Adipose tissue mitochondrial function is modulated by antioxidants

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Cover illustration: CARS and 2-PE fluorescence microscopy image of fresh isolated inguinal white adipose tissue by Alexandra Paul

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To Alba and Biel, the loves of my life

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ABSTRACT

Antioxidants are widely used as reactive oxygen species (ROS) scavenging agents in experimental research and in conditions where oxidative stress plays a primary role. However, the effect of antioxidant supplementation on white and brown adipose tissue functionality is understudied, and the role of ROS and/or antioxidant treatment during adipose tissue browning, a process in which the adipocytes' mitochondrial density and activity increase, is largely unknown. In **paper I**, by using antioxidants and ROS-sensitive fluorescent probes in cultured β3-AR-stimulated adipocytes, we observed that 24-48-hour antioxidant treatment increases the mitochondrial ROS production associated with reduced respiration and increased glycolysis. Moreover, treatment of mice with the antioxidant N-acetylcysteine (NAC) blunted the β3-AR agonist-induced browning response of white adipose tissue and reduced the mitochondrial activity in brown adipose tissue even in the absence of β3-AR stimulation. Previous studies have shown positive effects of prolonged NAC treatment on whole-body metabolism in mice. In light of these seemingly contradictory results, we hypothesize that chronic antioxidant exposure, in a dose-dependent manner, can lead to so-called mitohormesis. Indeed, in **paper II**, by treating mice with a set of different NAC doses across a defined time course, we found that prolonged supplementation with a high dose of NAC leads to increased mitochondrial function of white adipose tissue, reduced fat mass and improved insulin sensitivity. In summary, this thesis demonstrates that the adipose tissue response to antioxidant treatment in mice is biphasic and tightly connected to the adipose tissue type, the dosage and the treatment duration. This thesis also provides an alternative explanation for previously reported controversial findings where antioxidants (such as NAC) have exerted deleterious effects on health. Finally, the results of this thesis provide new insights into the appropriate design of antioxidant treatment studies: optimizing treatment dosage and duration may be the key to achieve success with antioxidant therapy.

Keywords: Adipocyte; Antioxidant; Browning; Reductive stress; Hormesis

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SAMMANFATTNING PÅ SVENSKA

I denna studie har vi undersökt effekten av antioxidantbehandling på fettvävens mitokondriefunktion. Våra huvudsakliga fynd är att antioxidanter kan, beroende på dosering och behandlingslängd, öka den mitokondriella produktionen av fria syreradikaler. Detta är dock inte nödvändigtvis av ondo utan kan över tid leda till förbättrad mitokondriefunktion, åtminstone i fettväven.

Fettväv som ökar sin mängd aktiva mitokondrier blir bättre på att ta upp fett och socker från blodbanan, vilket minskar risken för att kärl och andra organ förfettas. I mitokondrierna omvandlas näringsämnen till kemisk energi i en syrekrävande process som kallas cellandning. I denna process bildas även fria syreradikaler som kan orsaka skadlig oxidativ stress. Det är därför vanligt att kosttillskott innehåller antioxidanter som kan neutralisera dessa syreradikaler. Antioxidanter används även i forskningssammanhang för att försöka förbättra sjukdomstillstånd i vilka man tror att oxidativ stress är en bidragande orsak, som till exempel diabetes och neurodegenerativa sjukdomar. I flera vetenskapliga studier har det dock visat sig att antioxidantbehandling inte har några positiva effekter och i vissa fall har till och med negativa utfall rapporterats.

I delarbete I fann vi att antioxidantbehandling paradoxalt nog kan öka den mitokondriella produktionen av fria syreradikaler i odlade fettceller. Denna effekt är kopplad till en sänkt cellandning. Vi fick liknande resultat i möss; möss behandlade med antioxidanten N-acetylcystein under två veckor fick försämrad mitokondriefunktion i fettväven än kontrollmössen. I andra musstudier har det dock rapporterats att långtidsbehandling med N-acetylcystein ökar fettvävens mitokondriemängd samt förbättrar kroppens ämnesomsättning. I delarbete II förklarar vi dessa motsatta resultat genom att påvisa en så kallad hormetisk effekt av N-acetylcystein i musfettväv. Hormesis innebär att en giftig substans eller en stressor, i ett visst dosintervall, kan ge fördelaktiga effekter till följd av att cellen anpassar sig. Våra data tyder på att den paradoxala ökningen av fria syreradikaler till följd av N-acetylcysteinbehandling kan uppreglera fettvävsmitokondriernas egna antioxidantsystem samt produktion av fler och/eller effektivare mitokondrier, vilket resulterar i sänkt fettmassa och förbättrad ämnesomsättning.

Resultaten från denna studie ger oss således fördjupad kunskap om möjliga utfall av antioxidantbehandling samt en ny förklaringsmodell för tidigare, tillsynes motsägelsefulla fynd.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. *Peris, E., Micallef, P., Paul, A., Palsdottir, V., Enejder, A., Bauzá-Thorbrügge, M., Olofsson, C. S., and Wernstedt Asterholm, I.* Antioxidant treatment induces reductive stress associated with mitochondrial dysfunction in adipocytes. *J Biol Chem 294, 2340-2352 (2019).*

II. *Peris, E., Bauzá-Thorbrügge, M., Micallef, P., Bartesaghi, S., Benrick, A. and Wernstedt Asterholm, I.* Prolonged N-acetylcysteine treatment induces mitohormesis in adipose tissue. *Manuscript.*

Publications not included in this thesis

- *Mishra, D., Richard, J.E., Maric, I., Porteiro, B., Häring, M., Kooijman, S., Musovic, S., Eerola, K., López-Ferreras, L., Peris, E., Grycel, K., Shevchouk, O.T., Micallef, P., Olofsson, C.S., Wernstedt Asterholm, I., Grill, H.J., Nogueiras, R., Skibicka, K.P.* Parabrachial Interleukin-6 Reduces Body Weight and Food Intake and Increases Thermogenesis to Regulate Energy Metabolism. *Cell Reports, 26 (11), pp. 3011-3026 (2019).*
- *Komai, A.M.*, Musovic, S.*, Peris, E., Alrifaiy, A., El Hachmane, M.F., Johansson, M., Asterholm, I.W., Olofsson, C.S.* White adipocyte adiponectin exocytosis is stimulated via β3-adrenergic signaling and activation of Epac1: Catecholamine resistance in obesity and type 2 diabetes. *Diabetes, 65 (11), pp. 3301-3313 (2016).* * contributed equally.
- *Svahn, S.L., Väremo, L., Gabrielsson, B.G., Peris, E., Nookaew, I., Grahnemo, L., Sandberg, A.-S., Asterholm, I.W., Jansson, J.-O., Nielsen, J., Johansson, M.E.* Six tissue transcriptomics reveals specific immune suppression in spleen by dietary polyunsaturated fatty acids. *PLoS ONE, 11 (5), art. no. e0155099 (2016).*

CONTENT

ABBREVIATIONS

2-PE	2-photon excitation
APN	Adiponectin
ATP6	ATP synthase Fo subunit 6
AUC	Area under the curve
β-AR	Beta adrenergic receptor
BAT	Interscapular brown adipose tissue
cAMP	Cyclic adenosine monophosphate
CARS	Coherent anti-Stokes Raman spectroscopy
cDNA	Complementary DNA
CM-H_2DCF-DA	2',7'-dichlorofluorescein diacetate
COXI	Cytochrome c oxidase subunit 1
COXIV	Cytochrome c oxidase subunit 4
CREB	cAMP response element-binding protein
DEXA	Dual-energy X-ray absorptiometry
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FFA	Free fatty acid
GLUT4	Glucose transporter type 4
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GWAT	Gonadal white adipose tissue
HFD	High fat diet
HRP	Horseradish peroxidase
IWAT	Inguinal white adipose tissue
IP	Intraperitoneal
KEAP1	Kelch-like ECH-associated protein 1
mRNA	Messenger RNA
MYF5	Myogenic factor 5
NAC	N-acetyl-L-cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NDUF8B	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8
NOX4	NADPH oxidase 4
NRF2	Nuclear factor erythroid 2–related factor 2

OCR	Oxygen consumption rate
OGTT	Oral glucose tolerance test
OPO	Optical parametric oscillator
OXPHOS	Oxidative phosphorylation
PEPCK	Phosphoenolpyruvate carboxykinase
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGC1β	Peroxisome proliferator-activated receptor gamma coactivator 1-beta
PKA	Protein kinase A
PPARγ	Peroxisome proliferator-activated receptor gamma
PRDM16	PR domain containing 16
PRDX	Peroxiredoxin
qRT-PCR	Quantitative real-time polymerase chain reaction
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RXR	Retinoic acid receptor
SDHB	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit
SOD	Superoxide dismutase
SVF	Stromal vascular fraction
TPP+	Triphenylphosphonium cation
TR	Thyroid hormone receptor
TRX	Thioredoxin
UCP1	Uncoupling protein 1
WAT	White adipose tissue
WT	Wild-type

1 INTRODUCTION

Obesity is defined by the World Health Organization as an excessive fat accumulation in the body and is considered a major risk for diseases such as diabetes, cardiovascular diseases (mainly heart diseases and stroke) (Lavie, Milani et al. 2009), musculoskeletal disorders (especially osteoarthritis) (Wearing, Hennig et al. 2006) and several cancers (including endometrial, breast, ovarian, prostate, liver, gallbladder, kidney, and colon) (Stone, McPherson et al. 2018). However, it is not increased adiposity *per se*, but rather the obesity-associated adipose tissue dysfunction that increases the risk of developing co-morbidities. Dysfunctional adipose tissue contributes to the metabolic syndrome, whereas healthy adipose tissue, regardless of its size, is protective through its ability to remove excess nutrients from the blood stream. To increase our knowledge about the pathogenesis of obesity-related diseases and to identify new therapeutic targets it is, therefore, important to delineate mechanisms that regulate adipose tissue functionality.

1.1 ADIPOSE TISSUE AND ADIPOCYTES

The adipose tissue is distributed throughout the body and plays a central role in the regulation of whole-body energy homeostasis. It can expand and shrink dramatically to store and release energy to accommodate the body's need. Adipose tissue displays thus an extreme flexibility compared to other tissues and organs. In mammals, the adipose tissue is classified as either white or brown containing respectively, white adipocytes, which are the primary site of triglyceride/energy storage, and brown adipocytes, which are mainly responsible of thermogenesis i.e. dissipating energy as heat (Gesta, Tseng et al. 2007). In humans, adipose tissue accounts for around 20 % of the total body weight and the amount increases to more than 40 % in obesity (Sepa-Kishi and Ceddia 2018).

1.1.1 WHITE ADIPOSE TISSUE

The classical fat cell, the white adipocyte, is a spherical, large and expandable cell that usually contains one large lipid droplet accounting for 95 % of the cell volume. Its mitochondrial content is relatively low and its main role is to store excess energy in the form of triglycerides and, when required, deliver energy back to the body through lipolysis leading to the release of glycerol and fatty acids. The adipocytes also have a very important role as

hormone/adipokine producers. For instance, the adipokines leptin and adiponectin contribute to the regulation of whole-body energy balance and metabolism (Balistreri, Caruso et al. 2010). Other cell types but adipocytes account for around 50 % of the total cell population in adipose tissue. They are collectively termed the stromal vascular fraction (SVF). The SVF includes preadipocytes, various immune cells such as macrophages and lymphocytes, endothelial cells and pericytes (Bourin, Bunnell et al. 2013) and these SVF cells provide the necessary means for normal adipose tissue growth and homeostasis.

Anatomically, white adipose tissue (WAT) comprises two major depots: the subcutaneous and the visceral WAT. In humans, the subcutaneous WAT is mainly found in the abdominal, gluteal and femoral regions, while the visceral WAT is found around internal organs in the mesentery and the omentum. The same anatomical division holds true for mice. However, mice have a rather large fat depot surrounding their gonads, the gonadal WAT (GWAT), which is a visceral depot not found in humans (Chusyd, Wang et al. 2016). Although having the same lineage of origin, different anatomical location of adipose tissue is associated with different functionality, connected to the protein expression signature in both humans (Vidal 2001) and mice (Gesta, Blüher et al. 2006). Importantly, excess visceral WAT is highly correlated with increased risk of metabolic disorders such as insulin resistance and type 2 diabetes (Item and Konrad 2012), whereas increased subcutaneous WAT has been connected to low risk of the metabolic syndrome (Kissebah and Krakower 1994) (Figure 1).

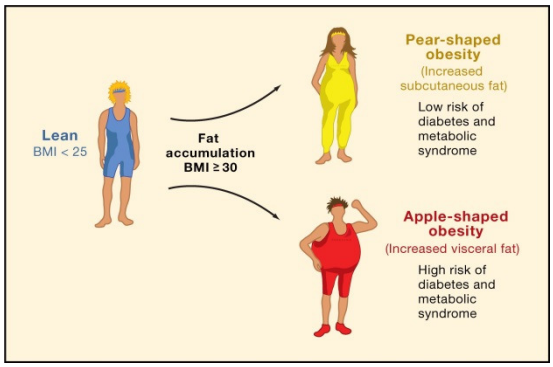

Figure 1. Fat distribution influences risks associated with obesity in humans. Gesta et al., published in Cell (2007).

1.1.2 BROWN ADIPOSE TISSUE

In contrast to white adipocytes, brown adipocytes are ellipsoid-shaped small cells with multilocular lipid droplets. The brown color of brown adipose tissue (BAT) is primarily due to a high mitochondrial content of the resident adipocytes, which specifically express uncoupling protein 1 (UCP1) also known as thermogenin. Indeed, BAT is a specialized organ that is critically important for heat generation and lipid oxidation (Richard and Picard 2011). In humans, it is present in newborns (Cannon and Nedergaard 2004) but decreases quickly and has been considered insignificant in adults. However, more recent studies indicate that human BAT is not as insignificant as once believed (Nedergaard, Bengtsson et al. 2007), but may regulate whole-body metabolism even in adulthood (Cypess, Lehman et al. 2009). In rodents, BAT is mainly located in the interscapular region and plays an important role in temperature regulation and metabolism across their whole life.

Interestingly, fate-mapping studies combined with cell sorting analysis have shown that distinct mesenchymal progenitors give rise to white and brown adipocytes. Skeletal muscle cells and preformed brown adipocytes share a common ancestry and originate from myogenic factor 5 (MYF5)-expressing precursor cells, while white adipocytes originate from precursor cells that are MYF5 negative (Timmons, Wennmalm et al. 2007). Brown and white adipocytes display in many ways opposite functions although both contain lipids and play a crucial role in whole-body metabolism.

1.2 BROWNING

Browning of WAT can be defined as a process where there is a significant increase in adipocyte UCP1 levels. The resultant adipocytes are referred to as beige, brite (brown-to-white), convertible, ectopic, inducible or recruitable adipocytes (Nedergaard and Cannon 2014). Simply speaking, adipose tissue browning is thus the process of turning white adipocytes into brown-like adipocytes that display increased mitochondrial content, increased UCP1 levels and elevated metabolic rate.

Adipose tissue browning can be triggered by cold temperature, food components, gene modifications or drugs. The list of browning agents is growing rapidly, and more than 50 agents have been identified (Wu, Cohen et al. 2013). The first report of a browning process shows increased UCP1 expression in clusters of GWAT adipocytes in BALB/c mice exposed to cold temperature (Young, Arch et al. 1984). The browning process has thus been

studied during many years, but it was not until the last decade that the term "browning" became broadly used. The current paradigm is that browning can be triggered by two different pathways (Nedergaard and Cannon 2014):

- Through pharmacological or sympathetic nerve system-mediated activation of β-adrenergic receptors (β3-AR) in white adipocytes.
- Through pharmacological induction of the peroxisome proliferator-activated receptor γ (PPARγ) pathways.

Both these pathways involve three master regulators of the browning process: PPARγ, PR domain containing 16 (PRDM16) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α).

In 2014, a new mechanism driving cold-induced browning process *in vivo* is suggested (Qiu, Nguyen et al. 2014). In brief, Qiu and colleagues propose that cold temperature is associated with an increased local production of catecholamines by WAT-resident M2-polarized macrophages. These catecholamines stimulate β3-ARs leading to increased expression of thermogenic genes in adipocytes. However, this suggested mechanism remains controversial (Fischer, Ruiz et al. 2017). Moreover, it is not clear whether cold temperature exposure and pharmacological activation of the β3-AR stimulate the same cell populations that finally results in browning (Jiang, Berry et al. 2017). Thus, different browning agents are likely triggering distinct pathways in different adipocytes and/or precursor cells leading to slightly different browning processes although the final result may appear similar. One of the most used methods to study the browning process and its consequences is however pharmacological stimulation of the β3-AR. Amongst several options, a compound named CL316,243 is widely used because of its high specificity and potency (Peng, Gennemark et al. 2015).

1.2.1 BEIGE ADIPOCYTES

The beige adipocyte is considered to be a distinct cell type that has mixed characteristics from both white and brown adipocytes. They are spherical cells, smaller than white adipocytes with increased mitochondrial content and multilocular lipid content. Upon stimulation they can activate their thermogenic capacity through increased UCP1 expression. In mice, beige adipocytes are dispersed mainly across subcutaneous white adipose tissue while fewer are to be found in visceral depots (Wu, Boström et al. 2012). Furthermore, beige adipocytes have been suggested to possess a distinct gene expression signature as compared to white and brown adipocytes (Wu,

Boström et al. 2012, Harms and Seale 2013), although the full validity of this signature has been questioned and more flexible borders between adipocyte cell types have been described (de Jong, Larsson et al. 2015).

