 7.4 at 25 ºC. The reaction was started by the addition of cytochrome c previously reduced with sodium borohydride [115]. The activity was measured as the disappearance of reduced cytochrome c at 550 nm.

3.4.4. Determination of mitochondrial nitrites

The method involves the use of the Griess diazotization reaction to spectrophotometrically detect nitrite formed by the spontaneous oxidation of NO under physiological condition. This determination was carried out after the mitochondrial isolation, using a Griess Reagent Kit (Molecular Probes, G-7921), as described in manufacturers instructions. Briefly, sulfanilic acid is quantitatively converted to a diazonium salt by reaction with nitrite in acid solution. The diazonium salt is then coupled to N-(1-naphthyl)ethylenediamine, forming an azo dye that can be spectrophotometrically quantitated based on its absorbance at 548 nm.

3.4.5. Mitochondrial activity of SOD

The Superoxide Dismutase (SOD, both Mn and Cu/Zn) activity was measured using a SOD Assay Kit-WST (Sigma-Aldrich, 19160, Switzerland), after the mitochondrial isolation, following the kit instruction. SOD Assay Kit-WST allows very convenient SOD assaying by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2Htetrazolium, monosodium salt) that produces a water-soluble formazan dye upon

reduction with a superoxide anion. The rate of the reduction with O2 are linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the IC50 (50% inhibition activity of SOD or SOD-like materials) can be determined by a colorimetric method. Since the absorbance at 440 nm is proportional to the amount of superoxide anion, the SOD activity as an inhibition activity can be quantified by measuring the decrease in the color development at 440 nm. Results were expressed as inhibition rate percentage (SOD activity).

3.4.6. PTP opening

Each well was filled with 0.5 mg/ml of mitochondrial solution, 10 µl of a respiratory substrate and 0.25 µM of the fluorochrome Oregon green (Invitrogen) in a final volume of 100 µl. Firstly, baseline fluorescence was measured without adding calcium, for a number of cycles. Then, 0.50 µM Cl_2Ca pulses were added, which will join the Green and produce fluorescence. Calcium is retained and accumulated in the mitochondria in the presence of ATP. Calcium overload in mitochondria causes PTP opening, leading to a large release of calcium that is quantitated with the fluorometer. 1 mM cyclosporine A (CsA) (Sigma-Aldrich) was also used as the reference mPTP inhibitor. Experimental groups were: control (mitochondria from white and beige fat depots of non-treated ZL and ZDF rats) challenged with calcium overload alone; melatonin (mitochondria from white and beige fat depots of melatonin-treated ZL and ZDF rats); CsA (mitochondria from white and beige fat depots of non-treated ZL and ZDF rats) challenged with calcium in the presence of cyclosporine A) and melatonin + CsA (mitochondria from white and beige fat depots of melatonin-treated ZL and ZDF rats) challenged with calcium in the presence of CsA as the inhibitor. Four replicates were made for each sample.

With each calcium addition, the mitochondrial PTP opened temporary to reduce the stress caused by the calcium excess, and closed later. When the calcium retention capacity was reached, the PTP opened permanently, inducing a mitochondrial swelling. Therefore, a well mitochondrial state ensures the temporary opening and a greater calcium retention capacity. The CsA addition avoids the PTP opening, so we use it as a control. As for the PTP opening, a greater level indicates a bigger opening, related with detrimental effects at mitochondrial level.

The sensitivity of the mitochondrial PTP to calcium was evaluated fluorometrically by monitoring the calcium retention capacity of mitochondria supplemented with Oregon green (Molecular Probes, O6806). Four pulses of calcium were added until pore opening, and the specificity was assessed by adding 1 µM Cyclosporine A (Sigma-Aldrich), the standard inhibitor of PTP.

3.5. Protein expression analysis

100-200 mg of white or beige inguinal adipose tissues were homogenized in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4) without Triton X-100 and homogenized with a Teflon pestle. Homogenates were centrifuged (3000 *g* x 15 min, 4ºC) and the fat cake was removed from the top of the tube. Then, Triton X-100 was added to a final concentration of 1%. After incubating at 4°C for 30 minutes, extracts were cleared by centrifugation at 15,000 *g* for 15 minutes at 4°C. One hundred mg of total protein were analyzed by SDS-PAGE (sodium dodecylsulphate polyacrylamide gel electrophoresis. The gels for immunoblot analyses were transferred to a nitrocellulose membrane (Bio-Rad Trans-Blot SD; Bio-Rad Laboratories).

The membranes were cut at UCP1 and PGC-1amolecular weight level (33 kDa), and blots were reacted with a 1:2000 dilution of anti-UCP1 produced in rabbit (Sigma Aldrich, U6382), in blocking solution (PBS, 5% non-fat milk) and anti- PGC-1aproduced in rabbit.β-actin antibody generated in mouse (Santa Cruz Biotechnology, SC-81178) was used as a control. Horseradish peroxidase-labelled secondary antibodies were goat anti-mouse IgG and goat anti-rabbit IgG (1:1000, Sigma Aldrich). Proteins were visualized by enhanced chemiluminescence (ECL kit, GE Healthcare Life Sciences).

3.6. Determination of low-grade inflammation parameters

To evaluate systemic subclinical inflammation, plasma levels of two mostly representative circulating inflammatory cytokines were measured [201, 238, 239].

The Milliplex rat Cytokine Panel 6-plex (Millipore Corp., Billerica, MD, USA) was used to measure circulating levels of IL-6 and TNF-a. The assay was performed according to the manufacturer′s instructions. Briefly, 50 μL of working solution containing multiple microbeads, labeled with specific antibodies against each of the aforementioned cytokines, were added into each well, washed twice with 200 μL of Linco-Plex wash buffer and filtered to dryness. Then 25 lL thawed plasma aliquots, diluted 1:4 with the specific Linco-Plex sample diluents, were added to each well and incubated for 60 min at room temperature. After a wash step (twice) with 200 lL Linco-Plex wash buffer, the beads were incubated with 25 μL of the detection antibody cocktail for 30 min at room temperature, each antibody specific to a single cytokine. After another two time wash step with 200 μL Linco-Plex wash buffer, the beads were incubated with 25 μL of the streptavidin–phycoerythrin solution for 30 min at room temperature and washed twice again. The beads were resuspended in each well with 100 μL of the Linco-Plex Sheath fluid, and the concentration of each

cytokine was determined using the array reader. A parallel standard curve was constructed for each cytokine. Levels are expressed in pg/mL.

Levels of CRP were determined by using the ultrasensitive assay (Kamiya Biomedical Company, Seattle, WA, USA) following the manufacturer's instructions. Briefly, 100 μL of appropriate blank, standards, and samples (diluted 1: 200 with assay buffer) were added to appropriate wells in 96-well plates. The plates were incubated for 1 hr at room temperature and then washed and thereafter 100 μL of anti-rat CRP-HRP conjugate (second antibody for CRP) was added to each well. The plates were incubated for 30 min in the dark, at room temperature. The horseradish peroxidase (HRP) substrate,3,3,5,5-tetramethylbenzidine was added to the CRP plate and incubated for 15 min in the dark. The reaction for CRP was terminated with a stop solution (100 μL). The yellow color developed was read at 450 nm using a microplate reader.

3.7. Determination of oxidative stress biomarkers

To assess circulating pro-oxidant/antioxidant balance, levels of plasma lipid peroxidation (LPO), one of the most widely used indicators of oxidant status, were measured. LPO was estimated both in the basal (endogenous) state and after a challenge with co-incubation of plasma with$FeSO_4/H_2O_2$ (0.42/0.73 mol/L), which produce hydroxyl radical by the mean of Fenton reaction [(240)]. LPO was assayed by a modification of the method of Yagi [(241)], (a modification of the thiobarbituric acid (TBA)-reactive substances method). Briefly, three modifications were made to improve the specificity of the plasma LPO measurement. First, water-soluble substances, which also react with TBA to yield the same product as LPO, were removed by including a phosphotungstic acid (0.1 g/L) and sulfuric acid (35 mmol/L) step to precipitate lipids and proteins. The supernatant was discarded. Second, reading the fluorescence at 553 nm rather than at 532 nm prevented bilirubin interference. Finally, adding acetic acid into the TBA reagent to adjust the pH to 3.0 eliminated sialic acid interference. The fluorescence was red at 553 nm with 515 nm excitation with a Perkin-Elmer LM-5 spectrofluorometer (Norwalk, CT, USA). The amount of LPO was expressed in terms of malondialdehyde equivalents (MDA)(μmol/L of plasma), as calibrated with freshly diluted 1,1′, 3, 3′-tetramethoxypropane. The intra- and inter-assay CVs of this assay were 4.2% and 4.0%, respectively. Assays were performed in duplicate with a sample volume of 100 μL.

3.8. Serum irisin levels

Commercially available irisin enzyme-linked immunosorbent assay (Aviscera Bioscience, Santa Clara, CA, USA) was used to measure serum irisin concentrations. The assay was carried out in

duplicate, following the manufacturer′s instructions. The sensitivity was 1 ng/mL; the intraassay CV, 4–6%; and the interassay precision, 8–10%.

3.9. Hematoxylin-eosin staining

Adipose tissues were fixed in 10% buffered formaldehyde and subsequently treated for the histological study by dehydration (increasing alcohol concentrations, from 80% to absolute alcohol), mounting in xylene and immersion in paraffin. The paraffin blocks were cut into 4-mm sections for hematoxylin-eosin (H-E) staining.

4. Statistical analysis

Data are expressed as means ± S.E.M. Means were compared among groups by using a two-way ANOVA followed by the Mann-Whitney test. SPSS version 15 for Windows (SPSS, Michigan, IL) was used for the data analyses. A p <0.05 was considered statistically significant, and levels of significance were labeled on the figures as follows: *** P < 0.001; ** P < 0.01; * P < 0.05, and ###, P < 0.001; ## P < 0.01; # P < 0.05.

RESULTS

MELATONIN AMELIORATES LOW-GRADE INFLAMMATION AND OXIDATIVE STRESS IN YOUNG ZUCKER DIABETIC FATTY RATS

Agil A, Reiter RJ, Jiménez-Aranda A, et al. Melatonin ameliorates low-grade inflammation and oxidative stress in young Zucker diabetic fatty rats. J Pineal Res 2013; 54:381-388

The aim of this study was to investigate the effects of melatonin on low-grade inflammation and oxidative stress in young male ZDF rats, an experimental model of metabolic syndrome and T2DM.

ZDF rats (n=30) and lean littermates (ZL) (n=30) were used. At 6 week of age, both lean and fatty animals were subdivided into three groups, each composed of 10 rats: naïve (N), vehicle treated (V), and melatonin treated (M) (10 mg/kg/day) for 6 weeks. Vehicle and melatonin were added to the drinking water. To study the pro-inflammatory state, plasma levels of interleukin-6 (IL-6), TNF-α, and C- reactive protein (CRP) were evaluated. Also, oxidative stress was assessed by plasma lipid peroxidation (LPO), both basal and after Fe^{2+}/H_2O_2 inducement.

As expected, the N-ZDF group exhibited higher levels of IL-6 (112.4 ± 1.5 pg/mL) than ZL animals (89.9 ± 1.0, $P < 0.01$). Melatonin treatment lowered IL-6 levels by 10% (to 98.3 ± 1.6, $P < 0.05$) in ZDF rats, but no changes were observed in ZL animals (Fig. 13).

