Reactive species and diabetes: counteracting oxidative stress to improve health
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Oxidative stress is at the very core of metabolism. Reactive species behave as true second messengers that control important cellular functions. However, under pathological conditions, abnormally large concentrations of these species may lead to permanent changes in signal transduction and gene expression. Attenuation of oxidative stress as a way to improve several diseases such as diabetes has flourished as one of the main challenges of research. The lack of evidence to prove the benefits from antioxidant compounds has led to boost these strategies. Inhibition of reactive oxygen species (ROS) production through the development of inhibitors against NADPH oxidase and mitochondria offers an alternative approach to conventional antioxidant therapies. There is a need to understand oxidative stress process to implement health-disorder approaches.

Introduction: oxidative stress, reactive species and antioxidant systems
Oxidative stress has been generating much interest primarily because of its accepted role as a major contributor to the aetiology of both normal senescence and severe pathologies with serious public health implications such as diabetes, obesity, atherosclerosis, etc. The triggering factors for oxidative stress may be diverse, ranging from genetic or environmental factors to pure stochastic events such as metabolic fluctuations [1].

Oxidative stress can result from diminished levels of antioxidants but can also result from increased production of reactive species. The consequences of oxidative stress can include: firstly, adaptation of the cell or organism by upregulation of defence systems, which may first, completely protect against damage; second, protect against damage to some extent but not completely; or third, ‘overprotect’ (e.g. the cell is then resistant to higher levels of oxidative stress imposed subsequently). Secondly, cell injury, which involves damage (oxidative damage) to any or all molecular targets: lipids, DNA, proteins, carbohydrates, etc. Thirdly, cell death as the cell may first, recover from the oxidative damage by repairing it or replacing the damaged molecules, or second, it may survive with persistent oxidative damage or third, oxidative damage, especially to DNA, that may trigger cell death, by apoptosis or necrosis [2].

There are different types of reactive species: reactive oxygen species (ROS, thus, oxygen-containing molecules that are highly reactive in tissues), reactive chlorine species (RCN) and reactive nitrogen species (RNS). All these reactants contain free radicals as well as non-radicals. Low concentrations of these reactive species are necessary for normal cell redox status, cell function and intracellular signalling [3,4**]. However, in some disease states, free radicals are produced in excess and can damage DNA, proteins, carbohydrates and lipid constituents and compromise cell function leading to the development of type 2 diabetes, atherosclerosis, obesity, arthritis and a number of other diseases.

ROS include superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^-$), nitric oxide (NO), hypohalite and peroxyxynitrite (ONOO$^-$). Within mammalian cells, the only enzymes whose sole function is to generate ROS appear to be the NADPH oxidases, located on the cell membrane of polymorphonuclear cells, macrophages and endothelial cells [5–8]. Other enzyme systems capable of transferring electrons to molecular oxygen to produce superoxide include nitric oxide synthases (NOS), cyclooxygenases, lipoxygenases, cytochrome P450 reductases and xanthine oxidases (XO) (reviewed by Selemidis et al. [9]). During energy transduction in the mitochondrial electron-transport chain, a small number of electrons ‘leak’ to oxygen prematurely, forming the oxygen free radical superoxide [10]. Given that ROS are highly reactive and short-lived species, it is tempting to suggest that their effects should be greatest in immediate area surrounding their locus of production. It is conceivable that mitochondrial membrane constituents, including the complexes of the respiratory chain and phospholipids constituents, could be not only important sources of ROS, but also major targets of ROS attack [11]. Furthermore, mitochondrial ROS generation showed tissue-substrate and site-specific characteristics [12] which should explain the differences observed in
the literature regarding the quantity of mitochondrial ROS formed under specific conditions or the more relevant sites for their generation, etc.

Epigenetic mechanisms that control gene expression and promote the accumulation of oxidative DNA damage through alterations on the methylation or oxidation of CpG dinucleotides also seem to be involved in age-related and oxidative-stress-related disorders such as Alzheimer’s disease [13†], suggesting that new genetic pathways may also be involved in ROS generation.

