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Peroxynitrite-Driven Mechanisms in Diabetes and Insulin Resistance – the Latest Advances

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Abstract

Since its discovery, peroxynitrite has been known as a potent oxidant in biological systems, and a rapidly growing body of literature has characterized its biochemistry and role in the pathophysiology of various conditions. Either directly or by inducing free radical pathways, peroxynitrite damages vital biomolecules such as DNA, proteins including enzymes with important functions, and lipids. It also initiates diverse reactions leading eventually to disrupted cell signaling, cell death, and apoptosis. The potential role and contribution of this deleterious species has been the subject of investigation in several important diseases, including but not limited to, cancer, neurodegeneration, stroke, inflammatory conditions, cardiovascular problems, and diabetes mellitus. Diabetes, obesity, insulin resistance, and diabetes-related complications represent a major health problem at epidemic levels. Therefore, tremendous efforts have been put into investigation of the molecular basics of peroxynitrite-related mechanisms in diabetes. Studies constantly seek new therapeutical approaches in order to eliminate or decrease the level of peroxynitrite, or to interfere with its downstream mechanisms. This review is intended to emphasize the latest findings about peroxynitrite and diabetes, and, in addition, to discuss recent and novel advances that are likely to contribute to a better understanding of peroxynitrite-mediated damage in this disease.

Keywords

Diabetes; diabetic complications; insulin resistance; lipid peroxidation; nitrotyrosine; peroxynitrite

INTRODUCTION

The possible role of peroxynitrite and the correlation of peroxynitrite-mediated damage with pathophysiological mechanisms in various disease models have been extensively studied in the past decade. Several disease conditions are associated or accompanied with proinflammation or low-grade inflammatory processes to such an extent that both superoxide and NO production are elevated, making the chance of peroxynitrite generation extremely high. The physiological and pathological implications of peroxynitrite are

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numerous and are the focus of several well established research groups and investigators. Diseases that are major health problems such as neurodegenerative disorders, cardiovascular diseases, infectious problems and inflammation, as well as diabetes and its related conditions and complications (for reviews see [1-8]), are studied to address potential peroxynitrite-mediated mechanisms and the damaging effects of peroxynitrite in protein oxidation/nitration, in hopes of finding novel therapeutic approaches to mitigate peroxynitrite-related oxidative stress processes.

The past few years have provided a large number of studies and excellent, wide-ranging reviews on the contribution of peroxynitrite to cell signaling and major pathophysiological conditions and diseases [4, 7, 9-10]. In this recent review, we would like to focus on the major lines of evidence and findings in diabetes mellitus and its complications. We emphasize the importance of complex peroxynitrite biochemistry in light of the latest novel findings in diabetes, including type 1 models, complications, type 2 models, metabolic syndrome, and insulin resistance studies. Our intention is also to highlight some of the new postulated mechanisms that may link lipid peroxidation and peroxynitrite-mediated damage and can explain the frequent co-occurrence of these events.

This review was written in response to the growing interest in the relationship of oxidative stress and peroxynitrite to diabetes outside of the community of free radical biochemists. As such, we attempt to bridge the gaps between the highly mechanistic, largely *in vitro* investigation of the kinetic probabilities of different radical reactions occurring, and the primarily *in vivo* analysis of the correlation between loosely defined biomarkers of oxidative stress and diabetes progression. Several investigators have already begun to integrate these two approaches, applying novel techniques to rigorously identify specific free radicals and their biological compartmentalization and role *in vivo* in diabetes models – the pool of these contains a few hundred papers at least. The literature presented here reviews the biochemistry of free radicals in relation to diabetes, specifically focusing on peroxynitrite and its possible products, as well as recent work using specific and novel approaches. We feel these papers represent important steps in advancing to a much more mechanistic and nuanced analysis of the role of such specific oxidative metabolites like peroxynitrite in diabetes.

THE CHEMISTRY OF PEROXYNITRITE – ONE- AND TWO-ELECTRON OXIDATION MECHANISMS IN BIOLOGY

The production of peroxynitrite, a potent oxidant and its role in *in vivo* endothelial damage was proposed in 1990 [11]. Since then, several studies and investigators have provided solid evidence to support the possibility of the *in vivo* formation of this molecule, once a controversial subject. In addition, they have unraveled its role in a diversity of pathological conditions.