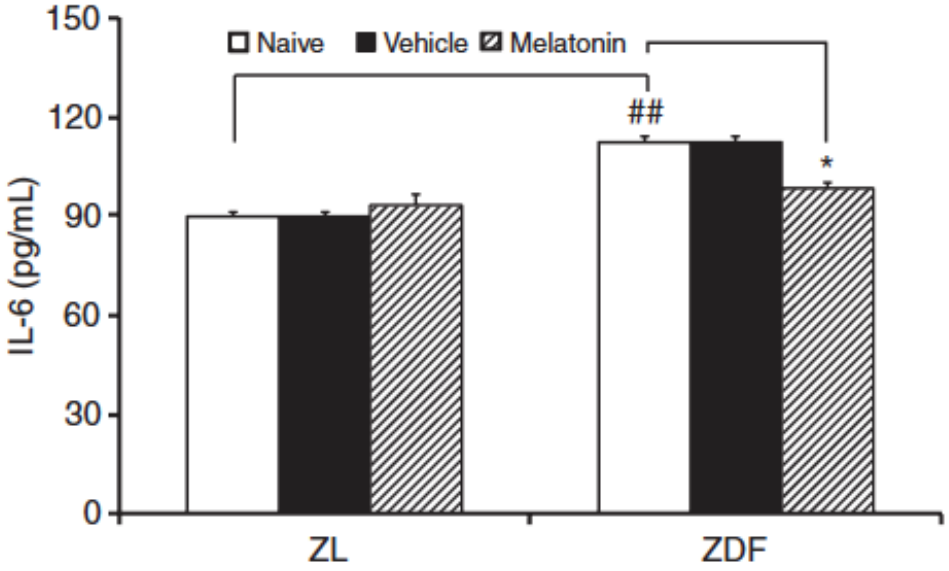


Figure 13. Effect of melatonin treatment on circulating IL-6 levels. ZL, Zucker lean; ZDF, Zucker diabetic fatty. Fasting values were measured in naıve (N), vehicle- and melatonin (M)-treated ZL and ZDF rats. Values represent the means ± S.E.M. of 10 animals/group. The level of significance between the groups is represented by the symbols above the histograms (##$P < 0.01$ N-ZDF versus N-ZL rats; *$P < 0.05$ M-ZDF versus N-ZDF rats).

As shown in Fig. 14, ZDF rats had elevated TNF-α levels (11.0 ± 0.1 pg/mL) when compared with ZL animals (9.7 ± 0.4, $P < 0.01$) and melatonin treatment curtailed TNF-α levels in obese ZDF group by 10% (to 9.5 ± 0.4, $P < 0.05$). No changes were observed in ZL rats in response to melatonin administration.

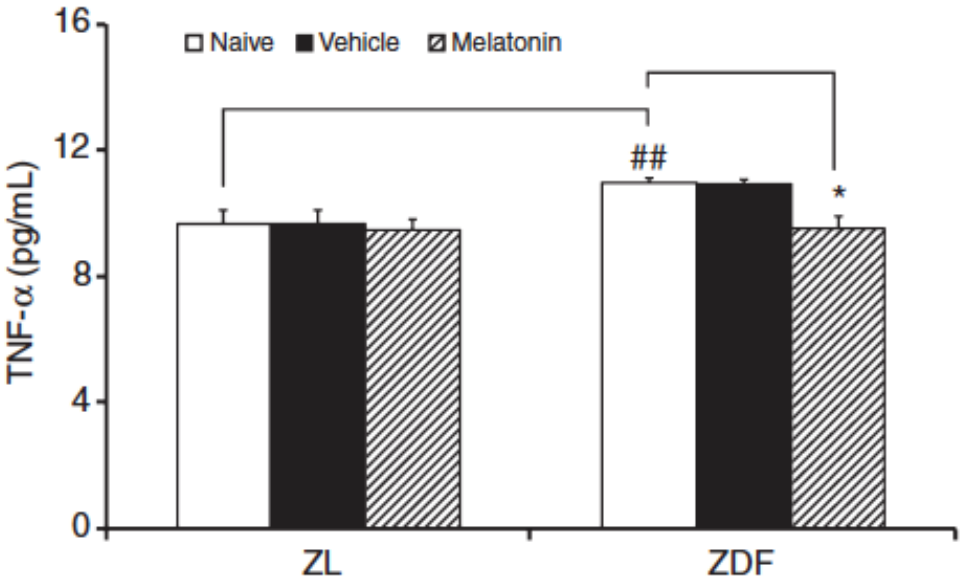


Figure 14. Effect of melatonin treatment on circulating TNF-α levels. ZL, Zucker lean; ZDF, Zucker diabetic fatty. Fasting values were measured in naıve (N), vehicle and melatonin (M)-treated ZL and ZDF rats. Values represent the means ± S.E.M. of 10 animals/group. The level of significance between the groups is represented by the symbols above the histograms (##$P < 0.01$ N-ZDF versus N-ZL rats; *$P < 0.05$ M-ZDF versus N-ZDF rats).

Plasma CRP values were clearly higher in ZDF rats (828 ± 16.0 μg/mL) than in lean rats (508 ± 21.5; $P < 0.001$). Melatonin treatment of ZDF rats reduced CRP levels by 21% (to 651 ± 26.4; $P < 0.01$) (Fig. 15). Melatonin treatment did not affect levels of these inflammatory biomarkers in ZL animals.

As shown in Fig. 16, basal plasma lipid peroxidation (LPO), expressed as MDA equivalents, was higher in ZDF rats (3.2 ± 0.1 versus 2.5 ± 0.1 μmol/L in ZL rats; $P < 0.01$). Melatonin treatment decreased these levels by 15% (to 2.7 ± 0.2; $P < 0.05$) without affecting lipid peroxidation values in ZL animals. Moreover, plasma lipid susceptibility to oxidation by Fe^{2+}/H_2O_2 was higher in ZDF rats (8.7 ± 0.2 versus 5.5 ± 0.3 μmol/L in ZL; $P < 0.001$) (Fig. 17). Melatonin treatment abated these levels in both ZL (by 15.2%, to 4.5 ± 0.3 μmol/L; $P < 0.01$) and ZDF rats (by 39%, to 5.4 ± 0.4 μmol/L; $P < 0.001$).

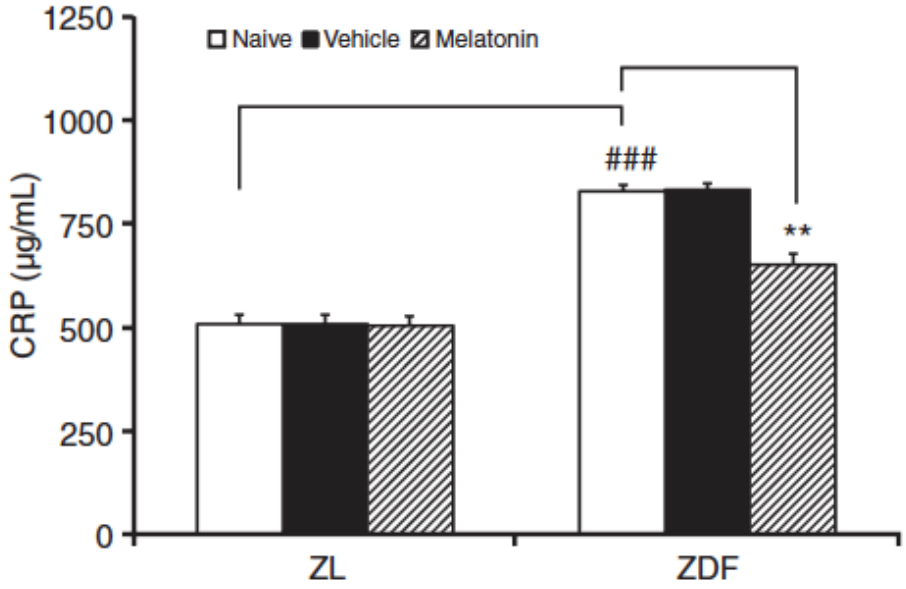


Figure 15. Effect of melatonin treatment on circulating (CRP) levels. ZL, Zucker lean; ZDF, Zucker diabetic fatty. Fasting values were measured in naıve (N), vehicle- and melatonin (M)-treated ZL and ZDF rats. Values represent the means ± S.E.M. of 10 animals/group. The level of significance between the groups is represented by the symbols above the histograms (###P < 0.001 N-ZDF versus N-ZL rats; **P < 0.01 M-ZDF versus N-ZDF rats).

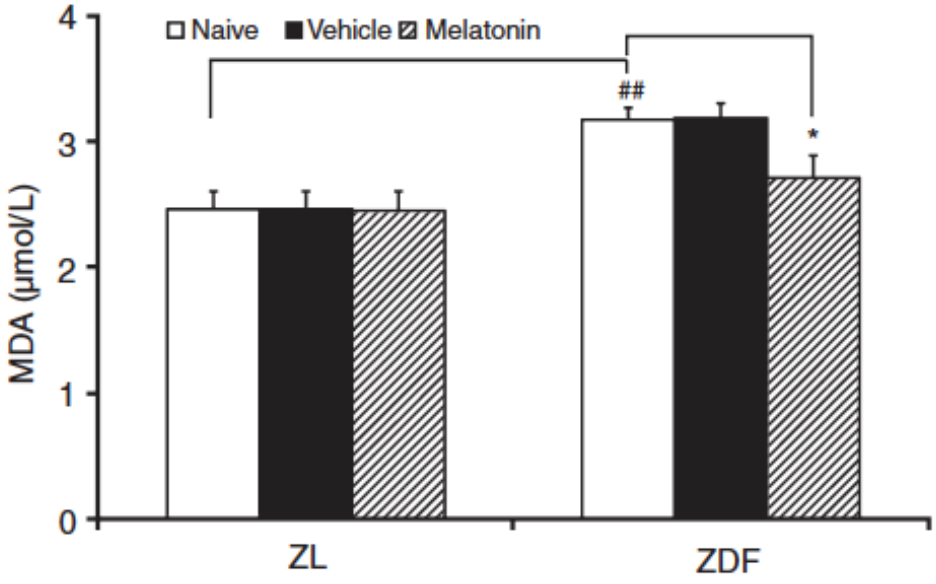


Figure 16. Effect of melatonin treatment on basal plasma LPO. ZL, Zucker lean; ZDF, Zucker diabetic fatty. Lipid peroxides were measured as malondialdehyde (MDA) equivalents in naıve (N), vehicle- and melatonin (M)-treated ZL and ZDF rats. The level of significance between the groups is represented by the symbols above the histograms (##P < 0.01 N-ZDF versus N-ZL rats; *P < 0.05 M-ZDF versus N-ZDF rats).

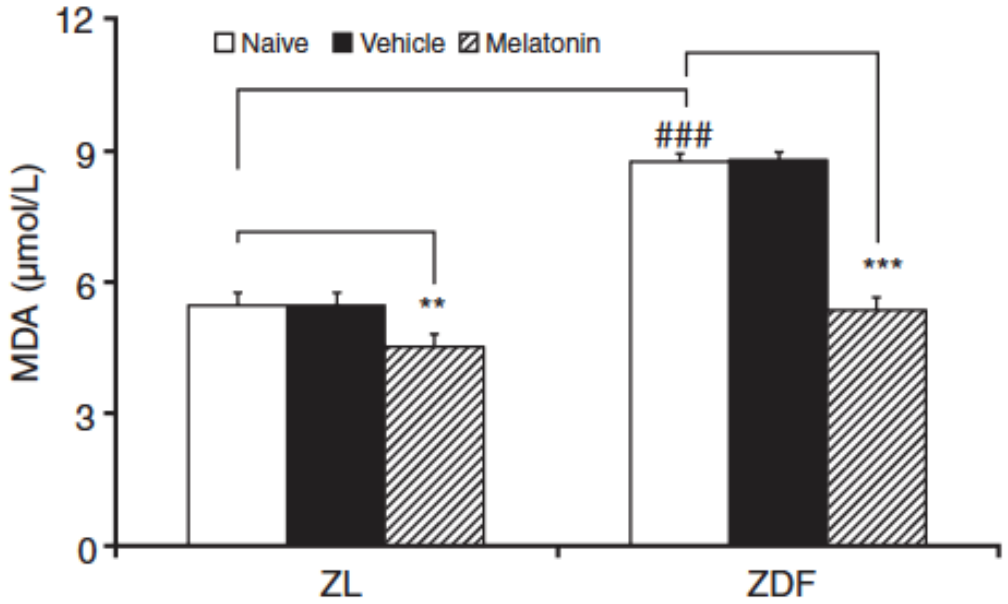


Figure 17. Effect of melatonin treatment on Fe^{2+}/H_2O_2-induced plasma lipid peroxidation (Fe^{2+}/H_2O_2-LPO). ZL, Zucker lean; ZDF, Zucker diabetic fatty. Lipid peroxides were measured as malondialdehyde (MDA) equivalents in naıve (N), vehicle- and melatonin (M)-treated ZL and ZDF rats. Values represent the means ± S.E.M. of 10 animals/group. The level of significance between the groups is represented by the symbols above the histograms (###P < 0.001 N-ZDF versus N-ZL rats; **P < 0.01 M-ZL versus N-ZL; ***P < 0.001 M-ZDF versus N-ZDF rats).