Several studies have demonstrated that the mitochondrial generation of ROS increases in moderately hypoxic conditions [14,15], which is paradoxical, because oxygen is a substrate for ROS generation. Interestingly, mutants without a functioning respiratory chain did not show this increase in ROS. Moreover, under severe hypoxic conditions, this response was also eliminated (reviewed by Turrens [16]). No increase in mitochondrial ROS emission was observed with isolated mitochondria at any level of oxygen tension meaning that ROS generation in response to hypoxia in cells is not intrinsic to the mitochondrial respiratory chain alone, but may involve other factors that are not occurring in the isolated and intact mitochondria system [11,17]. Still more research is needed to clarify the extent of ROS formation in response to hypoxia, a clear situation involved in a wide range of diseases.

‘Antioxidant’ is defined as any substance that when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate [18]. Tissue antioxidant defences against ROS and radicals have been extensively studied and reviewed elsewhere [4,9,19–21]. These defences include both enzymatic and non-enzymatic systems.

The primary antioxidant enzymes include superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GPX) and thioredoxin. Thus, SOD catalyzes the dismutation of $O_2^-$ to O$_2$ and the less reactive H$_2$O$_2$ ($O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$). Unlike $O_2^-$, which remains near the site of its production, H$_2$O$_2$ can diffuse across membranes and through the cytosol. Since H$_2$O$_2$ is a powerful oxidizing agent, cells express abundant CAT, GPX and thioredoxin that transform H$_2$O$_2$ to water and molecular oxygen (2H$_2$O$_2$ → 2H$_2$O + O$_2$) [22]. GPX is a selenium-containing tetrameric enzyme that reduces H$_2$O$_2$, lipoperoxides and other organic hydroperoxides to their corresponding hydroxylated compounds using glutathione as a hydrogen donor (ROOH + 2GSH → ROH + GSSG + H$_2$O).

Serum high-density lipoprotein (HDL)-associated paraoxonase 1 (PON1) has received much attention during the last years as it reduces oxidative stress in lipoproteins, but also in macrophages and in the atherosclerotic lesion, whereas paraoxonase 2 (PON2), which is present in tissues, but not in serum, acts as an antioxidant at the cellular and not humoral level. Both PON1 and PON2 protect against atherosclerosis development, and this phenomenon could be related to their anti-oxidative properties [23].

Nonenzymatic antioxidants include, amongst others, vitamins such as vitamin C, vitamin E and β-carotene; minerals such as selenium, copper, zinc; reduced glutathione and numerous phytochemicals, involving a broad spectrum of plant-based nutrients that work synergistically to combat free radical processes [19,24,25].

The ratio GSH/GSSG is a good measure of oxidative stress of an organism. In fact, the capacity of glutathione to regenerate the most important antioxidants is linked with the redox state of the glutathione disulphide–glutathione couple (GSSG/2GSH) [21,26,27].

In addition to these very well-known antioxidant enzymatic and non-enzymatic systems, there are compounds that have a relatively low specific anti-oxidative activity, that is, on a molar basis, but, when present at high concentrations, can contribute significantly to the overall ROS scavenging activity. The most prominent examples of such high-level, low-efficiency antioxidants are free amino acids, peptides and proteins [3].

The aim of this article was to review recent reports on ROS mechanism’s of action and their relevance in human physiology and pathology paying peculiar attention to their role in type 2 diabetes development and to the different strategies carried out to improve diabetes and other oxidative-stress-related diseases. We also demonstrated that despite the high battery of biomarkers for oxidative stress, there are not yet adequately validated markers of the onset, progression and/or regression of any oxidative stress associated chronic diseases.

**Signal transduction**

It is a well-known feature that cells are capable of generating ROS endogenously and constitutively. In fact, it is generally accepted that ROS generated can function as true second messengers and can mediate important cellular functions such as cell growth, differentiation and programmed cell death (Figure 1).

