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Lipid Peroxidation by-Products and the Metabolic Syndrome

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1. Introduction

About twenty-one percent of the air we breathe is composed of oxygen, and our life will not be possible without it. Oxygen is however a toxic, highly reactive molecule which was originally released in atmosphere as a waste product of the first photosynthetic organisms. Its accumulation on Earth indeed led to a massive extinction of living species. Few organisms survived and some developed the ability to use this toxic oxygen to improve the production of energy from carbohydrates. This "oxidative metabolism" was however a double-edge sword as the use of intracellular oxygen generates deleterious oxidative damages. To protect themselves toward this toxicity, those organisms consequently developed several "antioxidants" protection mechanisms which helped them maintain a balance between oxidative damage and efficient use of oxygen to produce energy.

When antioxidant defences are reduced and/or oxidative mechanisms increased, uncontrolled oxidation of cell targets leads to the accumulation of reactive oxygen species (ROS) and a state of "oxidative stress", often deleterious for the cells. This stress is involved in the pathophysiology of several human diseases, and especially in the development of metabolic diseases, even if its causative role remains questionable. A definite increase in oxidative stress biomarkers can be found in obese and diabetic humans as well as in animal model of these diseases. Accumulation of ROS can be deleterious by itself or can induce the oxidation of proteins, nucleic acids and lipids, generating secondary by-products. The specific reactivity of ROS towards polyunsaturated fatty acids (PUFAs) present in cell membranes induces lipid peroxidation, a noxious mechanism producing toxic aldehydes. Among them, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) have been extensively studied. Originally simple markers of lipid peroxidation, these aldehydes have demonstrated causative roles in the impairment of cellular functions: activation of signalling pathways, apoptosis, and modification of enzyme function. In addition to being hallmarks

of oxidative damage, lipid aldehydes could be mediators of oxidative insults, propagating tissue injury and activating cellular stress signalling pathways. Several studies demonstrated the association of obesity and diabetes with lipid peroxidation by-products, and the role of aldehydes in impairment of insulin function and signalling was recently pointed out.

This chapter aims to review the diverse implications of lipid peroxidation by-products in the pathophysiology of metabolic diseases, from evidence of their production during obesity and diabetes to the cellular mechanisms of their toxicity and protection against their deleterious effects.

2. Lipid peroxidation by-products

Under conditions of oxidative stress, excessive production of reactive oxygen species promotes the peroxidation of polyunsaturated fatty acids (PUFA). The resulting accumulation of hydroperoxides, unstable molecules, leads to their non-enzymatic degradation in many compounds, including aldehydes. Quantification of lipid peroxidation in biological samples has been extensively performed with the thiobarbituric acid (TBA) test. TBA detects malondialdehyde (MDA), an end-product of nonenzymatic PUFA oxidative degradation, which has therefore been used for decades as a marker of lipid peroxidation (Gutteridge, 1982). Another aldehyde: acrolein, first attracted attention because of its formation during tobacco combustion and its ubiquitous presence in the environment (Dong & Moldoveanu, 2004). Because of its carcinogenic potential, its role in smoking-related diseases has received extensive attention; however, acrolein is also produced endogenously though lipid peroxidation and its link with oxidative-associated pathologies is now well established. 4-hydroxy-2-alkenals are specific by-products of the oxidation of omega-3 and omega-6 fatty acids. 4-hydroxy-2-nonenal (HNE) is derived from the oxidation of polyunsaturated fatty acids of the n-6 series, mainly linoleic and arachidonic acids, while 4-hydroxy-2-hexenal (HHE) results from the peroxidation of polyunsaturated fatty acids of the n-3 series (mainly docosahexaenoic, eicosapentaenoic and linolenic acid). The peroxidation of arachidonic acid via 12-lipoxygenase leads to the formation of 4-hydroxy-2-dodecadienal (HDDE) (Guichardant et al., 2006).
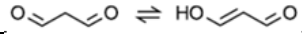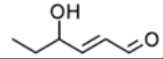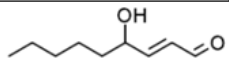
Name	Molecular Weight (Da)	Molecular Formula	Skeletal Formula
Acrolein (prop-2-enal)	56.1	C_3H_4O	
Malondialdehyde (propanedial, MDA)	72.1	$C_3H_4O_2$	
4-hydroxy-2-hexenal (HHE)	114.1	$C_6H_{10}O_2$	
4-hydroxy-2-nonenal (HNE)	156.2	$C_9H_{16}O_2$	
4-hydroxy-2-dodecadienal (HDDE)	196.0	$C_{12}H_{20}O_2$	

Table 1. α,β-Unsaturated aldehydes produced during polyunsaturated fatty acids oxidation

2.1. Chemistry and reactivity

Acrolein, MDA and 4-hydroxy-alkenals are α,β-unsaturated aldehydes, a class of compounds sharing the general structure C=C–C=O. They are characterised by an aldehyde group (C=O) on carbon 1 and a conjugated double bond (C=C) between carbons 2 and 3 (Table 1). In this structure, the oxygen atom of the carbonyl group increases the polarity of the double bond, which makes α,β-unsaturated aldehydes potent electrophiles. Acrolein has the simplest structure composed of 3 carbons, MDA is a dicarbonyl compound and 4-hydroxy-2-alkenals are characterized by the presence of a hydroxyl group on carbon 4. HHE, HNE and HDDE only differ by the length of their carbon chain and the presence of an additional double bond for the HDDE. In the case of MDA and 4-hydroxy-alkenals, the presence of a second oxygen atom makes the double bond even more reactive.

These aldehydes are part of the "*reactive electrophile species*" able to form covalent adducts with the nucleophilic groups present in DNA, proteins and phospholipids. In physiological conditions, they spontaneously react with the thiol group of glutathione to form Michael adduct by attack of the nucleophilic group of glutathione to the double bond of aldehydes. They can also react with thiol groups present on cysteine residues of certain proteins, leading to impairment of their biological activity. Under certain conditions, especially alkaline pH, aldehydes react with the amine groups present in proteins, nucleic acids and aminophospholipids, leading to Michael adducts. On the other hand, the reaction between a primary amine group and the carbonyl group of the aldehyde leads to the formation of Schiff bases (Schaur, 2003).

Aldehydes produced during lipid peroxidation are precursors of Advanced Lipoxidation End products (ALEs). Together with Advanced Glycation End products (AGEs) generated during glycoxidation, they accumulate in cells and tissues. The "carbonyl stress" is a result of this adduct accumulation, which induces protein dysfunctions and consequent pathological events such as inflammation and apoptosis (Negre-Salvayre *et al.*, 2008).