A simultaneous flux of nitric oxide and superoxide anion overproduction in a given system with close proximity to one another leads to the formation of peroxynitrite in a diffusion controlled fashion [12]. Superoxide anion and nitric oxide are not necessarily toxic but normally exist in a fine balance with physiological levels participating in several signaling

events [13-14]. If overproduced in a pathophysiological case, in a diseased organ or tissue, efficient defense mechanisms such as superoxide dismutases (SOD) are available to neutralize superoxide. NO is able to diffuse rapidly, eventually being converted to nitrate [15]. Problems can occur when the formation of these species happens within very close proximity, a few molecules of distance apart, and NO production is increased several folds, diffusing to different places; it is difficult to kinetically outcompete the reaction between superoxide and NO *in vivo*. Various studies indicate slightly different rate constants, but they are commonly reported to be in the range of 10^9, which is essentially a diffusion controlled condition [12, 16]. This phenomenon rendered the focus of biological research moving from hydroxyl radical and the possibility of Fenton reaction towards peroxynitrite mediated biochemical pathways and described NO as a Janus-faced molecule and peroxynitrite as a biological oxidant [11, 17]. Though free radical production from peroxynitrite was initially controversial, currently peroxynitrite biochemistry is well described and widely accepted by many investigators and research groups. The molecule is considered to be a strong oxidant and can react with biomolecules as direct targets or in the absence of those, through its various ways of decomposition will likely mediate biologically relevant free radical reactions. These modes of action, and the factors that influence which pathways are most likely to occur in different *in vivo* situations, are detailed below.

At neutral pH, peroxynitrite decomposes by a homolytic cleavage giving rise to hydroxyl radicals ($^{\bullet}OH$) and nitrogen dioxide ($^{\bullet}NO_2$) in about 30 % and nitrate in about 70 % [18-21]. These species can then further initiate various free radical reactions (both oxidation and nitration). They can damage biomembranes, DNA, start lipid peroxidation processes, and modify proteins [22-26]. Protein modification often occurs either by oxidation or nitration of tyrosines, cysteins (thiols), or tryptophanes. These residues can be key functional parts in active sites of enzymes, receptors, and other proteins. The decomposition is considered very slow at neutral pHs but has much more relevance at low acidic pHs, which can occur, for example, in inflamed tissues, ischemia, or in phagosomes.

At neutral pH, the reaction of peroxynitrite with thiols and heme proteins, as well as with CO_2, becomes significantly more important than spontaneous decomposition because both the concentrations of these biomolecules and their rapid reactions will favor this route [27-28]. The result is a greatly reduced half-life of peroxynitrite (in the millisecond range) and the two electron-oxidation route of the targets. Functionally important parts of enzymes such as thiols or methionine can be oxidized, which can lead to loss of function. Peroxynitrite also reacts with the *in vivo* often ubiquitous carbon dioxide; this reaction gives ~35 % carbonate radical anion ($CO_3^{\bullet -}$) and $^{\bullet}NO_2$, and ~65 % nitrate through a rapidly decomposing intermediate product [29-33]. CO_2 will efficiently compete with other biological targets for peroxynitrite; therefore, most of the reactions will occur from carbonate radical anion and nitrogen dioxide, resulting in one electron oxidation mechanisms [34]. Fig. (**1**) shows the summary of the above mentioned biological reactions of peroxynitrite.

The numerous studies of peroxynitrite-mediated oxidation of biotargets *in vitro* and *in vivo* provided a very good background to support the theory of how free radical production from this toxic oxidant can play a role in various conditions. While carbonate radical anion

and $^{\bullet}NO_2$ will mediate most of these reactions in a neutral biological milieu, spontaneous decomposition and hydroxyl radical will also contribute at low pHs and in certain pathological conditions or hydrophobic environments [34]. Conversely, while hydroxyl radical reacts mostly at a diffusion limited rate oxidizing amino acids or DNA bases, carbonate radical is considerably more selective. Tyrosine in a hydrophilic environment, for example, is more accessible to this charged species, and carbonate radical anion reacts about a hundred times faster with Tyr than nitrogen dioxide [29]. Carbonate radical anion also increases the efficiency of $^{\bullet}NO_2$ for protein tyrosine nitration [33, 35-36]. The *in vivo* consequence can be a very efficient protein tyrosine nitration due to the combination of these radicals.

Compartmentalization, biomembranes, and the heterogenous nature of cells further complicate the reactions of peroxynitrite-derived species in biology. Uncharged species such as the peroxynitrous acid or nitrogen dioxide can diffuse through membranes, while charged ones such as the carbonate radical anion or peroxynitrite anion are unable to do so [37-40]. It has also been demonstrated that peroxynitrite can travel through anion channels in erythrocytes [41-42], reaching various parts and components of the cell. Therefore, the diversity of the cellular environment including membranes, protein structures and domains, and phospholipids makes peroxynitrite chemistry and biochemistry rather complex. Due to different permeability and diffusibility of the different peroxynitrite-derived radicals, the intra- or extracellular production site of these species will strongly determine and influence the outcome and the possible targets in a given biological system. A very thorough demonstration of these possibilities was shown for the first time in Augusto *et al*.'s studies [40]. The group demonstrated how peroxynitrous acid and nitrogen dioxide penetrated the cell membrane in a macrophage cell line model and oxidized intracellular protein tyrosine and thiol residues. The addition of bicarbonate/carbon dioxide resulted in an inhibition of the formation of these residues, due to the quick reaction of peroxynitrite with carbon dioxide. Moreover, they also discussed how the results reflect on physiological conditions in a biological environment and how different radicals play a role in biotarget oxidation when peroxynitrite is produced extra- vs. intracellularly. Another very thorough investigation shows kinetic predictions and gives important considerations for reactive nitrogen species chemistry in biology [43]. Various studies (demonstrating both *in vitro* and *in vivo*) have since emphasized the important role of hydrophobic and hydro-philic compartments, lipid membranes, and organelles, such as mitochondria in the diverse chemical biology of peroxynitrite and its derived species [37, 39-40, 44-45]. Yet, in clinical medicine and medicinal chemistry, these considerations, the variability and the limitation of the reactions mediated directly by peroxynitrite or peroxynitrite-derived species are still often overlooked.