Some of the signalling pathways affected by ROS involve the activation of several non-receptor protein kinases (PTKs) belonging to the Src family (Src kinases) and Janus kinase (JAK-STAT). Thus, activated Src binds to cell membranes by myristilation and initiates mitogen-activated protein kinase (MAPK), NF-κB and phosphoinositide 3-kinases (PI3K) signalling pathways. In addition, the protein tyrosine phosphatases (PTPs) are
probably the best characterized direct targets of ROS. Experiments revealed that ROS induce a release of calcium from intracellular stores, resulting in the activation of kinases, such as PKC, a member of serine/threonine kinases. However, probably the most significant effect of ROS on signalling pathways has been observed in the MAPK pathways, especially through JNK and p38 MAPK [28]. In addition, experimental studies on the upregulation of MAPKs by H2O2 treatment have shown that the activation of each signalling pathway is type-specific and stimulus-specific.

As already mentioned above, probably the most significant effect of ROS on signalling pathways has been observed in the MAPK pathways. This involves activation of nuclear transcription factors. These factors control the expression of protective genes that repair damaged DNA, power the immune system, arrest the proliferation of damaged cells and induced apoptosis. The nuclear transcription factor NF-κB is involved in inflammatory responses and ROS have been implicated in its activation via TNF-α and IL-1. AP-1 is important for cell growth and differentiation and its activity is induced in response to certain metals in the presence of H2O2. p53 is a gene whose disruption is associated with more than half of all human cancers. The nuclear factor of activated T cells (NFAT) regulates cytokine formation, muscle growth and differentiation, angiogenesis and adipogenesis. Various ROS/metals are known to increase intracellular calcium, and this may represent a probable mechanism by which ROS/metals activate NFAT. hypoxia-inducible factor (HIF-1) regulates the expression of many cancer-related genes.
It has been demonstrated that HIF-1 influences mitochondrial function, suppressing both the tricarboxylic acid cycle (TCA) and respiration by inducing pyruvated dehydrogenase kinase 1 (PDK1). PDK1 regulation in hypoxic cells promotes cells survival [29]. Figure 1 summarizes the main signalling pathways and transcription factors activated by ROS.

Under pathological conditions, however, abnormally large concentrations of ROS/RNS may lead to permanent changes in signal transduction and gene expression, typical for disease states.

**Biomarkers of oxidative stress**

Methodologically, different strategies are available to determine alterations of the oxidative/anti-oxidative state, namely the direct detection of reactive species, the determination of antioxidants, assessment of total anti-oxidative capacity and the quantification of molecules altered by the prevalence of ROS/RNS.

The only technique that can detect free radicals directly is electron spin resonance (ESR). However, this method is still too insensitive to detect free radicals with very short half-lives, such as superoxide anions and hydroxyl radicals in biological systems. Additionally, techniques are available to directly measure the production of ROS by using fluorescent probes [30], chemiluminescent detection [31] or measuring oxidase activity (such as that of NADPH oxidase) [32]. However, free radical activity is most usually assessed by indirect methods based on the feature that although short-lived, ROS leave a detectable trace of modified oxidative products, and an array of these damaged end products can be detected using a variety of assays. Examples of ROS-damaged biological molecules include oxidized proteins, oxidized lipids, oxidized low-density lipoproteins (oxLDL), oxidized carbohydrates (glycated products), oxidized nucleic acid bases and enzymatic markers of oxidative stress, such as myeloperoxidase (MPO), an endogenous generator of oxidants. The number of different antioxidants in plasma, serum, urine or other biological samples makes it difficult to measure each antioxidant separately. Therefore, as an alternative approach, several methods have been developed to determine the total anti-oxidative capacity of these biological samples as reviewed elsewhere [2,19,21,22,33]. There are also several plasma molecules that can be used as indirect biomarkers of oxidative stress such as the plasma retinol-binding protein-4 (RBP4), whose levels have been demonstrated to be modulated by the ingestion of selenium [34] or the asymmetric dimethylarginine (ADMA), a metabolic by-product of continual protein modification processes in the cytoplasm of all human cells that interferes with L-arginine in the production of NO and whose levels are increased in oxidative stress [35] (Table 1).

Stable isotope methodologies offer a number of possibilities for the nutritional assessment of many different processes and metabolic pathways [36]. The use of $^{13}$C as a tracer offers all the advantages of stable isotopes and has been widely applied for measuring various types of metabolic processes. In fact, the 2-keto[1-(13)C]isocaproate breath test can be used as a good marker of mitochondrial oxidation and therefore, constitutes a good marker/method to evaluate the mitochondria function [37].