This study shows, for the first time, that chronic oral melatonin administration improves circulating biomarkers of systemic low-grade inflammation and oxidative stress in young ZDF rats. These, in concert with other reported actions in this animal model, including increased plasma adiponectin levels, reduced leptinemia and plasma free fatty acids, likely explain the improvements on insulin resistance and β-cell dysfunction, which ultimately leads to better glycemic control. Therefore, these results demonstrated that oral melatonin administration ameliorates the pro-inflammatory state and oxidative stress, which underlie the development of insulin resistance and their consequences, metabolic syndrome, diabetes, and cardiovascular disease.

MELATONIN INDUCES BROWNING OF INGUINAL WHITE ADIPOSE TISSUE IN ZUCKER DIABETIC FATTY RATS

Jiménez-Aranda A, Fernández-Vázquez G, Campos D, et al. Melatonin induces browning of inguinal white adipose tissue in Zucker diabetic fatty rats. J Pineal Res 2013; doi: 10.1111/jpi.12089

The aim of this study was to investigate the effects of melatonin inducing browning of inguinal WAT in ZDF rats. Thermogenic parameters were analyzed (thermal images, UCP1 and PGC1-α expression). Melatonin limits obesity in rodents without affecting food intake and activity, suggesting a thermogenic effect. Identification of brown fat (beige/brite) in white adipose tissue prompted us to investigate whether melatonin is a brown-fat inducer. We used Zucker diabetic fatty rats, a model of obesity-related type 2 diabetes and a strain in which melatonin reduces obesity and improves their metabolic profiles. At 5 weeks of age, ZDF rats and lean littermates were subdivided into two groups, each composed of four rats: control (C) and those treated with oral melatonin in the drinking water (10 mg/kg/day) for 6 weeks. Melatonin induced browning of inguinal WAT in both ZDF and ZL rats.

To test our hypothesis, we initially investigated whether melatonin treatment could induce inguinal WAT browning in ZDF and ZL rats. Whereas 75% of nontreated C-ZL rats showed beige depots, none of C-ZDF rats exhibited beige areas in the inguinal WAT (Fig. 18).

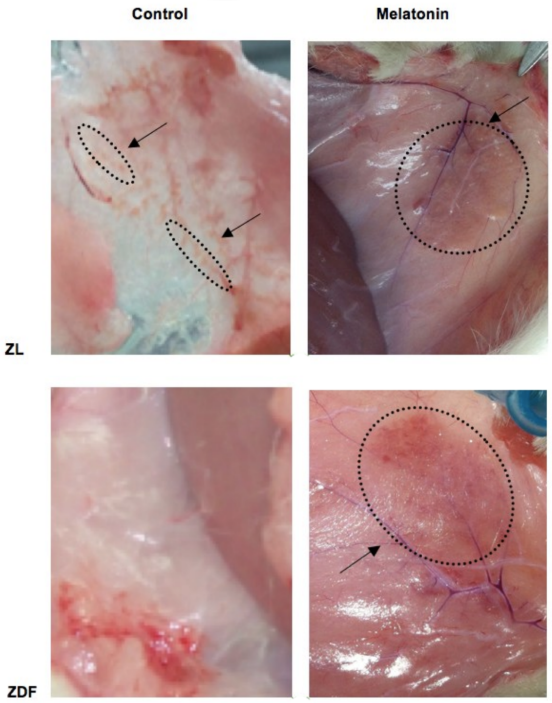


Figure 18. Macroscopic appearance of brownish fat depots observed in the inguinal subcutaneous fat after treatment of Zucker lean (ZL) and Zucker diabetic fatty (ZDF) rats with melatonin.

Melatonin induced browning of inguinal WAT in obese rats (Fig. 18, lower panel) and increased browning in ZL rats (upper panel).

Beige adipocytes are thermogenic fat cells. Accordingly, it is expected that the inguinal skin temperature would correlate with the degree of browning of the underlying fat. As shown in Fig. 19, (upper panel), inguinal skin temperature was similar in both nontreated lean and obese groups of animals. Chronic melatonin treatment raised inguinal temperature in both lean and obese rats, with the temperature being higher in ZL animals (control, 34.6±0.04°C; melatonin, 35.9±0.16°C; $P < 0.01$). Melatonin also sensitizes the thermogenic response to cold exposure and raised their inguinal skin temperature from 0.77±0.02°C to 1.30±0.02°C in ZL and from 0.15±0.05°C to 0.43±0.12 in ZDF rats (Fig. 19, lower panel).

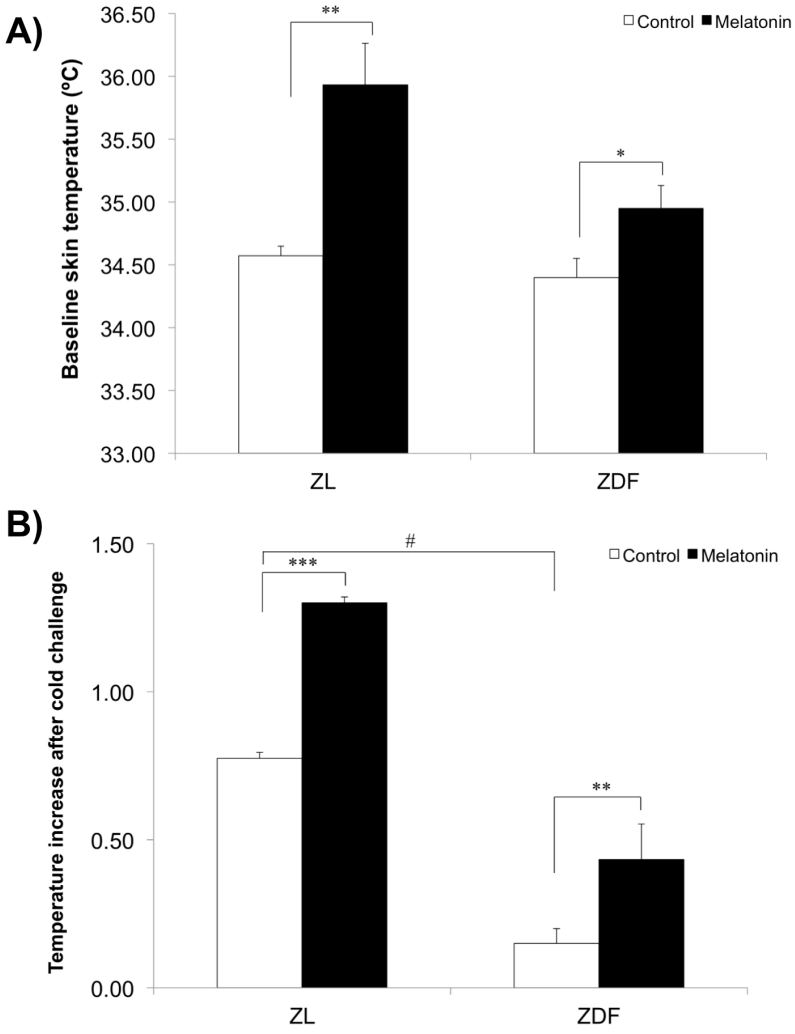


Figure 19. Effect of melatonin treatment on the skin temperature of inguinal area of lean (ZL) and Zucker diabetic fatty (ZDF) rats; upper panel shows basal temperature, while the lower panel documents the temperature increase after acute cold exposure. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, #$P < 0.05$.

This thermogenic effect of melatonin is also apparent in the thermal images shown in Fig. 20.

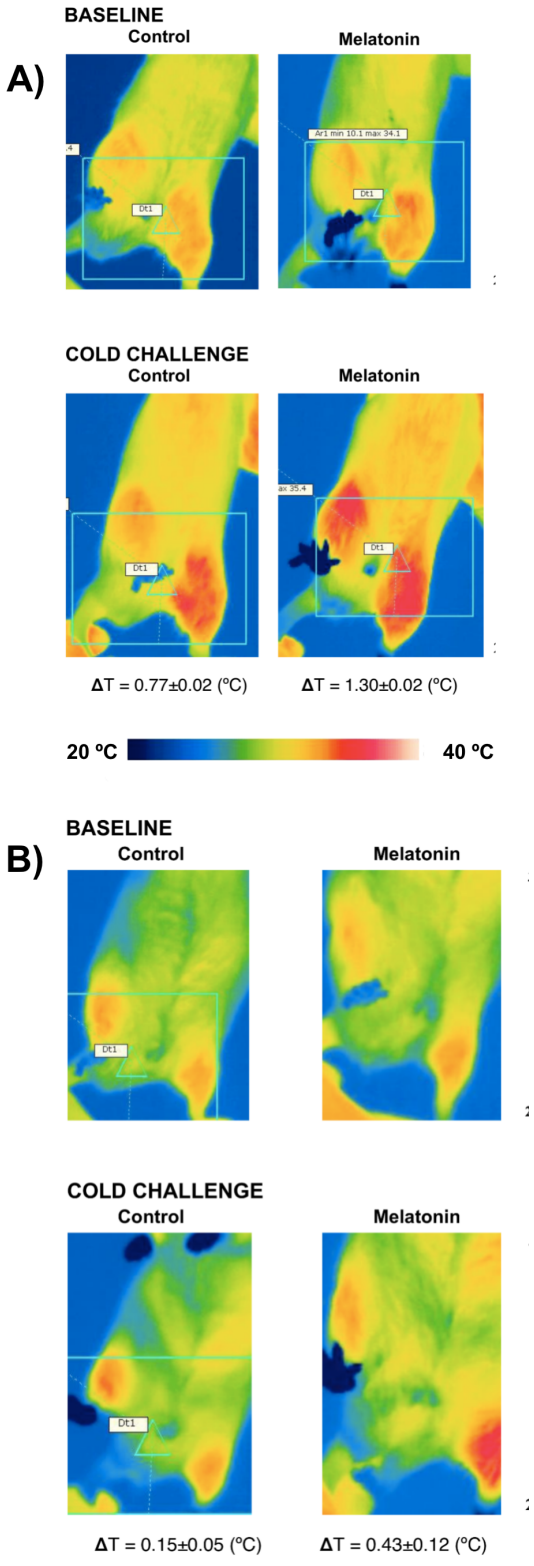


Figure 20. (A) Thermal images of the inguinal area in lean (ZL) rats. Effect of melatonin treatment, before (upper panel) and after cold exposure (lower panel). (B) Thermal images of the inguinal área in Zucker diabetic fatty (ZDF) rats. Effect of melatonin treatment, before (upper panel) and after cold exposure (lower panel).

White- and brown-induced adipocytes possess different functions and also distinct microscopic characteristics. Whereas white adipocytes store triacylglycerols, brown and beige cells burn fat dissipating their chemical energy as heat. White cells are larger and endowed with a single fat droplet, with the nucleus in the periphery; they contain relatively few mitochondria. Beige cells are smaller, with multilocular fat droplets, and are enriched with highly active mitochondria containing abundant cytochromes, which render them brown. Hematoxylin and eosin staining of inguinal fat depots showed that melatonin treatment induced the appearance of patches of multilocular brownfat-like cells in WAT in ZDF rats (Fig. 21, lower panel) and also increased the proportion of these cells in the WAT of ZL animals (Fig. 21, upper panel).