There are studies showing that beige adipocytes share lineage of origin with white adipocytes (they are both $MYF5^-$) (Seale, Bjork et al. 2008), but upon stimulation they can transform their morphology to acquire the appearance and protein expression profile of brown adipocytes (Seale, Conroe et al. 2011). However, some beige cells have been shown to originate from the $MYF5^+$ lineage, suggesting heterogeneity within beige adipocytes (Sanchez-Gurmaches, Hung et al. 2012) (Figure 2).

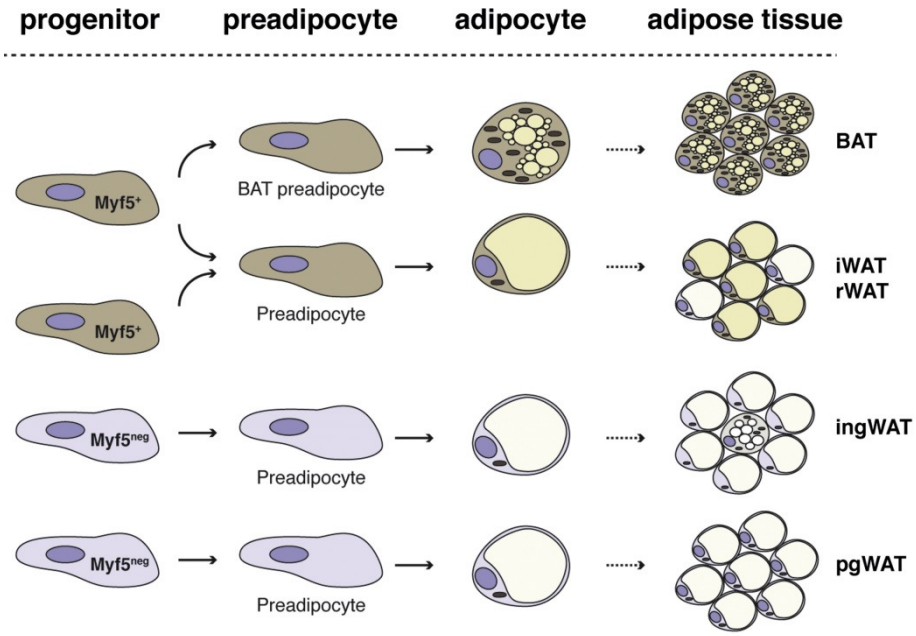

*Figure 2. Model of adipose tissue (BAT, interscapular WAT (iWAT), retroperitoneal WAT (rWAT), inguinal (ingWAT) , perigonadal (pgWAT) adipocyte development based on lineage analysis, image based on Sánchez-Gurmaches et al., in Cell Metabolism (2012) *Different acronyms are used on this figure compared to this thesis.*

Besides the origin of beige adipocytes, their formation is also debated. There are studies supporting the notion that beige adipocytes are recruited via *de novo* adipogenesis (Wu, Boström et al. 2012), but there are also several studies supporting the so-called transdifferentiation theory where beige

adipocytes originate from white adipocytes that are reprogrammed into brown-like adipocytes (Barbatelli, Murano et al. 2010).

Regardless of their lineage of origin and their formation process, beige adipocytes have become of great interest as a potential therapeutic target to reduce obesity-related co-morbidities. Beige adipocytes may reduce body weight via increased non-shivering thermogenesis (Kopecky, Rossmeisl et al. 2001, Kazak, Chouchani et al. 2015, Ikeda, Maretich et al. 2018) and/or increased oxidative capacity associated with improved whole-body metabolism (Granneman, Li et al. 2005, Duteil, Metzger et al. 2014).

1.3 MITOCHONDRIA

Mitochondria are cytoplasmic organelles found in most eukaryotic organisms. Their main role is energy biogenesis, typically providing 90 % of the cellular energy *via* the electron transport chain/ATP production. Therefore, they are usually termed the powerhouse of the cell. However, they are also involved in other cellular processes such as apoptosis, Ca^{2+}-signaling and redox homeostasis (Duchen and Szabadkai 2010, Spinelli and Haigis 2018).

Mitochondria have a double membrane arrangement which separates the organelle into four distinct compartments: the outer membrane, the intermembrane space, the inner membrane, and the matrix. The outer membrane is quite permeable and controls diffusion of molecules into the space between the outer and inner membranes. The intermembrane space contains proteins that play major roles in mitochondrial energetics and apoptosis. In contrast to the outer membrane, the inner membrane is highly impermeable and most ions and molecules require transporters to cross. It contains around 20 % of the total mitochondrial protein composition, amongst which are protein transporters into the matrix (e.g. translocase of the inner membrane) and the enzymes of the electron transport chain. In order to expand the capacity for chemical reactions, the area of this membrane is increased by several *cristae*, i.e. folds in the inner membrane. The matrix contains most of the enzymes that are responsible for the reactions of the citric acid cycle (McCarron, Wilson et al. 2013).

Mitochondria cannot be generated *de novo*, thus new organelles arise from pre-existing ones, through a multi-step process which involves both fusion and fission events. This is a tightly regulated process, dependent on the activity of both mitochondrial and nuclear factors. PGC1α is a major

regulator of mitochondrial biogenesis, activating different transcription factors such as nuclear respiratory factor 1 and 2, that further promote transcription of key mitochondrial enzymes (Jornayvaz and Shulman 2010). The cyclic adenosine monophosphate (cAMP) level is one of the upstream signals that regulate the expression of PGC1α. Interestingly, β3-AR activation results in increased cAMP levels, leading to the protein kinase A (PKA)-dependent activation of the cAMP response element-binding protein (CREB), which in turn upregulates PGC1α expression, resulting in new mitochondria.

The mitochondrial content and function in adipocytes are tightly linked to the cell type. White adipocytes contain few mitochondria that primarily functions as ATP-producers, but they also provide key intermediates needed for *de novo* lipogenesis such as glycerol 3-phosphate and acetyl-CoA (Cedikova, Kripnerov et al. 2016). Beige adipocytes, prior activation, have a slightly higher mitochondrial content with similar basal UCP1 levels as white adipocytes (Wu, Boström et al. 2012). Upon stimulation, their UCP1 level increases together with an increment in mitochondrial content (Velazquez-Villegas, Perino et al. 2018). Brown adipocytes contain many mitochondria, whose activity is dedicated mostly to non-shivering thermogenesis (Cedikova, Kripnerová et al. 2016).

1.3.1 UCP1

Non-shivering thermogenesis in brown (and beige) adipocytes' mitochondria is essentially dependent on UCP1. This protein is a 33kDa monomer, composed by 306 amino acids, that is encoded by nuclear DNA. The structure of the UCP1 gene is highly conserved in mouse, rat and human: six exons encompass the coding sequence and each exon encodes a transmembrane domain (Ledesma, de Lacoba et al. 2002). Its sequence is also highly homologous among species, suggesting an important role in metabolism. It is mainly expressed in brown adipocytes but also in activated beige adipocytes (Nedergaard, Golozoubova et al. 2001, Wu, Boström et al. 2012) and in other cell types such as thymocytes (Adams, Carroll et al. 2008).

UCP1 acts as a proton transporter over the mitochondrial inner membrane. It mediates the passive re-entry of protons into the mitochondrial matrix, producing heat and concomitantly decreasing the yield of ATP .Thus, UCP1-mediated uncoupling of the electron transport chain enables the oxidative metabolism to run at maximal rate leading to increased heat production

(Labbe, Caron et al. 2015). Moreover, UCP1 is activated by fatty acids and inhibited by ATP (Jastroch, Divakaruni et al. 2010) and the *Ucp1* gene is under extensive transcriptional control: in response to cold temperature or overfeeding, activation of β-ARs initiates a cAMP signal transduction pathway that activates *Ucp1* transcription mediated by three nuclear receptors: PPARγ, thyroid hormone receptor (TR) and retinoic acid receptor (RXR) (Divakaruni and Brand 2011) and the essential coactivator PGC1α (Puigserver, Wu et al. 1998) as shown in Figure 3.

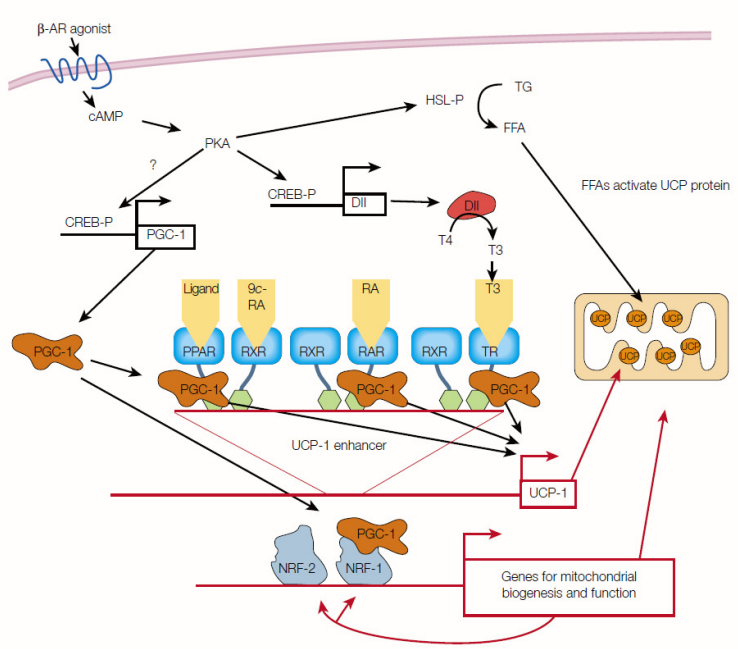

Figure 3. Ucp1 transcription regulation through β-AR activation. Lowell et al., published in Nature (2000).

1.3.2 REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are chemically reactive species containing oxygen, generated by cells. There are radical molecules such as superoxide ($O_2^{-\bullet}$), peroxyl ($RO^{\bullet}$) and hydroperoxyl ($HO_2^{\bullet}$) and non-radical ones e.g. hydrogen peroxide (H_2O_2), but the degree of reactivity is independent of having unpaired electrons (Stowe and Camara 2009). In adipocytes, there are many ROS sources:

- Mitochondria, which harbor the bulk of oxidative pathways, contain many enzymatic complexes that potentially can transfer single electrons to oxygen and convert it into superoxide anion in a process referred to as electron leak (Jastroch, Divakaruni et al. 2010).
- Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is described as a major contributor of ROS in adipocytes (Han, Umemoto et al. 2012).
- Nitric oxide synthase can, under certain pathological conditions, be uncoupled to produce superoxide (Le Lay, Simard et al. 2014).

The basic initiating step in ROS production is the single reduction (transferring of one electron) of molecular oxygen into superoxide (Andreyev, Kushnareva et al. 2015). This reaction is the rate-limiting step in the whole cascade. Once formed, it is involved in several reactions that, in turn, generate other ROS such as hydrogen peroxide, hydroxyl radical ($OH^{\bullet}$), peroxynitrite ($ONOO^{-}$) and hypochlorous acid (HOCl) (Pizzino, Irrera et al. 2017).

ROS have long been considered inevitable by-products of cellular processes. 0.15 to 2 % of the consumed O_2 during respiration is reduced by single electrons, generating superoxide (Brand 2010). ROS can react with DNA, proteins and phospholipids leading to an irreversible damage to the cell. However, in recent years, accumulating evidence indicates that ROS also serve as critically important signaling molecules in cell proliferation and survival (Ray, Huang et al. 2012). Therefore, a tight control of ROS production and elimination is required for cell homeostasis.

1.3.3 REDOX REGULATION

Systems to control ROS and the redox state under physiological conditions are spread throughout the cell. Indeed, it is equipped with enzymatic and nonenzymatic antioxidants that eliminate ROS and maintain redox homeostasis.

Superoxide dismutases (SOD) are a major class of enzymatic antioxidants, which catalyze the dismutation of $O_2^{-\bullet}$ to H_2O_2 without any support of reducing equivalents for their ROS decomposition activity. There are multiple isoforms that can be found in different cellular compartments (Trachootham, Lu et al. 2008). SOD1, which contains Cu and Zn and is found in the cytoplasm, mitochondrial intermembrane space, nucleus, and

lysosomes, accounts for 70-80 % of the total cellular SOD activity (Halliwell 1989). SOD2, a key mitochondrial antioxidant enzyme which contains Mn, is mainly found in the mitochondrial matrix (Fukui and Zhu 2010). SOD3 is an extracellular Cu/Zn dismutase that is expressed in a limited number of tissues (Ookawara, Imazeki et al. 1998).

Hydrogen peroxide, the resulting product of SOD activity, can be neutralized by three different defense systems: catalases, thioredoxins (TRX) / peroxiredoxins (PRDX) and/or glutathione-based.

Catalase is an enzyme that catalyzes dismutation of hydrogen peroxide to oxygen and water, and is present in all aerobic cells. It is located in peroxisomes and is one of the most efficient enzymes known, resulting in reaction rates approaching the diffusion-controlled limit (Vainshtein, Melik-Adamyan et al. 1981).

The second system for hydrogen peroxide clearance includes the tandem TRX / PRDX that, as a whole system, scavenge hydrogen peroxide and use NADPH as a reducing agent. Similarly to SODs, there are multiple isoforms of PRDX that can be found in different subcellular locations (Wood, Schroder et al. 2003). Briefly, hydrogen peroxide is reduced to water by PRDX, in a two-step reaction. The resulting oxidized form of PRDX is reduced back by TRX, which in the end will be reduced by NADPH (Hanschmann, Godoy et al. 2013). Interestingly, PRDX3, in the mitochondrial matrix, contributes to around 90 % of the total ROS removal (Andreyev, Kushnareva et al. 2015).

The last system is based on glutathione. Glutathione (γ-glutamylcysteinyl-glycine) is a thiol-containing tripeptide that is found at high concentrations (1–10 mM) in all eukaryotes and many prokaryotic species (Sikanyika, Aragao et al. 2019). Glutathione can exist in a reduced state (GSH) or an oxidized state (GSSG), which consists of two GSH molecules that are linked together by a disulfide bond. Different subcellular locations harbor different glutathione pools. For example, the cytosolic glutathione pool is highly reduced, with a GSH:GSSG ratio in the order of 100:1, whereas the ratio in the mitochondrial matrix is around 30:1 (Zhang, Limphong et al. 2012). GSH is exclusively produced in the cytosol and is further transported into the mitochondrial matrix (Calabrese, Morgan et al. 2017). Similar to peroxiredoxins, hydrogen peroxide in the mitochondria can be reduced by glutathione peroxidase, using GSH as substrate, producing GSSG. This oxidized molecule can be reduced back to GSH using NADPH as a reducing equivalent by glutathione reductase.

The hydrogen peroxide removal systems rely on the NADPH/$NADP^+$ redox state to be efficient. Thus, optimal mitochondrial bioenergetics function is required for the proper antioxidant activity of these systems. Moreover, there is a complex interaction between PRDXs, TRXs and the glutathione systems in the mitochondrial matrix; a slight change in the balance of one of these systems may change the redox state of the whole cell and thereby affect many cellular functions including metabolic processes.

1.4 MITOCHONDRIAL DYSFUNCTION

Mitochondrial dysfunction is a term that has been used to define the incapacity of mitochondria to generate and sustain sufficient ATP levels, through oxidative phosphorylation (OXPHOS), in response to energy demands (Kusminski and Scherer 2012). However, it can also be applied to describe all maladaptive physiological responses of mitochondria involving processes such as substrate catabolism, calcium buffering, iron transport, intracellular mitochondrial shape and location, apoptosis, and ROS production. Specifically in white adipocytes, the alterations in mitochondrial function can e.g. involve impaired oxidative capacity, elevated ROS production and/or altered mitochondrial turnover (Boudina and Graham 2014).

Oxidative stress is the specific term to describe the mitochondrial dysfunction that includes an elevation in ROS production and/or incapacity of the cellular defense system to neutralize ROS (Schieber and Chandel 2014). Main consequences of oxidative stress in WAT are impairment in adipogenesis leading to adipocyte hypertrophy, tissue inflammation and insulin resistance (Castro, Grune et al. 2016). Etiology of obesity-associated type 2 diabetes has been connected to excessive nutritional overload, leading to increased lipid-induced ROS formation (Kusminski and Scherer 2012) which, if sustained over time, can lead to chronic oxidative stress that results in e.g. insulin resistance (Houstis, Rosen et al. 2006) and reduced adiponectin secretion (Wang, Wang et al. 2013).

On the other hand, reductive stress, a less appreciated concept than oxidative stress, is defined as an excess of reducing equivalents (NADPH and/or GSH) in the presence of intact oxido-reductive systems (Brewer, Mustafi et al. 2013). However, similarly to oxidative stress, it has been connected to mitochondrial dysfunction (Zhang, Limphong et al. 2012), insulin resistance (Kobayashi, Matsuda et al. 2009) and etiology of many diseases (Pérez-Torres, Guarner-Lans et al. 2017).

Overall, both alteration of the redox homeostasis, oxidative or reductive stress, have deleterious effects on mitochondrial function that if chronically sustained, have negative implications in metabolism.

