Reactive oxygen species in diabetic nephropathy: friend or foe?

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ABSTRACT

Based on the numerous cellular and animal studies over the last decades, it has been postulated that reactive oxygen species (ROS) are important secondary messengers for signalling pathways associated with apoptosis, proliferation, damage and inflammation. Their adverse effects were considered to play a leading role in the onset and progression of type 1 and type 2 diabetes mellitus as well as in the complication of diabetic disease leading to vascular-, cardiac-, neuro-degeneration, diabetic retinopathy and diabetic nephropathy. All these complications were mostly linked to the generation of the superoxide anion, due to a prolonged hyperglycaemia in diabetes, and this anion was almost ‘blamed for everything’, despite the fact that its measurement and detection in life systems is extremely complicated due to the short lifespan of the superoxide anion. Therefore, a tremendous amount of research has been focused on finding ways to suppress ROS production. However, a recent report from Dugan et al. shed new insights into the life detection of superoxide generation in diabetes and raised the question of whether we treat the diabetes-related complications correctly or the target is somewhat different as thought. This review will focus on some aspects of this novel concept for the role of ROS in diabetic nephropathy.

Keywords: AMPK, diabetic nephropathy, ROS, superoxide anion

INTRODUCTION

Diabetic disease is characterized by chronic hyperglycaemia. Numerous studies have shown that in diabetes mellitus, there exists an accumulation of advanced glycated end-products (AGEs) [1, 2], increased oxidative stress [3], enhanced angiotensin II levels [4] and activation of inflammatory mechanisms, which play a critical role in the development of diabetes and diabetic complications. The adverse effects of most of those factors have often been linked to the generation of reactive oxygen species (ROS) [5, 6]. It has been proposed that oxidative stress plays a crucial role in the progression and severity of diabetic disease [6]. It has also been hypothesized that ROS generation is involved in the destruction of the pancreatic β-cells and the onset of type 1 diabetes, whereas type 2 diabetes is mostly related to the late effects of the ROS generation as age-related metabolic disorder. Indeed, studies using isolated β-cells [7] and animal models [8] demonstrate that the accumulation of ROS due to the prolonged hyperglycaemia is possibly the leading cause for the destruction of the pancreatic β-cells in type 1 diabetes or for the insulin resistance in type 2 diabetes [6]. Diabetic nephropathy is a late diabetic complication leading ultimately to renal failure and end-stage kidney disease. It is characterized by mesangial expansion and an accumulation of the extracellular matrix due to the transforming growth factor (TGF)-β-mediated enhanced expression of various extracellular matrix proteins causing thickening of the glomerular basal membrane, glomerular and interstitial fibrosis, glomerular and tubular hypertrophy and podocyte foot process effacement leading to albuminuria [9, 10].

The question of whether oxidative stress is primarily responsible for the diabetic complications was widely investigated, but remained unanswered. Studies using anti-oxidative vitamins, such as vitamin C and E, targeting the overall cellular redox status have been very disappointing. The recent report of Dugan et al. presents a novel way of live detection of superoxide generation in the kidney and this finding could likely explain the hitherto inefficient renoprotective effect reported in the antioxidant studies.
generated by enzymes such as a NAD(P)H oxidase complex [11], uncoupled nitric oxide synthase [12], mono-oxygenase cytochrome p450 or xanthine oxidase (XO) [13]. Several reactive oxygen intermediates can be generated: the free radical superoxide anion (O$_2^-$), the non-radical hydrogen peroxide (H$_2$O$_2$), the highly reactive hydroxyl free radical (·OH), peroxynitrite (ONOO$^-$) and singlet oxygen (¹O$_2$) (Figure 1). Among all ROS, attention has mainly been focused on superoxide anion production. It is accepted that oxidative stress is generated when an imbalance between the amounts of the pro-oxidants and the anti-oxidative cellular machinery occurs, thus inducing a potential cellular damage [14]. Considerable amounts of ROS are generated in different cellular compartments enhancing collectively the levels of the cellular oxidative stress, but the chief source of ROS within the cell is the mitochondrion [15]. Mitochondrial superoxide anion generation (O$_2^-$) are generated by the respiratory chains I–III of the electron transport chain (ETC) [16]. The superoxide anion is routinely produced by oxidative phosphorylation when an oxygen molecule (O$_2$) binds electrons leaking either from complex I or complex III [16]. It has been shown that mitochondrial ROS generation differs depending on the physiological state of the cell (low or high ATP synthesis), the oxygen supply and the concentration of the enzyme or protein containing electron carriers that are present in the redox form, and can react with the $O_2$ to produce O$_2^-$ [16].