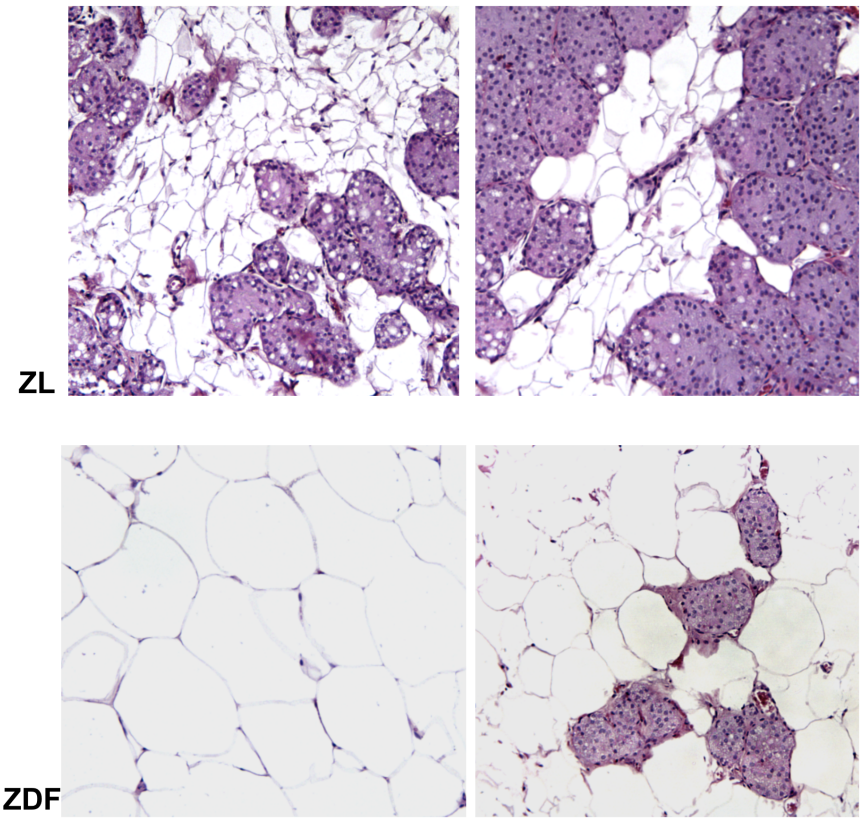


Figure 21. Hematoxylin and eosin stained inguinal fat depots. Melatonin treatment induced the appearance of nests of multilocular brown-like fat cells in Zucker diabetic fatty (ZDF) rats and increased the proportion of these cells in lean (ZL) animals (x40).

Because mitochondrial functionality is crucial in energy metabolism, we investigated whether melatonin treatment influences enzyme activities in isolated mitocondria from different experimental groups of animals. For this purpose, citrate synthase (CS), the rate-limiting enzyme of the tricarboxylic acid cycle in the matrix of mitochondria, and cytochrome c oxidase (complex IV), a

prototype component of the electron transfer chain in the inner membrane, were measured. Activities are expressed as nmol.min/mg of mitochondrial protein (Fig. 22). Chronic melatonin treatment increased the activity of CS in both inguinal depots, WAT and beige, of lean rats (by 1.59, $P < 0.05$ and by 2.12, $P < 0.01$, respectively). Melatonin also increased the activity of CS in induced brown areas of inguinal fat tissue of obese diabetic fatty rats (by 1.26 $P < 0.01$) (Fig. 22, upper panel). Cytochrome C oxidase activity was significantly increased in obese and lean animals in both white and beige depots, with higher value being measured in brown areas (Fig. 22, lower panel).

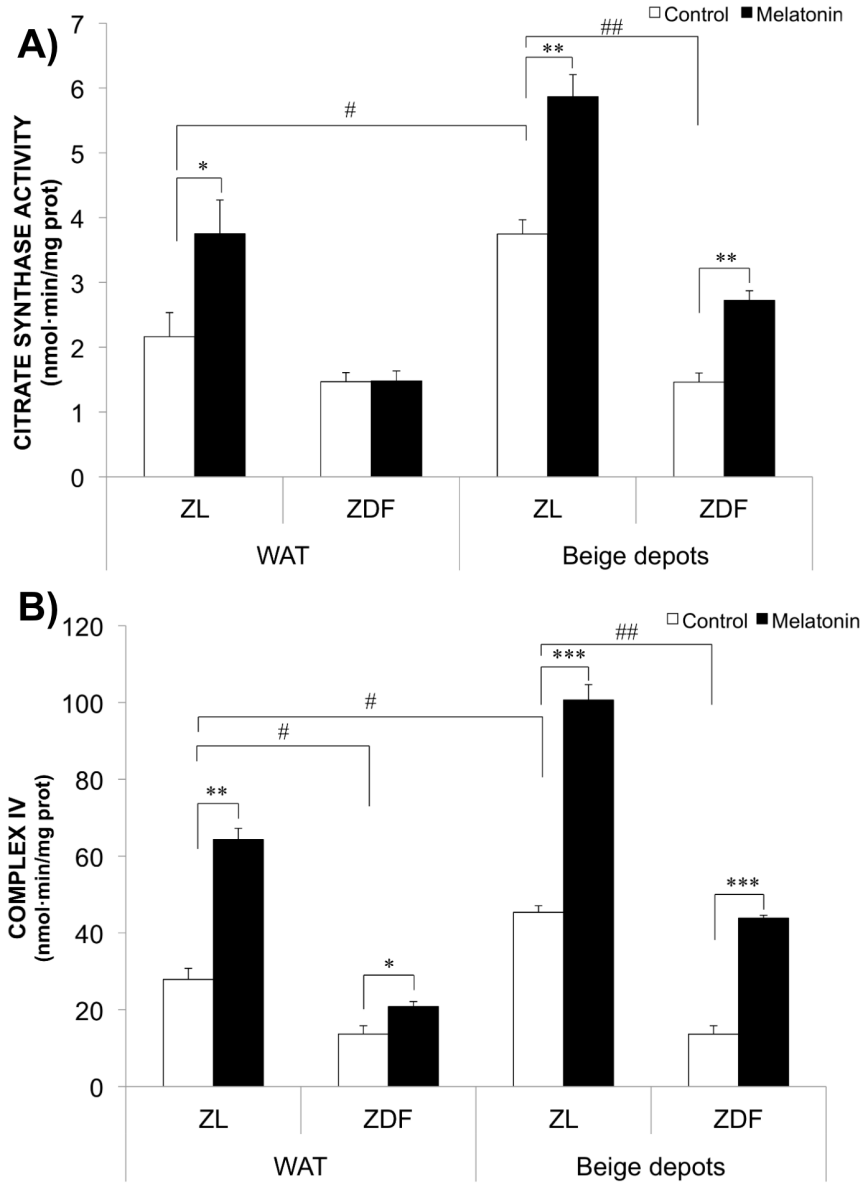
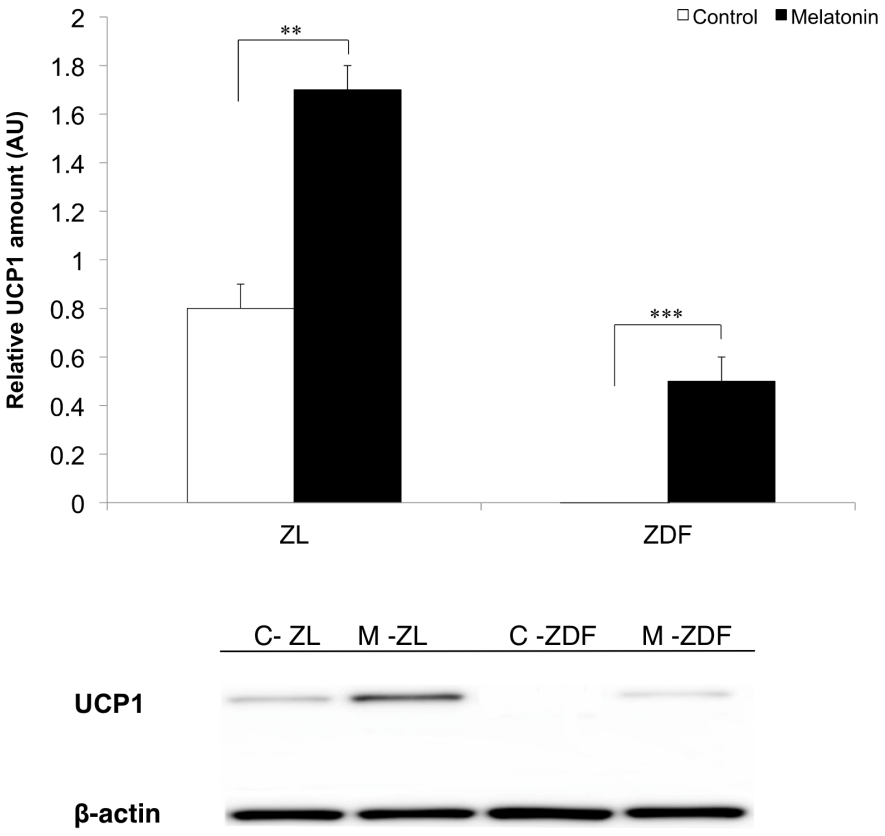


Figure 22. Citrate synthase (upper panel) and cytochrome c oxidase (complex IV) activities in isolated mitochondria from inguinal WAT and beige areas of nontreated and melatonin-treated ZL and ZDF rats. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ##$P < 0.01$, #$P < 0.05$.

The molecular signature that identifies brown and brown-like adipocytes is UCP1. Therefore, we measured the amounts of UCP1 in extracts from beige areas of the inguinal fat depots of both lean and obese rats in response to melatonin treatment using Western blot. As expected from the optic microscopy images, UCP1 was detected in beige regions of nontreated lean animals, and melatonin treatment increased these levels ~2-fold (Fig. 23, upper panel). UCP1 was undetectable in the inguinal fat of nontreated obese ZDF rats. Melatonin treatment clearly induced the expression of UCP1 in these animals (Fig. 23, upper panel). Peroxisome proliferator-activated receptor γ-cofactor-1 (PCG-1α) is a master nuclear transcription factor that controls the expression of the thermogenic gene program, including the expression of UCP1 gene. Western blot analysis of extracts from beige areas of the inguinal fat of lean animals revealed relatively elevated expression of PGC-1α, which was significantly higher in lean melatonin-treated rats ($P < 0.01$) (Fig. 23, lower panel). In contrast, the amounts of PGC-1α were lower in nontreated obese animals that only contained white adipocytes; melatonin treatment originated a ~2-fold rise of PGC-1α levels, consistent with the appearance of beige areas (Fig. 23, lower panel).