1.5 ANTIOXIDANTS

Antioxidant supplementation has been suggested to assist the cellular defenses to overcome the excess of ROS in several pathologies. Vitamins and cofactors such as vitamin C, E or carnitine together with polyphenols and carotenoids, commonly used as supplements, have been tested due to their high availability in food sources (Abdali, Samson et al. 2015). These supplements have been studied in a broad range of diseases, such as diabetes/metabolic syndrome (Abdali, Samson et al. 2015), atherosclerosis (Packer, Weber et al. 2001), cancer (Sayin, Ibrahim et al. 2014) or processes where ROS are considered to play an important role e.g. physical exercise (Ristow, Zarse et al. 2009).

More sophisticated compounds, that have a direct effect on mitochondria redox status, have also been studied. MitoQ, a derivative of ubiquinone conjugated to triphenylphosphonium (TPP^+), is accumulated in the mitochondrial matrix and has been used in animal models and in patients (Oyewole and Birch-Machin 2015).

N-acetylcysteine (NAC) has been suggested to possess antioxidant properties both by direct reaction of its sulfhydryl group with ROS or by increasing cellular GSH levels (Kelly 1998, Mokhtari, Afsharian et al. 2017). It has been broadly studied in animal models and patients in a wide range of conditions such as chronic obstructive pulmonary disease, HIV, cancer and insulin resistance (Kelly 1998, Fulghesu, Ciampelli et al. 2002, El Midaoui, Ismael et al. 2008).

Antioxidant treatment studies in patients and mouse models have reported conflicting results i.e. positive effects where harmful oxidative stress is reduced (Udupa, Nahar et al. 2012), no impact on ROS levels (Choi and Ho 2018) or deleterious outcomes such as increased levels of oxidative stress markers in blood, prevention of health-promoting effects of physical exercise, or increased melanoma progression (Kleinveld, Demacker et al. 1992, Ristow, Zarse et al. 2009, Le Gal, Ibrahim et al. 2015).

For NAC specifically, there are experimental mouse studies where an improvement in mitochondrial function by reduction of oxidative stress is

reported (Wright, Renoir et al. 2015) whereas in *in vitro,* there are reports showing that NAC causes reductive stress, leading to mitochondrial dysfunction (Zhang, Limphong et al. 2012).

Overall, the administration method, treatment length and dosage appear extremely important for the final outcome of antioxidant supplementation.

1.6 HORMESIS

Hormesis is defined as an adaptive response of cells and organisms to a moderate (usually intermittent) stress (Mattson 2008). It is a process that was first observed in the 19th century in yeast exposed to small doses of toxins, which oppositely to what one expected, showed increased growth rate and metabolism. It was not until 1943 the term hormesis, using the Greek word for "excite", was used to describe this U-shaped dose–response relationships (Southam 1943). The hormesis concept has thereafter been applied to different aspects of cellular behavior, but mitochondrial hormesis (mitohormesis) was not mentioned until 2006 (Tapia 2006). Analyzing previously published data, Tapia suggests that intermittent induction of ROS in mitochondria (mild oxidative stress), by different interventions such as: dietary restriction, exercise, consumption of pro-oxidant or mitochondrially injurious biochemical compounds might exert protective effects through beneficial cellular adaptations to these ROS increases. Since then, mitohormesis has been shown in *C. Elegans* using DNA mutagens (Yang and Hekimi 2010), using antioxidants (Oh, Park et al. 2015) and glucose restriction (Schulz, Zarse et al. 2007), in cardiac and skeletal muscles using statins (Bouitbir, Charles et al. 2012), myoblasts using antioxidants (Singh, Charles et al. 2015), in liver and fibroblasts from genetically modified mice (inducible SOD2 model) (Cox, McKay et al. 2018) and very recently in mice using radiation as a pro-oxidant agent (Zhang, Humes et al. 2018). Interestingly, nutrient restriction can lead to mitohormesis in adipocytes but treatment with antioxidants prevents this hormetic effect (Lettieri Barbato, Tatulli et al. 2015) (Figure 4). Thus, there are conflicting reports regarding the effects of antioxidant treatment also on mitohormesis.

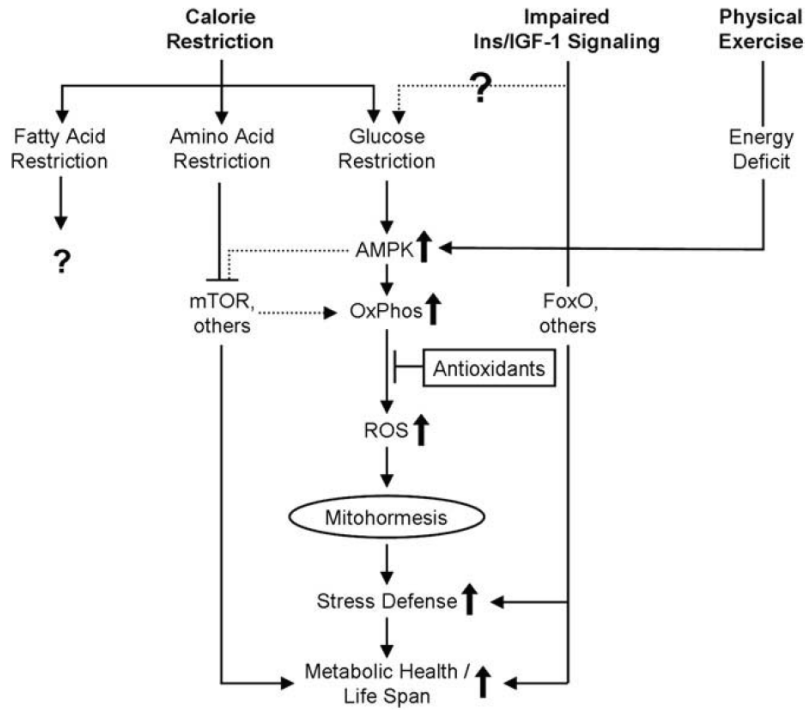

Figure 4. Mitohormesis suggested mechanism of action. Ristow et al., published in Experimental gerolontogy (2010).

2 AIM

The general aim of this thesis is to elucidate the effect of antioxidant supplementation on mitochondrial function in white and brown adipose tissue.

The specific aims are:

1. To delineate the role of ROS in chronic β3-AR activation-induced browning of white adipose tissue by the use of antioxidants as scavenging agents.

2. To establish the pro-oxidative effect of antioxidants and their effect on mitochondrial function in white and brown adipocytes.

3. To evaluate the effect of N-acetylcysteine supplementation on mitochondrial function of adipose tissue and whole-body metabolism.

3 METHODS

In our studies, we have used a broad range of resources and analysis methods. We have performed *in vitro* and *in vivo* experiments, using C57BL/6J mice, primary cells and immortalized cell lines. We have studied functional processes with live cells and tissues, such as ROS production and cellular respiration. We have also analyzed cellular lipid and mitochondrial content in freshly dissected tissues. Moreover, we have analyzed the outcomes of the experiments in processed tissues and cells using commercially available kits to determine the levels of GSH, lactate, glycerol, free fatty acid (FFA) or insulin. These kits require tissue or cell processing to purify or concentrate samples for analysis.

In this section, I will address the significant or controversial aspects of the most relevant methods and resources that I developed and used during this project. For descriptions of all methods, please read methods section of papers I and II.

3T3-L1 adipocytes

The murine 3T3-L1 cell line was established in 1974 and is a widely used *in vitro* adipocyte model that has been employed to study e.g. white adipocyte differentiation, lipid metabolism and endocrine function (Greenberger and Aaronson 1974). Untreated 3T3-L1 cells have fibroblast morphology and can be differentiated into lipid-storing adipocytes through a 10-day differentiation protocol. Their initial morphology i.e. multipolar and elongated shape is sequentially transformed into polygonal and rounded structure with multilocular lipid droplets (Figure 5).

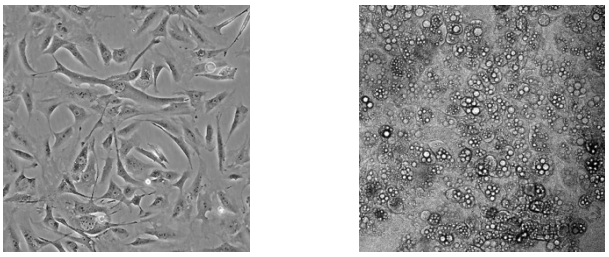

Figure 5. 3T3-L1 untreated (left) and after differentiation process (right).

The differentiation protocol used in this project is essentially the same as described by Kohn and colleagues (Kohn, Summers et al. 1996). In brief,

addition of a differentiation cocktail containing insulin, dexamethasone and phosphodiesterase inhibitor, activates adipogenic processes resulting in the production of adipocyte-related proteins including the β3-AR (Komai, Musovic et al. 2016). Importantly, treatment of mature 3T3-L1 adipocytes with the β3-AR agonist CL316,243 induces a browning program as judged by upregulation of *Prdm16*, *Pgc1α* and *Ucp1* (Duteil, Metzger et al. 2014). Thus, this model can be used to study, at least, some aspects of the browning process.

The overall advantage of using an immortalized cell line as a model is its higher degree of reproducibility, i.e. its homogenous genotypic and phenotypic characteristics, compared to primary cells that typically show greater variability. On the other hand, during their immortalization process they might lose some characteristics that are key characteristics to the model of interest.

Isolated primary adipocytes are non-attaching cells that are very fragile, and thus very difficult to handle and use in fluorescence microscopy or culture. Moreover, isolated adipocyte precursors, although being primary cells from the stromal vascular fraction, have to be differentiated *ex vivo* which gives them similar morphology as 3T3-L1 adipocytes and simultaneously also introduces an additional source of variability.

Primary brown adipocytes

Although there are several immortalized brown cell lines (Klaus, Choy et al. 1994, Klein, Fasshauer et al. 2002), isolated and differentiated primary brown preadipocytes are more likely to resemble 'real' brown adipocytes and thereby we believe those cells are the better option to elucidate the mechanism for our observed effect of antioxidant treatment on brown adipose tissue *in vivo*. The method of isolation and culture of brown adipocytes was adapted from the method described by Néchad and colleagues (Néchad, Kuusela et al. 1983). Brown adipose tissue from young mice (younger than 4 weeks), is required since there is a negative influence of age on the abundance of brown preadipocytes. Studies were performed in completely differentiated adipocytes (based on occurrence of multilocular lipid droplets) between days 8 and 9 from start of differentiation; only cultures in which >90 % of cells displayed adipocyte morphology were used.

Intracellular ROS generation in cultured adipocytes

ROS are not one single entity but represent a broad range of chemically distinct oxidant molecules with different biological reactivity. One of the

most widely used tools to analyze ROS are fluorescent probes/dyes. They are reducing agents that become fluorescent when reacting with any oxidant molecule. Therefore, we cannot simply attribute their fluorescence to a specific reactive molecule but rather generalize the discussion to multiple ROS and speculate about the most probable substrate based on the probe cellular localization (Kalyanaraman, Darley-Usmar et al. 2012). However, probe-based fluorescence analyses together with measurements of cellular enzymes that possess well-established substrates facilitate the identification of the specific ROS compound that is most likely to be responsible for the ROS-associated fluorescence. The use of fluorescent probes for measuring ROS is complex since their nature as reducing agents interferes with cellular biochemistry in a dynamic manner. Their use requires therefore extensive titration to determine the minimum needed working concentrations (Polster, Nicholls et al. 2014), microscopy imaging to check cellular locations and distribution of fluorescent dyes and, most importantly, extra caution when drawing conclusions from measurements. Although other indirect methods could be used to estimate ROS levels, such as measurement of ROS-related damage e.g. protein oxidative modifications, it would be very difficult to determine the ROS origin and to follow changes in ROS production over time. Moreover, transiently increased ROS levels do not necessarily lead to measurable damage. Thus, we strongly believe that fluorescent probes, despite their complexity, are the best available option for ROS measurements in this project.

CM-H_2DCF-DA is a chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate, a cellular probe that becomes fluorescent when it is oxidized by the loss of two electrons (Ex/Em: 495/520 nm) in a two-step process. The chloromethyl presence provides better cellular retention of the probe. Indeed, this probe is commonly used to measure H_2O_2 and other oxidants, or to monitor stimulation-induced changes in redox signaling in cultured cells (Wang, Si et al. 2010, Wojtala, Bonora et al. 2014). Although this probe cannot identify specific oxidative species, it is - because of its homogeneous intracellular distribution through passive diffusion - a good indicator for the general ROS production.

MitoSox Red, also called mito-hydroethidine or mito-dihydroethidium, is a fluorescent dye that measures $O_2^{-\bullet}$ production in mitochondria (Ex/Em: 520/580 nm). It is simply hydroethidine, a fluorescent probe, conjugated to triphenylphosphonium, which enables its accumulation in the mitochondrial matrix i.e. the positively charged MitoSox redistributes across the plasma and mitochondrial membranes according to its Nernst potential. Although some concerns about its subcellular location (Polster, Nicholls et al. 2014) and

reactivity with other oxidative species, it is considered suitable for measuring mitochondrial ROS, mostly $O_2^{\bullet-}$, at least in normally polarized mitochondria (Kalyanaraman, Darley-Usmar et al. 2012).

In order to quantify the effect of β3-AR stimulation on general ROS production, fluorescence images with laser scanning confocal microscopy (LSM 700 from Zeiss) were obtained at baseline followed by sequential measurements after addition of CL316,243 using different concentrations of CM-H_2DCF-DA. After the minimum working concentration had been stablished, experiments in 12-well plates were performed and the total fluorescence level at every time point was quantified by calculating averages of 12-point readouts per well, covering the whole area of the well.

Similarly, for titration and localization of MitoSox fluorescence together with the evaluation of the possibility of nuclear MitoSox staining, we used laser scanning confocal microscopy. The analysis of the resulting images, indicate that MitoSox nuclear-related fluorescence (Polster, Nicholls et al. 2014) is unlikely to contribute to the observed differences in the MitoSox measurements across groups.

2-photon excitation fluorescence and coherent anti-Stokes Raman scattering microscopy

Imaging lipids and mitochondria in freshly dissected tissues without any isolation or digestion is an extremely powerful tool that allows accurate measurements of cellular lipid content and mitochondrial density/activity.

Coherent anti-Stokes Raman scattering microscopy (CARS) uses the inherent properties of stored lipids in cells, without any exogenous labeling. The specific vibration at 2845 cm^{-1} of carbon-hydrogen bonds in the alkylic chains of the highly dense triglycerides in lipid droplets is probed in a CARS process by overlapping two picosecond-pulsed laser beams at wavelengths 817 nm and 1064 nm in the focal plane of an inverted optical microscope.

Rhodamine 123 is a cationic fluorescent compound that binds specifically to mitochondria (Darzynkiewicz, Traganos et al. 1982) and displays fluorescent activity proportionally to the mitochondria's membrane potential (Huang, Camara et al. 2007). It has an excitation wavelength that peaks at 505 nm and an emission wavelength at 560 nm. It can be excited with a laser emitting at 817 nm following a 2-photon excitation (2-PE) process (Tehrani, Pendleton et al. 2017). By the use of an infrared wavelength laser, we reach more penetration in the sample together with reduced photobleaching.

A small piece of adipose tissue (<1 mm^3) is collected from the same anatomical region in each animal and stained with Rhodamine 123. Indeed, taking adipose tissue pieces from the same region within a depot is critically important because of the heterogenic nature of adipose tissue. By means of dichroic mirrors and high optical-density filters, the forward-scattered CARS signal and backscattered 2-PE fluorescence signal are recorded using single photon counting detector technology. Multiple planes, each with 512x512 pixels, are recorded resulting in 3D images of the tissues. Three different areas are recorded per sample. Quantitative data from the CARS and 2-PE fluorescence images are determined using ImageJ software. Regions of interest are defined by outlining the cell from autofluorescent images or by viewing z-stacks of the individual images. A voxel counting procedure is used to determine the number of voxels in the defined cell that meet a threshold intensity setting in the different analyzed channels (663 nm for CARS, 514 nm for Rhodamine 123) revealing cell size, lipid and mitochondrial content. In each image, 5 cells were analyzed.

Gene expression analysis

In order to quantify the messenger RNA (mRNA) expression levels of a gene, tissue or cells of interest were lysed and RNA was isolated using a commercial kit from Promega. When adipose tissue is processed, prior to isolation, an extra lipid elimination step is required. The concentration and quality of the isolated RNA was determined by absorbance measurement. Thereafter, the RNA was transcribed into complementary DNA (cDNA) generating the starting material for the quantitative real time polymerase chain reaction (qRT-PCR). Through continuous PCR cycles, a fluorescent (SYBR green)-tagged amplicon of the gene of interest was generated in a geometrical progression fashion, and its concentration was measured by a fluorometer. The threshold cycle, C_T, indicates the fractional cycle number at which the amount of amplified target gene reaches a fixed threshold. It is inversely proportional to the expression of the target gene. For quantification purposes, there are two different methods:

- Absolute quantification of a gene in the lysate can be calculated by interpolation of the readout in a calibration curve. This method is however not commonly used since accurate standard samples are required and it is usually unnecessary to know the absolute transcript copy number.
- Relative quantification describes the change in expression of the target gene relative to either a standard sample (standard curve method) or the C_T-

value of a reference gene (comparative C_T method) and then often relative to a reference group such as an untreated control or a sample at time zero in a time-course study.