2.2. Cellular effects

2.2.1. Cytotoxicity

Since α,β-unsaturated compounds are strong electrophiles, they exhibit a high cytotoxic and mutagenic potential and have consequently been extensively studied for their effects on cell viability. HNE, HHE and acrolein indeed induce cell death, but the lethal concentration 50 (LC50), concentration that induces the death of 50% of the cells, is subject to variation, depending on the aldehyde, exposure duration and cell type (Table 2). The LC50 for a long exposure (>16 hours) to HNE or HHE is however consistently found 20-60 µM in several cell types, including human lymphoma Jurkat cells, lens epithelial cells, hamster V79-4 cells and muscle cells (Table 2). For acrolein, the same treatment gives a range of LC50 of 5-100 µM in human fibroblasts, human neuroblastoma cells, PC12 chromaffin cells and lymphocytes. MDA-induced cell death is less documented, even if

the LC50 range for MDA is found around 1 mM in cortical, endothelial cells and fibroblasts. MDA was also reported to induce cell cycle arrest, which is to relate to cell damage and death (Ji *et al.*, 1998). HDDE appears to be the most toxic lipid aldehyde with a LC50 in endothelial cells in the submicromolar range.

Name	LC50, μM	References
Acrolein (prop-2-enal)	5-100	Poirier *et al.*, 2002; Luo *et al.*, 2005; Jia *et al.*, 2009*b*, 2009*a*
Malondialdehyde (propanedial, MDA)	600-3000	Michiels & Remacle, 1991; Hipkiss *et al.*, 1997; Cheng *et al.*, 2011
4-hydroxy-2-hexenal (HHE)	20-60	Liu *et al.*, 2000; Choudhary *et al.*, 2002; Pillon *et al.*, 2010; Li *et al.*, 2011
4-hydroxy-2-nonenal (HNE)	20-60	Liu *et al.*, 2000; Choudhary *et al.*, 2002; Pillon *et al.*, 2010; Li *et al.*, 2011
4-hydroxy-2-dodecadienal (HDDE)	0.22	Riahi *et al.*, 2010

Table 2. Range of lethal concentration 50 for long-term treatment (>16 hours) with α,β-unsaturated aldehydes. LC50 values were calculated from the indicated references.

On the opposite, very little cell death is detectable for short term treatments (<4 hours), likely because aldehyde-induced cell death involves apoptosis mechanisms not yet occurring during this short period of time. Several studies indeed reported that cell death is induced by aldehydes through apoptosis for low concentration and both apoptosis and necrosis for high doses (Luo *et al.*, 2005; Liu *et al.*, 2010). Acrolein-induced necrosis was described in few studies (Luo *et al.*, 2005), but mitochondrial-driven cell death seems to be the canonical road and was widely studied. Acrolein-induced apoptosis was indeed confirmed in several cell types through DNA fragmentation, phosphatidylserine externalization, poly(ADP-ribose) polymerase cleavage and activation of caspases (Pan *et al.*, 2009; Roy *et al.*, 2010). Last but not least, hydroxyalkenals are potent activators of apoptosis. They both induce DNA fragmentation and activation of caspases in very different cell types (Choudhary *et al.*, 2002; Vaillancourt *et al.*, 2008). In addition, HHE has been shown to decrease the expression/phosphorylation of Bcl-2, while increasing that of Bax, leading to apoptosis of human renal epithelial cells (Bae *et al.*, 2011; Bodur *et al.*, 2012).

Interestingly, the toxicity of aldehydes is highly correlated to their ability to form covalent adducts on proteins. In muscle cells, the lethal concentration 50 (LC50) for 12 different aldehydes was calculated, including HHE and HNE (Pillon *et al.*, 2010). This LC50 was strongly correlated with their respective potency to form covalent adducts on albumin *in vitro* (Figure 1). This demonstrates that the cytotoxicity and likely other biological effects of aldehydes mainly occur through chemical adduction of other biomolecules.
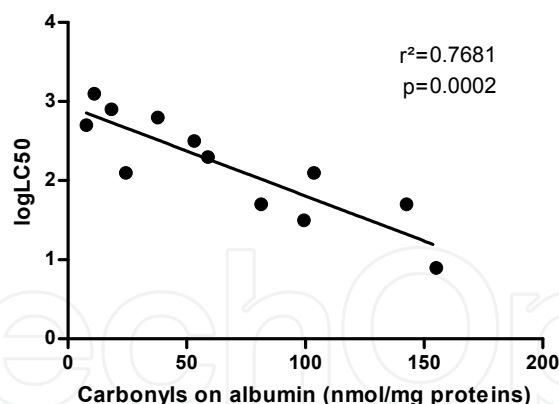
Figure 1. Toxicity is correlated to the adduction ability. Viability of muscle cells was measured in response to 12 different aldehydes[1]. The calculated LC50 was then correlated to their ability to form covalent adducts on bovine serumalbumin (Pillon et al., 2010).

2.2.2. Oxidative stress and ROS production

The classical sequence of events is that oxidative stress triggers lipid peroxidation which in turns produces aldehydes by-products. However, an interesting paradigm was pointed out by several groups: lipid aldehydes are able to induce the production of ROS, and this is thought to be of importance in their deleterious effects. For instance, acrolein treatment produces nitric oxide (Misonou *et al.*, 2006) and induces generation of intracellular oxidants (Luo *et al.*, 2005; Wang *et al.*, 2011). Similarly, accumulation of intracellular ROS was described in cells treated with MDA (Cheng *et al.*, 2011); and unsurprisingly, the 4-hydroxyalkenals HHE and HNE share the same ability. HHE induces ROS in neurons and tubular epithelial cells (Long *et al.*, 2008; Bae *et al.*, 2011) and HNE induces mitochondrial oxidative stress in neurons, vascular muscle, liver and skeletal muscle cells (Uchida *et al.*, 1999; Lee *et al.*, 2006; Pillon *et al.*, 2012). The source of ROS was suggested to be mitochondria, as several aldehydes have been shown to induce a significant decrease in mitochondrial membrane potential (Uchida *et al.*, 1999; Luo *et al.*, 2005).
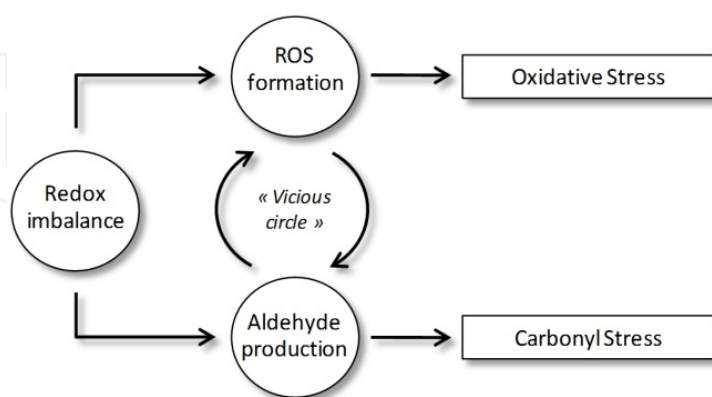

Figure 2. The vicious circle linking ROS and lipid aldehyde production

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[1] 4-hydroxy-2-hexenal, 4-hydroxy-2-nonenal, 4-hydroxy-2-dodecenal, 4-hydroxy-2-hexenal dimethylacetal, 4-hydroxy-2-nonenal dimethylacetal, 4-hydroxy-2-dodecenal dimethylacetal, trans-2-hexenal, trans-2-nonenal, trans-2-dodecenal, hexanal, nonanal, dodecanal

This concept is reinforced by the fact that an increase in glutathione pool can prevent the deleterious effects of HNE on both adduct formation and ROS production (Pillon *et al.*, 2012) and that dysfunction of glutathione S-transferase, a major enzyme for aldehyde detoxification, leads to excess 4-hydroxy-2-nonenal and oxidative stress (Kostyuk *et al.*, 2010; Curtis *et al.*, 2010). Based on this body of evidence, lipid by-products can be seen as parts of a vicious circle in which increased ROS production generates aldehydes which further amplify the generation of oxidative species and so on (Figure 2).