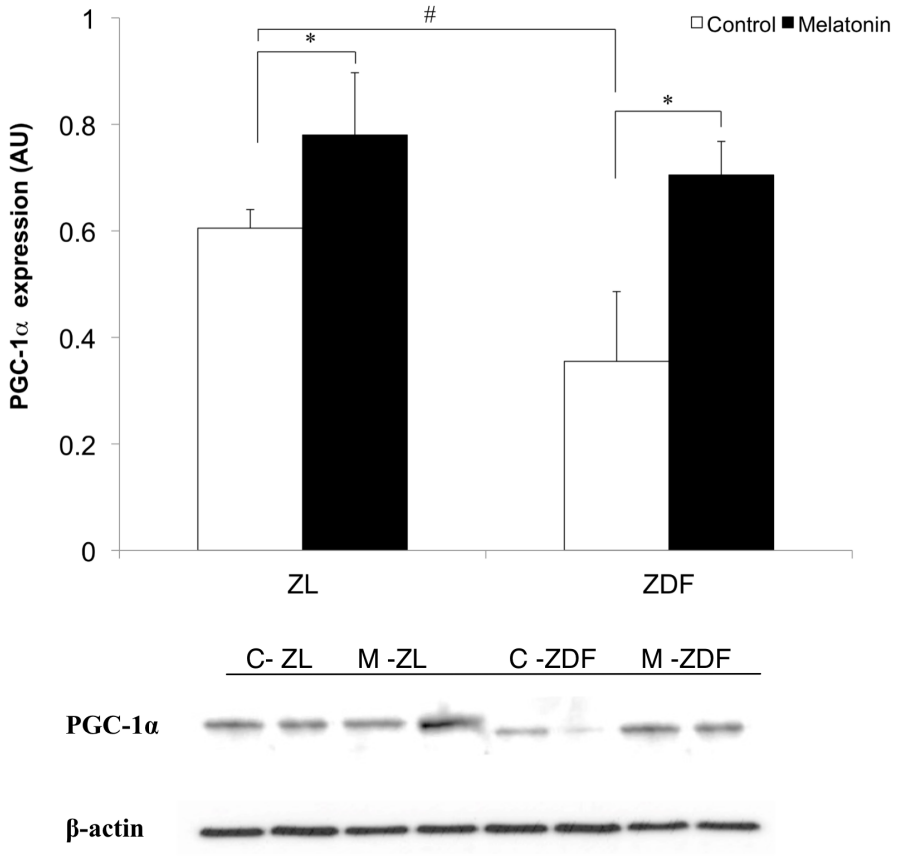


Figure 23. Effect of melatonin treatment on thermogenic protein levels, UCP1 (upper panel) and PGC-1α (lower panel) in the inguinal fat tissue as measured by Western blot. ZL, Zucker lean; M-ZL, melatonin-treated ZL rats; C-ZDF, nontreated obese rats; M-ZDF, melatonin-treated obese rats. ***P < 0.001, **P < 0.01, *P < 0.05, #P < 0.05.

Because melatonin may influence the locomotor activity of rats, we investigated whether melatonin influences circulating irisin levels along with animal′s locomotor activity. As shown in Figure 22, neither circulating irisin nor the spontaneous locomotor activity was significantly affected by melatonin treatment. As expected, lean rats have higher locomotor activity and higher irisin levels than their obese littermates (Fig. 24).

In conclusion, we have demonstrated that chronic melatonin treatment in rats behaves as a white fat browning inducer with thermogenic properties, driving WAT into a brown-fat-like function in ZDF rats. Also, melatonin treatment does not modify rat physical activity, so it is presumed that it can potentiate the thermogenic effect of exercise. This may contribute to melatonin's control of body weight and its metabolic benefits. All these observations, together with its high pharmacological safety profile, make melatonin a potentially useful tool for a stand-alone or adjunct therapy for obesity.

	Irisin (ng/ml)	Locomotor activity	
		Crossed squares/5 min	Rears/5 min
C-ZL	107±3.2	52.0±1.47	27.5±1.10
M-ZL	113±1.7	56.5±1.70	25.0±0.87
C-ZDF	94±2.6*	38.2±1.25*	15.7±1.10*
M-ZDF	107±4.08	44.5±1.97	15.5±1.68

Figure 24. Effect of melatonin treatment on circulating irisin levels and locomotor activity. C-ZL, nontreated Zucker lean rats; M-ZL, melatonin-treated ZL rats; C-ZDF, nontreated Zucker diabetic fatty rats; M-ZDF, melatonin-treated ZDF rats. *P < 0.05 vs C-ZL.

MELATONIN IMPROVES MITOCHONDRIAL FUNCTION IN INGUINAL WHITE ADIPOSE TISSUE OF ZÜCKER DIABETIC FATTY RATS

Jiménez-Aranda A, Fernández-Vázquez G, Mohammed-Aquel Serrano M, et al. Melatonin improves mitochondrial function in inguinal white adipose tissue of Zucker diabetic fatty rats. J Pineal Res 2014 (Submitted)

The aim of this study was to investigate the effects of melatonin on white and beige adipose tissue of Zucker rats, considering the mitochondrial function. Oxygen consumption, ADP:O, nitrite levels and SOD activity was evaluated. Also, the PTP opening was studied.

Mitochondrial dysfunction in adipose tissue may contribute to obesity-related metabolic derangements such as T2DM. At 6 weeks of age, ZDF rats and lean littermates (ZL) were subdivided into two groups, each composed of four rats: control (C-ZDF and C-ZL) and treated with oral melatonin in the drinking water (10 mg/kg/day) for 6 weeks (M-ZDF and M-ZL). After the treatment period, animals were sacrificed, tissues dissected and mitochondrial function assessed in isolated organelles.

Mitochondrial functionality is crucial in energy metabolism, so we investigated whether melatonin treatment influences mitochondrial respiration in isolated mitochondria from white and beige depots of inguinal adipose tissue, in both lean and obese diabetic animals.

The rate of the oxygen uptake, proportional to oxygen flux (expressed as pmol/min.mg protein) increases sharply after addition of ADP, followed by a rapid reduction due to depletion of ADP, which has been phosphorylated to ATP (state 3). As showed in figure 25A, mitochondria isolated from melatonin-treated ZL and ZDF animals have a slightly reduced oxygen flux in state 3 compared with non-treated controls, in both fat depots, white (15.6 ± 0.9 in C-ZL vs. 14.2 ± 1.0 in M-ZL rats, *N.S.*; 15.8 ± 0.2 in C-ZDF vs. 13.9 ± 0.2 in M-ZDF rats, $p< 0.05$) and beige (15.4 ± 0.03 in C-ZL vs. 12.6 ± 0.05 in M-ZL rats, $p<0.05$; 15.8 ± 0.1 in C-ZDF vs. 14.3 ± 0.062 in M-ZDF, $p< 0.05$). All control groups have similar respiration rates in state 3.

As showed in figure 25B, mitochondria from white fat depots of both ZL and ZDF melatonin-treated animals have a reduced leak respiration, i.e., in the absence of ATP formation (state 4) (28 % lower in ZL, from 4.70 ± 0.37 to 3.38 ± 0.3; $p < 0.05$ and 35 % lower in ZDF, from 4.97 ± 0.16 to 3.23 ± 0.23; $p< 0.01$). This effect of melatonin is much lower in mitochondria from beige fat in ZL (12.2 % decrease, from 5.69 ± 0.16 to 5.0 ± 0.12; $p< 0.05$) and no change in ZDF rats (from 4.99 ± 0.11 to 5.19 ± 0.10; N.S.).

The respiratory control ratio (RCR), which is the best global indicator of mitochondrial function *"in vitro",* increased in the white fat of melatonin-treated ZL (from 3.31 ± 0.06 to 4.19 ± 0.06, $p < 0.01$) and ZDF (from 3.18 ± 0.07 to 4.31 ± 0.09, $p < 0.01$) rats (Fig. 26). However,

melatonin treatment reduced the RCR of mitochondria from beige depots of both ZL (from 2.71 ± 0.06 to 2.52 ± 0.03, $p < 0.05$) and ZDF rats (from 3.16 ± 0.09 to 2.75 ± 0.06, $p < 0.05$). As expected, mitochondria from beige fat have lower RCRs than organelles from white depots (Figure 26). In all tested experimental conditions, the values for RCR were greater than 2.2, indicating an optimal mitochondrial preparation and well-coupled isolated mitochondria. As we did not found any beige depots in the inguinal fat of C-ZDF rats, we considered the same results for both depots in the C-ZDF group.

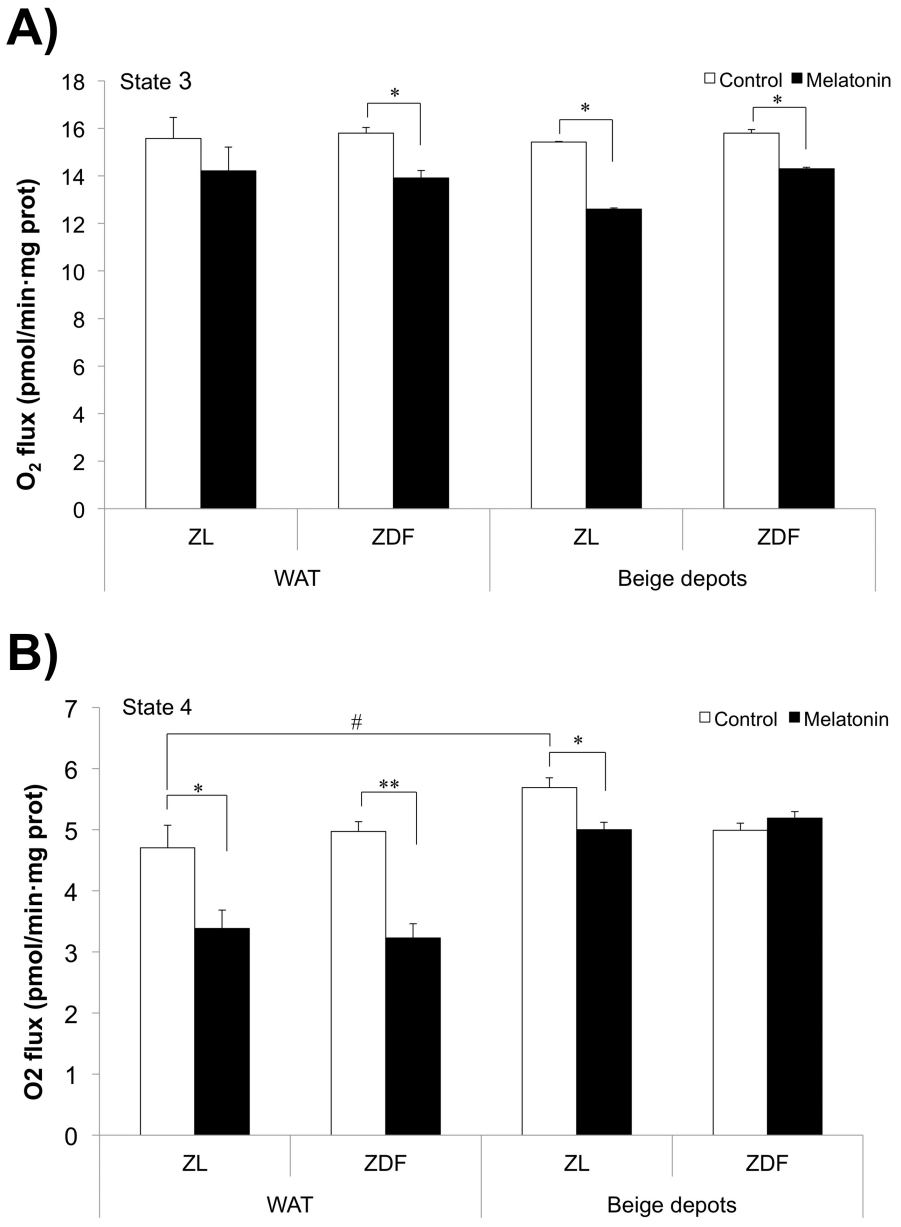


Figure 25. Effect of melatonin treatment on the respiratory states in isolated mitochondria from white (WAT) and beige fat depots. The upper panel shows the state 3 (oxygen flux while producing ATP in response to ADP pulses in the presence of substrates). The lower panel documents the state 4 or leak respiration (oxygen

flux in the absence of ATP synthesis). Glutamate/Malate were used as respiratory substrates. Values are the means ± S.E.M. ZL, Zücker lean rats; ZDF, Zücker diabetic fatty rats. ** p < 0.01, * p< 0.05, # p<0.05.