In this work the comparative C_T method was used and the gene-expression levels relative to untreated control were calculated using the $2^{-\Delta\Delta CT}$ formula (Livak and Schmittgen 2001). To use this method, two conditions need to be met:

- Equal and high primer efficiency for all genes. To validate primer efficiency, a standard curve is created by serial dilutions (commonly 1:10) of a cDNA sample and qRT-PCR for each sample in duplicate is performed. Linear regression of results, allows calculating efficiency, which optimally should be found between 90 and 110 %. To keep constant efficiency across genes and samples one should also use the same transcription polymerase (in our case Fast SYBR-Green Master Mix) throughout the experiment.
- Constitutive expression of the reference gene in all groups i.e. the expression should not be altered by the experimental conditions. Thus an equal amount of sample should, independently of the experimental group, result in the same C_T value.

Oxygen consumption rate

One of the most informative analyses of mitochondrial function is quantification of cellular respiration, since it directly reflects electron transport chain performance/impairment and depends on many sequential reactions from glycolysis to oxidative phosphorylation. Oxygen consumption rate (OCR) is classically measured by the use of a Clark-type electrode, which is time-consuming, limited to whole cells or mitochondria in suspension and tied to manual injections of reagents (Yepez, Kremer et al. 2018). The XF Seahorse instruments have, on the other hand, an integrated automatic drug delivery system and allow OCR measurements in several samples at the same time in a 24 or 96-well plate format. Samples can be either isolated mitochondria, cultured cells or even tissues.

OCR analysis of isolated mitochondria provides useful information about their electron transport chain functionality without cellular interferences. In our case, we used succinate as the electron provider and rotenone to block

electron backflow to complex I to measure basal respiration of isolated mitochondria from adipose tissue.

Cultured cells have the advantage of providing the machinery necessary for metabolizing and generating natural substrate to the mitochondria. By sequentially adding oligomycin (that blocks ATP synthase), FCCP (that uncouples mitochondria) and finally a mixture of antimycin A and rotenone (that completely stop electron flow through the transport chain) to plated cells, different mitochondrial characteristics can be calculated from the OCR data (Figure 6).

Measurements of cellular respiration in white adipose tissue (Dunham-Snary, Sandel et al. 2014) allow mitochondrial status analysis without possible disturbances due to the cellular or mitochondrial isolation procedure. In our experimental conditions, the diffusion of drugs into the tissue pieces was however very heterogeneous. Thus, we could only with sufficient confidence record basal respiration while other mitochondrial parameters could not be accurately calculated.

Seahorse is an extremely sensitive instrument that requires careful data analysis to remove non-responding wells or technical outliers. There can be significant variation between plates, i.e. a small variation in e.g. the amount of injected solutions and the quality of the starting material can lead to completely different absolute OCR values. To minimize this inter-plate effect, we therefore report ratios of OCR levels i.e. normalization to control OCR level and data are normalizing to the cell number for each well. As shown in Figure 6, basal respiration is calculated by subtracting the average of the last 3 measurements, which correspond to the non-mitochondrial respiration, to the average of the first 3 measurements. Then an average value is obtained for each group and finally values are normalized to a control group, thus obtaining a ratio. Statistical analyses are performed on these ratios.

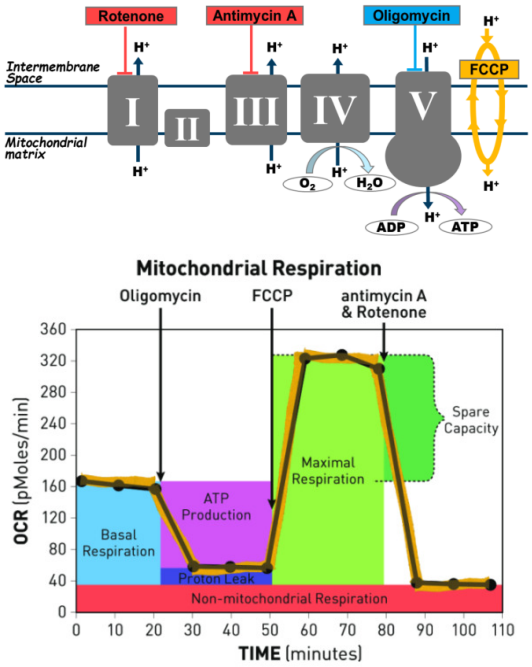

Figure 6. Agilent Seahorse XF Cell Mito Stress test: (top) modulators of the electron transport chain are used to (bottom) calculate different respiration parameters that can be used to delineate the mitochondrial function of cultured cells, published by Agilent (2019).

Body composition

Dual-energy X-ray absorptiometry (DEXA) is a tool for determining body composition *in vivo*, providing measures of fat mass, bone-free lean tissue mass, total-body bone mineral, and total-bone mineral density. DEXA has been adapted to accurately measure body composition in anesthetized rodents by using specialized software in conjunction with clinical whole-body DEXA machines (Nagy and Clair 2000). It provides an accurate measurement on total body fat, but cannot accurately provide information about the fat distribution. Thus, other measurements such as weighing individual adipose depots and/or CT scans are necessary to fully draw conclusions about fat distribution.

NAC treatment

NAC has been used in therapeutic practices for more than 40 years. It was first used as a mucolytic agent, but soon new potential applications were found and nowadays it has more than 10 different clinical uses (Lasram, Dhouib et al. 2015) with a broad dosage range. Moreover, it has been studied

in many different conditions e.g. fatigue reducing agent in sports (Petersen, McKenna et al. 2012) or type-2 diabetes (Ho, Chen et al. 1999), and in different models i.e. cell lines (Liu, Liu et al. 2017), *C. Elegans* (Oh, Park et al. 2015), rodents (El Midaoui, Ismael et al. 2008) and humans (Kleinveld, Demacker et al. 1992). In *in vivo* use, different administration ways are reported e.g. intraperitoneal injection (IP) (Wright, Renoir et al. 2015), supplementation of drinking water (Ma, Gao et al. 2016), food supplementation (Ikonne, Vann et al. 2019) or tablets (Fulghesu, Ciampelli et al. 2002). In rodents, it is usually administered through drinking water, with doses going from 1 g/L (Jang and Sharkis 2007) to 20 g/L (Pinniger, Terrill et al. 2017). In *in vitro* models, the concentration range of NAC used is quite broad: from 5 μM (Calzadilla, Sapochnik et al. 2011), up to 20 mM (Toyoda, Hayashi et al. 2004). However, 1 mM is one of the most commonly used concentrations, and this concentration of NAC has proven to effectively scavenge ROS (Inoguchi, Li et al. 2000, Maheshwari, Misro et al. 2011, Ali, Qadir et al. 2017). Therefore, we decided to use 1 mM NAC in our *in vitro* experiments.

In paper I, our initial aim was to study the role of ROS in adipose tissue browning in mice and to use NAC as our primary antioxidant. Thus, to start on the most conservative side to avoid potential deleterious effects that were reported with higher doses (>6.5 g/L), we picked the lowest reported dose (1 g/L in drinking water) and short pretreatment (1 week) (Palmer, Doctor et al. 2007, Sceneay, Liu et al. 2013). NAC supplementation was continued during the 10-day β3-AR stimulation period, but the total NAC exposure was still on the lower side compared to the majority of published studies. In paper II, our objective was to explore different doses and treatment length to evaluate the impact of both these factors on mitochondrial function in adipose tissue. We decided to use a very low dose, 0.5 g/L, which to our knowledge has not been tested in previously published studies, a middle range dose, 2 g/L, shown to be protective against high fat diet (HFD)-induced metabolic disorders (Ma, Gao et al. 2016) and a high dose, 10 g/L, reported to have deleterious effects (Palmer, Doctor et al. 2007).

Statistical analysis

GraphPad Prism 8 (GraphPad Software, San Diego, CA) was used for statistical analysis. Results in this thesis are represented as mean values ± SEM, expressed either as fold-change relative to controls or as absolute values. Comparisons were performed using one-way analysis of variance, two-way analysis of variance, or two-tailed Student's t test depending on the experimental layout. Data were log-transformed as necessary to achieve normal distributions; and $p<0.05$ was considered significant.

4 RESULTS AND DISCUSSION

Antioxidant treatment induces reductive stress associated with mitochondrial dysfunction in adipocytes (Paper I)

Browning is a process that can be triggered by β3-AR activation leading to the conversion of white adipocytes into beige adipocytes which have increased mitochondrial content and express higher levels of UCP1 (Nedergaard and Cannon 2014). Treatment of adipocytes with CL316,243, a potent and selective β3-AR agonist (Bloom, Dutia et al. 1992, Yoshida, Sakane et al. 1994, Li, Zhu et al. 2005), increases the intracellular levels of cyclic AMP, driving activation of the PKA complex leading to hormone-sensitive lipase phosphorylation (activation) and thereby the breakdown of triglycerides to FFAs and glycerol, i.e. lipolysis (Arner and Langin 2007). Elevated fatty acid levels increase the ROS production from non-mitochondrial sources such as NADPH oxidases in adipocytes (Han, Umemoto et al. 2012). Furthermore, β-adrenergic stimulation promotes OXPHOS in adipocytes (Duteil, Metzger et al. 2014) and consequentially mitochondrial ROS, as a by-product of oxidative metabolism, will also increase. We thus argue that β3-AR stimulation will, at least transiently, increase ROS levels in adipocytes. NAC is an antioxidant that can scavenge ROS both directly and indirectly as a precursor of glutathione (Kelly 1998, Mokhtari, Afsharian et al. 2017). Vitamin E, similarly, can directly scavenge oxidant radicals and alter redox status of the cell, i.e. glutathione cellular levels (Packer, Weber et al. 2001). Altogether, we hypothesize that such increase in ROS stimulates signaling pathways that trigger β3-AR activation-induced adipose tissue browning and consequently pretreatment with NAC and vitamin E should reduce the browning response.

ANTIOXIDANT EFFECTS IN 3T3-L1 ADIPOCYTES

NAC and vitamin E alter glutathione levels in 3T3-L1 adipocytes

To investigate the effect of antioxidant treatment in adipocytes' redox status, we treated 3T3-L1 adipocytes with different antioxidants. As shown in Figure 7, NAC and glutathione ethyl ester raised the levels of reduced glutathione by respectively, 20 and 10 %, shifting the redox balance towards a more reduced status. Interestingly, vitamin E treatment increased GSH to similar levels of direct glutathione ester treatment. NAC treatment has been shown to increase systemic GSH levels mainly through hepatic GSH synthesis (Atkuri, Mantovani et al. 2007), but here we thus demonstrate that adipocytes also increase their GSH levels in response to NAC treatment.

Similarly, vitamin E treatment has been shown to increase GSH levels in erythrocytes in patients (Jain, McVie et al. 2000) and in liver in rats (Scott, Kelleher et al. 1977), but the mechanism of this upregulation has not been established. Our results indicate that vitamin E affects the redox status of adipocytes directly, and increase the GSH levels in adipocyte to a similar extent as Scoot and colleagues reported for liver and kidney.

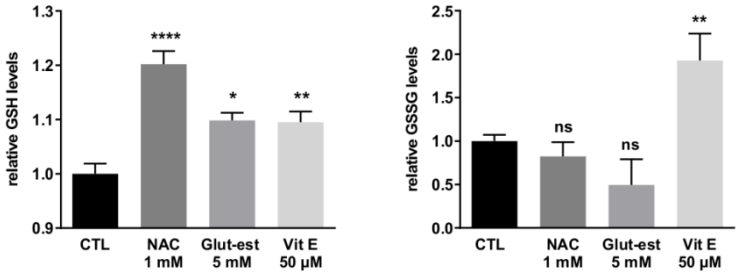

*Figure 7. (left) Relative GSH and (right) GSSG levels in 3T3-L13 adipocytes treated with or without NAC, glutathione ethyl ester and vitamin E for 48 h. (n=4/group, *p<0.05, **p<0.01 and ****p<0.0001).*

The absolute levels of GSSG are at least 10 times lower than the GSH levels in 3T3-L1 adipocytes and are calculated from measurements of total glutathione and GSH (page 2349 in Paper I: Experimental procedures; Measurements of GSH and GSSG). We believe that these facts, combined with the reduced accuracy when estimating GSSG levels that are as low as we observe, make the ratio calculations very unreliable (SD in GSSG are 5 to 7 times larger than in GSH levels). A slight variation in glutathione and GSH readouts during the analysis (optical density measurement) could thus easily have a strong impact of the estimated GSSG level and thereby lead to a misleading GSH/GSSG ratio. For this reason, we did not report the GSH/GSSG ratios. Rather our main conclusion is that the increase in GSH levels drives a change in the cells' redox state since GSSG levels do not change (except for vitamin E). In fact, even though GSH levels increase in all antioxidant treated groups only vitamin E generates a significant change in the calculated ratio, and this is surprisingly a decrease.

β3-AR activation has no effect on glutathione levels in 3T3-L1 adipocytes

β3-AR stimulation of cultured adipocytes had no effect on the intracellular GSH or GSSG levels (Figure 8). This indicates that either adipocytes rapidly restore their GSH levels (that might have been decreased due to ROS generated by NADPH oxidases and/or from mitochondrial sources) or

changes in GSH are too small to be detected by this method. Surprisingly, when NAC-pretreated cells are stimulated with CL316,243, GSH levels are similar to the levels of untreated cells. From these data, we can infer that both compounds can interact, resulting in decreased GSH levels, either by an elevation of ROS levels or interference in GSH production.

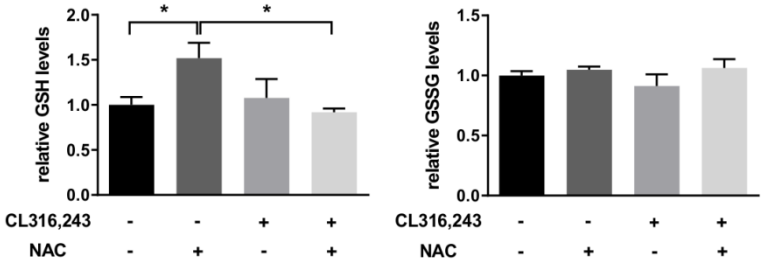

*Figure 8. (left) Relative GSH and (right) GSSG levels in cultured adipocytes treated with vehicle or 1 mM NAC for 48 h exposed to 1 µM CL316,243 or vehicle for. (n=4/group, *p<0.05).*

β3-AR activation increases ROS and Ucp1 production in 3T3-L1 adipocytes

To confirm that the expected CL316,243-induced browning response is in place in our 3T3-L1 adipocytes, we analyzed *Ucp1* mRNA expression. Indeed, CL316,243 treatment led to increased *Ucp1* levels. However, contrary to our hypothesis, NAC pretreatment had no effect on *Ucp1* expression as shown in Figure 9.

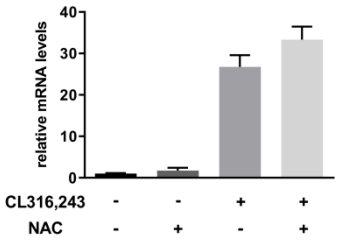

Figure 9. Ucp1 expression in cultured adipocytes pretreated with or without 1 mM NAC for 24 h after 60' incubation with 1µM CL316,243. (n=6/group).

Palmitate can stimulate NADPH oxidase 4 (NOX4)-derived ROS production in adipocytes (Han, Umemoto et al. 2012). To elucidate the possibility of a similar effect through β3-AR-induced lipolysis, we measured transient effects on the ROS production in 3T3-L1-derived adipocytes using CM-H_2DCF-DA

for 60 minutes. The ROS production increased 3-fold with adrenergic induction but, surprisingly, NAC pretreatment did not show any effect on the total ROS production in these lipolytic cells (Figure 10).

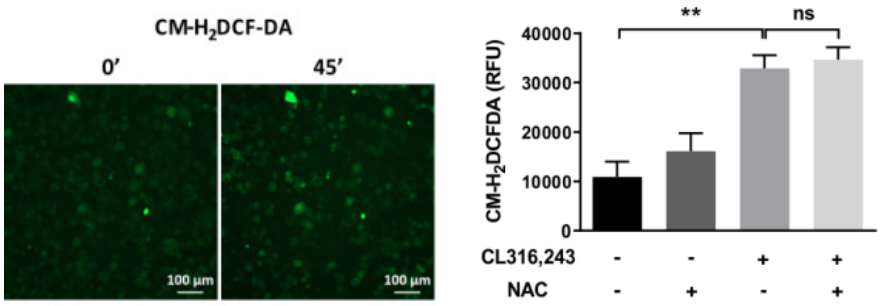

*Figure 10. (left) Representative ROS emission images (CM-H_2DCF-DA) of cultured adipocytes before and 45' after incubation with 5 µM CL316,243 and (right) quantification of the ROS emission of cultured adipocytes pretreated with or without 1 mM NAC for 24 h after 60' incubation with 1 µM CL316,243. (n=3/group, experiment repeated three times, **p<0.01).*

Next, we decided to selectively analyze the mitochondrial ROS production to investigate whether there is an effect of NAC treatment on the mitochondrial GSH pool leading to reduced ROS levels locally (that would not be picked up by measuring the total ROS levels by CM-H_2DCF-DA). Indeed, it surely was an effect by NAC treatment, but rather than seeing a decrease, NAC increased the mitochondrial ROS production in CL316,243-stimulated cells. While this result may appear surprising, it has been previously reported that increased GSH levels can lead to a too reduced state in the mitochondria that increases the ROS production through reduction of oxygen to superoxide anion (Korge, Calmettes et al. 2015). Moreover. since ROS has been shown to inhibit respiration in adipocytes (Wang, Si et al. 2010), we analyzed the oxygen consumption and the glycolytic activity (lactate production) in NAC pretreated cells with and without CL316,243 stimulation. We found reduced oxygen consumption rate (OCR) and increased lactate production in NAC-treated cells independently of adrenergic stimuli (Figure 11). Through analysis of the OCR in response to oligomycin, we found that this NAC-induced decrease in respiration is not due to reduced uncoupled respiration but rather is an effect from reduced oxidative phosphorylation.