2.2.3. Signalling pathways and transcription factors

Consistent with the extensive work done on cytotoxicity, the stress signalling pathways have been primarily pointed out as the main intracellular route activated by aldehydes. The mitogen-activated protein kinases (MAPKs) are indeed activated by several aldehydes including MDA, acrolein and HNE. MDA activates c-Jun N-terminal kinases (JNK) and extracellular signal-regulated kinases (ERK) (Cheng *et al.*, 2011) and acrolein-induced apoptosis occurs through activation of p38 and ERK (Tanel & Averill-Bates, 2007). The activation of these three MAPK has also been described following treatment with HNE (Uchida *et al.*, 1999; Zarrouki *et al.*, 2007; Pillon *et al.*, 2012) and HHE (Je *et al.*, 2004; Bae *et al.*, 2011). Overall, most studies investigated the cytotoxic effects of aldehydes and thus focused on cellular stress pathways; therefore, very little data is available regarding their potential effects on other pathways. Only very recent work shows that HNE interfere with insulin signalling pathway through oxidative stress and adduction of IRS1 and Akt (Demozay *et al.*, 2008; Shearn *et al.*, 2011; Pillon *et al.*, 2012).

Aldehydes regulate gene expression by activating the signalling pathways described above, or by direct modification of transcription factors. Unsurprisingly, aldehyde production regulates the expression of several antioxidant enzymes such as NAD(P)H quinone oxidoreductase-1, Heme oxygenase-1 and glutathione S-transferase (GST). This occurs through the activation of the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which drives the expression of these antioxidant enzymes. Aldehyde-induced Heme Oxygenase-1 expression is indeed mediated by the Nrf2 pathway in HUVECs (Lee *et al.*, 2011) and Nrf2 silencing significantly attenuates the induction of this same gene by acrolein (Zhang & Forman, 2008).

Beyond the expression of antioxidant enzymes, cellular growth, apoptosis and inflammatory responses can be induced by aldehydes, involving the activation of the nuclear factor kappa B (NF-κB) family. The effects of acrolein on NF-κB activation are controversial, but its activation by HNE and HHE is well documented. HHE and HNE induce NF-kappaB activation through IKK/NIK pathway, leading to IκB phosphorylation and subsequent proteolysis (Page *et al.*, 1999; Je *et al.*, 2004; Lee *et al.*, 2004). HNE has moreover been shown to induce DNA-binding of NF-κB in vascular smooth muscle cells (Ruef *et al.*, 2001).

The peroxisome proliferator-activated receptor (PPAR) family regulate the expression of genes that encode proteins involved in energy balance. They act as ligand-activated

transcription factors and are responsive to the lipid status of the cell, therefore important during high fat diets and obesity. HNE is an intracellular agonist of PPARβ/δ while HHE do not activate this receptor (Coleman *et al.*, 2007). Through this pathway, HNE significantly elevates adiponectin gene expression, concomitant with increased PPAR-γ gene expression and transactivity. Meanwhile, HDDE acts through PPARδ signalling pathways to regulate glucose transport in vascular endothelial cells subjected to hyperglycemia (Riahi *et al.*, 2010) and HNE stimulates insulin secretion from Beta cells through interaction with PPARδ (Cohen *et al.*, 2011).

2.3. Concentration in plasma and tissues

Little data is available concerning aldehydes levels in biological fluids, except for MDA and HNE which have been widely used as lipid peroxidation markers. The concentration of MDA in the plasma of healthy subjects is around 2-5 μM and increases up to 2-fold in type 2 diabetic patients (Figure 4). In the specific context of metabolic diseases, MDA is positively correlated with BMI and waist circumference in obese patients (Furukawa *et al.*, 2004). Depending on the study, HNE concentration has been found to range from 50 nM to 10 μM under normal conditions. This significant variability in concentration according to the authors could be explained partly by the method used (LC/MS, GC/MS ...) and also by the difficulty to measure such reactive derivatives. If HNE has been widely studied, there is however scarce data in the literature regarding the pathophysiological concentrations of HHE and HDDE. Plasma HHE concentration was however found to be around 9 nM in human, and dramatically increases to reach 90 nM after several weeks of a diet rich in omega-3 fatty acids (Calzada *et al.*, 2010). Our group recently showed that HHE concentration was 20 nM in humans and 7 nM in rats and that it increases in both type-2 diabetes patients and type-1 diabetic rats, reinforcing existing evidence for a role of lipid aldehydes in metabolic diseases.

Tissue	Aldehyde	Concentration (μM)	References
Plasma	MDA	1 – 6	See figure 4
	HHE	0.006 – 0.090	(Calzada et al., 2010; Pillon et al, unpublished)
	HNE	0.007 – 11	(McGrath *et al.*, 2001; Selley, 2004; Syslova *et al.*, 2009)
Exhaled breath condensate	HNE	0.25 - 5	(Syslova *et al.*, 2009; Manini *et al.*, 2010)
	MDA	0.63 - 14	(Syslova *et al.*, 2009; Manini *et al.*, 2010)
Ventricular Fluid	HNE	0.2 - 120	(Lovell *et al.*, 1997)
Pancreatic Islets	HNE	23 – 35	(Miwa *et al.*, 2000)

Table 3. Concentration range of aldehydes in healthy plasma and tissues

One should keep in mind that all current quantification methods (HPLC, GC) only assay the free fraction (*i.e.* unreacted) of lipid aldehydes present in samples. Being very reactive, this is certainly not representative of the amount indeed produced from lipid peroxidation, which rapidly react with neighbour targets to form covalent adducts and thus, other non-quantitative methods estimating the amount of HNE have been used. For example, detection of protein adducts by immunohistochemistry has shown a significant increase in HHE and HNE proteins adducts in Parkinson's disease and in chronic liver disease (Yoritaka *et al.*, 1996; Paradis *et al.*, 1997). Of particular interest for this chapter, HNE adducts on plasma albumin are increased in type 2 diabetes (Toyokuni *et al.*, 2000).