DIABETES, DIABETIC PATHOGENESIS AND PEROXYNITRITE

It is well known that the number of patients with this chronic disease is on the rise – and by 2030 is expected to double, reaching approximately 300 million according to the World Health Organization [46]. Around 90 % are type 2 diabetic, usually with long years of metabolic syndrome combined with cardiovascular risk factors, insulin resistance and obesity. Metabolic syndrome-related conditions and complications such as arteriosclerosis, associated cardiac problems like stroke and infarction, and cardiomyopathy are among the

leading causes of death for these patients in the United States and worldwide [46]. The microvascular complications such as nephropathy and retinopathy lead to renal failure, cataract formation, and seriously impaired vision [47-48]. Peripheral neuropathy is another member of late complications and can be associated with juvenile or type 2 diabetes, leading to impaired sensation, numbness, and often painful conditions. The potential role of peroxynitrite has been investigated by a large number of groups supporting the involvement of this molecule in type 1 and type 2 diabetic models - STZ-mice, NOD mice, ob/ob and high fat diet mice - as well as in diabetic complications including cardiovascular problems, neuropathy, retinopathy, and nephropathy [5, 9, 49-52].

A body of work focuses on the diabetes pathogenesis itself, where the emphasis is on β-cell death in type 1 diabetes, as this type of diabetes is characterized by the total loss of β-cells in the pancreas, mainly due to autoimmune processes. A myriad of factors have been proposed to participate in the triggering mechanism including cytokines, free radicals, and peroxynitrite itself.

In an *in vitro* model system, when Langerhans islet cells from rodents or human patients are exposed to peroxynitrite, insulin secretion is inhibited, with concomitant DNA damage and eventually apoptosis [53]. Similar results were obtained in studies using nitric oxide donors or various ROS generating environments [54-56]. Because of DNA damage, the activation of the enzyme poly-ADP-ribose polymerase (PARP) as a downstream mechanism is also under the scope of intense investigation. PARP is a protein involved in a number of cellular processes related mainly to DNA repair and programmed cell death. One important function of PARP is assisting in the repair of single-strand breaks, on the other hand, upon DNA cleavage by enzymes involved in cell death (such as caspases), PARP can deplete the ATP pool of a cell in an attempt to repair the damaged DNA. ATP depletion in a cell leads to lysis and cell death [57-59].

In *in vivo* animal models, iNOS often mediates a possibly at least partially peroxynitrite-driven mechanism. Although first discovered in macrophages, the enzyme is over-expressed in various tissues, including the pancreas, [60] and knocking out iNOS protects against chemically induced diabetes [61]. Genetic disruption or pharmacological inhibition of iNOS are also shown to be protective in different models including the non-obese diabetic (NOD) mice [62]. Furthermore, apart from the above mentioned *in vitro* models, the islets from a NOD mice pancreas show significant nitrotyrosine staining compared to healthy controls [62], suggesting a correlation between this biomarker and the consequent protein oxidation and damage in β cell death. The application of peroxynitrite scavengers as well as PARP inhibitors also reduces nitrotyrosine damage in the Langerhans, further confirming this correlation [62-64]. A few years ago, a unifying mechanism was proposed in relation to diabetes and diabetic complications where, probably through the decomposition of peroxynitrite, excess superoxide production from the mitochondria (due to hyperglycemia) induces single strand breaks, and therefore, PARP activation. A key enzyme in the glycolytic pathway, GAPDH is modified by PARP, which leads to the impairment of various biochemical pathways involved in oxidative stress, such as the polyol pathway and aldose reductase activation [65].