Despite the high battery of biomarkers previously described and summarized in Table 1, the current challenge is sorting out which markers or combinations of markers are predictive of human diseases. Ideally one would wish to demonstrate that modulation of a biomarker by a specific antioxidant intervention is predictive of modulation of incidence of some major chronic disease endpoint in humans. To accomplish this, further investigation is needed.

**ROS and human diseases**

Some of the most relevant physiological conditions or diseases associated with increased cell oxidative stress and with a high impact on public health are ageing, obesity, hypertension, atherosclerosis, vascular alterations, metabolic syndrome, inflammation, chronic obstructive pulmonary disease, asthma, several neurological disorders such as Alzheimer, Parkinson and Schizophrenia, AIDS, obstructive sleep apnea, polycystic ovarian syndrome, liver diseases, chronic kidney diseases (CKD), rheumatoid arthritis, cataracts, tumours (breast cancer) ischaemia and diabetes [3,4*,22,38–40].

Type 2 diabetes is associated with a decreased uptake of glucose into muscle and adipose tissue, which leads to chronic extracellular hyperglycaemia resulting in tissue damage and pathophysiological complications, involving heart disease, atherosclerosis, cataract formation, retinopathy and others. In fact, these microvascular and macrovascular complications are major causes of disability and death in patients with diabetes mellitus.

ROS/RNS act as a double-edged sword in modulating insulin signalling. On one hand, they are generated in response to insulin and they are required for insulin to exert its full physiological actions, but, on the other hand, increasing evidence implicates ROS and RNS as negative regulators of insulin signalling, rendering them putative mediators in the development of insulin resistance, a common endocrine abnormality that accompanies obesity and which is a risk factor of type 2 diabetes [4*,41*]. Increased oxidative stress seems to be an important alteration triggering several others and the major culprit of diabetes and its associated complications such as kidney disease [42,43].
There are four main hypotheses about how hyperglycaemia causes diabetic complications: first, activation of protein kinase C (PKC) isoforms; second, increased hexosamine pathway flux; third, increased polyol pathway flux: aldose reductase mediates conversion of glucose to sorbitol and excess sorbitol causes oxidative damage and activates stress genes; fourth, increased advanced glycation end-product (AGE) formation, which bind to specific cell surface receptors (RAGE) and lead to postreceptor signalling and further generation of ROS. AGEs also activate intracellular transcription factors such as NF-κB which induces PKC, sorbitol and transcription of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). Activation of these intracellular molecules can produce ROS. In this context, AGE formation is probably a significant contributor to the onset of diabetic complications, mainly atherosclerosis [44]. All these observations seem to reflect a single hyperglycaemia-induced process of overproduction of O$_2^-$ by the mitochondrial electron-transport chain. In this sense, under normal conditions, the key sites of superoxide generation so that complex II becomes the primary source of electrons that contribute to O$_2^-$ formation under diabetic conditions [45]. There is a very recent study that placed the aetiology of insulin resistance in the context of mitochondrial bioenergetics by demonstrating that mitochondrial H$_2$O$_2$ emission serves as both a gauge of energy balance and a regulator of cellular redox environment, linking intracellular metabolic balance to the control of insulin sensitivity [40,46]. Thus, attenuation of mitochondrial H$_2$O$_2$ emission completely preserves insulin sensitivity despite a high-fat diet [46*].

Hyperglycaemia also activates NADPH oxidase, which has been implicated as the major source of ROS generation in the vasculature in response to high glucose and AGEs. However, recent evidence suggests that basal ROS production via NADPH oxidase may upregulate antioxidant enzyme defences. Thus, NADPH oxidase could also act as a dually faceted mechanism with transient activation providing a feedback defence against excessive ROS generation through the activation of receptor tyrosine kinases and the redox-sensitive Nrf2-Keap1 signalling pathway whereas prolonged NADPH oxidase activation leads to eNOS uncoupling.
mitochondrial dysfunction and impaired antioxidant defences owing to depletion of intracellular NADPH and, therefore, contributing to oxidative stress in diabetes [47].