3. Insulin secretion and type-1 diabetes

3.1. Lipid aldehydes and insulin secretion

Oxidative stress associated with hyperglycemia is suspected to participate in beta cell dysfunction in terms of insulin synthesis and/or secretion. Diabetic animals display increased levels of lipoperoxidation in pancreas, and HNE concentration was reported to reach up to 35 μM in pancreatic islets of diabetic rats (Miwa *et al.*, 2000). In addition, HNE-modified proteins are increased in the pancreatic beta-cells of Goto-Kakizaki rats, a genetic model of non obese type 2 diabetes (Ihara *et al.*, 1999), as well as in type 2 diabetic patients (Sakuraba *et al.*, 2002). HNE and other lipid peroxidation by-products such as 2-hexenal and 2-butenal inhibit glucose-induced insulin secretion in isolated rat islets. Both glucose utilization and glucose oxidation are blunted in islets after treatment with aldehydes suggesting that they impair glucose-induced insulin secretion through an interference with glycolytic pathway and citric acid cycle (Miwa *et al.*, 2000). Another piece of evidence comes from the exposure of beta cells to NO donors and to interleukin-1 beta, which leads to generation of oxidative stress and lipoperoxidation by-products. MDA and HNE produced under this condition are involved in the activation of an apoptotic program, contributing to the reduction in the beta cell mass (Cahuana *et al.*, 2003). Peroxynitrite indeed triggers lipoperoxidation in the beta-cell line RIN-5-F, and the resulting protein carbonylation is a key factor linking NO-dependent lipoperoxidation and apoptosis (Cahuana *et al.*, 2003). Alloxan, a toxic glucose analogue, has been widely used to generate rodent models of type-1 diabetes, as it selectively destroys insulin-producing cells in the pancreas. Alloxan-induced diabetic rats exhibit increased lipid peroxidation associated with defects in insulin secretion, which can be prevented by the antioxidant S-allyl cysteine therefore restoring insulin secretion and ameliorating the glycaemic control (Augusti & Sheela, 1996).

3.2. Lipid aldehydes and the beta cells: Doctor Jeckyll or Mr Hyde?

HNE was shown to elicit various physiological or physiopathological responses: high concentrations elicit beta cell death and defect in insulin secretion, while lower concentrations act as signalling mediators. In INS-1E beta-cells, elevated glucose levels increase the release of arachidonic acid and linoleic acid from membrane phospholipids and

promote their peroxidation to HNE. At non cytotoxic concentrations, HNE behaves as an endogenous ligand for nuclear receptor PPAR-δ, stimulating insulin secretion in beta-cells (Cohen *et al.*, 2011). In rat islet beta-cell-derived RINm5F cells, a recent report demonstrates the involvement of the transient receptor potential (TRP) cation channels in the HNE-induced insulin secretion. Short-term (1h) exposure to HNE induces a transient increase in intracellular calcium concentration and triggers insulin secretion. HNE induces calcium influx through activation of TRP channels (amongst which TRPA1) which appears to be coupled with the L-type voltage-dependent calcium channel, and ultimately insulin secretion (Numazawa *et al.*, 2012). Lipid aldehydes should therefore be considered either as detrimental (>10 μM) or as beneficial (sub micromolar range) depending on their actual tissue concentration.

3.3. Direct adduction of the insulin polypeptide

Under conditions of oxidative stress, insulin, a polypeptidic hormone composed of 51 amino acid residues, is exposed to direct oxidative insult or to modification by lipoperoxidation by-products. Several amino acids are putative sites of adduction, and thus, covalent binding of lipid aldehydes affect the biological actions of this hormone. This applies to acrolein and methylglyoxal, whose fixation on insulin has been shown to reduce both hypoglycemic effects in rats and glucose uptake in 3T3-L1 adipocytes (Jia *et al.*, 2006; Medina-Navarro *et al.*, 2007). HHE and HNE, toxic aldehydes generated during lipid peroxidation, also modify the B-chain of human insulin *in vitro*, predominantly at the His B5 and His B10 residues via Michael adduction (Figure 3). Adduct formation affects the biological activity of insulin *in vivo*, decreasing its hypoglycemic effect in mice and stimulation of glucose uptake in adipose and muscle cells (Pillon *et al.*, 2011).
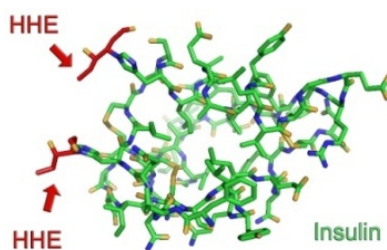

Figure 3. Structure of insulin monomer displaying two HHE adducts on histidine residues. From Pillon et al, 2011.

4. Obesity, insulin resistance and type-2 diabetes

4.1. Lipid peroxidation by-products association with obesity

Obesity is a major factor in the development of metabolic syndrome. After consumption of an energy-dense (i.e. high-fat) diet, plasma HNE levels increase rapidly and significantly within minutes (Devaraj *et al.*, 2008). When consumed regularly, this diet promotes obesity, which suggests a role for HNE very early in the development of obesity. On the other hand, levels of circulating HNE tend to decrease when obese people are maintained on calorie

restriction (Johnson *et al.*, 2007), demonstrating that lipid peroxidation is tightly linked to high fat diet and obesity. Furukawa et al. reported that increased oxidative stress in accumulated fat is an important pathogenic mechanism of obesity-associated metabolic syndrome. Production of ROS is indeed selectively increased in white adipose tissue of obese mice and associated with a blunted expression of antioxidant enzymes. In good agreement, fat accumulation correlates with systemic lipid peroxidation in humans (Furukawa *et al.*, 2004), and the plasma concentration of MDA is 1.8 fold higher in subjects with a BMI above 40 kg/m^2 compared to lean individuals (Olusi, 2002). Diet-induced obesity increases tissue and plasma accumulation of ALEs (protein–acrolein and protein–HNE adducts for example), suggesting that obesity is associated with an increase in the formation of lipid peroxidation-derived aldehydes (Baba *et al.*, 2011). A significant accumulation of HNE was noticed in the white adipose tissue of obese mice, where the adipocyte fatty acid binding protein (AFABP also known as aP2) is the soluble protein most highly modified by HNE in this tissue (Grimsrud *et al.*, 2007). In obese mice roughly 7% of the AFABP in adipose tissue is covalently modified by HNE resulting in a decreased binding affinity for fatty acids. Lipid peroxidation is however not restricted to adipose tissue since HNE is also elevated in skeletal muscles of Otsuka Long-Evans Tokushima fatty (OLETF) rat, a model for hyperphagic obesity (Morris *et al.*, 2008). Intracellular triglyacylglycerols accumulate in the muscle of obese humans where it is considered as a pathogenic factor in the development of insulin resistance. In obese compared to endurance-trained subjects, the lipid peroxidation to intracellular triacylglycerols ratio was 4-fold higher suggesting that obesity is associated with increased muscle lipid peroxidation (Russell *et al.*, 2003; Vincent *et al.*, 2006).

Chronic, low grade inflammation of white adipose tissue is a hallmark of obesity and a major contributor to oxidative stress and lipid peroxidation (Wellen & Hotamisligil, 2003). In the expanding adipose tissue, hypertrophied adipocytes contribute to the inflammation by up-regulating the expression and release of pro-inflammatory cytokines. In 3T3-L1 adipocytes, HNE can dose-dependently increase the expression of the inducible cyclooxygenase (COX-2) (Zarrouki *et al.*, 2007) and that of the plasminogen activator inhibitor-1 (PAI-1). In the meantime, HNE decreases the expression of the anti-inflammatory, insulin-sensitizing hormone adiponectin (Soares *et al.*, 2005; Wang *et al.*, 2012), therefore linking lipid peroxidation by-products and chronic inflammation.