ROS may also be produced through different enzymatic systems. The XO is generated due to a H$_2$O$_2$-induced transformation of xanthine dehydrogenase to XO [17]. It is present in high levels in epithelial cells and its expression is also increased in injured or diseased tissues. It utilizes molecular oxygen as an e$^-$ acceptor rather than NAD$^+$ during xanthine catabolism. In addition to the O$_2^-$ generation XO, is also a significant producer of H$_2$O$_2$ and ‘OH [13]. Besides ROS, XO has also been shown to generate nitric oxide (NO) [18] and the highly reactive OONO$^-$ via nitrite reduction [19]. Nuclear factor kappa-B (NF-κB), activator protein-1 (AP-1), cytokines and oxygen tension have been reported to activate XO [13]. Inducible NOS is activated by the inflammatory cytokines, growth factors and endotoxins, and can also produce O$_2^-$ and OONO$^-$ at low availability of i-arginine in addition to NO [20]. OONO$^-$ is a powerful oxidant more reactive than its precursors NO and O$_2^-$. Once generated, it can induce post-translational modification of proteins, lipids and amino acids changing their structure and causing enzyme inactivation [21]. In between the ROS producing enzymes, the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are particularly important as their sole biological function is the generation of ROS. The NAD(P)H (Nox) oxidases are found in neutrophils and macrophages but their distribution is not only restricted to those cell types. NAD(P)H oxidase is present in renal mesangial proximal tubular, vascular smooth muscle cells and podocytes [22, 23]. NAD(P)H oxidases are O$_2^-$ and H$_2$O$_2$-generating enzymes and consist of membrane bound subunits p22$^{phox}$ and gp91$^{phox}$ (Nox2) /Nox1/Nox4 and cytosolic p47$^{phox}$, p67$^{phox}$, p40$^{phox}$ and Rac 1/2 subunits, and are activated as well by numerous factors including cytokines, angiotensin II [24], AGEs [25] and advanced oxidation protein products [26] leading to an activation of NF-κB and AP-1 transcription factors [27]. Therefore, in the cell exists an interplay and cross-talk mechanisms between specific ROS and ROS sources such as mitochondria and cellular NADPH oxidases, which are not yet completely understood. These observations demonstrate that cytosolic ROS, such as NAD(P)H oxidase, could contribute to the progression of diabetic nephropathy in addition to those generated from the mitochondria.

**HYPERGLYCAEMIA REDUCES MITOCHONDRIAL SUPEROXIDE PRODUCTION IN DIABETES**

The generation of ROS and in particular of the O$_2^-$ production was typically measured under *in vitro* conditions from isolated mitochondrial proteins, which has little if any physiological relevance or from isolated mitochondria under conditions assumed to represent the *in vivo* situations. That is a difficult task because the system as described above is extremely complicated with many interactions and cross-talks, and is presumably even more complex under pathophysiological conditions such as diabetes mellitus. Importantly, the produced O$_2^-$ is unstable in aqueous solutions, with a very short-life span of only a few seconds, and is rapidly converted to H$_2$O$_2$ by dismutation via the mitochondrial superoxide dismutase MnSOD (SOD2) [16]. Thus, most of the previous analyses were most likely performed under improper conditions.