DIABETIC COMPLICATIONS AND CONSIDERATIONS ABOUT PEROXYNITRITE AND NITROTYROSINE FORMATION

Regardless of oral treatments or insulin injection, almost half of the patients with either type of diabetes develop late complications, such as retinopathy, nephropathy, peripheral neuropathy, or various forms of vascular and cardiac problems. These complications can lead to more severe conditions including ulcers, renal failure, cataract, and blindness, as well as endothelial dysfunction, atherosclerosis, and myocardial injury [66-70]. The possible contribution from peroxynitrite and its derived species has been intensely investigated by a number of groups studying various complications.

In diabetic neuropathy, one of the first studies where the possible role of peroxynitrite-derived species was discussed showed that preventing superoxide formation in epineurial arterioles of the sciatic nerve restored endothelium-dependent vasodilation [71]. Later on, a body of studies focused on potent peroxynitrite decomposition catalysts to improve neuronal function and counteract peripheral neuropathy in the disease, using the streptozotocin-diabetic model, the NOD mice, as well as the leptin deficient ob/ob mice [72-77]. Furthermore, focusing on human patients, nitrosylated proteins have been suggested as a new biomarker for peroxynitrite related stress in diabetic subjects with macroangiopathy [78]. As diabetes is associated generally with chronic low-grade inflammation, the potential role of iNOS has been investigated in neuropathy as well. iNOS knockout mice treated with streptozotocin showed improved function in multiple manifestations of peripheral neuropathy, including normal nerve conduction velocities and less severe small-fibre sensory neuropathy [79]. In relation to peroxynitrite-mediated downstream mechanisms, several lines of evidence prove the role of PARP activation in diabetic neuropathy [52, 80-81]. These studies include a range of PARP inhibitors and knockout mice experiments. The possibility for new therapeutic strategies to ameliorate oxidative stress and peroxynitrite-related damage in neuropathy was covered and discussed in comprehensive overviews [5, 9, 59].

Studies on diabetic nephropathy first reported increased levels of nitrotyrosine in the kidney of human patients [82]. One of our previous studies suggested the involvement of nitric oxide and peroxynitrite in pathological changes of various tissues, including the diabetic kidney [83]. Similarly to neuropathy, DNA damage and the activation of PARP in peroxynitrite-mediated cytotoxicity were studied in the pathogenesis of nephropathy as well [84-85]. Peroxynitrite also seems to play a role in glomerular lesions in the kidney of diabetic rats [86], possibly through the JAK/STAT signaling pathway [87]. As the peroxynitrite-induced damage – PARP activation – metabolic changes pathway probably works both ways, the inhibition of aldose reductase by the compound fidarestat ameliorated oxidative/nitrosative stress and PARP activation in tissues that are involved in complications [88], such as the diabetic kidney as well as in human mesangial cells in a high glucose environment [89].

In diabetic retinopathy, the role of PARP activation has also been proposed, together with the involvement of NADPH oxidase, iNOS, and peroxynitrite [90]. In a streptozotocin-induced long term model, nitrotyrosine formation was abundant in the microvessels of the

retina, and more alarming, this nitrotyrosine accumulation was not normalized after 6 months of glycemic control [91]. This failure to normalize protein oxidation/nitration in the retina, and the accumulation of these oxidized protein aggregates, may be at least partially responsible for the resistance of diabetic retinopathy. In *in vitro* studies, indications for peroxynitrite-mediated oxidative changes were also found where retinal endothelial cells were exposed to high glucose [92]. Furthermore, at the site of pathological changes in the retina, in the retinal microvasculature, peroxynitrite has been shown to contribute to permeability changes. Also, lipid peroxidation and peroxynitrite were linked to retinal ischemia/reperfusion injury [93-94]. *In vivo* studies compared various rat and mice models of diabetes in regard of how early diabetes-related changes occur in the retina [95]. iNOS inhibitors such as aminoguanidine or peroxynitrite scavengers, like the compound FP15, and furthermore, PARP inhibitors also successfully corrected some major alterations in this complication [96-98].

As peroxynitrite is able to interfere with several bio-molecules in the endothelium, vascular smooth muscle, and in the cardiac tissues; its contribution has also been postulated in cardiovascular complications of diabetes [99-103]. The various cardiac problems - cardiomyopathy, hypertension, infarction, and endothelial dysfunction - related to long term diabetes are still among the major complication problems. Observations in human aortic endothelial cells confirmed that high glucose *via* peroxynitrite can cause tyrosine nitration, which in this case lead to important functional changes, inactivating enzymes such as prostacyclin synthase, in association with apoptosis [104]. Others confirmed as well that peroxynitrite is a major trigger leading to cardiomyocyte apoptosis *in vitro* and *in vivo* [105]. In high glucose-perfused hearts, iNOS upregulation was observed together with increasing NO and superoxide production. These conditions favor peroxynitrite formation, indicated by elevated nitrotyrosine levels, and furthermore, apoptosis [99]. Moreover, evidence was found for increased nitrotyrosine and a correlation between the degree of pathological change and nitrotyrosine levels in human patients, in heart biopsies [101]. Peroxynitrite was shown to be able to oxidize the zinc-thiolate complex of eNOS, which can lead to an important vicious cycle, as the enzyme gets uncoupled, producing both super-oxide and NO simultaneously, resulting in more peroxynitrite formation [106]. The same research group showed direct S-glutathiolation of p21 ras by peroxynitrite in endothelial cells, which event mediates its direct activation, as well as endothelial insulin resistance caused by oxidized LDL [107-108]. As DNA strand breaks and peroxynitrite are usually linked, regardless of the site or tissue, PARP activation was intensively studied in cardiovascular and endothelial dys-function and heart failure as well [109-114]. Streptozotocin-diabetic rats showed increased peroxynitrite production in the aorta, cardiac hypertrophy, protein carbonylation or nitrotyrosine formation [115-116]. Similarly to studies in other complications, aminoguanidine or other NOS inhibitors, peroxynitrite scavengers or PARP inhibitors were shown to be effectively attenuating the pathological changes in cardiac studies as well [64, 115-119].