Since hyperglycaemia-induced oxidative stress occurs in nonnucleated cells lacking mitochondria and NADPH oxidase, another mechanisms of ROS formation in such cells could be glucose auto-oxidation. Glucose itself, as well as its metabolites, is known to react with H₂O₂ in the presence of iron and copper ions to form OH⁻. Other sources of ROS in diabetes are XO, whose role has been suggested to be tissue dependent, and lipoxygenases [4**].

β-Cells are particularly sensitive to ROS because they are low in free radical quenching (antioxidant) enzymes such as CAT, glutathione peroxidase and SOD. Therefore, the ability of oxidative stress to damage mitochondria and markedly blunt insulin secretion is not surprising. It is also relevant to mention that ROS derived from glucose metabolism, such as H₂O₂, act as metabolic signalling molecules for glucose-stimulated insulin secretion (GSIS) in β-cells. Induction of antioxidant enzymes protects β-cells from oxidative damage and possible cell death, thus minimizing oxidative damage-related impairment of insulin secretion. However, on the other hand, the induction of antioxidant enzymes by the Nrf2 activation blunts glucose-triggered ROS signalling, thus resulting in reduced GSIS [48].

Thus, it is clear that excessive production of free radicals causes damage to biological material and is an essential event in the aetio-pathogenesis of various diseases such as diabetes and metabolic syndrome. However, the question that has risen in the past years is whether uncontrolled formation of ROS is a primary cause or a downstream consequence of the pathological processes [4**]. In other words, it is still not clear what comes first, the chicken or the egg.

Finally and despite the unifying hypothesis that maintains that hyperglycaemia stimulatory effect on ROS generation in the mitochondria and/or NADPH oxidase leads to several diabetic complications, diabetes is also associated with a decline in the levels of endogenous antioxidants such as vitamin E, vitamin C, selenium, taurine or CoQ10 [4**,49–51], raising the possibility that changes in these and other antioxidants might also contribute to the severity of oxidant-mediated damage in type 2 diabetes.

**Suppressing oxidative stress**

If oxidative damage contributes significantly to disease pathology, then actions that decrease it might be therapeutically beneficial. In fact, attenuation or complete suppression of oxidative stress as a way to improve several diseases has flourished as one of the main challenges of research in the last years. Thus, several approaches have been carried out in order to either decrease the high levels of ROS generated or boost the endogenous levels of antioxidants.

Numerous trials have examined the effects of supplementation with different antioxidants on oxidative stress [21,52–54]. In this sense, the adherence of a Mediterranean diet (not confounded by genetic or shared environmental factors) [55] or energy-restricted diets with high content in fruits [56] seems to have beneficial effects on oxidative stress and therefore, they could also be useful strategies for the improvement of oxidative stress-related disorders. However and despite the initial positive and beneficial effects observed in some of these studies, other clinical studies investigating antioxidant effects have been often disappointing given the consistent and promising findings from experimental investigations, clinical observations and epidemiological data [19,57*]. Possible reasons relate to (1) the type of antioxidant used (some of the antioxidants examined were ineffective and non-specific and dosage regimen or duration of therapy were inefficient), (2) patient cohort included in trials, (3) the trial design itself and (4) inappropriate or insensitive methodologies to evaluate oxidative state [58] which underlines the urgent need for the development of sensitive and specific biomarkers to correctly assess the oxidant status of patients.

On the other hand, inhibition of ROS production through the development of inhibitors against the main sources of ROS generation offers an alternative approach to conventional antioxidant therapies (Figure 2). Thus, NADPH oxidase, as the main source of ROS production in endothelial cells and directly involved in hypertension and cardiovascular disease [59], has been suggested as a potential target for decreasing ROS generation. A number of clinically important drugs used for the treatment of hypertension, hypercholesterolemia and coronary artery disease such as the statins, AT1 (angiotensin II receptor type 1) antagonists and ACE inhibitors have been shown to decrease NADPH oxidase-derived superoxide and ROS production [9]. Furthermore, it has been recently described that the newer-age diazeniumdiolate NONOate class of NO donors suppressed vascular NADPH oxidase-dependent superoxide production, making these NO donors suitable for oxidative stress attenuation in atherosclerosis and, for even, reverse atherosclerosis [57*].