Current existing data based on such approaches and interventions concluded that in diabetic conditions, the chronic hyperglycaemia, via the increased flux through oxidative phosphorylation, leads to an elevated production of O$_2^-$, thus inducing the adverse effects observed in diabetic nephropathy as cellular apoptosis, hypertrophy effects, mesangial matrix expansion, podocytes injury and activation of the redox-sensitive transcriptional factors such as NF-κB [9, 28].

However, a very recent report by Dugan et al. [29] for the first time studied the superoxide production in streptozotocin (STZ)-induced diabetes in life animals. Applying a method recently developed for life-time imaging of O$_2^-$ generation in the brain [30], the authors detected the O$_2^-$ in control and diabetic murine kidneys by *in vivo* real-time trans-cutaneous fluorescence. Surprisingly, the analysis demonstrated an opposite finding as expected: the detected levels of O$_2^-$ generation in diabetic kidneys were significantly lower than in the non-diabetic animals [29]. This novel observation was also confirmed by confocal microscopy, showing reduced glomerular and tubular levels of the oxidized dihydroethidium in diabetic kidney sections in comparison with non-diabetic controls [29]. Moreover, even using *ex vivo* measurement via electron paramagnetic resonance analysis the authors found that high glucose reduced the mitochondrial O$_2^-$ production in kidney cortical homogenates from diabetic mice compared with the healthy mitochondria, and a high glucose did not affect the O$_2^-$ amounts in healthy cortical homogenates [29]. This important novel finding was confirmed in various animal models of type 1 diabetes, unmistakable demonstrating that a
reduced generation of the mitochondrial superoxide anion exists in diabetes mellitus [29], a finding which was for so many years elusive.

**SOD2 REDUCTION DOES NOT EXACERBATE DIABETIC NEPHROPATHY**

Three different superoxide dismutases exist encoded from Sod1, Sod2 and Sod3 genes. SOD1 is ubiquitously expressed, SOD3 is characteristic only for some tissues, including the kidney, and SOD2 is present only in the mitochondria.

Sod2−/− mice are neonatal lethal [31] when the Sod2+/− mice which have a normal life span, no aging acceleration, but increased DNA mutations and a higher incidence of cancer development [32]. Although it is anticipated that the increased O2−−− production in Sod2+/− mice could enhance the severity of diabetic disease, the analysis of Dugan et al. showed [29] that an induction of type 1 diabetes mellitus with multiple STZ application in Sod2+/− mice, despite the elevated mitochondria O2−−− neither increased the albuminuria nor mesangial matrix expansion compared with diabetic wild-type mice [29]. Therefore the reduction of the mitochondrial SOD2 does not exacerbate diabetic nephropathy. This finding also points that the increased O2−−− generation (as in Sod2+/− mice) is likely not necessarily harmful in diabetes mellitus and causative for the development of diabetic nephropathy.

There are also controversial data regarding the SOD2 activity in diabetes mellitus. Some studies reported an increased activity of SOD2 and glutathione peroxidase in diabetic hearts, accompanied with an ameliorated catalase and reduced glutathione activity [33], whereas others reported that the decreased SOD2 levels in diabetes correlated with enhanced DNA damage and impaired glucose regulation [34]. Thus, the mechanisms of activation or inhibition under diabetic conditions of SOD2 in type 1 and type 2 diabetes mellitus need to be investigated in more detail in light of the new findings.