In the light of all these studies, it is important to note that careful consideration should be given regarding whether 3-nitrotyrosine indicates functional alteration on enzymes and a mechanistic contribution to the pathological condition or if it is only a biomarker of the

oxidative stress process in the disease [120]. Although nitrotyrosine is not exclusively formed from the reaction with peroxynitrite, it indicates the involvement of $Tyr^{\bullet}$ and protein oxidation, and is still probably the most valid marker to suspect at least a contribution from peroxynitrite-modulated mechanisms. It is a post-translational modification of protein tyrosine residues, which can lead to loss [121-122] or gain of function [123-125] or even no change in function of the protein [126]. In general, the nitrotyrosine formation yield in biology is relatively low, site specific, and affecting only a few Tyr residues of a given protein, which raises the question of relevancy in certain cases. Protein methionine and cystein residues can also be oxidized, contributing to loss of function. Hence, the influence of protein tyrosine nitration on enzyme function may be a finely modulated mechanism in diabetes as well. It should always be evaluated whether nitration, nitrosylation, oxidation or other peroxynitrite-derived species dependent mechanisms play a role in the disease progression at molecular levels or only serve as markers of oxidative stress. Nevertheless, the accumulating data suggest a strong correlation between 3-nitrotyrosine formation and important pathophysiological consequences in diabetes.

NOVEL FINDINGS ABOUT PEROXYNITRITE IN DIABETES, INSULIN RESISTANCE AND RELATED CONDITIONS

The past decade has seen the continuation of research on the currently recognized and established pathways, as well as novel investigations that further indicate and confirm the correlation of peroxynitrite-mediated damage in various diabetes related conditions.

The evaluation of PARP inhibition as a downstream mechanism stayed in the focus of research [127]. In recent studies, PARP inhibition was found to counteract cataract formation and early retinal changes [128], on the other hand, the aldose reductase inhibitor fidarestat suppressed ischemia/reperfusion induced changes in the retina of rats [129]. Inhibition of PARP was also efficient to abrogate multiple manifestations of type 1 diabetic early nephropathy [130] where rats were streptozotocin-diabetic for ~ 3 months, as well as in a longer term model to successfully ameliorate kidney disease [131], where rats were diabetic for about 6 months. PARP gene deficiency or inhibition successfully ameliorated neuropathic pain [132]. Novel research papers and reviews discuss the role of the peroxynitrite-PARP pathway in human diseases, in diabetic vascular complications, and in experimental neuropathy, and moreover, show a novel side of this mechanism in gestational diabetes and diabetic pregnancies [133-135].

The various diabetic model studies have been expanded to focus on type 2 diabetes and metabolic syndrome-like situations, including high-fat diet fed mice, leptin deficient ob/ob mice, or type 2 diabetic rats. The relationship between nitrosative stress and peripheral neuropathy has been evaluated in the ob/ob model [77, 136] and in high-fat diet induced obesity [137]. The pathogenesis and the potential treatments in neuropathy have been recently reviewed [138-139]. New results support the role of superoxide, nitric oxide, peroxynitrite, and PARP in diabetic retinopathy as well [140]. In relation to diabetes, early intervention of protein tyrosine nitration helps to prevent neovascularization in ischemic retinopathy [141].

