

Oxidative stress as a therapeutic target in diabetes: revisiting the controversy

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SUMMARY

Oxidative stress has been repetitively shown to be a hallmark of many diseases linked with metabolic or vascular disorders. Therefore diabetes represents an ideal candidate for studying the consequences of oxidative stress and its treatment. Indeed diabetes constitutes a multiple source of free radicals, starting very early in the disease process and worsening over the course of disease. In view of the typical characteristics of diabetes, oxidative stress is expected to have a double impact, on both metabolic and vascular functions. It is therefore particularly disappointing to note the dramatic failure of clinical trials with antioxidants, although it must be pointed out that such studies have not been performed with only diabetic patients. This review describes the many different aspects of oxidative stress in diabetes and proposes possible explanations for the apparent lack of efficacy of antioxidant treatments in patients. Some verifications seem warranted before a definitive conclusion can be drawn about the validity of this therapeutic concept.

Key-words: Oxidative Stress · Antioxydants · Diabetes · Insulin Resistance · Diabetic Microangiopathy.

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RÉSUMÉ

Le stress oxydant, cible thérapeutique de diabète : un nouveau regard sur la controverse

La multiplicité des sources de formation de radicaux libres fait du diabète une maladie particulièrement sujette au stress oxydant. Au cours des années récentes, d'innombrables démonstrations ont d'ailleurs été rapportées, de même qu'il a été montré que ce processus apparaît aussi dans l'obésité et l'intolérance au glucose. Ceci suggère que l'hyperglycémie n'est sans doute pas seule en cause. A l'inverse, et presque sans exception, toutes les études cliniques de grande envergure ont montré l'inefficacité des traitements anti-oxydants, aussi bien sur le plan métabolique que vasculaire. Ce résultat pose donc à nouveau la question fondamentale : le stress oxydant est-il une cible thérapeutique à développer dans le diabète ?

Cet article se propose d'attirer l'attention sur les risques et les raisons scientifiques d'une interprétation hâtive ou même fautive de résultats cliniques obtenus jusqu'ici presque exclusivement avec la vitamine E (parfois ajoutée de vitamine C). Choix de l'anti-oxydant, dosages, co-administration de molécules sont quelques uns des aspects qui doivent être clarifiés avant de pouvoir conclure à une exclusion définitive de cette approche thérapeutique, en faveur de laquelle il existe un nombre grandissant d'évidences fondamentales.

Mots-clés : Stress oxydant · Antioxydants · Diabète · Insulino-résistance · Micro-angiopathies diabétiques.

The importance of a process such as oxidative stress (OST) in pathology is dependent on both its sources and its targets. From that point of view diabetes is an exquisite candidate to study the biological impact of OST and the potential efficacy of its correction. Indeed diabetes adds to the many environmental causes, which are common to many diseases, some extremely active sources generating reactive oxygen species (ROS) as a consequence of high glucose but also, as will be seen, several other factors.

It is suffice to observe the infinite amount of scientific contributions devoted to this topic to have an idea of the apparent evidence for a cardinal role played by OST in diabetes and its vascular complications. Recently OST has even been suggested to be the unifying link between the various molecular disorders in diabetes [1]. However important questions have been raised from both a theoretical view and from deceiving clinical results in most trials using antioxidants (AOS) [2]. The present review will therefore not add to the long list of excellent exhaustive reviews on the nature and postulated mechanisms of OST ; it rather intends to analyze the problem of OST in diabetes from a critical point of view and tries to evaluate the pros and contras of two main aspects of this problem : the strength of the concept and the pertinence of defining AOS therapy as a valuable treatment for such patients. References are therefore cited as examples, but are not exhaustive.