**ROBUST INCREASE OF OTHER ROS SPECIES IN DIABETES**

In spite of the overall reduced O2−−− presence in diabetic tissues, in agreement with previous reports Dugan et al. found that diabetes is associated with a robust increase in H2O2 levels in urine as well as an elevated glomerular nitrotyrosine and 8-hydroxy-deoxyguanosine (OHdG) staining [29, 35–37]. The detection of 8-OHdG in urine is accepted as a marker for the generalized oxidative stress in the body [36], thus the locally increased renal levels of the 8-OHdG are representative for an
enhanced cellular oxidative stress in the diabetic kidney. The increased levels of 8-OHdG in the urine of diabetic patients correlate with the progression of the diabetic nephropathy [38]. Similarly, the H$_2$O$_2$ levels were increased in urine, consistent with the oxidative stress. Furthermore, the elevated nitrotyrosine levels in diabetic mice unveiled an increased nitrosative stress. It is shown that under prolonged hyperglycaemia and hypoxia, the NO could be a source of the powerful oxidant OONO$^-$ also detected in diabetic complications [39]. OONO$^-$ is a powerful inducer of apoptosis and its levels are considered as a collective index of the reactive nitrosative stress (RNS) [37, 38]. It has been shown that OONO$^-$ could mediate the rapid mobilization of Ca$^{2+}$ in mitochondria, thus inducing mitochondrial apoptosis via calpains [39]. Indeed, Dugan et al. detected an increased mitochondrial DNA mutations rate (D17 deletion), which was independent of the mitochondrial superoxide generation, but was mostly related to the diabetic state [29]. On the other hand, mtDNA mutations could be as well associated with the increased amounts of the other ROS and RNS in diabetic tissues by causing mitochondrial dysfunction, as shown [39]. Thus, in diabetes mellitus, low levels of mitochondrial superoxide and significant amounts of some other ROS species simultaneously exist which may differentially govern the development of diabetic complications.

**Reduced Levels of O$_2^{ullet-}$ Are Associated with Inhibition of the AMP-Activated Protein Kinase Activity in Diabetes: Renoprotective Role of AMPK**

Using several mouse models of type 1 diabetes and in vivo and ex vivo analysis, the well-conducted study of Dugan et al. demonstrated that high glucose levels inhibited the generation of mitochondrial O$_2^{ullet-}$ leading to reduced mitochondrial biogenesis, reduced pyruvate dehydrogenases complex (PDH) activity, down-regulation of the gene and protein expression of the peroxisome-proliferator-activated receptor gamma co-activator 1α (PGC1α), and inhibition of the mitochondrial ETC activity, due to an inactivation of a single enzyme—the AMP-activated protein kinase (AMPK) [29]. It has been known that PGC1α is a key regulator of mitochondrial functional capacity and is regulated by factors that control energy and nutrient homeostasis [40, 41], therefore its suppression could contribute to the reduced mitochondrial biogenesis and density. Suppressing the O$_2^{ullet-}$ generation with rotenone, a mitochondrial complex I and O$_2^{ullet-}$ blocker inhibited the AMPK phosphorylation at the absence of high glucose. On the other hand, the application of 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an activator of AMPK, reversed the O$_2^{ullet-}$ production, increased the PDH activity and the PGC1α expression, and restored the mitochondrial function [29]. Interestingly, the activation of the AMPK by AICAR, was also able to repair mitochondrial DNA fragmentation, even if it was uncoupled from the reduced superoxide generation. The albuminuria, mesangial expansion and high TGF-β levels, which are markers for diabetic nephropathy [9], were increased in STZ and AKITA diabetic mice, and were reversed by AICAR-dependent activation of AMPK [29]. Moreover, activated AMPK suppressed the generalized ROS and RNS production, as seen by normalizing the urinary H$_2$O$_2$ excretion and reducing the glomerular nitrotyrosine and 8-OHdG immunoreactivities in diabetic kidney [29]. Thus, AMPK functions not only as a master energy sensor of the cell, but also as a ‘master ROS sensor’ in the cell. It could be anticipated that the enzyme activity is modulated by the different O$_2^{ullet-}$ levels, thus during the early and late diabetic stage the O$_2^{ullet-}$ levels and the AMPK activation could vary depending on the glucose concentrations.