Beside the inducible form of nitric oxide synthase (iNOS), studies extended to the other constitutive isoforms of the enzyme (eNOS/nNOS) to investigate their potential role to the peroxynitrite pathway in diabetes related patho-physiological changes. nNOS gene deficiency prevented diabetes-induced peroxynitrite injury, as diabetic nNOS −/− mice had near normal nitrotyrosine and PARP levels in dorsal root ganglia neurons but not in peripheral nerve, which is indicative of different roles of nNOS-derived NO in peroxynitrite formation in these two tissue-sites of peripheral diabetic neuropathy [142]. The nNOS knockout mice also developed motor and sensory nerve conduction velocity deficits as well as thermal hypoalgesia, which indicates that nNOS is required for maintaining the normal peripheral nerve function and small sensory nerve fibre innervation. In a db/db mouse model, nNOS expression was reduced in the sciatic where the compound rosuvastatin reversed the reduction of nNOS expression by mechanisms that involved phosphoinositide 3-kinase and phosphorylation of Akt [143]. In the same study, co-treatment of diabetic mice with rosuvastatin and a selective nNOS inhibitor blunted beneficial effects of rosuvastatin on conduction velocity deficits, thermal sensitivity, and nerve vascularity, which also suggests an important role for nNOS-derived NO in peripheral nerve function.

Interestingly, a study that recently examined renal biopsies from patients showed that in human diabetic nephropathy there is an overexpression of eNOS – the endothelial form of nitric oxide synthase in the diseased tissue [144].

Others are focusing on the role of iNOS as a player mediating chronic low-grade inflammation in diabetes and, furthermore, contributing to insulin resistance through peroxynitrite as a possible intermediate. Even after the diabetes has developed, a transition metal-independent hydroxyl radical generation was observed with direct *in vivo* and EPR approaches in a streptozotocin-induced rat model. The specific iNOS inhibitior 1400W reduced lipid radical and hydroxyl radical production, indicating peroxynitrite and its decomposition as initiators of protein- and lipid damage [145]. It is possible that a lower pH in a chronic inflammatory condition favors the generation of hydroxyl radicals, which in turn starts lipid peroxidation. These lipid radicals then initiate tyrosyl radical formation and, hence, increase the possibility of nitration in a hydrophobic environment.

As it has been demonstrated that targeted disruption of iNOS protects against obesity-related insulin resistance [146-147], more studies are currently investigating the pathways with potential peroxynitrite involvement in relation to insulin signaling and insulin resistance, an important condition accompanying metabolic syndrome and type 2 diabetes. These novel studies showed that peroxynitrite may also contribute to insulin resistance. Exogenous peroxynitrite from SIN-1 as a donor, for example, in the muscle resulted in increased nitration of insulin receptor or IRS-1 and Akt [148]. The stimulation of peroxynitrite catalysis improved insulin sensitivity in a high fat diet-fed mice model [149]. Furthermore, in a lipid infusion model, tyrosine nitration of insulin signaling proteins was observed [150]. Although *in vitro* studies have shown before that peroxynitrite can induce tyrosine nitration of IRS-1 [151], this study shows for the first time that protein tyrosine nitration regulates hepatic insulin signaling and disturbs glucose metabolism *in vivo*. Other investigators have previously also observed iNOS induction in diet-induced obesity and in *ob*/*ob* mice models, in association with enhanced *S*-nitrosation of IRS-1 and its reduced protein level in muscle

[152]. Pharmacological or genetic blockade of iNOS prevented the reduction in the IRS-1 protein level in these two models of insulin resistance. Similar mechanisms were suggested in other inflammation and obesity related models [153]. The same aforementioned considerations apply in the case of insulin resistance and phosphorylation studies as well – at the mechanistic level, very interestingly, various studies showed inactivation and decreased tyrosine phosphorylation [154], while others demonstrated enhanced Tyr phosphorylation of proteins after nitration [155-156]. These investigations point out a finely modulated regulatory mechanism balancing tyrosine phosphorylation and nitration.

It is noteworthy that in relation to all these studies, novel *in vitro* works are focusing on the possible mechanisms of how protein tyrosine nitration, dimerization, and hydroxylation happens in hydrophobic compartments such as lipoproteins and cell membranes. The studies clarify the kinetic possibility, the role and relevance of peroxynitrite and peroxynitrite-derived radicals in these reactions, as well as provide a possible link between lipid peroxidation and protein oxidation/nitration [157-158]. When protein tyrosine nitration and/or dimerization caused by biological oxidants happen, it usually requires the formation of a $Tyr^{\bullet}$ as an intermediate. In the case of an *in vivo* system, where proteins are often associated with hydrophobic compartments, membranes for example, the participation of unsaturated fatty acids is quite possible, as they often serve as targets for an initial free radical attack. In the latest study, it has been demonstrated that lipid-derived radicals mediate the one-electron oxidation of tyrosine to $Tyr^{\bullet}$, which can afterward react with another $Tyr^{\bullet}$, or with nitrogen dioxide to yield 3,3'-dityrosine or 3-nitrotyrosine [158]. This data indicates that lipid peroxide radicals ($LOO^{\bullet}$) can mediate tyrosine oxidation processes in hydrophobic biocompartments and provide a new mechanistic insight to understand protein oxidation and nitration in lipoproteins and biomembranes. The findings provide a new biochemical background to test the relationship of peroxynitrite and nitration, as well as lipid peroxidation in pathological conditions like obesity, metabolic syndrome, diabetes, and insulin resistance (a summary scheme is shown on Fig. **1**). In these various models of chronic metabolic disturbances, or the excess lipid accumulation models like the infusion, the elevated levels of free fatty acids, toxic lipid metabolites, and oxidation/nitration of proteins are often observed together, in time and location. These processes may harm several proteins and contribute to the development of oxidative imbalance, while worsening the disease conditions such as poor glucose metabolism or insulin resistance. Therefore an optional new therapeutic way may be to optimally block both lipid peroxidation and protein nitration processes by pharmacological inhibition or possible scavenging and neutralization of the species.