Oxidative stress: a brief reminder

OST depicts the existence of products called free radicals (molecules possessing an unpaired electron) and reactive oxygen species, which are formed in normal physiology but become deleterious when not being quenched by a cascade of antioxidants systems. This can result either from an overproduction of ROS or from the inactivation of the AOS, thus shifting the OST/AOS balance in favour of stress. Excellent descriptions of the individual molecules and their scavengers can be found in a number of recent reviews [3-5]. ROS oxidize various types of biomolecules, finally leading to cellular lesions by damaging DNA or stimulating apoptosis for cell death. Some ROS are considered more important than others, such as superoxide, hydroxyl radicals or peroxides. However not all oxygen-containing radicals have high oxidative potential. ROS are neutralized by a battery of AOS, which can be divided into mainly two categories : enzymes (ex : superoxide dismutase SOD, glutathione peroxidase GPx and catalase) and non-enzymatic systems (ex : glutathione GSH, vitamins A, C and E). Some are located in cell membranes, others in the cytosol, others in blood plasma. Due to its location in mitochondria and its position in the antioxidant chain, SOD is usually considered as particularly important since even modest decreases in SOD are sufficient

to provoke cell damage [6, 7]. Quantitatively, however, albumin and uric acid are the main AOS.

Diabetes: multiple sources of oxidative stress

Hyperglycemia

Hyperglycemia generates oxidative stress

Hyperglycemia, defining established diabetes, can induce OST by various mechanisms ; excessive levels of glucose reaching the mitochondria lead to an overdrive of the electron transport chain, resulting in overproduction of superoxide anions normally scavenged by mitochondrial SOD. When the latter fails OST develops and it was recently proposed that this mechanism is responsible for the activation of all major pathways underlying the different components of vascular diabetic complications (glycation, PKC activation, sorbitol pathway) [8]. The *in vitro* supplementation of SOD-like drugs corrected most of these defects, supporting the importance of these mechanisms [9, 10]. It has also been proposed that uncoupling mitochondrial NOS by hyperglycemia would be involved [11].

Another mechanism whereby high glucose can stimulate OST is the autoxidation of glucose in the presence of transition metals as well as the generation of ROS during the process of glycation [12]. Indeed the development from Schiff base to Amadori to advanced glycation endproducts (AGEs) is accompanied by ROS-generating reactions at various steps [13, 14]. It was also proposed that carbonyl stress, rather than OST, involving both sugars and lipids would be the relevant source of OST in diabetes [15]. Once AGEs are formed they bind to various receptors termed RAGE and this step is also generating ROS [16].

Hyperglycemia reduces antioxidant potential

OST acts on signal transduction and, via nF-kB, affects gene expression. Thereby the expression of antioxidant enzyme can be reduced. Moreover hyperglycemia can simply inactivate existing enzymes by glycating these proteins ; glycation of SOD, for example, also leads to DNA cleavage [17-20]. This finding suggests that OST may develop by insufficient AOS activity, even if ROS production is within a physiological range.

Other factors generating oxidative stress

Hormones

Most type 2 diabetes patients are hyperinsulinemic for a long period. Insulin can stimulate OST by various mechanisms : the hormone induces production of H₂O₂ when activating its receptors and, although hydrogen peroxide is not a strong oxidant itself, it can indirectly activate oxidative reactions. Insulin also stimulates the sympathetic nervous sys-

tem, which leads to activation of neurotransmitters and their enzymatic systems, several of which induce OST. For example diabetic vessel walls contain high levels of NAD (P) H oxidase, which may be activated by prenylation of p21rac [21, 22]. Leptin is another hormone reportedly stimulating OST [23].

Lipids

Increased fasting and postprandial plasma levels of triglycerides, free fatty acids and cholesterol are common in type 2 diabetes. They are known to generate ROS [24, 25]. In the vessel wall, import of-or local formation of oxLDL is a cardinal mechanism involving OST in the atherosclerotic process.

Varia

Angiotensin II generates OST in blood vessels by stimulating NADH oxidase and is claimed to mediate the effect of hyperinsulinemia.

A major source of OST in vascular pathophysiology is the alternance of ischemia/reperfusion, since the hypoxic period characterizing ischemia is followed by a brutal oxidative burst upon refilling of the vessels with blood during reactive hyperemia. Thus, diabetic patients suffering from complications such as arteritis or diabetic foot experience numerous daily repetitive episodes of ischemia/reperfusion. This phenomenon is even more frequent in patients suffering snoring and/or sleep apnea and exciting data were recently reported linking degradation of glycemic control with oxidative stress generated along these night episodes, which are particularly frequent in patients presenting with obesity [26, 27].