AMPK is a multitasking heterotrimeric enzymatic complex, known as the master energy sensor in the cell and the whole body and is conserved from yeast to humans (for a review see [42]). Several upstream kinases such as the tumour suppressor LKB1, and in some cells the calmodulin-dependent kinase kinase (CaMKKbeta), were implicated in the AMPK activation by phosphorylation of the alpha catalytic subunit at threonine (Thr-172) [42]. Metabolic stress, hypoxic conditions and the hormones insulin, leptin and adiponectin can modulate the activity of the enzyme [42]. In the liver, activation of AMPK results in enhanced fatty acid oxidation and decreased glucose production (reviewed in [43]). AMPK is inhibited in obesity and diabetes [44]. High glucose levels are associated with a decrease in AMPK activity in both skeletal and β-cells, while low glucose levels in fact improve AMPK activity [31, 44]. Both adiponectin and leptin are able to activate the AMPK, and both are found to be reduced in diabetes. Moreover, recently Sharma et al. [35] showed that restoring the adiponectin levels in diabetes mellitus is renoprotective and improved the podocyte foot processes effacement and albuminuria, by decreasing the oxidative stress in an AMPK-dependent manner. In fact, the current report confirmed that the AMPK activation is renoprotective and suppressed the oxidative stress from other ROS, but this oxidative stress was not related to the increased mitochondrial superoxide, as probably was expected before. Studies on rosiglitazone, which increased the adiponectine levels and reversed the insulin sensitivity, effectively suppressed albuminuria in type 2 diabetic patients could also now be linked to its action as an AMPK activator through adiponectin [45]. Therefore, AMPK had already received much attention as a relevant therapeutic target in disease associated with obesity, diabetes and the metabolic syndrome (reviewed in [46]).

The exact mechanisms of the AMPK and superoxide anion interplay are yet to be identified. The study by Dugan et al. [29], delighted a new AMPK regulatory mechanism of diabetes, demonstrating that the renoprotective and anti-diabetic role of AMPK, are related to its control of the mitochondrial superoxide O$_2^{ullet-}$, and vice versa [29], thus driving the feed-forward loop, namely a key function in the regulation of the mitochondrial superoxide generation [29]. On the other hand, AMPK activation suppress NAD(P)H/Nox signalling [47] playing an ample role for ROS generation (Figure 2). The most commonly used anti-diabetic drug in type 2 diabetes mellitus, metformin, has also been reported to suppress hyperglycaemia via an AMPK-dependent mechanism [48]. Metformin was also able to reverse the mitochondrial superoxide production
and improve the diabetic nephropathy in diabetic mouse models of Dugan et al. [29]. Thus, the treatments of diabetes by general antioxidants did not show satisfactory results, or were only partially effective in diabetic nephropathy. In fact, the antioxidants were inhibiting the other ROS, as at the same time support low levels of mitochondrial ROS and the AMPK activity. Instead, the search should be focused on antioxidants, which are at the same time activators of AMPK. Several medicaments such as ginsenoside Re are found to have anti-diabetic and anti-hyperlipidemic activity. Quan et al. [49] found that ginsenoside Re application effectively lowered blood glucose levels and lipid levels through activation of AMPK. Furthermore, the ability of anti-AGE agents and ANGII receptor blockers to affect the AMPK activity in diabetes should be extensively studied, as AT1-receptor blockers were protective in patients with renal disease [50].

CONCLUSIONS AND PERSPECTIVES

The findings of Dugan et al. [29] collectively represent a kind of a novel understanding of the role of mitochondrial superoxide generation in diabetes. High levels of mitochondrial superoxide are protective and restore the renal damage and function in an AMPK-dependent manner. Yet, these innovative findings do not correlate with the current widely accepted view that generation of the mitochondrial superoxide is an 'unwanted' second messenger involved in the progression of diabetic disease and diabetes-associated complications. The inhibition of AMPK activity in diabetic mice contributes to the severity of diabetes. Thus, the novel findings, reported by Dugan et al. [29], initiate a new understanding that mitochondrial ROS suppression is the cause of diabetic complications and the rescue of the superoxide production is beneficial and may be even renoprotective in diabetes mellitus and reduces albuminuria. Defining more precisely the mechanisms and the proper methods to distinguish between the different ROS species could be of great benefit for the strategy to combat diabetes mellitus and diabetic complications such as diabetic nephropathy. Thus, we are forced to re-order the puzzle pieces of the 'friend' or 'foe' ROS in diabetic disease.

CONFLICT OF INTEREST STATEMENT

None declared.

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