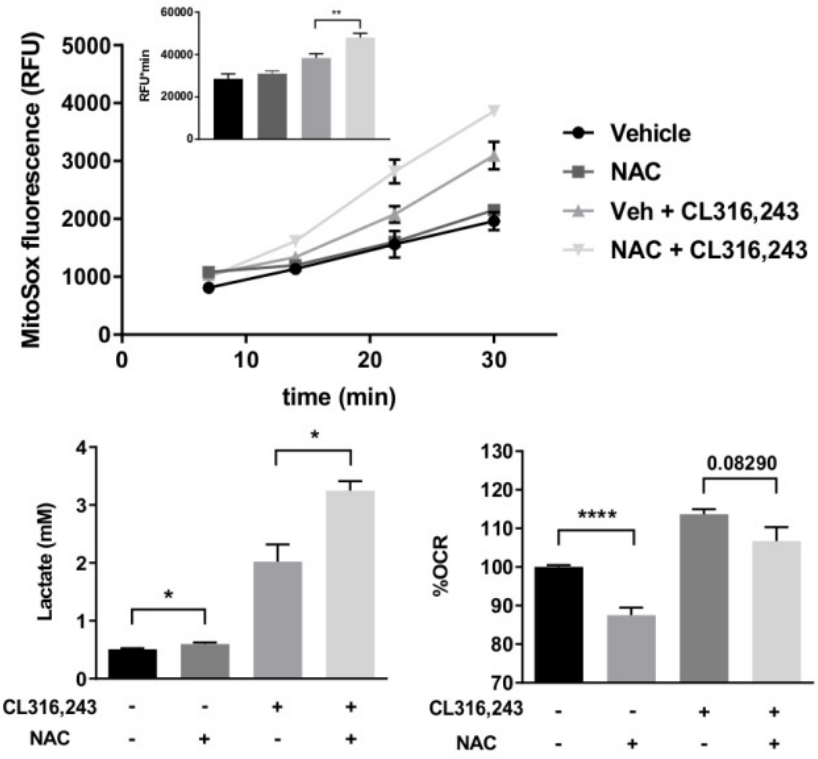

*Figure 11. (top) Area under the curve (AUC) quantification of MitoSox Red fluorescence of cultured adipocytes pretreated with or without 1 mM NAC for 24 h, 30' after incubation with 5 µM CL316,243. (n=3/group, **p<0.01). (bottom-left) Lactate levels in medium from cultured adipocytes incubated for 24 h with or without 1 mM NAC in combination with CL316,243 (n=3/group, *p<0.05). (bottom-right) Oxygen consumption rate of cultured adipocytes treated in presence or absence of 1 mM NAC for 48 h and thereafter exposed to 1 µM CL316,243 or vehicle during 20' (n=12/group, ****p<0.0001).*

There were no morphological changes or signs of cell death through visual inspection indicating that the reduced respiration in NAC-treated cells is a successful adaptation that limits the buildup of too high mitochondrial ROS levels. However, Wang et al. shows that shorter-term NAC treatment actually can scavenge ROS and this effect is associated with increased oxygen consumption. To elucidate this discrepancy, we analyzed basal OCR in 3T3-L1 adipocytes with different NAC concentrations during 30 minutes and a fixed dose for 24 and 48 h. Interestingly, the adipocytes display a completely opposite behavior depending on the exposure time. Short-time exposure leads to an increase in OCR i.e. under this condition NAC is likely acting as a ROS scavenger, leading to increased respiration (Figure 12).

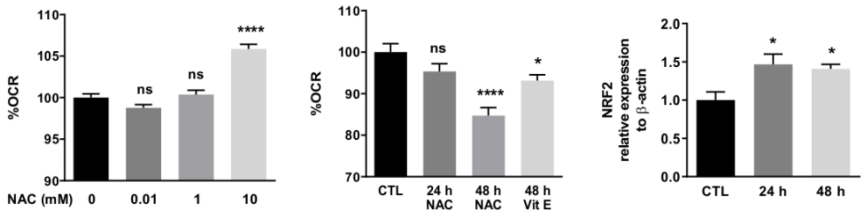

*Figure 12. (left) OCR of cultured adipocytes 30' after NAC treatment, at indicated concentrations (n=10/group, ****p<0.0001). (center) OCR of cultured adipocytes treated with 1 mM NAC or 10 µM vitamin E (n=12/group, *p<0.05, ****p<0.0001). (right) NRF2 protein levels in cultured adipocytes treated with 1 mM NAC at the indicated time points (n=4/group, *p<0.05).*

Longer-term treatment (≥ 24 h) with NAC, on the other hand, resulted in reduced cellular respiration most probably due to a compensatory response to put a lid on the rising mitochondrial ROS levels. In line with this assumption, NAC treatment leads to activation of the Kelch-like ECH-associated protein 1 (KEAP1)/Nuclear factor erythroid 2-related factor 2 (NRF2) pathway, a protective mechanism triggered by increased ROS-levels (Ma 2013), resulting in elevated levels of NRF2 protein (Figure 12) that in turn induce increased expression of cytoprotective proteins.

The difference between short- and long-term effects of NAC could be explained on the basis of NAC acting as an antioxidant, scavenging ROS in the acute exposure setting but triggering reductive stress i.e. increased ROS production, due to increased mitochondrial GSH levels, and thus reduced oxygen consumption and activation of the KEAP1/NRF2 pathway when treatment is prolonged.

Vitamin E effects in 3T3-L1 adipocytes

Vitamin E is a member, together with vitamin C and thiols (e.g. glutathione), of the so called antioxidant protective network of the cell, as shown in Figure 13 (Packer, Weber et al. 2001).

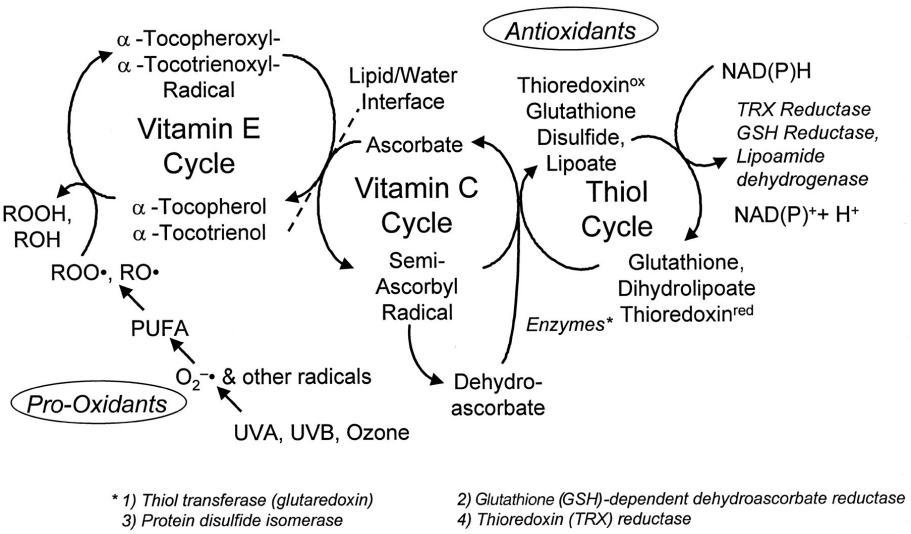

Figure 13. The antioxidant network showing the interaction among vitamin E, vitamin C and thiol redox cycles. Packer et al., published by The Journal of Nutrition (2001).

Vitamin E had similar consequences in cultured adipocytes as NAC although to a lesser extent i.e. vitamin E increased glutathione levels (as shown in Figure 7), that further increased β3-AR stimulation-induced mitochondrial ROS production associated with reduced OCR. Interestingly, vitamin E treatment at a lower dose (1 μM) than we used, has been shown to have no impact in mitochondrial function in murine mesangial cells, but when conjugated to TPP^+ to specifically target mitochondria, it reduces oxygen consumption by 30 % due to reduced oxidative phosphorylation associated with increased glycolysis (Reily, Mitchell et al. 2013). Thus, we propose that the higher vitamin E concentration and treatment duration in our adipocyte studies could lead to sufficient mitochondrial vitamin E accumulation to cause a negative impact on oxygen consumption. Considering the interconnection between vitamin E and the thiols in the antioxidant network, we hypothesize that treatment with vitamin E, through increased GSH mitochondrial levels, leads to similar consequences as treatment with NAC i.e. reduced basal OCR and increased mitochondrial ROS production.

To summarize this section, β3-AR activation in 3T3-L1 adipocytes leads to an increase in ROS and *Ucp1* expression, but 24 h antioxidant pretreatment does not prevent these effects. Furthermore, this NAC preconditioning of adipocytes increases the mitochondrial ROS levels associated with reduced oxygen consumption and elevated glycolysis. In conclusion, exogenous

antioxidants can, at least in adipocytes, lead to increased mitochondrial ROS production, likely through an elevation of mitochondrial GSH levels; therefore, they cannot be used as a tool to study the role of ROS in β3-AR activation-induced browning of adipose tissue.

ANTIOXIDANT EFFECTS IN MOUSE ADIPOSE TISSUE

NAC treatment leads to impaired fatty acid clearance in mice

To elucidate whether the observed effect of antioxidants on mitochondrial ROS production *in vitro* could translate into a disturbance of the browning process *in vivo*, we treated mice with either regular or NAC-supplemented drinking water for a week. Then, we injected animals for 10 days with either vehicle or CL316,243 to induce browning of IWAT. Tissues and samples were collected at 3 h, 24 h and 10 days (daily injection) post injection.

At all timepoints, no differences in total body weight across the four groups were observed. Furthermore, there were no differences in body weight-normalized weight of IWAT, GWAT or BAT between animals injected with CL316,243 on regular and NAC-supplemented water. Relative gene expression analysis of browning markers in the collected adipose tissues, showed no effect of NAC treatment in CL316,243 injected animals after 3 and 24 h. These data suggest that our antioxidant treatment regimen does not interfere with the acute browning response.

Short-term NAC treatment (1.5 hours) has been shown to reduce ROS and lipolysis in cultured human adipocytes (Krawczyk, Haller et al. 2012). To test whether lipolysis is affected by antioxidant treatment in our experimental setting, mice were treated for a week with water or NAC and further injected with either vehicle or CL316,243. NAC had no effect on serum glycerol levels, neither in vehicle nor β3-AR-stimulated mice (Figure 14). Interestingly, serum FFA levels of NAC-treated mice were significantly higher compared to control animals receiving regular water. Thus lipolysis, as judged by glycerol levels, was not influenced by NAC treatment. However, the higher FFA levels suggest reduced fatty acid clearance, which - given our *in vitro* data - points towards a reduced mitochondrial metabolism in NAC-treated mice.

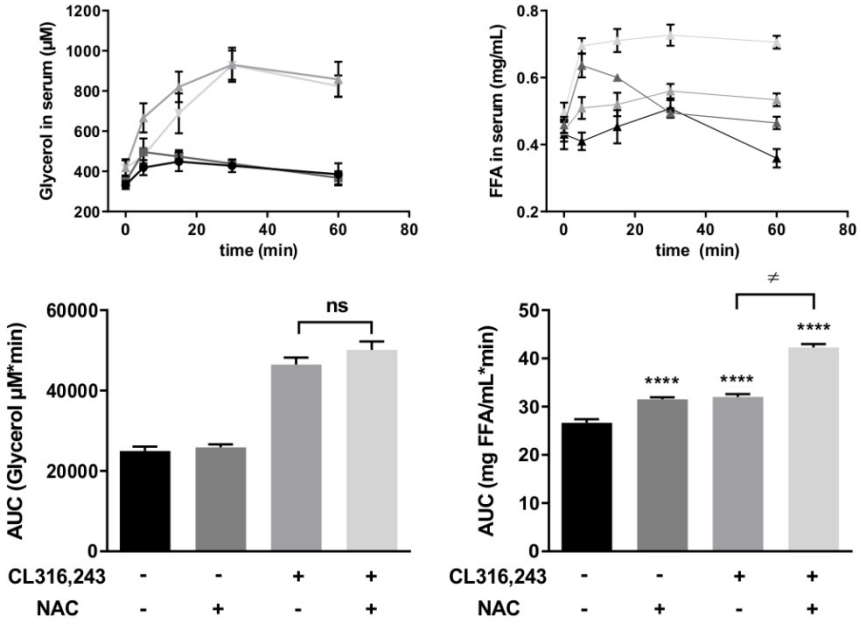

*Figure 14. (left) Quantification, of a 60-minute time course, of glycerol and (right) FFA levels in serum of mice pretreated for 1 week with either water or NAC 1 g/L solution injected with either vehicle or 1 µg of CL316,243 per g of body weight and area under the curve (AUC) (n=5/group, ****p<0.0001, ≠p<0.0001).*

NAC treatment reduces browning of IWAT

After 10 days of daily CL316,243 treatment (i.e. 2.5 weeks of antioxidant treatment), NAC reduced the CL316,243-induced mRNA and protein expression of several browning and mitochondrial markers in IWAT (*Ucp1*, *Pgc1α*, Cytochrome c oxidase subunit 4 isoform 1 (*CoxIV*) and mitochondrially encoded ATP synthase membrane subunit 6 (*Atp6*). Furthermore, the COXIV protein expression was reduced in NAC-treated mice even in absence of β3-AR activation (Figure 15). These data are thus consistent with mitochondrial dysfunction in white adipose tissue of NAC-treated mice.

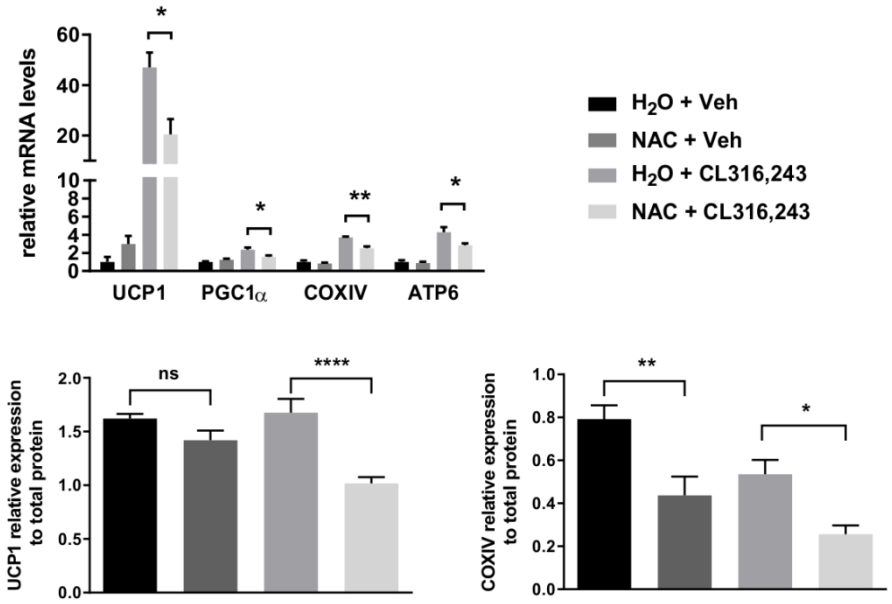

*Figure 15. (top) Relative mRNA expression of Ucp1, Pgc1α, CoxIV and Atp6 in IWAT in response to the different treatment regimens in C57/BL6J mice, (n=10/group, *p<0.05, **p<0.01). (bottom-left) Relative UCP1 and (bottom-right) COXIV protein expression in IWAT (n=5/group, *p<0.05, **p<0.01 ****p<0.0001).*

To further explore the effect of NAC on adipocyte mitochondrial function, we analyzed lipid and mitochondrial density in freshly dissected mouse IWAT samples using combined CARS and 2-PE fluorescence microscopy. Stimulation of β-ARs in IWAT is typically associated with a reduction in lipid density (Granneman, Li et al. 2005) combined with increased number of mitochondria per cell (Barneda, Frontini et al. 2013). Through image analysis, both these effects could be observed in CL316,243-treated animals, but NAC preconditioning blunted this reduction in lipid density and mitochondrial increased density/activity (Figure 16). The CL316,243-induced difference in mitochondrial density/activity was significant between groups also when mitochondrial density/activity were analyzed as absolute areas, while the lipid density area was no longer different between groups arguing that reduced mitochondrial density/activity is the primary mechanism underlying the blunted IWAT browning in NAC-treated mice.