Nitric oxide (NO), is both a scavenger and a prooxidant when it is attacked by radical such as becoming transformed into peroxynitrite [28]. It may represent an important contributor to OST because NO levels are frequently elevated in early stages of diabetes.

Diabetes: targets of oxidative stress

Because diabetes is characterized by defects in both metabolic and vascular domains, this disease represents a privileged situation for OST exerting harmful effects.

Effects of OST on diabetic metabolism

The development from prediabetes to fasting hyperglycemia is now considered to be due mainly to the development of cell failure, a process being aggravated by the duration of the disease. An implication of OST has been first suggested when it was found that alloxan and STZ, used to induce diabetes in animals, destroyed pancreas by OST. In fact, OST induces β -cell death; this is favoured by an obvious low antioxidant potential of native β -cells [29, 30]. *In*

vitro, OST decreases the insulin gene promoter activity in HIT cells [31]. It was recently found that addition of a SOD mimetic increased human islet survival [32]. OST may be the mediator whereby FFA induce β -cell apoptosis. Moreover amyloid deposition in the pancreas is linked with OST [33].

OST can also impair the internalisation of insulin by endothelial, thus limiting hormone delivery to targets tissues and interfere with GLUT-4-mediated glucose transport [34, 35].

Effects of OST in blood vessels

Because they are located at the interface between blood and tissue, vessels walls are particularly exposed to OST. Not only do they have constitutive ROS-generating enzymes (cyclooxygenase COX 1, lipoxygenases, NADH oxidase, cyt P450, eNOS) but they also contain extravasated cells such as monocytes when atherosclerotic damage is present. When these cells are activated, NADH(P)H oxidase and myeloperoxidase are stimulated. Activated leucocytes/monocytes as well as glycation of endothelial cells induce OST, which favours the expression of adhesion molecules and subsequent cell infiltration. In diabetic capillaries activated leucocytes stick to the endothelium, plug the vessel and stimulate permeability. Endothelium-produced NO can be transformed into the oxidant peroxynitrite but, although this substance can induce apoptosis, its relevance *in vivo* is controversial [36, 2]. There is also evidence that glucose can directly scavenge NO and, although there are data showing stimulation of NO formation by high glucose, several studies have indeed shown that acute hyperglycemia reduces endothelial-dependent vasodilatation [37, 38]. Interestingly it has been found that tetrahydrobiopterin, an important cofactor of NO synthesis, is reduced in insulin resistant, fructose-fed rats, generating superoxide and reducing endothelial vasodilatation [39]. Cyclic strain, exerting tension on vessel walls, generates OST in endothelium and secretes PAI-1, an inhibitor of the fibrinolytic system largely involved in the metabolic syndrome and in diabetes [40]. Such a mechanism may be an important contributor to the development of atherosclerotic lesions at arterial bifurcations. In organs like the heart, OST may lead to cardiomyocyte apoptosis.

Finally, a provocative hypothesis has recently been proposed, implying the competitive inhibition by hyperglycemia of DHA (dehydroascorbate, the uncharged form of vit C) uptake at the level of the glucose transporters. By preventing entry of DHA and consequent reconversion into ascorbic acid, cells would lose their antioxidant potential. Since DHA uptake occurs in microvessels, this defect might be the common denominator of the typical small vessel complications of diabetes [41]. More extensive data can be found in several recent reviews [42-44].

An important aspect must be evoked, because it could influence the outcome of AOS treatments: the basal antioxidant equipment can vary drastically among cell types. Thus the reaction to hyperglycemia-induced OST is different in cells from large (smooth muscle cells) *vs* small vessels (pericytes); this difference can be observed even between cell types of the same vessel (endothelial cells *vs* pericytes) [45, 46].