SUMMARY

Past years showed an increasing number of studies and the investigation of peroxynitrite in diabetes and its related conditions in further and deeper details. The major approaches, mechanisms, and possible prevention was discussed in this review to a certain extent, focusing on the major lines of evidence of how peroxynitrite, this powerful biological oxidant, contributes to many aspects of diabetes, metabolic syndrome, and insulin resistance, and how the markers and footprints related to peroxynitrite and its derived species seem to correlate with the progression of all complications. Careful evaluation and interpretation of

the data should always be carried out to emphasize whether the relationship is mechanistic or serves as a correlative biomarker. These studies continuously contribute to a better understanding of the biochemical mechanisms underlying these conditions, and also provide possible therapeutic options (through inhibition, scavenging, or downstream effectors related to peroxynitrite) to at least partially ameliorate the disease progression. They are highlighting the importance and the multi-faceted nature of peroxynitrite, and that the actions of this molecule, including the modulation of lipid peroxidation and protein nitration, are in fine tuned balance. Further understanding the precise chemistry and the biochemical pathways, with present and future medicinal chemistry studies in such a common disease as diabetes and in the midst of an increasing obesity crisis, are extremely important and will certainly lead to novel paradigms, and therefore, new treatment designs in the battle against this disease and all its related, long-term affecting conditions.

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ABBREVIATIONS

1400W	N-(3-(Aminomethyl)benzyl)acetamidine
4-HNE	4 - hydroxynonenal
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
IRS-1	insulin receptor substrate - 1
NOD mice	non-obese diabetic mice
NOS	nitric oxide synthase
PARP	poly-ADP-ribose polymerase
ROS	reactive oxygen species
SIN-1	molsidomine
SOD	superoxide dismutase
STZ	streptozotocin

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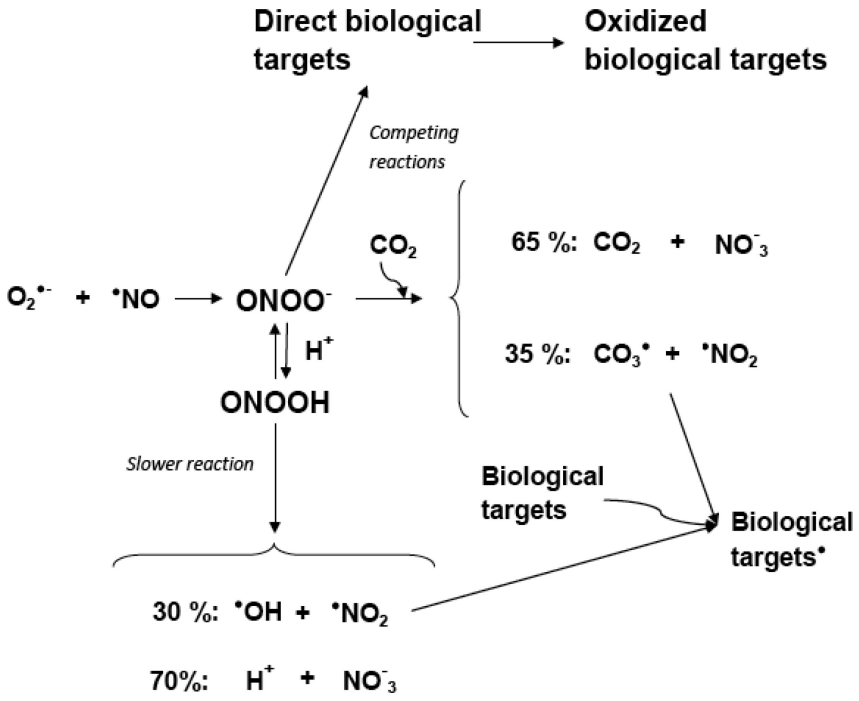

Fig. (1).