4.2. Lipid peroxidation by-products association with type-2 diabetes

Epidemiological studies demonstrates that fasting glycemia is positively correlated with oxidative stress markers such as 8-epi-PGF2α and TBARs and negatively correlated with plasma glutathione (Trevisan *et al.*, 2001; Menon *et al.*, 2004). In type-2 diabetic individuals, 8-epi-PGF2α is positively correlated to the HOMA index for insulin resistance (Gopaul *et al.*, 2001) and urinary acrolein correlates with glycated haemoglobin HbA1c (Daimon *et al.*, 2003). This was confirmed in animal models of insulin resistance which exhibit increased markers of oxidative stress, such as plasma F2-isoprostanes (Laight *et al.*, 1999a). In parallel,

antioxidant defences are reduced during an oral glucose tolerance test in normal and non-insulin-dependent diabetic subjects (Ceriello *et al.*, 1998), and diabetes is associated with decreased vitamin C and glutathione (Maxwell *et al.*, 1997; Dierckx *et al.*, 2003). Reciprocally, an intensive treatment of diabetes improves circulating levels of H_2O_2 and MDA (Wierusz-Wysocka *et al.*, 1995); and improved insulin sensitivity resulting from exercise and/or dietary restriction is associated with reduced levels of lipid peroxidation products (Reviewed by Vincent *et al.*, 2007). On the other hand, insulin sensitivity can be improved through antioxidant or carbonyl scavenging treatment (Kamenova, 2006; Vincent *et al.*, 2009), demonstrating the tight link existing between oxidative stress, oxidation by-products and insulin resistance.
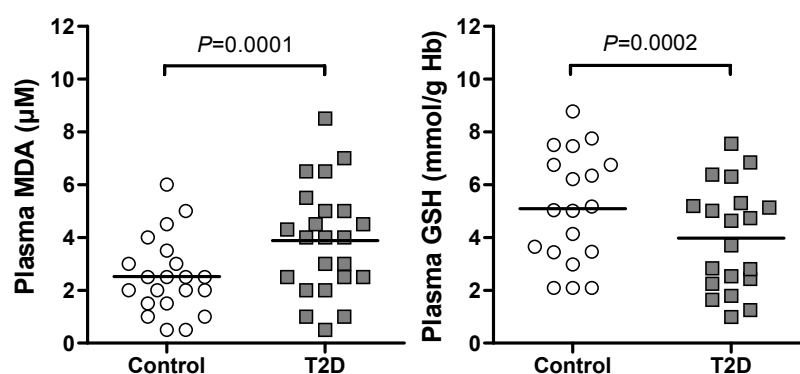

Figure 4. Plasma MDA concentration (μM) and blood GSH content (mmol/g haemoglobin) in healthy and type-2 diabetic (T2D) individuals. Results are a meta-analysis from 23 different publications; one dot represents the mean value obtained in one study[2]. In average, MDA is significantly increased by 60% while GSH is decreased by 25% in T2D compared to healthy subjects (paired student t-test, n=21).

Among α,β-unsaturated aldehydes, only MDA and to some extend HNE have been studied as oxidative stress biomarkers in diabetes, and they are indeed both increased up to 2-fold in both human (Figure 4) and animal models of type-2 diabetes (Wierusz-Wysocka *et al.*, 1995; Dierckx *et al.*, 2003). It has moreover been demonstrated that Type-2 diabetes duration is independently associated with increased levels of lipid peroxidation (Nakhjavani *et al.*, 2010), and our group recently showed an increase in HHE concentration in type-2 diabetes patients, reinforcing existing evidence for the specific role of lipid aldehydes in insulin resistance.

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[2] Results from Wierusz-Wysocka et al., 1995; Vijayalingam et al., 1996; Feillet-Coudray et al., 1999; Rábago-Velasco et al., 2000; Rizvi & Zaid, 2001; Seghrouchni et al., 2002; Dinçer et al., 2002; Dierckx et al., 2003; Duman et al., 2003; Pasaoglu et al., 2004; Memişoğullari & Bakan, 2004; Skrha et al., 2005; Kurtul et al., 2005; Ozdemir et al., 2005; Mahboob et al., 2005; Saxena et al., 2005; Sampathkumar et al., 2005; Kuppusamy et al., 2005; Lapolla et al., 2007; Sathiyapriya et al., 2007; Singhania et al., 2008; Jain et al., 2009; Nakhjavani et al., 2010; Narasimhan et al., 2010; Shinde et al., 2011; Huang et al., 2011; Bahadoran et al., 2011; Zhang et al., 2011; Pácal et al., 2011; Calabrese et al., 2011 and Rasic-Milutinovic et al., 2012

Aldehydes concentration is increased in several tissues during diabetes, as revealed by increased levels of HNE in pancreas, liver, brain and heart. Pancreatic islets from type-2 diabetic patients are positively stained with HNE, suggesting that oxidative stress through lipid peroxidation could contribute to the reduced beta-cell mass and islet-cell injury (Sakuraba *et al.*, 2002). An increased level of HNE-modified proteins is reported in the pancreatic beta cells of Goto Kakizaki rats as a result of hyperglycaemia (Ihara *et al.*, 1999); and accumulation of HNE is observed in liver of diabetic rats due to the impairment of HNE-metabolizing enzymes (Traverso *et al.*, 1998, 2002). Diabetic mice under a high fat diet exhibit increased HNE adducts levels in temporal lobes relative to control (Lyn-Cook *et al.*, 2009), and HNE conjugation of GLUT3, the glucose transporter present in neurons, is increased in the hippocampus of diabetic rats subjected to stress (Reagan *et al.*, 2000). When db/db obese mice are fed a Western diet containing 21% fat and 0.15% cholesterol, they develop obesity, hyperglycemia, and insulin resistance. In this situation, HNE is significantly elevated in the left ventricular myocardium of diabetic mice compared to their lean littermates (Yamashita *et al.*, 2010). Finally, hyperglycemic Zucker Diabetic Fatty (ZDF) rats exhibited a 8-fold increase in plasma HDDE concentration compared to their lean non diabetic counterparts (Riahi *et al.*, 2010).

4.3. Causative role for lipid peroxidation by-products in the metabolic syndrome

This body of evidence linking oxidative stress with the metabolic syndrome is however only based on correlations and do not decipher the mechanisms and/or the causative role of oxidative stress in diabetes. There are consequently two main hypotheses:

1. First is chronic hyperglycemia which then leads to oxidative stress. In this case, lipid peroxidation products would be by-products of this oxidative stress and a consequence of diabetes, even if contributing to the progression of the disease and its complications.
2. First is oxidative stress which precedes the development of diabetes and plays a causative role in its development. In this hypothesis, deregulation of antioxidant defences and increased oxidative stress would lead to accumulation of secondary by-products consequently inducing insulin resistance.

We will focus in this chapter on the second hypothesis for which evidences have been recently accumulating from human, animal and cell culture studies.