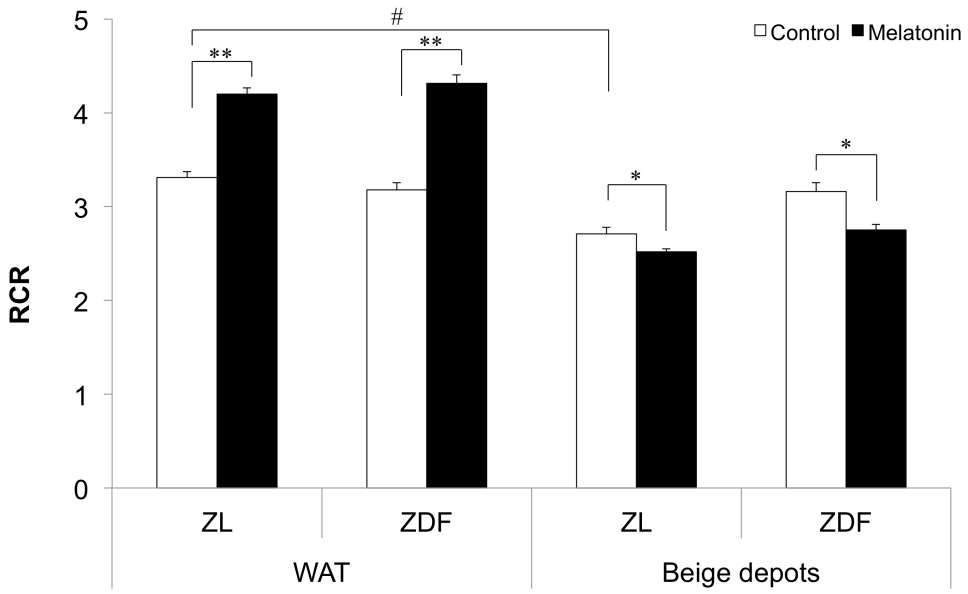


Figure 26. Respiratory control ratio (RCR) in isolated mitochondria from white (WAT) and beige fat depots. RCR is defined as the ratio state3/state4. Values are the means ± S.E.M. ZL, Zücker lean rats; ZDF, Zücker diabetic fatty rats. ** p < 0.01, * p< 0.05, # p<0.05.

One of the major effects of melatonin in mitochondria is related to combat free radical production and their clearance. The effect of melatonin treatment on mitochondrial oxidative status was analyzed by measurement the nitrites levels and the superoxide dismutase (SOD) activity. The nitrites levels in white fat depots were increased in diabetic obese (C-ZDF) rats (from 24.1 ± 4.6 µmol/mg protein in C-ZL to 31.5 ± 1.3 in C-ZDF; p < 0.05) (Fig.27). Albeit not significant, animals treated with melatonin have lower nitrites levels (20.7 ± 0.7 in C-ZL vs. 27.2 ± 3.4 µmol/mg protein in C-ZDF). Melatonin treatment significantly reduced nitrites amounts in mitochondria of beige fat (from 33.4 ± 4.7 to 22.5 ± 3.8 µmol/mg protein in ZL, p < 0.05 and from 31.5 ± 1.3 to 28.1 ± 3.1 µmol/mg protein in ZDF rats, N.S.).

The SOD activity was higher in beige depots compared with white depots in ZL rats (36.5 ± 1.3 % for white and 58.5 ± 1.3 % for beige depots; p < 0.01) (Fig. 28). Melatonin-treated animals have higher SOD activity in white fat of lean animals (from 40.3 ± 6.7 to 51.3 ± 1.6; p < 0.05) and a tendency to increase in their beige depots (Fig. 28). No statistically significant changes were observed in both white and beige areas of obese animals.

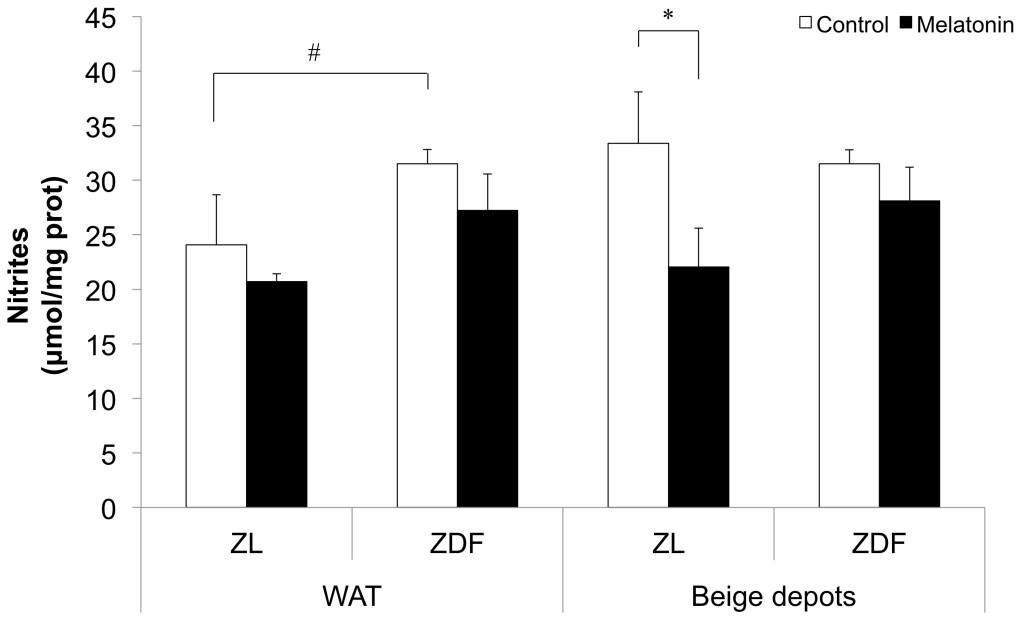


Figure 27. Effect of melatonin on nitrite levels in mitochondria isolated from white (WAT) and beige fat depots. Values are the means ± S.E.M. ZL, Zücker lean rats; ZDF, Zücker diabetic fatty rats. * p< 0.05, # p<0.05.

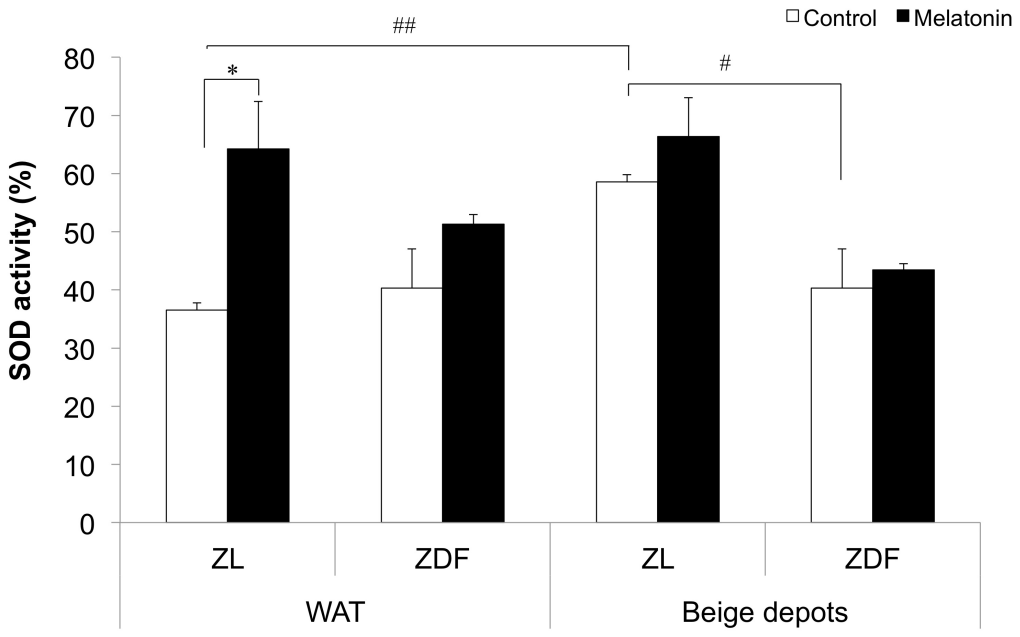


Figure 28. Effect of melatonin treatment on superoxide dismutase (SOD) activity in mitochondria isolated from white (WAT) and beige fat depots. Values are the means ± S.E.M. ZL, Zücker lean rats; ZDF, Zücker diabetic fatty rats. * p< 0.05, ## p< 0.01, # p< 0.05.

The activity (opening) of the mitochondrial permeability transition pore (mPTP), the gatekeeper of cell death, was induced by raising the Ca^{2+} concentration in the incubation media. The possible inhibitory effect of melatonin treatment on Ca^{2+}-induced mPTP was studied and compared with that of cyclosporine A (CsA), the classical mPTP inhibitor (Fig. 29).

Also, we investigated if melatonin treatment sensitizes to or have an additive effect to the inhibition of mPTP caused by CsA. Melatonin pretreatment in vivo causes a notorious inhibition of Ca-induced opening of mPTP in isolated mitochondria "in vitro" from both types of fat, white and beige, in both lean and obese rats (by 73.4 %, $p < 0.001$ in white ZL fat; by 45 %, $p < 0.01$ in white adipocytes from ZDF rats; by 60 % in beige adipocytes from ZL rats, $p < 0.001$ % and by 65 % in beige adipocytes from obese ZDF rats). CsA inhibited the activity of mPTP by 90, 83, 86 and 84 % in mitochondria from white ZL, white ZDF, beige ZL and beige ZDF rats, respectively. In mitochondria from melatonin-treated animals, CsA provoked even significant greater inhibition (94, 83, 95 and 94 %) of mPTP (Fig. 29).

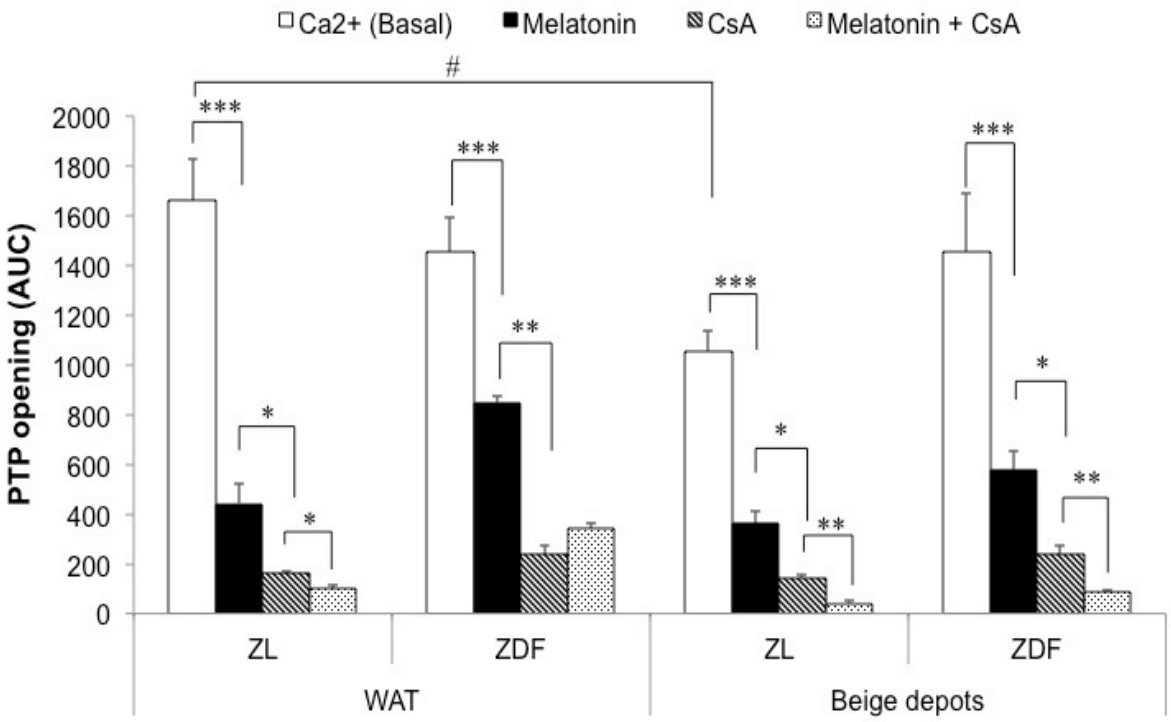


Figure 29. Effect of melatonin treatment on the Ca^{2+}-induced permeability transition pore (PTP) in mitochondria isolated from white (WAT) and beige depots. The effect of cyclosporine A (CsA) was included as the classical inhibitor of mitochondrial PTP. Values are the means ± S.E.M of the area under the curve. ZL, Zücker lean rats; ZDF, Zücker diabetic fatty rats. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, # $p < 0.05$.