One area of investigation that has been the focus of much recent interest in the past years is to address mitochondria, and more specifically, to analyze the potential beneficial effects of modulating mitochondrial ROS generation in order to treat or prevent the development of several oxidative-stress associated disorders [40,60**,61]. In this sense, it has been observed that lipoic acid reduces O₂⁻ production by decreasing oxygen...
consumption in isolated rat liver mitochondria [62]. Furthermore, Anderson et al. [46] demonstrated that attenuating mitochondrial H$\textsubscript{2}$O$\textsubscript{2}$ emission, either by treating rats with a mitochondrial-targeted antioxidant or by genetically engineering the over-expression of CAT in mitochondria, completely preserves insulin sensitivity despite a high-fat diet.

Finally, the discovery of new transcription factors, proteins and/or pathways that may have protective roles against oxidative stress such as the X-box-binding protein (XBP1), a major endoplasmic reticulum stress-linked transcriptional factor that positively regulates CAT expression [63] or the Bcl-2 family, which includes pro-apoptotic and anti-apoptotic factors acting at mitochondrial and microsomal membranes modulating mitochondrial bioenergetics in a direction that diminishes $O_2^-$ generation within mitochondria [64], will successfully contribute to the full understanding of the mechanisms involved in the regulation of ROS metabolism and therefore, will help to identify those processes that promote oxidative excess which, in turn, will contribute to find new efficient therapies against oxidative stress.

**Conclusions**

‘Living with the risk of oxidative stress is a price that aerobic organisms must pay for more efficient bioenergetics’. Thus, oxidative stress seems to be at the very core of metabolism and what is more worrying, oxidative stress is at the very core of every metabolic alteration. Attenuation or complete suppression of oxidative stress as a way to improve several diseases such as type 2 diabetes has flourished as one of the main challenges of research in the last years. The lack of evidence to prove the benefits from usage of antioxidant vitamins, minerals or drinks and foods with bioactive compounds to prevent several oxidative-stress-related diseases, has lead to boost these strategies. In fact, inhibition of ROS production through
the development of inhibitors (natural or chemical) against the main sources of ROS generation offers an alternative approach to conventional antioxidant therapies. Thus, NADPH oxidase and mitochondria have been postulated as the main targets to reduce ROS production. However, there are some limitations in this field, since there are not yet adequately validated markers of the onset, progression and/or regression of any oxidative stress associated chronic diseases. There is, therefore, an urgent need to investigate in this direction too.

All the studies herein presented are a good testimony that current research is focussed on understanding the underlying mechanisms that lead to the development of oxidative stress which would be useful for preventing and treating diabetes and other oxidative-stress-related illnesses instead of being just focussed on alleviating the symptoms of these diseases.

Acknowledgements
Investigations about oxidative stress and human health are being supported by the Línea Especial about Nutrition, Obesity, and Health (University of Navarra LE/97) and the Health Department of the Government of Navarra in Spain (22/2007).

References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest


A very exhaustive and elegant review on free radicals and antioxidants in normal physiological conditions and human diseases. The question whether excessive formation of free radicals is a primary cause or a downstream consequence of tissue injury is also reviewed in this article.


This is the first study that describes the possibility that early life hypermethylation or hyper-methylation of specific genes can impact gene expression and imprint susceptibility to oxidative DNA damage in the aged brain. This study associates epigenetic mechanisms with oxidative stress.


A recent and very exhaustive review regarding the cellular and molecular mechanisms by which ROS and RNS are thought to participate in normal mechanisms by which ROS and RNS are thought to participate in normal negative regulation of insulin signalling is reviewed in this article.


This study evidenced that the use of mitochondrial-targeted antioxidants represents a potentially effective counterbalance strategy for treating insulin resistance and other diseases associated with chronic metabolic imbalance.


Here, it is hypothesized that NONOates provide a novel means of suppressing NADPH oxidase-dependent oxidative stress to restore vascular NO levels to prevent, and even reverse, atherosclerosis.


An updated review that proposed mitochondria as a possible target for improvement of adipose tissue failure by bioactive components such as vitamin B3, retinoids, long-chain omega 3 fatty acids, conjugated linoleic acid and polyphenols.