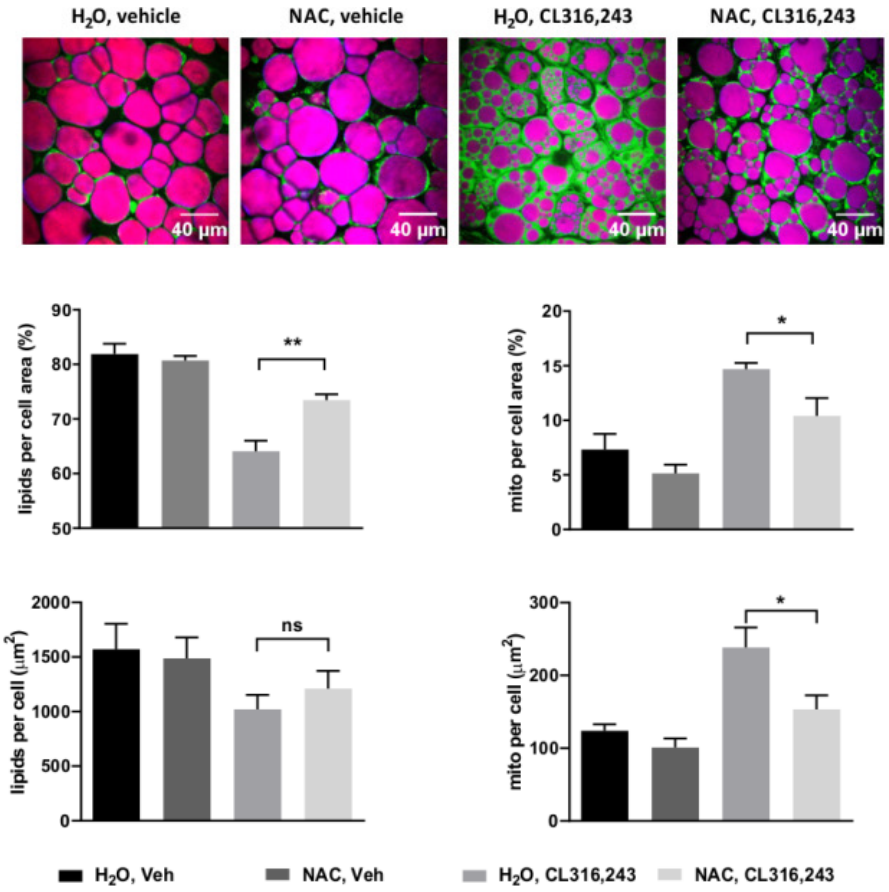

*Figure 16. (top) Representative images of fresh pieces of IWAT of C57/BL6J mice pretreated for 1 week with either water or NAC 1 g/L solution followed by 10 days injection with either vehicle or 1 µg of CL316,243 per g of body weight of CARS (lipids, in purple) and 2-PE fluorescence microscopy (mitochondria, in green). (middle) Relative and (bottom) absolute quantification of (left) lipid content and (right) mitochondrial density/activity in experiments depicted (n=5/group, *p<0.05, **p<0.01).*

The average adipocyte size did not differ between groups, but there was considerable variability in cell size leading to larger variability in lipid density and mitochondrial density/activity when these parameters are analyzed as absolute area as opposed to % of cell area. No changes in morphology of lipid droplets could be observed in cells treated with NAC alone, neither with regard to total density nor number of droplets per cell (data not shown).

Another interesting consequence of NAC treatment in white adipose tissue was the increase in relative IWAT and GWAT weights (normalized to body weight (Figure 17). Oxidative stress has been shown to contribute to an upregulation of phosphoenolpyruvate carboxykinase (PEPCK) in hepatocytes (Ito, Oumi et al. 2006). Indeed, *Pepck*, the rate-limiting enzyme for glyceroneogenesis and essential for re-esterification of fatty acids in adipose tissue (Hanson and Reshef 2003), was increased 3.5-fold by NAC treatment in IWAT. This increase in *Pepck* may at least in part explain the increased fat pad weight in NAC-treated control animals and could also be regarded as a secondary adaptation to reduce the risk of the lipotoxic effects from increased circulating fatty acid levels.

We suggest that NAC-induced mitochondrial reductive stress in IWAT (as we demonstrated in 3T3-L1 adipocytes) interferes with both the components necessary to accomplish a complete β3-AR activation-induced browning response and the systemic FFA clearance, resulting in an increased FFA reuptake by WAT.

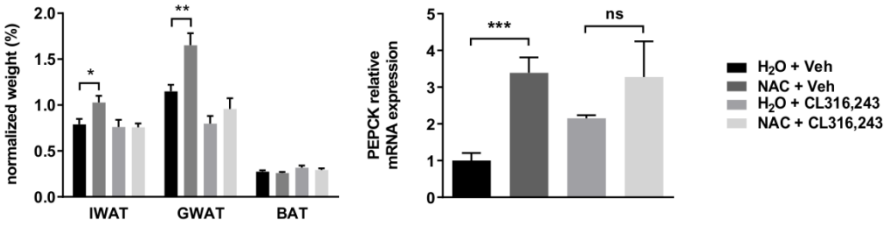

*Figure 17. (left) IWAT, GWAT and BAT weight (normalized to total body weight) in response to the different treatment regimens in C57/BL6J mice (n=10/group, *p<0.05, **p<0.01). (right) Relative Pepck levels in IWAT 10 days after CL316,243 treatment (n=10/group, ***p<0.001).*

Effect of vitamin E treatment on IWAT browning

To study whether the observed effect on white adipose tissue browning in NAC-treated mice could be reproduced with other antioxidant that also has an effect in glutathione levels, we investigated the effect of vitamin E treatment on CL316,243-induced browning in mice.

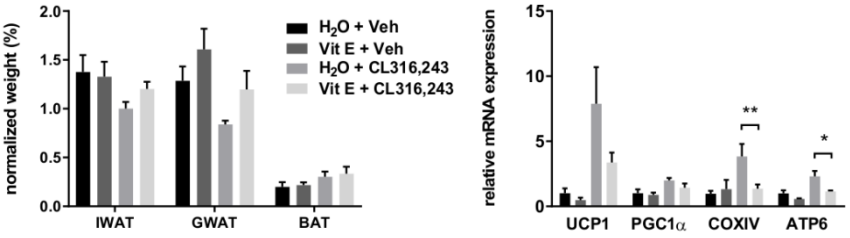

*Figure 18. (left) IWAT, GWAT and BAT weight, normalized to total body weight, in response to the different treatment regimens in C57/BL6J mice (n=10/group). (right) Relative mRNA expression of Ucp1, Pgc1α, CoxIV, and Atp6 in IWAT, post chronic CL316,243 treatment (n=10/group, *p<0.05, **p<0.01).*

Vitamin E-treated mice did not show an increase in fat pad weights and no significant effect on *Ucp1* and *Pgc1α* mRNA expression in IWAT (Figure 18). However, vitamin E treatment reduced the CL316,243-induced expression of the mitochondrial markers *Atp6* and *CoxIV* in IWAT. Thus, similarly to what we observed *in vitro*, vitamin E has a weaker effect on a white adipose tissue compared to NAC in this regard. Thus, both NAC and vitamin E interfered with CL316,243-induced mitochondrial changes, but NAC had a more potent effect than vitamin E. We hypothesize that the indirect effect of vitamin E treatment on mitochondrial glutathione levels, as we showed previously in 3T3-L1 cells, is not strong enough to exert sufficient reductive stress to cause the same negative impact as NAC treatment in adipose tissue.

NAC treatment induces mitochondrial dysfunction in BAT

NAC treatment had no effect on body weight-normalized BAT weight either in controls or in CL316,243 injected animals (Figure 19). Although no differences could be observed in *Upc1* and *Pgc1α* mRNA levels, UCP1 protein levels were upregulated in BAT of CL316,243 treated animals in line with previous studies (Park, Jung et al. 2015, Xiao, Goldgof et al. 2015). Importantly, this upregulation was slightly blunted in NAC-treated animals (Figure 19). Furthermore, NAC treatment blunted the CL316,243-induced increase in *Atp6* and *CoxIV* mRNA levels in BAT. This effect on *CoxIV* mRNA expression was however not observed at the protein level.

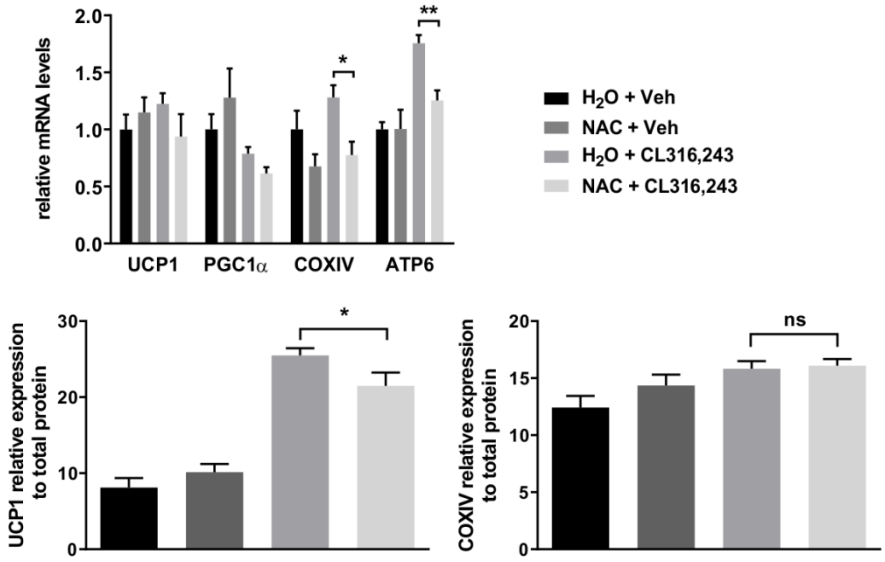

*Figure 19. (top) Relative mRNA expression of Ucp1, Pgc1α, CoxIV, and Atp6 in BAT in response to the different treatment regimens in C57/BL6J mice, (n=10/group, *p<0.05, **p<0.01). (bottom-left) Relative UCP1 and (bottom-right) COXIV protein expression in IWAT (n=5/group, *p<0.05).*

Moreover, NAC completely suppressed the CL316,243-induced reduction in lipid density and, even in the absence of CL316,243 stimulation, reduced the mitochondrial density/activity of freshly dissected BAT as judged by combined CARS and fluorescence microscopy (Figure 20).

The difference in mitochondrial density/activity between NAC and their corresponding controls remained significant in CL316,243 treated mice even if when measured as absolute area. Similar to IWAT, lipid density measured as absolute area/cell and average brown adipocyte size did not differ between groups suggesting that the NAC-induced difference in mitochondrial density/activity is the primary driver of the observed BAT phenotype. Rhodamine 123 is a probe that is commonly used to locate mitochondria by fluorescence microscopy (Johnson, Walsh et al. 1980), but can also be used to monitor the membrane potential of mitochondria (Baracca, Sgarbi et al. 2003), i.e. mitochondrial activity. The reduced Rhodamine 123 staining of freshly dissected BAT samples in NAC-treated mice may thus be due to both reduced mitochondrial density and blunted activity. However, measurements of the relative protein levels of four OXPHOS complexes in BAT showed no differences across the four groups thus arguing that it is the mitochondrial activity rather than the density that is affected. Activation of β3-ARs has

been shown to increase free fatty acid uptake and utilization in BAT *in vivo* (Warner, Kjellstedt et al. 2016). We thus suggest that these data provide a plausible explanation for the elevated CL316,243-induced FFAs levels in NAC-treated mice.

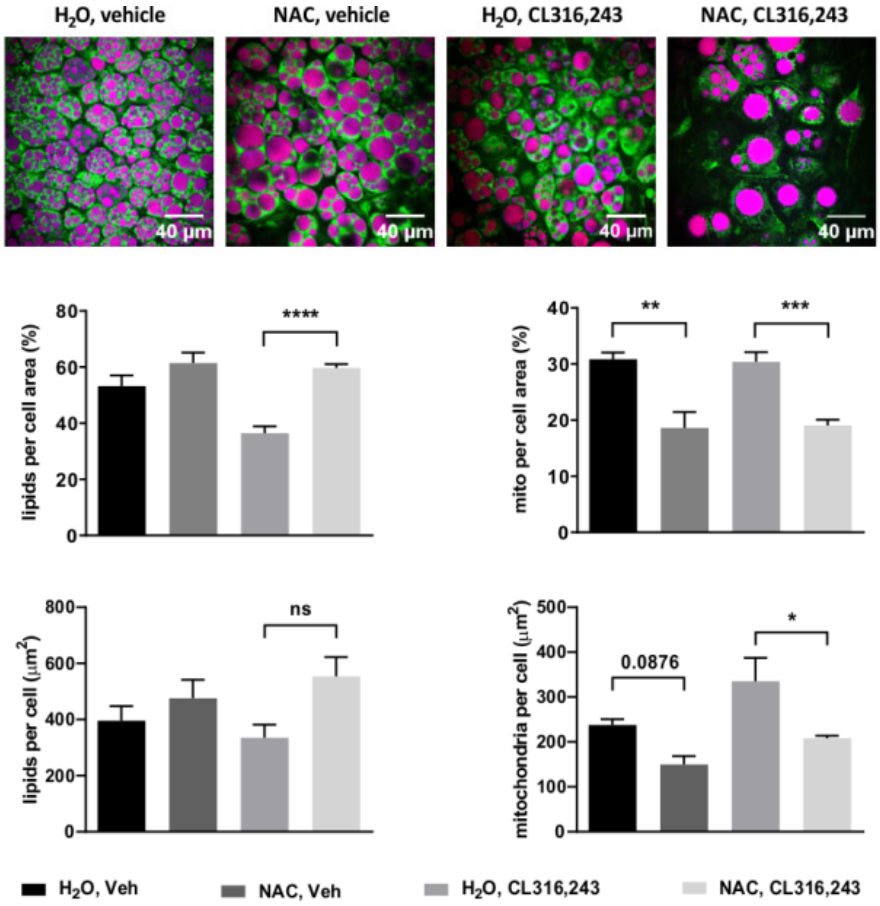

*Figure 20. (top) Representative images of fresh pieces of BAT of C57/BL6J mice pretreated for 1 week with either water or NAC 1 g/L solution followed by 10 days injection with either vehicle or 1 µg of CL316,243 per g of body weight of CARS (lipids, in purple) and 2-PE fluorescence microscopy (mitochondria, in green). (middle) Relative and (bottom) absolute quantification of (left) lipid content and (right) mitochondrial density/activity in experiments depicted (n=5/group, *p<0.05, **p<0.01).*

Data obtained from studies of 3T3-L1 adipocytes do not necessarily reflect the functionality of brown adipocytes. To further investigate the NAC-mediated mitochondrial dysfunction in brown adipocytes, we therefore

isolated and differentiated primary brown preadipocytes into mature brown adipocytes *in vitro*. In this model of brown adipocytes, NAC treatment reduced basal respiration, uncoupled respiration, ATP-production linked respiration and spare respiratory capacity independently of adrenergic activation as judged by oxygen consumption rate analysis (Figure 21).

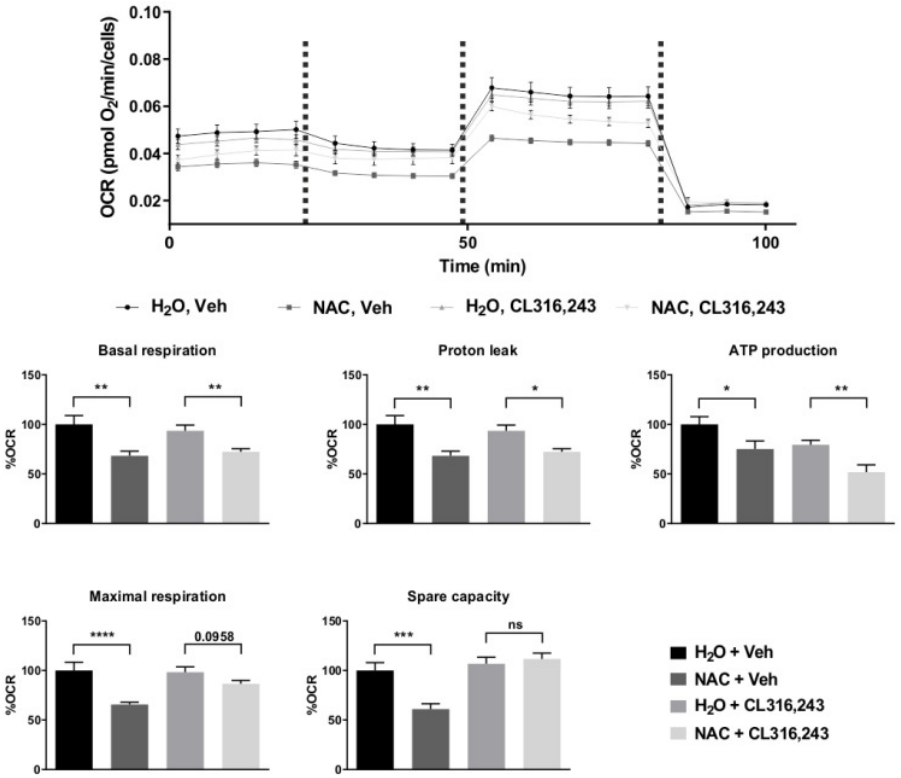

*Figure 21. (top) OCR of primary brown cultured adipocytes treated with 1 mM NAC for 48 h and 1 µM CL316,243 for 24 h analyzed using the Seahorse Technology; cells were sequentially treated (dashed lines show addition points) with 1 µM Oligomycin, 0.6 µM FCCP, and 0.5 µM Rotenone plus Antimycin A to assess mitochondrial function and (middle and bottom) calculate its main parameters (n=14/group, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).*

In conclusion, we believe that 1) the reduction in FFA clearance in NAC-treated mice, 2) the reduction in mitochondrial activity in BAT as judged by fluorescence microscopy in tissues, and 3) the reduction in oxygen consumption in cultured NAC-treated brown adipocytes, demonstrates that our chronic NAC treatment regimen leads to mitochondrial dysfunction in BAT.