Oxidative stress, through reactive oxygen species can positively and negatively regulate insulin signalling, depending on time, dose, model and free radical used (for review, see Bashan *et al.*, 2009). It is however admitted that prolonged oxidative stress impairs insulin signalling, insulin-induced GLUT4 translocation and glucose uptake. This occurs through several mechanisms including, but not limited to IRS inhibitory phosphorylation, MAPK activation and endoplasmic reticulum stress. This body of evidence for the role of oxidative stress in insulin resistance has been demonstrated in adipocytes, muscle, liver and cardiac cells (Rudich *et al.*, 1998; Bloch-Damti *et al.*, 2006; JeBailey *et al.*, 2007; Singh *et al.*, 2008*a*; Shibata *et al.*, 2010; Tan *et al.*, 2011). Even *in vivo*, a pro-oxidant challenge provokes the onset of type-2 diabetes in insulin resistant rats (Laight *et al.*, 1999*b*) and chronic methylglyoxal

infusion by minipump causes pancreatic beta-cell dysfunction and induces type-2 diabetes in Sprague-Dawley rats (Dhar *et al.*, 2011).

The role of lipid aldehydes *in vivo* during metabolic diseases is often indirectly assessed, and the most compelling evidence for a causative role of aldehydes comes from polymorphisms in the glutathione-S-transferase (GST) gene. This family of enzymes is responsible for the detoxification of aldehydes through conjugation to glutathione; and several deletion polymorphisms leading to blunted enzyme activity have been described. Patients carrying certain null GST polymorphisms had up to 3-fold increased incidence of type-2 diabetes mellitus compared to those with normal genotypes (Amer *et al.*, 2011). Accordingly, the expression of the GST4A is blunted in the adipose tissue of obese insulin resistant subjects (Curtis *et al.*, 2010).
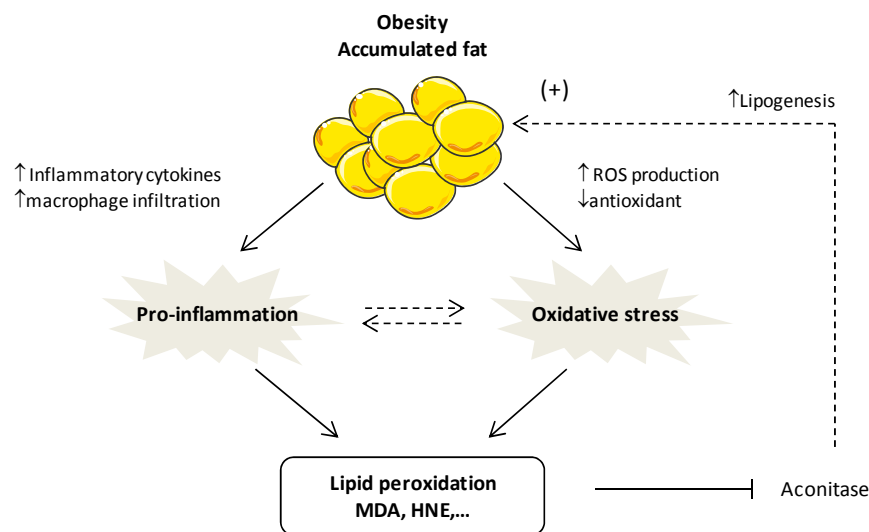

Figure 5. The obesity – lipid peroxidation vicious circle

This same observation was made in animals model, were it has been suggested that excessive production of HNE might be sufficient to cause obesity and the metabolic syndrome. Mice lacking the gene encoding the HNE-conjugating enzyme mGSTA4-4 develop obesity and insulin resistance, unsurprisingly associated with HNE accumulation in multiple tissues (Singh *et al.*, 2008*b*). Cell culture work further shows that dysfunction of glutathione S-transferase or its ablation by siRNA leads to excess HNE accumulation, increased protein carbonylation, oxidative stress, and mitochondrial dysfunction (Kostyuk *et al.*, 2010; Curtis *et al.*, 2010). On the other hand, overexpression of glutathione-S-transferase A4-4 protects against oxidative stress and HNE-induced apoptosis (Vaillancourt *et al.*, 2008). Additional findings suggest that HNE causes fat accumulation by promoting fatty acid synthesis and suppressing fatty acid beta-oxidation. Interestingly, the phenotype of mGSTA4-4 null mice is strain dependent: mGSTA4-4 null mice with 129/sv genetic background exhibit both increased accumulation of HNE and obesity while those with C57Bl6 genetic background are lean and HNE is unchanged (Singh *et al.*, 2008*b*). In good agreement, silencing of the mGSTA4-4 gene in the nematode Caenorhabditis elegans also results in an accumulation of lipid peroxidation by-products and a fatty phenotype (Singh *et*

al., 2009). When HNE is experimentally increased in the nematode, either by genetic deletion or through direct exposure, it promotes fat accumulation. The mechanism involves HNE inhibition of mitochondrial aconitase leading to an accumulation of malonyl CoA, precursor of fatty acid synthesis (Zimniak, 2010). Taken together, these data suggest that accumulation of lipid aldehydes and fat deposition could be mutually inductive leading to a vicious circle promoting fat accretion (Figure 5).

The direct causative role of lipid peroxidation by-products in insulin resistance has also been recently evidenced in cell cultures studies. A first study identified that methylglyoxal, an aldehyde by-product of glucose oxidation, can impair the insulin signalling pathways independently of the formation of intracellular reactive oxygen species (Riboulet-Chavey *et al.*, 2006). Then, focusing on lipid peroxidation products, two studies demonstrated that HNE can induce insulin resistance in adipocytes and muscle cell through inhibition of IRS and Akt signalling, as well as insulin-induced glucose uptake (Demozay *et al.*, 2008; Pillon *et al.*, 2012). These two studies identified carbonyl stress (notably IRS1 adduction) and ROS production as the possible mechanisms responsible for HNE effects; a third one being possibly the adduction of Akt2, which inhibits insulin-dependent Akt signalling in HepG2 cells (Shearn *et al.*, 2011). Altogether, these studies strongly suggest that excessive production of aldehydes might be sufficient to cause obesity, diabetes and the metabolic syndrome.

5. Preventing the deleterious effects of aldehydes

It has been known for decades that supplementation in α-lipoic acid in type-2 diabetic subject improves glucose tolerance and insulin sensitivity (Jacob et al., 1996). Similarly, increased intake of Vitamin E in obese and insulin resistant patients reduces fasting glycemia and the HOMA index for insulin resistance, and this is correlated with a decrease in the concentration of peroxides in plasma (Manning et al., 2004). This adds further evidence for a causative role of oxidative stress in the metabolic syndrome, and opens new therapeutic perspectives using antioxidant and/or scavenging of toxic aldehydes.