These results demonstrate that chronic oral melatonin is able to improve mitochondrial respiration, to reduce the oxidative status and susceptibility to apoptosis in white and beige

adipocytes. These effects of melatonin can contribute to prevent mitochondrial dysfunction and thereby to improve obesity-related metabolic derangements such as diabetes and dyslipidemia of ZDF rats.

DISCUSSION

Developed countries are facing an epidemic of interrelated metabolic diseases collectively referred to as the metabolic syndrome, the hallmarks of which include obesity, hyperlipidemia, hyperglycemia and insulin resistance. These symptoms are all independent risk factors of diabetes. Type 2 diabetes mellitus is a major public health problem worldwide, as a consequence of expanding pandemic of obesity. It also implies a very high risk for many of the most prevalent diseases such as cardiovascular disease, cancer and dementia. This causes a very high morbidity and mortality, as well as a huge economic burden. The search for safe and effective drugs that can improve this situation has proved unsuccessful.

Several studies suggest that melatonin, a natural molecule with high toxicological safety and low cost, could help improve the situation. But the scientific basis of its benefits on obesity and its metabolic complications are poorly understood. This neurohormone is produced by the pineal gland during the night, but also locally in many other tissues [236, 237]. Besides entrainment of circadian rhythms, melatonin and several of its metabolites are effective antioxidants and anti-inflammatory agents and also regulators of energy balance [10, 110, 124, 129].

Numerous studies have shown that melatonin administration in rodents reduces overweight associated to different experimental conditions (photoperiod and elongation pinealectomy) or physiological (aging) deficiency occurring with this neurohormone [126, 194]. Chronic administration of melatonin also limits weight gain in middle-aged rats and diet-induced obesity [126, 195] improving the visceral component and associated hyperinsulinemia and hyperleptinemia [194]. Our group demonstrated that melatonin improves overweight and dyslipidemia in young male ZDF rats. It also reduces body weight gain without affecting food intake. Moreover, this indoleamine alone reduces both fasting and sustained chronic hyperglycemia. Melatonin can exert its antihyperglycemic effect either by improvement of insulin action, by amelioration of insulin secretion, or both. Our group also showed that oral melatonin is accompanied by a decrease of hyperinsulinemia, reflecting and improvement of insulin resistance, as evidenced by a lower HOMA-IR index [108, 109].

With this background, we aimed to assess the melatonin influence on low-grade inflammation and adipose depots, locally and at mitochondrial level. This includes the molecular mechanisms by which melatonin improves overweight in an animal model of obesity and diabetes, the ZDF rat, in order to explore their potential application in the treatment of human obesity.

Our initial goal was to study the effect of melatonin as a preventive treatment for diabetes. The first results showed a positive effect exerted at chronic inflammation and oxidative stress level, without adversely affecting the physiological situation. Although our intention was to continue studying the effect at WAT and BAT, the findings in one of our tests redirect the course of our work. Analyzing the macroscopic characteristics of different WAT depots, we found that lean rats

possessed depots of a different adipose tissue included in the WAT inguinal area. Furthermore, we found that those rats treated with melatonin increased the size of these deposits. By observing the same area in obese rats, we found that only those rats treated with melatonin had this type of tissue. We performed a deep study at the morphological level and a comparison with tissues obtained from the same depot in control and treated animals. The second striking evidence was reported by optical microscopy (H&E staining), where we identified brown-like islands among WAT. This surprising finding led us to change the main objectives of our research and redirect it to the beige adipose tissue analysis.

At first we had doubts whether this new tissue was a different tissue or a BAT depot. Detailed analysis of each fat pad individually, together with recent findings from other groups, indicated that we were facing a completely different depot. From that initial moment when we realized that it was a singular tissue, the results led us through this amazing beige adipose tissue, showing the multiple possibilities that could provide its manipulation.

Although we have successfully characterized this beige adipose tissue and have compared it with the WAT, many questions are still unresolved. The origin of these beige adipocytes remains unclear: it could arise from pre-existing white fat cell trans differentiation or through an alternative differentiation pathway of Myf5-negative mesenchymal preadipocyte lineage.

A detailed study of beige adipose tissue, including adipocyte analysis at cell cultures, is among our new projects. Also, the ultraestructural examination of adipose depots performed by TEM is being considered. We have unpublished data confirming the different mitochondrial structure of WAT, BAT and beige adipose tissue, and some evidences suggesting that beige depots are an intermediate adipose type between that found in unilocular and in multilocular adipocytes.

In conclusion, the findings presented in these papers represent the beginning of a novel strategy for combating obesity and type 2 diabetes mellitus, where melatonin is the backbone of all the improvements observed.

2. Melatonin and inflammation

Daily oral administration of melatonin during a 6 week period to young male ZDF rats reduces insulin resistance by the HOMA-IR index, plasma oxidative stress, expressed as LPO (basal and induced), enhances L:A ratio by increasing adiponectinemia and by decreasing leptinemia, decreases free fatty acid concentration and thus lipotoxicity; plus amelioration of inflammation state by decreasing cytokines (TNF-α and IL-6) and CRP levels.

Mounting evidence indicates that both low-grade chronic inflammation and increased oxidative stress are present in obesity and metabolic syndrome [196-199]. It is well established that both features participate in the pathogenesis of metabolic syndrome, diabetes and their cardiovascular

complications [196-201]. Obesity-associated inflammation originates in WAT, mainly in visceral fat, in response to the positive energy balance [202]. Lipid overload to adipocytes causes an excessive fat cell enlargement and imposes an increase in the ER stress that triggers the inflammatory response, with macrophage infiltration, fat tissue hypoxia and adipocyte necrosis. Inflamed adipocytes release proinflammatory cytokines such as IL-6 that, in turn, activates macrophages to release other proinflammatory cytokines, including TNF-α. These cytokines, via paracrine/autocrine actions, self-feed fat tissue inflammation which limits adipocyte metabolic function by impairing lipogenic and antilipolytic actions of insulin and by interfering with lipid droplet formation. This leads to an increase in circulating FFA. Melatonin can reduce serum FFAs level through several mechanisms, such as weight loss, enhancement of insulin action and by increasing adiponectin levels. All of these factors contribute to abate lipolysis and to increase fatty acid oxidation. Hence, by decreasing circulating FFA levels, melatonin can improve insulin secretion and action and also diminish cardiovascular risk related to obesity and T2DM.

As previously mentioned, obesity is associated to a state of chronic inflammation [203], where an increase in cytokine (TNF-α, IL-6) production, as well as higher CRP levels are seen as a secondary effect of altered adipocytokine expression in WAT. Our findings are in accord with other researches; Gitto *et al*.[204] show that in septic newborns, melatonin's free radicals scavenge activity diminishes oxidative damage as well as CRP levels that in turn improve proinflammatory cytokine markers. Additionally, researchers report that rats, which were submitted to experimental thermal trauma and then received oral melatonin, had decreased values of CRP as well as MDA as marker of oxidative stress, attenuating the oxidative and inflammatory response [205, 206].

Conventional antioxidants (a combination of α- and γ-tocopherol) reduced significantly CRP and TNF-α levels in a placebo control study [207], but failed to do so with IL-6 levels; whilst others [208] report no changes at all in all proinflammatory parameters. Thus, more investigation in this field is needed, with concordance in the methodologies applied. In contrast, results presented in our study show melatonin's positive effect in cytokine and CRP levels, diminishing proinflammatory state.

Obesity-associated oxidative stress occurs mainly from oversupply of fatty acids and glucose to mitochondria. This leads to an overproduction of reactive oxygen (ROS) and nitrogen species (RNS), which exceed the anti-oxidant defense. Normal levels of free radicals are required for physiological cell signaling [197]. However, their overproduction induces oxidative stress that is toxic for biomacromolecules, and triggers and spurs the inflammatory response through the ability of ROS to activate nuclear factor kappa B (NF-kB)/IKK2, JNK/AP1 and protein kinase C signaling pathways. When activated, these pathways stimulate the expression of a set of genes encoding enzymes involved in inflammation, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) and proinflammatory cytokines. Also, these pathways may signal oxidative stress contribution to insulin resistance [243]. Therefore, 'metaflammation' and obesity-related oxidative

stress are intimately linked processes. During hyperglycemia, advanced glycation end products (AGEs) or lipoxidation end products are oxidants in vivo [244]. The results reported here showed melatonin's potent antioxidant capacity in basal plasma LPO, which is in agreement with other *in vitro* and *in vivo* investigations [125, 209, 210]. As it is demonstrated, melatonin for the first time has clear effect completely antagonizing Fenton reaction in young ZDF rats, returning it to baseline level. Additionally, it protects mitochondria from ROS overproduction [211], which is associated to a decrease in lipo- and glucotoxicity, amelioration of cellular senescence, tissue damage and dysfunction; all related to degenerative diseases like T2DM [210, 212, 213]. Therefore, its use as a pharmacological antioxidant treatment in degenerative diseases present in young obese subjects comes to high relevance for prevention of metabolic syndrome.

In conclusion, this study has shown that chronic oral melatonin treatment attenuates low-grade inflammation and oxidative stress. These, in concert with other reported actions in ZDF rats, including increased plasma adiponectin levels, reduced leptinemia and plasma FFA, would mean an improvement in key parameters of T2DM, with new potential applications of melatonin as treatment for attempting normoglicaemia by enhancing glucose tolerance and insulin sensitivity; and improving adipocytokine production resulting in a lesser proinflammatory and prooxidative state, that at the end diminishes the risk to develop T2DM in obesity or its CV complications in young ZDF rats.

3. Melatonin and browning

Before knowing the existence of beige adipose tissue, our objective was to study thermogenesis and BAT. In fact, we have some unpublished data showing that melatonin can promote BAT thermogenesis through activation of the mitochondrial uncoupling protein 1 (UCP1), either directly or by activating type 2 thyroxine 5´-deiodinase to increase intracellular tri-iodothyronine (T3) levels. The thermogenic effect of melatonin has gained strong interest because of expectations of its possible contribution to antiobesity, but so far all the thermogenic studies were focused on BAT. However, beige adipose tissue has gained importance in recent years for their peculiarities.

Beige adipocytes possess many of the morphological and functional characteristics of classical brown adipocytes, but they could play a more important role than the interscapular BAT [47]. A recent report [43] described that the identified brown fat depots in adult humans exhibit a molecular signature that is more comparable to that of murine beige than brown adipocytes. This work supports the hypothesis that the BAT present at birth is different from the thermogenic adipose tissue observed in human adults. Actually, in contrast to WAT and inguinal beige adipose tissue, which are easily accessible through biopsy procedures, human BAT is out of reach except for highly invasive surgery.

Consequently, we consider that beige adipose tissue provides better alternatives for its use, manipulation and recruitment than the classical BAT.

Several studies in rodents suggest that melatonin increase basal energy expenditure by activating thermogenesis "without trembling" in brown adipose tissue (BAT) and increased the mass of this tissue [20]. This hypothesis implies that melatonin, through specific receptors (membrane M1/M2 or RZR/ROR nuclear) could increase the mass of BAT stimulating the proliferation/differentiation of brown adipocytes and/or mitochondrial biogenesis, or prevent damage, reducing the fission thereof.