Finally OST may also affect vessel integrity by disrupting intercellular junctions through a stimulation of matrix metalloproteinases, in particular MMP-9 [47].

Diabetes: oxidative stress everywhere!

Evidence that OST is present in diabetes originates from the frequent observation that both ROS and AOS are increased. The latter is logically rather seen in early stages of diabetes and should be interpreted as a tentative compensation of cells against increasing OST [48, 49]. According to tissue and cell type, the nature of antioxidant elevation may vary, indicating specificities which, again, be important for therapeutic interventions.

Countless publications exist showing the existence of various indicators of OST *in vitro* and this has finally led to a lively ongoing debate about the pertinence and relevance of parameters such as TBARS, malondialdehyde, isoprostanes or nitrotyrosines as typical examples [50].

Oral intake of high glucose in animals increases TBARS and reduces the activity of hepatic enzymes susceptible to thiol group oxidation [51]. In humans, OST is also seen in postprandial periods in normal individuals but diabetic patients are unable to compensate for the increased ROS [52]. This increase may be attributed to acute effects of high glucose and/or lipids.

Increased OST is also found in the basal state in both types of diabetes, some studies suggesting that it is much more pronounced in type 2 than in type 1 diabetes [53]. Type 2 diabetics exhibit increases in TBARS and reduction in catalase activity but, surprisingly correlation was found in a recent study between TBARS and level or duration of hyperglycemia [48]. Plasma glutathione (GSH) levels are decreased and oxidized purines increase, illustrating DNA damage [54].

In blood vessels, increased levels of superoxide have been recorded in both arterial and venous segments [55].

Oxidative stress in prediabetes

Many studies report the presence of OST already in prediabetic stages; for example children have increased OST and SOD levels at the onset of type 1 diabetes [56]. In glucose-intolerant (IGT) subjects, the increased level of inflammatory cytokines is linked to oxidative processes [57]. Increased levels of vitamin A — but not vit E —, increased TBARS, increased isoprostanes as well as reduced AOS have all been reported in IGT [58-61]. Data on antioxidants

are variable since, according to the origin of the insult and tissue/cell type, antioxidants may even be increased [46, 62].

In view of data with insulin and some lipid fractions, it is likely that normoglycemic insulin-resistant subjects may already exhibit OST. Indeed obesity, characterized by hyperinsulinemia and dyslipidemia, is accompanied by elevated OST [63].

Interestingly, even in a healthy population, variations in insulin sensitivity are related to lipid hydroperoxyde levels and reduced catalase and vit E levels [64]. Again in the general population, various markers of glucose metabolism and of insulin resistance were associated with OST [65]. One should also remind that low vitamin E levels revealed to be better predictors of diabetes than age, BMI or smoking [66].

Thus, OST may be a very early event in the long history of diabetes, similar to what is seen for functional microvascular defects [67].

That OST can precipitate diabetes development is suggested by an experiment showing the appearance of fasting hyperglycemia within days after administration of a prooxidant to insulin resistant, obese Zucker rats [68]. It is therefore conceivable that chronic exposure of insulin resistant tissues to OST generated for example by the daily iterative postprandial periods might constitute an important factor in the etiology of diabetes. In this scenario diabetes, by virtue of adding hyperglycemia may essentially exacerbate a preexisting situation, as observed for functional microperfusion.

Oxidative stress: a key therapeutic target?

Several trials have shown that improving glycemic control does not necessarily improve accompanying oxidative stress [53]. One might infer from such observations that specific therapy directed towards OST is obvious. Moreover the large evidence for OST (only partially described here!) in insulin resistance and diabetes has logically prompted the use of AOS like a self-evident treatment. With few exceptions, large long-lasting clinical trials have been performed using vit E sometimes combined with vit C, so-called oxidative chain breakers. Shorter trials have also been achieved with other antioxidants such as lipoic acid.