5.1. Glutathione and enzymatic detoxification

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) which is present in high concentration in the cytoplasm of living cells: 5-50 nmol/mg proteins (Jungas *et al.*, 2002; Dominy *et al.*, 2007). GSH is an important coenzyme of several enzymatic reactions, and exerts its antioxidant activity mainly through regeneration of vitamin E, and also through direct interaction with free radical and aldehydes. GSH is obviously necessary for the activity of glutathione-S-transferases (GSTs), a family of enzymes responsible for detoxification of electrophile by-products, such as the ones derived from lipid peroxidation, and GSH exhibits indeed a high reactivity for HNE. Though HNE is electrophile enough to spontaneously react with GSH, this reaction is dramatically accelerated via the conjugation process catalyzed by GSTs (Alin *et al.*, 1985). As described above, mice lacking GSTs develop

obesity and insulin resistance (Singh *et al.*, 2008*b*), and humans carrying null polymorphisms for GST have a 3-fold increased risk of having type-2 diabetes (Amer *et al.*, 2011), pointing out the important role of GSH in the metabolic syndrome. As addition reaction to GSH contributes to the detoxification of aldehydes, pharmacological strategies to increase glutathione pools or GST activity should be protective against aldehydes. Several studies were indeed successful in protecting cultured cells from the deleterious effects of oxidative stress through an increase in intracellular pools of reduced glutathione. This strategy was particularly efficient to protect the cells against the deleterious effects of HNE (Yadav *et al.*, 2008; Jia *et al.*, 2009*b*; Pillon *et al.*, 2012).

In addition to glutathione-S-transferases, several enzymes are also responsible for the detoxification of aldehydes: aldehyde dehydrogenase, alcohol dehydrogenase (Hartley *et al.*, 1995), aldose reductase (Srivastava *et al.*, 2000) and fatty aldehyde dehydrogenase (Demozay *et al.*, 2008). These enzymes however participate to a lower extent in the metabolism of aldehydes, compared to glutathione and GST. Recent data indicate that if HHE and HNE are both metabolized via glutathione, the effectiveness of detoxification differs for these two molecules. By extension, aldehydes may be metabolized with different affinities and efficiencies by detoxification enzymes, which could explain some differences in their respective toxicities (Long *et al.*, 2010).

5.2. Scavenging

In chemistry, a scavenger is a chemical substance able to remove or inactivate impurities or unwanted reaction products. In living cells, a scavenger is, by extension, a molecule able to inactivate toxic compounds such as ROS and aldehydes, therefore preventing their deleterious effects. In the case of aldehydes, a scavenger would be a strong nucleophile molecule on which HHE, HNE or any other aldehyde would form a covalent adduct. Consequently, most of the aldehyde scavengers are amino- or sulphur-containing drugs such as N-acetyl-cysteine (NAC), hydralazine, S-adenosyl-methionine (SAM) aminoguanidine (AGD) and α-lipoic acid, the latter being tested in several human studies for treatment of type-2 diabetes.

5.2.1. α-lipoic acid

α-lipoic acid is a natural compound found in many foodstuffs (such as potatoes, broccoli and meat), but in rather low amount. The effect of lipoic acid was demonstrated in animal models, where it enhances insulin-stimulated glucose metabolism in skeletal muscle from insulin-resistant rat (Jacob et al., 1996). It was rapidly tested in several clinical studies which demonstrated its beneficial effects in type-2 diabetes through a decrease in fasted blood glucose, enhancement of glucose disposal, improved insulin sensitivity and decreased insulin resistance (Jacob *et al.*, 1995). These results were confirmed *in vitro*, where α-lipoic acid prevents the development of glucose-induced insulin resistance in adipocytes (Greene et al., 2001) and were reproduced by many independent studies. Treatment with lipoic acid decreases oxidative stress in both adipocytes and muscle cells (Rudich et al., 1999; Maddux

et al., 2001) and also decreases lipid peroxidation markers in insulin resistant rats (Thirunavukkarasu & Anuradha, 2004), suggesting that the improvement of insulin sensitivity is due to its antioxidant properties. Hence, the current literature supports the use of alpha lipoic acid for the treatment of diabetes complications and it consequently became the first antioxidant supplement used for the treatment of diabetes complications, being already approved in Germany for the treatment of diabetic neuropathy.

5.2.2. N-acetylcysteine (NAC)

N-acetylcysteine is a cysteine derivative and a potent antioxidant. Its properties are mainly due to its thiol group able to reduce free radicals as well as its role as a precursor in the formation of glutathione (Zafarullah *et al.*, 2003). NAC exhibits highly protective scavenging properties against aldehydes and protects against MDA increase and GSH decrease in animal models of insulin resistance. NAC is able to improve insulin sensitivity in healthy rats (Figure 6) and reverses insulin resistance and aldehyde-induced hypertension in rats (Haber *et al.*, 2003). In cell culture studies, NAC can prevent the insulin resistance induced by HNE in muscle cells (Pillon *et al.*, 2012), as well as the one induced by advanced glycation end products in adipocytes (Unoki *et al.*, 2007), thus confirming the important role NAC can play in improving both oxidative stress parameters and insulin resistance.
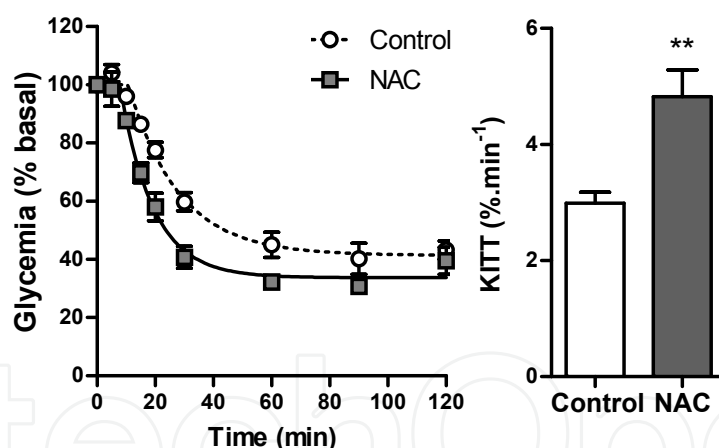

Figure 6. Insulin sensitizing effect of N-acetylcysteine. Wistar rats were given NAC in drinking water for one week (total intake was 225 mg.kg^{-1}.day^{-1}). Insulin sensitivity was calculated using a standard insulin tolerance test[3]. Results are average ± SEM from 5 different animals per group, expressed as percent of basal glycemia. From Pillon et al. unpublished results.

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[3] Animals fasted overnight were then injected intraperitoneally with 0.5 UI/kg body weight of insulin. Plasma glucose was measured from tail vein blood using a glucometer at 0, 20, 40, 60, and 120 min following the injection. Glucose disappearance rate for ITT (K_{ITT}; %/min) was calculated as: $K_{ITT}=(0.693\times100)/t_{1/2}$, where $t_{1/2}$ was calculated from the slope of the plasma glucose concentration, considering an exponential decrement of glucose concentration during the 20 min after insulin administration. Higher insulin sensitivity index (K_{ITT}) scores mean higher response of tissues to insulin.

Results from clinical trials in type-2 diabetic subjects show that NAC is able to decrease oxidative stress parameters, increase GSH and decrease plasma VCAM-1 (De Mattia et al., 1998); moreover, long-term N-acetylcysteine administration reduces endothelial activation and is proposed as a potential antiatherogenic therapy (Martina et al., 2008). Despite the fact that NAC can improve insulin sensitivity in women with polycystic ovary syndrome (Fulghesu *et al.*, 2002) and this body of evidence suggesting that NAC may slow down the progression of diabetic complications, to date no clinical trial demonstrated any significant benefit of its supplementation in diabetes.