NAC supplementation leads to reductive stress in mouse adipose tissue

Increased cellular ROS levels activate the NRF2/KEAP1 pathway, leading to increased levels of NRF2 (Bryan, Olayanju et al. 2013). This pathway stimulates the production of PRDXs (Miyamoto, Izumi et al. 2011) and SODs (Sun, Ren et al. 2015) that protect the cells from oxidative damage. IWAT *Sod*2 mRNA levels were upregulated in mice receiving both NAC and CL316,243 compared to their controls. Moreover, NAC-treated animals, independently of adrenergic activation, displayed significant upregulation of SOD2 protein expression levels compared to their controls. Similarly, but more dramatically, IWAT PRDX3 levels were also upregulated (Figure 22). Interestingly PRDX2, another peroxiredoxin enriched in the cytosol is equally expressed by all groups (Figure 22). In line with our ROS measurements in 3T3-L1 adipocytes, these PRDX data suggest that the increased reductive stress of IWAT in NAC-treated mice is localized to the mitochondria.

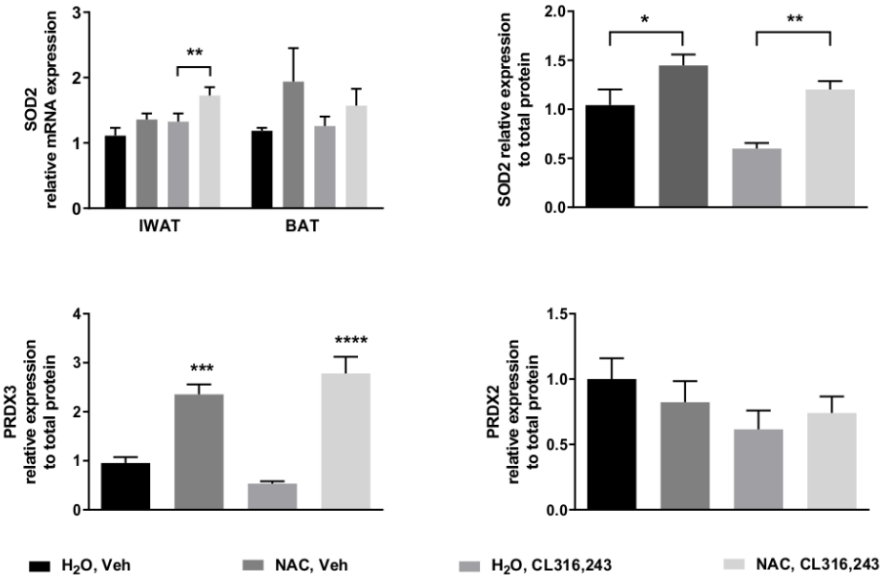

*Figure 22. (top-left) IWAT and BAT Sod2 mRNA expression after 10-day treatment with CL316,243 in C57/BL6J mice on either NAC-supplemented or regular water (n=5/group, **p<0.01). (top-right) SOD2, (bottom-left) PRDX3, and (bottom-right) PRDX2 protein levels in IWAT after 10-day treatment with CL316,243 in C57/BL6J mice on either NAC-supplemented or regular water (n=5/group, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

Thus, NAC, although being antioxidant, upregulates the production of mitochondrial enzymes that are responsible to scavenge superoxide anion and hydrogen peroxide, i.e. signs of increased oxidative stress. This pro-oxidant behavior of antioxidants may appear surprising, but it is well in line with a previous myoblast study where NAC treatment, especially at low ROS levels, leads to an alteration of the GSH homeostasis resulting in mitochondrial oxidation (Zhang, Limphong et al. 2012). The alteration of GSH homeostasis has been linked to chemical reduction of oxygen to superoxide anion, resulting in increased ROS levels, in a process referred to as reductive stress, in opposition to oxidative stress (Korge, Calmettes et al. 2015) although the outcome in both is elevated ROS production with similar consequences.

Oxidative stress is linked to increased mitochondrial fission (Ježek, Cooper et al. 2018), and reductive stress has been shown to increase the unfolded protein response in the endoplasmic reticulum. Under our experimental conditions, we could however not detect any effects of NAC treatment on either mitochondrial fission/fusion related genes expression or changes in *Xbp1* mRNA expression, a marker of endoplasmic reticulum stress (Wang and Kaufman 2016). One possibility is that we fail to observe such phenomena due to the transient nature of transcriptional regulation.

CONCLUDING REMARKS ON PAPER I

Our adipose tissue data confirms the occurrence of reductive stress in antioxidant-treated mice likely due to increased GSH levels in adipocytes, driving the reduction of molecular oxygen to superoxide in the adipocytes' mitochondria, i.e. elevation in ROS production. The adipocytes adapt to this pro-oxidant effect of chronic antioxidant treatment by activating the NRF2/KEAP1 pathway and reducing their mitochondrial oxidative metabolism as evident from e.g. the blunted β3-AR-activation induced browning of IWAT (Figure 23).

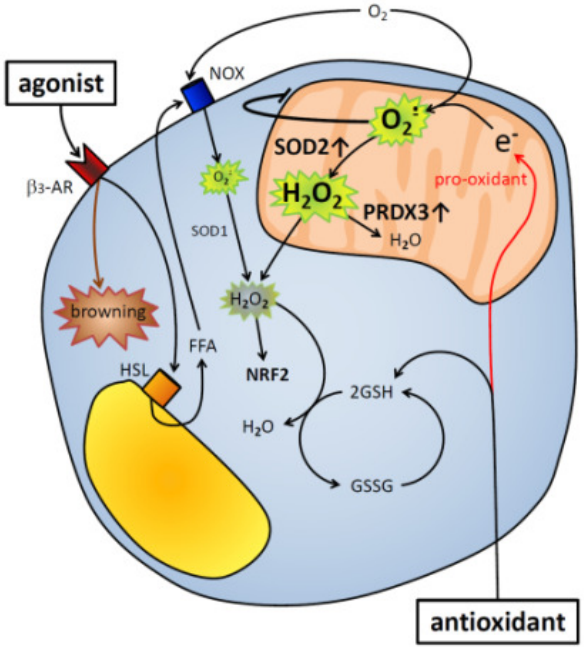

Figure 23. Model of antioxidant and β3-AR agonist effects on ROS and mitochondrial activity in adipocytes.

Prolonged N-acetylcysteine treatment induces mitohormesis in adipose tissue (Paper II)

Antioxidants are commonly used for their potential to fight oxidative stress in conditions where imbalance in ROS production and neutralization plays a central role. *In vitro* studies of myocytes (Singh, Charles et al. 2015) and tumor cells (Sceneay, Liu et al. 2013), or *in vivo* (Fulghesu, Ciampelli et al. 2002, Palmer, Doctor et al. 2007, Wright, Renoir et al. 2015, Ma, Gao et al. 2016, Pinniger, Terrill et al. 2017) in mice and humans have investigated the effects of NAC treatment using a broad range of concentrations and durations, from 2 weeks to 4 months, describing both beneficial results (e.g. protection against HFD-induced obesity, improvement of insulin sensitivity in women with polycystic ovarian syndrome or protection against neurological diseases) and deleterious effects (mitochondrial dysfunction, increased metastasis or reduced growth).

Temporary elevation of mitochondrial ROS levels (Lettieri Barbato, Tatulli et al. 2015, Zhang, Humes et al. 2018) or SOD2 levels (Yang and Hekimi 2010) trigger a coordinated response leaving the cell less susceptible to subsequent perturbations. We thus hypothesize that NAC treatment, although causing reduced mitochondrial function (as shown in paper I), also has the potential to enhance mitochondrial function in adipocytes in mice through mitohormesis. We also hypothesize that such hormetic effect of NAC is dose-dependent. Based on reports from previous studies, we chose three different concentrations of NAC in drinking water to cover a broad range of doses:

- Low dose, 0.5 g/L in drinking water, which is half of the dose used in the majority of mouse studies.
- Middle range dose, 2 g/L in drinking water, used e.g. in a study on HFD-fed mice and was shown to reduce weight gain (Ma, Gao et al. 2016).
- High dose, 10 g/L in drinking water to study the possibility of aberrant side-effects.

Based on the analysis of published studies, we wanted to follow NAC-treatment effects from relatively short (2 and 4 weeks) and chronic exposure (16 weeks).

SHORT-TERM NAC TREATMENT EFFECTS

2-week high dose NAC treatment leads to reduced weight gain and reduced mitochondrial function in BAT

Mice treated with the highest NAC dose (10 g/L) showed a 7.8 % lower body weight than controls after 2 weeks of treatment as shown in Figure 24. This effect was due to reduced weight gain, not detected in the lower dose groups. Body composition determined by DEXA revealed no differences in body fat percentage or bone mass. Body length was not different between control and NAC treated mice. Interestingly, the absolute lean mass was however lower in the 10 g/L NAC-group than in controls.

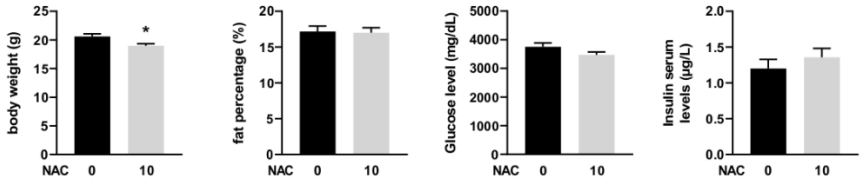

*Figure 24. (left) Total body weight and fat percentage of C57BL/6J mice treated for 2 weeks with either water or NAC 10 g/L solution (n=5/group, *p<0.05). (right) Fasting glucose and insulin levels of mice treated with either water or NAC 10 g/L solution (n=5/group).*

Short-term NAC treatment can lead to reductive stress in myoblasts (Singh, Charles et al. 2015). Moreover, ROS can reduce lean body mass by stimulating the expression and activity of skeletal muscle protein degradation pathways (Abrigo, Elorza et al. 2018). Thus, the observed reduction in absolute lean mass in the 10 g/L NAC-group could be attributed to reductive stress in muscle.

To further study the pro-oxidant effect of NAC in adipocytes that we established in paper I, we analyzed the functionality of isolated mitochondria from BAT of mice treated with 10 g/L of NAC for 2 weeks and their controls. There was a significant reduction of BAT mitochondrial OCR in NAC treated animals that we interpret as a sign of reductive stress. Mitochondrial oxidants have been shown to cause insulin resistance impairing the Glucose Transporter type 4 (GLUT4) localization to GLUT4 storage vesicles without changes in the oxidative phosphorylation machinery in an *in vitro* model (Fazakerley, Minard et al. 2018). In our study, at 2 weeks of NAC treatment, there were no significant differences in basal insulin and glucose levels (Figure 24). This suggest that, although the body is under reductive stress as evident from the mitochondrial dysfunction in IWAT

(shown in paper I), reduced mitochondrial activity in BAT and reduced lean mass gain, there are sufficient compensatory mechanisms available to sustain glucose homeostasis and whole-body insulin sensitivity under unchallenged conditions.

4-week high dose NAC treatment induces reductive stress in IWAT and BAT

To explore the possibility of a dose-dependent reductive stress effect of NAC in white and brown adipose tissue, we exposed mice to the three different doses during 4 weeks. Indeed, there was a dose- and tissue dependent effects of antioxidant treatment on PRDX3 and SOD2, two enzymes whose expression is induced by oxidative stress (Whitaker, Patel et al. 2013, Candas and Li 2014). IWAT PRDX3 protein expression was increased in the 10 g/L NAC group, while IWAT SOD2 protein levels remained similar between groups. In BAT, on the other hand, the PRDX3 expression was unaffected, but SOD2 was upregulated in a dose-dependent manner, reaching significance at 2 and 10 g/L (Figure 25). To understand the different response of IWAT and BAT to NAC-induced reductive stress, we analyzed mRNA expression levels of several mitochondrial-related genes. In IWAT, along with the upregulated expression of PRDX3 protein, there was a simultaneous upregulation of peroxisome proliferator-activated receptor gamma coactivator 1-beta (*Pgc1β*), a well-stablished regulator of mitochondrial biogenesis and fatty acid β-oxidation (Bouitbir, Charles et al. 2012) suggesting that a mitohormesis process has been triggered. In BAT, the upregulation of SOD2 protein was connected to a significant reduction of *Atp6* and cytochrome c oxidase subunit I (*CoxI*), two mitochondrial respiration related-genes, a possible sign of reduced activity in brown adipocytes.

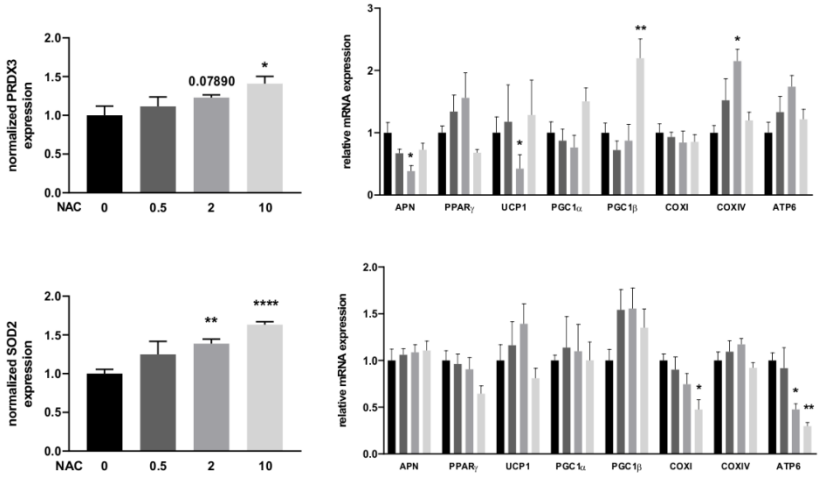

*Figure 25. (top-left) Relative protein expression of PRDX3 in IWAT and (bottom-left) relative protein expression of SOD2 in BAT (n=5/group, *p<0.05, **p<0.01, ****p<0.0001). Relative mRNA expression of adipocyte, mitochondrial and energy disposal related genes in (top-right) IWAT and (bottom-right) BAT after 4-week NAC treatment (n=5/group, *p<0.05, **p<0.01).*

As an alternative to a mitohormesis-based explanation for the increased expression of *Pgc1β,* the decrease in BAT mitochondrial activity can lead to a compensatory effect in IWAT, similarly to the effect shown in BAT-ablated mice (Piao, Zhai et al. 2018).

Clearly, the response to short-term NAC treatment is cell-specific, and thus different between white and brown adipocytes. Different basal levels of antioxidant endogenous enzymes due to these adipocytes types' different functions, might explain these protein expression differences after 4 weeks of NAC treatment.

CHRONIC NAC TREATMENT LEADS TO MITOHORMESIS

16-week high-dose NAC treatment leads to increased mRNA expression of browning markers in IWAT

After 16 weeks of NAC treatment, IWAT and BAT PRDX3 and SOD2 protein levels were no longer different between groups, implying that an adaptation to the reductive stress has occurred thus normalizing the levels of the main components of the previously upregulated mitochondrial endogenous antioxidant system. Alternatively, the reductive stress has become chronic and no longer triggers an adaptive increase in antioxidant

enzymes. The transcriptional response in IWAT of mice treated with the highest dose of NAC had a browning-like profile, with significant upregulation of *Ucp1* (also observed at the protein level in isolated mitochondria from IWAT (Figure 31)), *Pgc1α*, *Pgc1β*, *Cox1* and *Atp6* (Figure 26). The increased expression of *Pgc1α* and *β* suggest stimulation of mitochondrial biogenesis. Surprisingly, *Pparγ*, a marker of adipocyte differentiation and functionality (Chawla, Schwarz et al. 1994), was downregulated in IWAT, suggesting that NAC treatment downregulates adipocyte renewal or causes adipocyte dedifferentiation. In BAT, no major transcriptional differences could be observed, thus the expression of *Cox1* and *Atp6* expression had returned to normal levels in the highest NAC dose. However, there were some minor differences depending on the dose: *CoxIV* was downregulated in the lowest NAC dose in BAT, and in IWAT from mice treated with 2 g/L. One could attribute these differences to a delayed hormetic response at the lower doses of NAC or the existence of the minimal dose threshold to trigger the hormetic response, i.e. 0.5 and 2 g/L are on the left part of the U-shaped typical dose-response of a hormetic process.