Unfortunately, the final outcome of the large trials with major endpoints have been largely negative, while intermediate observations or smaller clinical trials have shown beneficial effects on surrogate end-points. Thus a series of large scale trials such as CHAOS, ATBC, GISSI, HPS, VEAPS but particularly HOPE and MICROHOPE failed to show any major improvement in long-term outcome. A very recent pooled analysis of trials using vit E confirmed the complete lack of change in mortality in patients at risk for coronary disease [69, 70]. Although it must be recalled that none of these trials addressed specific diabetic populations, the absence of effects in a situation where OST is at least expected

ted to play a prominent role, raises great questions about the pertinence of this therapeutic concept. However, before a firm negative conclusion can be made, we must analyze various reasons possibly explaining these results.

Is OST harmful?

In view of the large body of evidence for elevated OST in diabetes, this question might sound provocative. It is however a frequent trend in medicine to assimilate abnormal levels of a parameter with harmfulness. Although many studies show indeed an abnormal shift of the OST/AOS balance in favour of the former, there is still lacking proof that levels of OST observed in diabetic patients are harmful to tissues to an extent that its inhibition would save the organ structure or the biological function. Conceivably there could exist thresholds for OST harmfulness and this remains to be demonstrated. The fact that intensive OST can easily injure or kill cells *in vitro* must be considered with great caution because the culture conditions are frequently unphysiological in respect of oxygen environment or AOS levels in the medium. This renders any extrapolation to *in vivo* hazardous. It could thus be that the concept and the true role of OST are exaggerated.

The choice of the antioxidant

Most human trials were performed with vit E, which raises many questions as to this choice. Thus, there is no proof that the orally administered vit E reaches the adequate target cells in sufficient concentrations. Conversely there has been concern about the dosage (usually very high), because most AOS, including vit E can behave as prooxidants at higher dosage [71-73]. This is also seen in vascular physiology, where H₂O₂ modulates arteriolar tone in a bell-shaped fashion [74]. Finally, it has been suggested that vit E, for example, has other biological properties possibly responsible for the observed positive effects *in vitro* [75]. However a recent report using vit C also failed to show any improvement in glycemia, blood pressure, markers of oxidative stress and endothelial function in type 2 patients [76]. Thus, vitamins may have simply been the wrong choice! Alternatively pharmacological intervention with oxidant chain breakers may reveal insufficient and highlight the need for interfering directly with ROS production [77].

Do antioxidants have negative effects?

As seen above, AOS may act negatively when overdosed. However cells have learned to live with and to some extent to integrate ROS into their biochemistry [78]. Actually ROS are involved in the mediation of cell signals, where they may play positive roles in normal physiology: phagocytosis, insulin signalling or shear-stress induced vasodilatation are such examples [79, 80]. It could thus be that at least excessive concentrations of AOS interfere with physiological processes and alleviate the beneficial effects of these substances.

Monotherapy or combination?

What is the right choice?

Several reports have shown that, as might logically be anticipated, a combination of different AOS may be superior to monotherapy [81]. However, adding vitamin C to vitamin E has apparently not modified the outcome of large scale clinical trials. Moreover adding vit C, vit E or β -carotene to a combination of simvastatin and niacin has revealed extremely negative on cholesterol profile [82].

If we integrate the fact that, due to their native characteristics, not all cells and mechanisms might request the same therapeutic means, defining the adequate mixture of AOS in terms of both composition and dosages may constitute a difficult hurdle to cross; In view of the very evolving nature of diseases like diabetes, it might also preclude any kind of established standard AOS therapeutic schemes.

Conclusion

Probably few diseases exhibit so much evidence for exaggerated OST as does diabetes. This is comprehensive when one compares potential sources of oxidative stress with the pleiotropic pathophysiology of this disease. Therapeutic approaches performed in large scale trials with selected antioxidants have yielded disappointing results. We must however remain cautious because a) no such trial has been addressing specific diabetic patient populations and b) the actual experience is largely confined to vitamins, mainly alpha-tocopherol. There exist at least theoretical arguments susceptible to explain this discrepancy. Thus, before a final conclusion about inefficiency of AOS therapy can be drawn, a long-term, large scale clinical trial in type 2 diabetic patients with and without vascular complications is a prerequisite.

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