5.2.3. Aminoguanidine (AGD)

Aminoguanidine is a highly nucleophilic agent which reacts *in vitro* and *in vivo* with aldehydes, therefore protecting against the deleterious effects of ALE precursors (Peyroux & Sternberg, 2006). It is also an antioxidant able to quench hydroxyl radicals and *in vivo*. AGD in drinking water decreases lipid peroxidation in type-1 diabetic rats and rabbits (Ihm *et al.*, 1999). In experimental animal models of diabetes, AGD demonstrates significant effects in protecting against pathological complications, such as diabetic nephropathies, atherosclerosis and neurovascular complications (El Shazly *et al.*, 2009). Consequently, several clinical trials in humans have been designed to evaluate AGD efficiency but they demonstrate only mild effects and were not conclusive, partly because of side-effects, and of weak carbonyl scavenger effects in human vascular tissues (Bolton *et al.*, 2004).

5.2.4. Hydralazine

Primarily used as an antihypertensive drug, hydralazine exhibits a pronounced nucleophilicity and is consequently very efficient in scavenging several aldehydes (acrolein, HNE) and ketones, as well as aldehyde-adducted proteins (Burcham et al., 2002). It is also a powerful antioxidant, able to inhibit the generation of ROS (Münzel *et al.*, 1996). Its scavenging activity *in vivo* was demonstrated by its ability to reverse the formation of HNE and acrolein adducts on tissue proteins in atherosclerotic aortas of hypercholesterolemic animals (Vindis et al., 2006), but its effects on insulin resistance and diabetes are to date uncharacterized.

5.2.5. S-adenosyl-methionine (SAM)

In living organisms, SAM is endogenously synthesized from methionine in every cell, but the liver is the major site of its synthesis and degradation. SAM is an important precursor for cysteine and glutathione production (Lu, 2000) and its involvement in several metabolic pathways makes it essential for a wide spectrum of cellular processes. SAM inhibits both HNE production and adducts formation and efficiently prevents high-fat diet-induced non-alcoholic steatohepatitis in rats (Lieber et al., 2007), even if its direct effects on insulin resistance are still unknown.

6. Conclusion

Lipid peroxidation by-products are associated with metabolic diseases, but their primary role during obesity and diabetes is subject to debate. *In vitro* and *in vivo* animal studies highlighted that an aldehyde challenge, through a state of carbonyl stress, affects several steps involved in the development of obesity and type-2 diabetes (Figure 7). On insulin-sensitive tissues (muscle, adipose tissue), aldehydes lead to insulin resistance, while in pancreas, aldehydes impair insulin secretion. Together with the carbonylation of the insulin peptide itself, aldehydes could contribute to the defects in insulin action, leading to the metabolic syndrome.
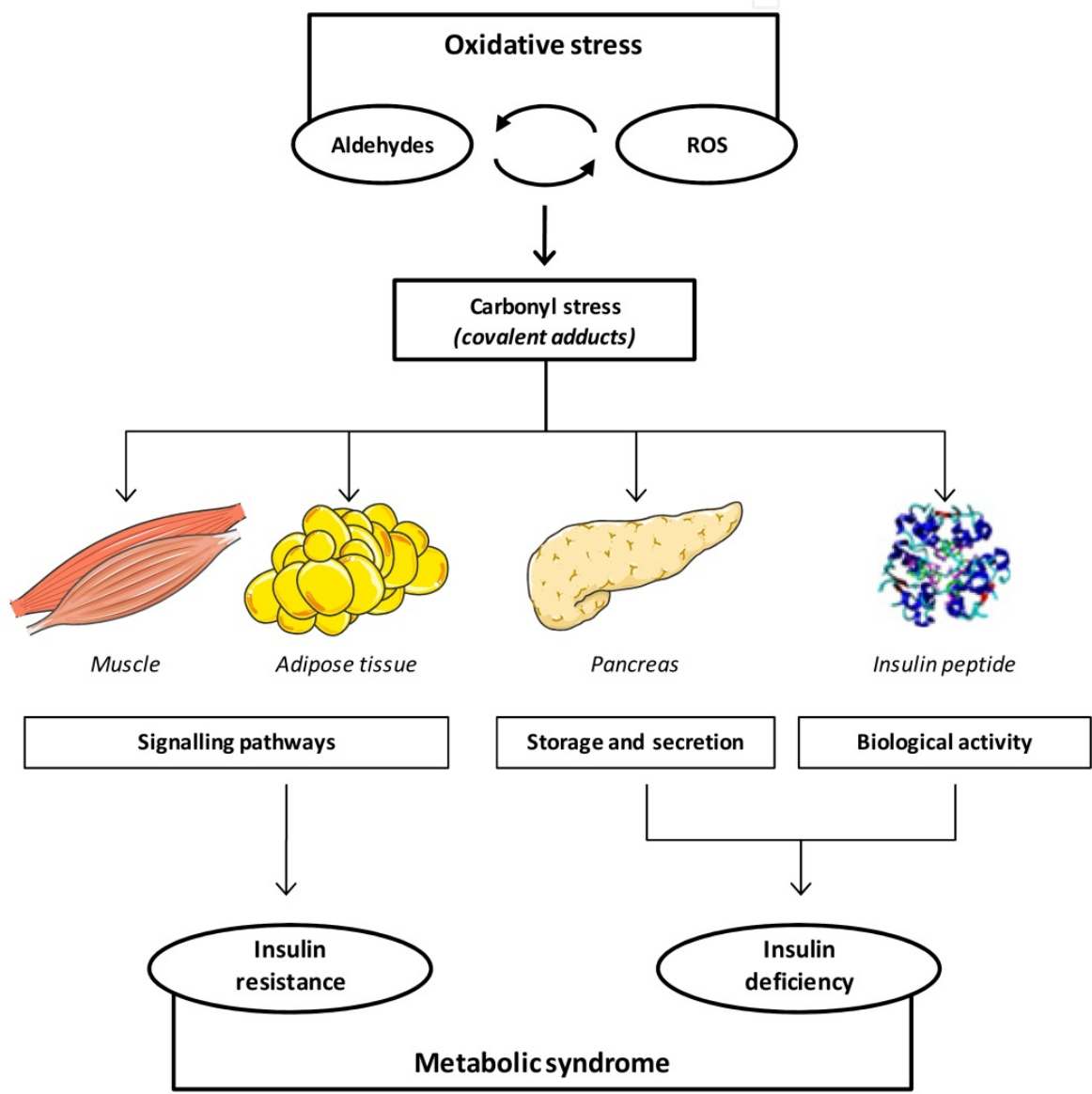

Figure 7. Lipid aldehydes effects leading to the metabolic syndrome.

Despite this evidence, the role of aldehydes is mitigated by the mild effect obtained with antioxidants and/or aldehyde scavengers in the treatment of diabetic complications in

human. "Oxidative stress" and its derivatives are nevertheless important in the metabolic syndrome, and prevention or treatment of some of its associated complications could be accessed through reduction of both ROS and toxic aldehydes by-products, as it is the case with lipoic acid.

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