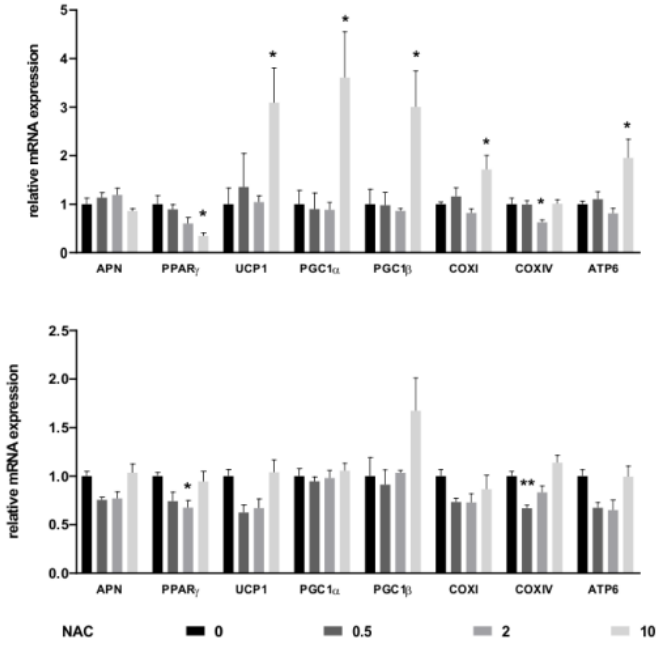

*Figure 26. Relative mRNA expression of adipocyte, mitochondrial and energy disposal related genes in (top) IWAT and (bottom) BAT after 16-week NAC treatment (n=5/group, *p<0.05, **p<0.01).*

16-week high-dose NAC treatment is associated with reduced fat mass and increased mitochondrial function in IWAT

Only the highest dose of NAC caused a difference in body weight and body mass composition. Although food consumption was similar between groups, 16-week 10 g/L NAC treatment led to a 22 % reduction in body weight and a 5.8 % reduction in body length compared to control (Figure 27).

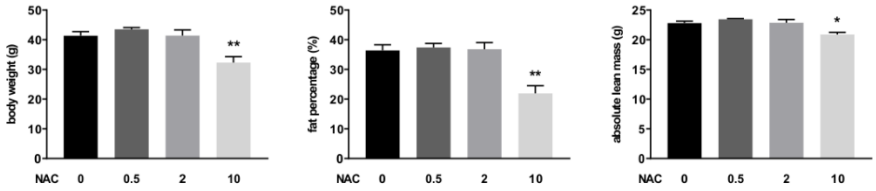

*Figure 27. (left) Body weight, (center) fat percentage, (right) lean mass (normalized to body length) of C57BL/6J mice treated for 16 weeks with either water or NAC 0.5, 2 or 10 g/L solution (n=5/group, *p<0.05, **p<0.01).*

The reduced body weight gain was mainly due to a reduction in fat mass, responsible for 88 % of the lower body mass. A reduction in lean mass gain was thus responsible for the remaining 12 %. Moreover, the white adipose tissue weight was severely affected by 10 g/L NAC treatment with a 38 % reduction in GWAT weight and a 53 % reduction in IWAT weight (Figure 28).

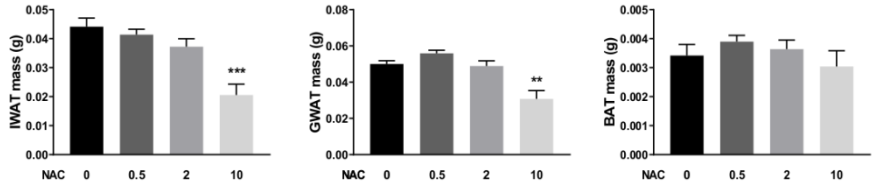

*Figure 28. (left) IWAT, (center) GWAT and (right) BAT weight of C57BL/6J mice treated for 16 weeks with either water or NAC 0.5, 2 or 10 g/L solution (n=5/group, **p<0.01, ***p<0.001).*

As judged by the mRNA expression, the primary target for a possible mitohormetic response of antioxidant treatment is white adipose tissue where chronic high-dose NAC treatment was associated with an upregulation of browning and mitochondrial genes. To further study the mitochondrial function in white adipose tissue at this stage, we performed OCR analysis of freshly dissected IWAT after 16 week-NAC treatment. IWAT samples from mice treated with the lowest dose of NAC showed a significant reduction in

OCR, while the OCR of IWAT samples from mice treated with higher doses was similar to the untreated control (Figure 29). This reduction in OCR in response to chronic low-dose NAC treatment can be interpreted as a sign of reductive stress, but the mitochondrial antioxidant enzymes PRDX3 and SOD2 were not different from controls. Altogether, we interpret these data as a sign of chronic reductive stress in IWAT from mice treated with the lowest NAC dose i.e the NAC dose is not high enough to trigger sufficient adaptation to the pro-oxidant effects of NAC leading to a sustained reduction of OCR limiting the mitochondrial ROS production.

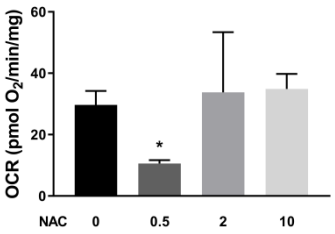

*Figure 29. IWAT OCR measured in fresh tissue samples from C57BL/6J mice treated for 16 weeks with either water or NAC 0.5, 2 or 10 g/L solution (n=3/group, *p<0.05).*

Based on the transcriptional response, we had anticipated increased IWAT OCR in mice treated with the highest NAC dose. OCR measurements of whole adipose tissue have however its limitations as discussed previously in the Methods section. We thus decided to perform a more thorough examination of the mitochondrial function in IWAT and BAT through analysis of isolated mitochondria. In support of a mitohormetic response in IWAT in mice on chronic high-dose NAC treatment, we found increased basal and ATP production-linked respiration in isolated IWAT mitochondria. The uncoupled respiration and the respiratory control ratio (RCR) showed however no difference likely due to large variation within groups. In contrast, isolated BAT mitochondria showed no difference in OCR in any parameter (Figure 30 and data not shown).

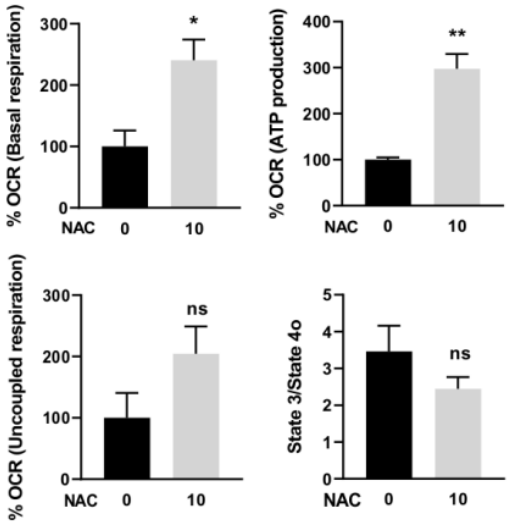

*Figure 30. (top-left) Basal respiration, (top-right) ATP production, (bottom-left) uncoupled respiration and (bottom-right) RCR measured in isolate mitochondria from IWAT samples from C57BL/6J mice treated for 16 weeks with either water or NAC 10 g/L solution (n=3/group, *p<0.05, **p<0.01).*

To delineate whether these changes in the mitochondrial performance can be attributed to changes in protein components of the electron transport chain, we analyzed OXPHOS proteins in isolated IWAT mitochondria (Figure 31). This analysis showed major differences between control mice and mice treated with the highest NAC dose; IWAT complex I and II protein members (NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8 (NDUF8B) and Succinate dehydrogenase [ubiquinone] iron-sulfur subunit (SDHB) respectively) were markedly upregulated in the NAC-treated group. Complex I and II are receiving electrons from respectively, NADH and $FADH_2$ that should increase during reductive stress. Thus, it is possible that changed activity of these complexes is driving the observed mitochondrial changes in IWAT. There was also a trend towards an upregulation of complex IV. Complex V was however ubiquitously expressed and was therefore used as a loading control for the relative quantification of UCP1. Even though we did not detect increased uncoupled respiration, the mitochondria from NAC-treated animals displayed a 5-fold increase in UCP1 protein expression. We attribute this discrepancy to the rather large variability in the OCR analysis. It is possible that the mitochondria function becomes more or less altered by the isolation procedure.

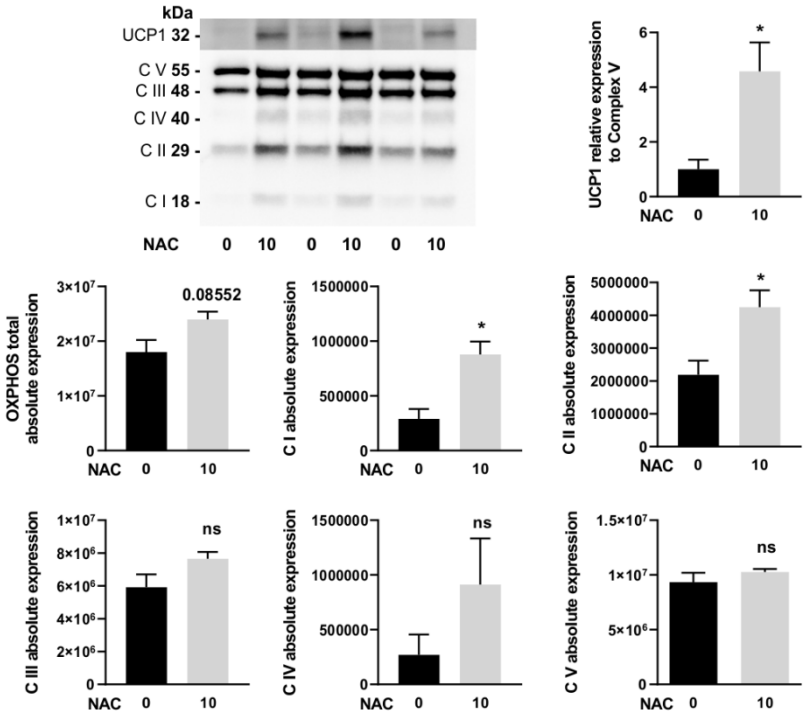

*Figure 31. (top-right) UCP1 and (top-left) OXPHOS complexes protein expression: representative blot and (middle and bottom) quantification from isolate mitochondria from IWAT samples from C57BL/6J mice treated for 16 weeks with either water or NAC 10 g/L solution (n=3/group, *p<0.05, **p<0.01).*

Similar to our study, 8-week treatment with daily NAC IP injections has been shown to reduce body weight in a dose-dependent manner (Kim, Ryu et al. 2006). This effect was in part explained by a reduction of GWAT weight, while IWAT weight remained unaffected (Kim, Ryu et al. 2006) possibly due to a different administration method and/or shorter treatment time compared to our study. The reduced GWAT weight gain detected by Kim and colleagues was attributed to NAC's inhibitory effect on adipocyte differentiation (Kim, Ryu et al. 2006). The downregulation of *Pparγ* mRNA expression we observed in IWAT adds support for such hypothesis, but we propose that the IWAT browning phenotype also plays a role for the reduced fat mass through increased energy expenditure. On the other hand, we saw no signs of enhanced mitochondrial functionality in BAT, the primary site for non-shivering thermogenesis. Moreover, whole-body energy balance is regulated by the brain implying that it is likely to also be a central component involved in the lowered body weight in NAC-treated mice.

16-week high-dose NAC treatment is associated with improved whole-body insulin sensitivity

To assess if the changes in body composition and IWAT mitochondrial function alter whole-body metabolism, we measured fasting glucose and insulin levels in mice treated for 16 weeks with of NAC. While basal glucose levels were similar between groups (data not shown), the insulin levels were affected in a NAC dose dependent manner. Mice receiving the highest NAC dose displayed a significant improvement in insulin sensitivity as judged by reduced fasting insulin levels and HOMA-IR index (Figure 32).

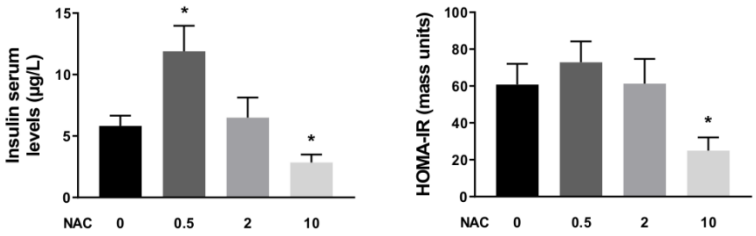

*Figure 32. (left) Fasting serum insulin levels and (right) calculated HOMA-IR indexes in C57BL/6J mice given water or NAC 0.5, 2 or 10 g/L solution (n=5/group, *p<0.05).*

Such NAC-induced improvement in insulin sensitivity has been reported previously in hyperinsulinemic patients (1.8 g/day for 6 weeks) (Fulghesu, Ciampelli et al. 2002), in an insulin resistant rat model (1-2 g/L, corresponding to 2 g/kg/day in drinking water) (El Midaoui, Ismael et al. 2008) and in high-fat diet fed mice (2 g/L in drinking water for 11 weeks) (Ma, Gao et al. 2016). In all these studies, there was a reversal of insulin resistance, attributed to NAC's antioxidant properties and the consequent anti-inflammatory effect. However, in our study mice are normoinsulinemic at the starting point with a completely unchallenged metabolic homeostasis, yet they are experiencing insulin sensitization together with a browning-like process in white adipose tissue when treated with 10 g/L of NAC for 16 weeks. Moreover, mice treated with the lowest dose displayed significantly increased insulin levels without a change in their glucose levels, i.e. a sign of insulin resistance (although not severe enough to change the HOMA-IR). This increase in insulin levels could be attributed to the lack of a mitohormetic response and thus the chronification of reductive stress as evident from the reduced IWAT respiration.

CONCLUDING REMARKS ON PAPER II

NAC treatment of unchallenged chow-fed mice results in a biphasic behavior of adipose tissue where the final outcome on mitochondrial function is tightly connected to both the adipose tissue type and the dosage. Short-term (2 weeks) high dose NAC treatment (10 g/L) increases the levels of the mitochondrial antioxidant enzymes in IWAT and BAT, and reduces mitochondrial respiration in BAT. After 16 weeks of high dose NAC treatment, BAT shows neither signs of compromised nor improved mitochondrial function indicating that BAT adapts, but does not supercompensate, to the reductive stress. IWAT, on the other hand, shows improved mitochondrial function in response to 16-week high dose NAC treatment as judged by both gene expression and mitochondrial analyses. Interestingly, mice kept on the lowest NAC dose (0.5 g/L), develop slight insulin resistance and display reduced IWAT OCR. We believe this negative outcome from low dose NAC treatment is due to chronic reductive stress and thus insufficient upregulation of e.g. mitochondrial antioxidant enzymes, OXPHOS proteins and mitochondrial biogenesis.

In conclusion, this study demonstrates that chronic NAC treatment, provided that the dose is adequate, can lead to a mitohormetic process in white adipose tissue associated with reduced fat mass and increased insulin sensitivity. Importantly, these results challenge the current paradigm where antioxidants primarily are thought to act as scavengers and thereby improve health by a simple reduction of oxidative stress. Moreover, our work may also explain the lack of effects or even the deleterious effects of NAC in some settings.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis we have studied the effects of antioxidant treatment on mitochondrial function in white and brown adipose tissue *in vitro* and *in vivo*. Our main conclusions from these studies are:

1. β3-AR activation in 3T3-L1 adipocytes leads to increased ROS production and increased UCP1 expression. These effects cannot be prevented by 24 h antioxidant pretreatment.
2. 24 h NAC pretreatment of 3T3-L1 adipocytes drives mitochondrial changes resulting in reduced basal oxygen consumption associated with elevated glycolysis. Thus, antioxidants cannot be used as tools to study the role of ROS in β3-AR-mediated responses of adipocytes.
3. 2-week NAC treatment (1 g/L in drinking water) of unchallenged chow-fed mice leads to reductive stress in adipose tissue, associated with reduced β3-AR agonist-mediated browning of IWAT and mitochondrial dysfunction in BAT.
4. NAC treatment of unchallenged chow-fed mice leads to a biphasic response where the effect on mitochondrial function in adipose tissue is tightly connected to the adipose tissue type and the treatment dose and duration.
5. Chronic NAC treatment can lead to a mitohormetic process in IWAT i.e. supercompensation to reductive stress leading to increased mitochondrial function (browning) associated with reduced fat mass and increased insulin sensitivity.

Antioxidant therapy is broadly used to treat conditions where oxidative stress is thought to play a central role. Antioxidants are also used in basic research due to their potential to scavenge ROS. The obtained results from clinical and experimental antioxidant studies are contradictory. We believe our data, which are summarized above, provide both a plausible explanation for many of these contradictory and/or controversial results and a new insight on how to better design antioxidant treatment studies. Nevertheless, further investigations are required to e.g. clarify the detailed mechanism that drives insulin sensitization or resistance depending on the dose, thus to understand the metabolic effects of antioxidants and the different effects that

antioxidants have on different adipose tissue types. Moreover, a more extensive study that includes also other types of antioxidants e.g. mitochondria-targeting antioxidants such as MitoQ, and other tissues (e.g. liver, pancreas or muscle) is highly recommended to evaluate possible pro-oxidative or hormetic effects. Overall, a better understanding of the consequences of chronic NAC treatment or a combination of antioxidants could pave the way for new treatments and applications.

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