Melatonin could promote BAT thermogenesis by activation and/or expression of mitochondrial UCP1 [214] or to act directly as uncoupler, given easy access to the mitochondria. PGC-1α [33] stimulates respiratory activity and mitochondrial biogenesis, through induction of UCP1 [215]. Therefore, melatonin could activate thermogenesis via PGC-1α. Also, the recent discovery of irisin [38] suggests that melatonin may increase energy expenditure through one irisin similar effect on adipocyte/pre-adipocytes of WAT.

We reported here that oral melatonin supplementation induces browning of the inguinal WAT in ZDF rats. This effect occurred without changing the locomotor activity of animals. Melatonin treatment not only induced the appearance of brown-like adipocytes in subcutaneous WAT, but also increased the thermogenic activity of this tissue as indicated by a temperature rise of the inguinal skin and also by the thermal camera records. Hence, thermal images of different adipose depots are a novel way to ensure the browning process that has shown a great relevance in this research. Western blot analyses of fat tissue extracts demonstrated that brownish areas expressed higher amounts of PGC-1α, the master regulator of the transcription of the thermogenic program. Moreover, melatonin-treated animals exhibited an increase of UCP1, the molecular effector of thermogenesis, in WAT beige depots of lean animals and induced its expression in obese rats, where it had been absent. Also, our data show that melatonin treatment sensitizes the thermogenic response to cold exposure, a well-recognized thermogenic stimulus.

It is well known that adrenergic activation by a variety of stimuli including cold exposure [150, 219, 220] or chronic exposure to supraphysiologic levels of catecholamines in patients with pheochromocytoma [221] leads to browning of WAT by acting through adrenergic receptors [20]. Whether beige adipocytes following adrenergic stimulation arise from pre-existing white fat cell trans-differentiation [219, 222, 223] or through an alternative differentiation pathway of Myf5-negative mesenchymal pre-adipocyte lineage is an unsolved question. However, it is clear that irisin-mediated exercise browning occurs through the activation of the thermogenic program of beige preadipocytes [224].

As previously noted, oral melatonin administration may induce white fat browning by multiple mechanisms, including sympathetic activation (leading to noradrenaline release) and acting directly on noradrenergic terminals where it releases the catecholamine. Regardless of the origin of

melatonin-induced new brown-like adipocytes in WAT, melatonin may also stimulate the thermogenic capacity of these cells by multiple mechanisms.

In rodents, Puig-Domingo et al [225, 226] demonstrated that melatonin specifically increases the activity of the BAT isoform of type 2 thyroxine 5´-deiodinase, without affecting serum thyroid hormone concentrations. This can lead to an intracellular increase of tri-iodothyronine (T3), which activates the expression of the thermogenic UCP1 [227]. Also, due to its amphiphylic properties, melatonin has easily access to the mitochondria where it reaches the highest subcellular concentrations [228] and where it may directly act as a mild uncoupler [115].

Some previously published reports indicate that melatonin increases locomotor activity [229, 230], although same authors failed to document this [231, 232]. Accordingly, melatonin could convert WAT into brown fat by way of irisin, a recently discovered exercise-induced myokine that selectively drives brown-fat-like development of white fat in mice and humans [38]. Our results demonstrated that melatonin´s browning action takes place in the absence of changes in both locomotor activity and circulating irisin levels. As expected, irisin levels and locomotor activity were higher in lean than obese rats. Therefore, higher levels of physical activity followed by higher irisin concentrations, could account for the presence of patches of brown-like adipocytes in the inguinal white fat of non-treated lean animals. Conversely, the absence of these thermogenic cells in obese rats may contribute to their obesity.

In conclusion, we have demonstrated that chronic melatonin treatment in rats behaves as a white fat browning inducer with thermogenic properties. Furthermore, we show that melatonin sensitizes fat cells to other thermogenic stimuli such as cold. Since melatonin treatment does not modify rat physical activity, it is presumed that it can potentiate the thermogenic effect of exercise. These observations, together with its high pharmacological safety profile, make melatonin a potentially useful tool for a stand-alone or adjunct therapy for obesity.

4. Melatonin and mitochondrial function

Mitochondria play an important role in adipocyte differentiation and body energy balance. Generalized mitochondrial dysfunction has been implicated in the development of insulin resistance in the pathogenesis of type 2 diabetes associated with obesity [216, 217]. This dysfunction is generally [2], but with tissue-specific differences [218] and is manifested in a reduction of the activity of the electron transport chain and ATP production. Adipocyte mitochondrial dysfunction can be cause and consequence of obesity and diabetes, since it results into less efficient substrate oxidation, increased oxidative stress and calcium permeability, raised endoplasmic reticulum stress, apoptosis and inflammation, that, in turn, all of them contribute to insulin resistance.

The observations that the mitochondria number in adipose tissue from obese people is reduced, suggests that impaired mitochondrial activity could predispose to obesity and mitochondria biogenesis is also altered in obesity. We also knew that prospective studies on mitochondrial function in WAT and beige adipose tissue were needed to fill many gaps in our current knowledge about mitochondrial dysfunction in adipose tissue, and its relationship with obesity and other associated complications. Hence, we studied and confirmed the improvement of WAT and beige mitochondrial function, as evidenced by better organelle respiration, less nitrites production and better antioxidant capacity, and inhibition of mitochondrial permeability transition pore (mPTP) after oral melatonin supplementation.

Our data showed melatonin treatment increased the respiratory control ratio (RCR) of mitochondria from white adipocytes in both lean and obese rats. The RCR is considered the best single measure of the function in isolated mitochondria because it encapsulates their main function. Melatonin treatment increased the RCR of mitochondria from white adipocytes through reducing proton leaking, as evidenced by a marked decrease in sate 4 of respiration despite a slight slowing of global oxygen flux in state 3. By a marked reduction of proton leaking together with a slight slowing of respiration, melatonin may contribute to reduce oxidative stress but keeping OXPHOS efficiency in mitochondria from WAT of both lean and obese-diabetic rats. Also, isolated mitochondria from white and beige subcutaneous adipose tissue of both lean and obese diabetic animals have equal respiratory capacities as demonstrated by the magnitudes of states 3 and 4 of respiration. This suggests that mitochondrial dysfunction in obesity and diabetes may be the result of altered biochemical environment "*in vivo"*, but when isolated and in the presence of the same biochemical medium, mitochondria have similar respirations no matter they originated from lean non-diabetic or obese diabetic animals. An additional explanation could be that we have evaluated mitochondrial respiration at an early stage of obesity and diabetes well before mitochondrial dysfunction can be present. Mitochondria from beige fat have a little higher uncoupled respiration, as expected because their thermogenic properties [(164)]. Chronic melatonin treatment also slightly reduces OXPHOS capacity of mitochondria isolated from beige adipocytes in both lean and obese rats, with same degree as in the case of white fat. However, mitochondria isolated from beige fat of melatonin-treated animals have a decreased RCR, due to a much less reducing effect on leak respiration than in the case of white adipose tissue. This can contribute to the thermogenic properties of melatonin in beige fat [(164)].

As for the oxidative status, the present study showed that melatonin administration could improve it. The production of reactive nitrogen species (RNS) has been found to increase in some pathological conditions, such as diabetes and obesity that further worsens the mitochondrial function because nitrosative damage [(177)]. Our results indicated that mitochondria from white adipocytes of obese diabetic animals exhibited significantly higher nitrites levels than of their lean littermates.

Melatonin treatment attenuates nitrites formation, especially in beige depots of lean rats and also increased the SOD activity in both adipose tissues of both phenotype rats. Attenuation of nitrites levels by melatonin can be regarded as the sum of indirect antioxidant effects via SOD activity, direct scavenger effect and of reduction of electron leakage. Beneficial effects of melatonin on adipocyte mitochondrial oxidative stress are in agreement with numerous reports in other tissues, pointing at the mitochondria as the natural target for the antioxidant actions of melatonin.

Finally, one of the most striking effects of melatonin treatment in our work is the inhibition of the Ca^{2+} -induced mitochondrial permeability transition pore (mPTP) in mitochondria isolated from both WAT and beige adipose tissues. Based on experiments with isolated mitochondria, calcium was thought to be the most important factor in mPTP induction. Our tests showed that the mPTP is less sensitive to increased calcium in melatonin treated mitochondria, indicating that melatonin would limit or reduce the mitochondrial damage. Oxidant stress mechanisms appear to be the dominant factors responsible for mPTP induction, which also justifies the protective effect of melatonin. The magnitude of melatonin inhibitory action on mPTP is closed to the observed with cyclosporine A (CsA) the classical inhibitor of mPTP [(233)] which impedes opening of PTP by binding and inactivating cyclophilin D. Thus, melatonin can protect mitochondria of adipose tissue from mitochondria-induced cell death, as has been reported in cardiomyocytes during ischemia/reperfusion [(234)], an effect that has been attributed to the melatonin capacity for directly inhibiting the mPTP, as occurred in neuronal cells [(235)].

In conclusion, the results of the present work demonstrated, for the first time, that melatonin treatment improved several mitochondrial functions of subcutaneous fat cells in Zücker diabetic fatty rats. Melatonin accomplishes improved mitochondrial homeostatic roles quite efficiently, since it is able to improve oxygen consumption, decreases RNS production and augments SOD. Thus, according to our novel findings, these results could help to broaden our understanding of the biological determinants of mitochondrial capacity and its role in diabesity.

Consequently, administering melatonin that enhance the formation of beige depots, could be an attractive mechanism to improve the mitochondrial function, a potentially useful future tool to reduce the dysfunction associated with T2DM and obesity, and may be beneficial in the future therapy for these pathologies.

CONCLUSIONS

CONCLUSIONS

1. Chronic oral melatonin treatment attenuates low-grade inflammation and oxidative stress, present in an experimental model of T2DM, the young ZDF rat. This contribute to support the concept that melatonin may be a helpful aid for the treatment of T2DM and the metabolic syndrome.

2. Chronic melatonin treatment in rats behaves as a white fat browning inducer with thermogenic properties. We have also shown that melatonin sensitizes fat cells to other thermogenic stimuli such as cold.

3. Chronic melatonin treatment in rats improves the bioenergetics function and reduces the cell damage. It is also worth noting that the improvement at mitochondrial level seen for the treated physiological situation (the M-ZL rats) might be an indicative of protective activity and reduced toxicity, which is desirable for any pharmacological treatment.

4. Melatonin accomplishes improved mitochondrial homeostatic roles quite efficiently, since it is able to improve oxygen consumption, decreases RNS production and augments SOD.

5. These observations together with its high pharmacological safety profile make melatonin a potentially useful tool for a stand-alone or adjunct therapy for obesity.

CONCLUSIONES

1. El tratamiento oral con melatonina atenúa la inflamación de bajo grado y el estrés oxidativo, presente en un modelo experimental de diabetes tipo 2, la rata ZDF. Esto contribuye a apoyar el concepto de que la melatonina puede ser útil para el tratamiento de la diabetes tipo 2 y el síndrome metabólico.

2. El tratamiento con melatonina se comporta como un inductor del "browning" con propiedades termogénicas. Además, hemos demostrado que la melatonina sensibiliza a los adipocitos frente a otros estímulos termogénicos como el frío.

3. El tratamiento con melatonina en ratas mejora la función bioenergética y reduce el daño celular. También vale la pena señalar que la mejora observada a nivel mitocondrial para la situación fisiológica tratada (las ratas M-ZL) podría ser un indicativo de la capacidad protectora y toxicidad reducida, lo cual es deseable para cualquier tratamiento farmacológico.

4. La melatonina lleva a cabo la mejora de funciones homeostáticas mitocondriales de forma muy eficiente, ya que es capaz de mejorar el consumo de oxígeno, disminuye la producción de RNS y aumenta la SOD.

5. Estas observaciones, junto a su alta seguridad farmacológica, hacen de la melatonina una herramienta potencialmente útil para una terapia independiente o complementaria para la obesidad.

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