Possible peroxynitrite reaction paths in a biological environment. NO and superoxide anion reacts in a diffusion controlled fashion to form peroxynitrite. The decomposition of peroxynitrous acid is considered slow at pH=7.4, to produce hydroxyl radicals. Peroxynitrite reacts with various biotargets *via* the two electron-oxidation mechanism. Carbon dioxide competes with this reaction, and may divert reactions to the one electron oxidation route where most of the biotargets will be oxidized by nitrogen dioxide and the carbonate radical anion. The scheme does not take compartmentalization, biomembranes, or non-homogenous milieu into account. Based on Augusto *et al*., *Free Radic Biol Med* 32:841-859, 2002.

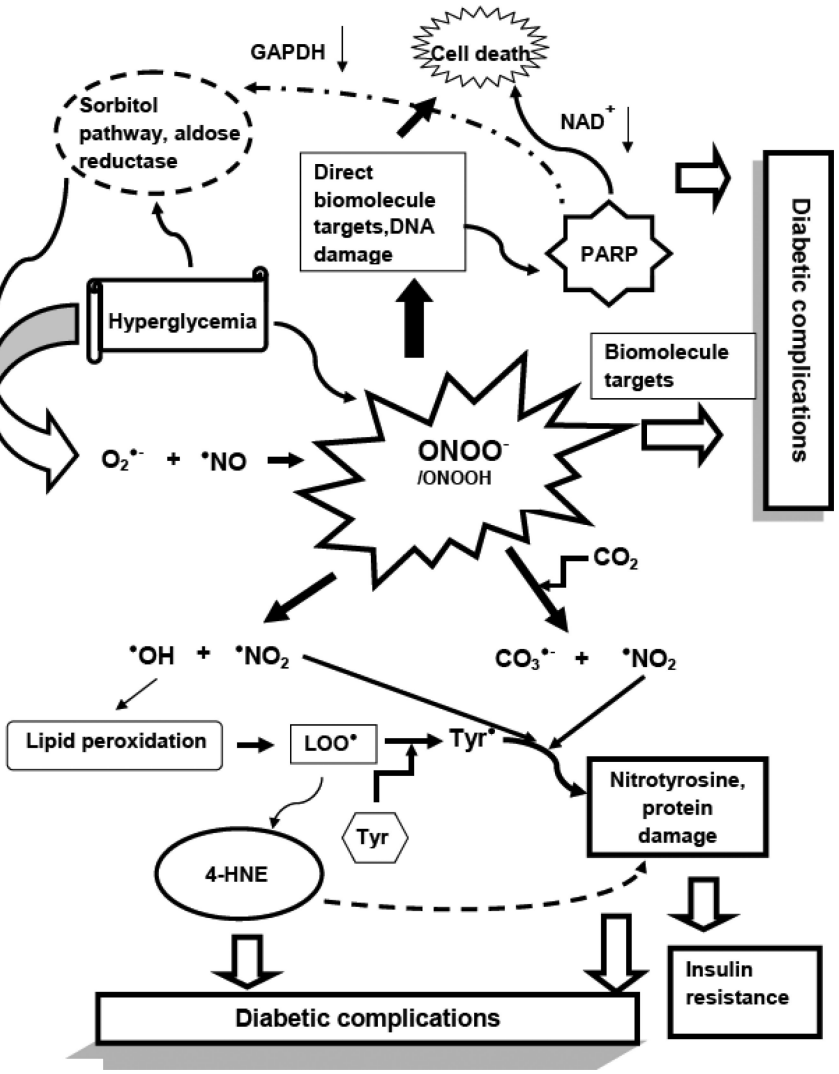

Fig. (2).

A short summary of peroxynitrite-mediated reactions and their possible relation to diabetic complications. Superoxide anion and nitric oxide reacts in a diffusion controlled fashion to form the potent oxidant peroxynitrite. The sources of superoxide in hyperglycemia can be several, including the activation of the polyol pathway and the enzyme aldose reductase as a major mechanism; mitochondrial oxidative stress, NADPH oxidases, uncoupled NOS and several others. NO levels can be elevated due to chronic low grade inflammation. Peroxynitrite affects biomolecules either directly, or by its homolytic cleavage or through its reaction with the ubiquitous CO_2. When DNA is targeted, causing single strand breaks, it activates PARP. PARP activation leads to loss of NAD^+ and ATP and eventually necrosis and cell death, contributing to diabetic pathogenesis or complications. From the various reactions of peroxynitrite, radicals can initiate lipid peroxidation, leading to the accumulation of lipid peroxide radicals ($LOO^{\bullet}$) and the toxic end product 4-hydroxynonenal (4-HNE). 4-hydroxynonenal has the ability to covalently bind to proteins and enzymes with vital role, interfering with their function. According to novel findings, lipid peroxide radicals may mediate the oxidation of tyrosine to Tyr radicals ($Tyr^{\bullet}$) in hydrophobic environments.

The tyrosyl radical then can react with $^{\bullet}NO_2$ to form nitrotyrosine, contributing to protein oxidation in tissue sites of diabetic complications.