Trends in Endocrinology & Metabolism

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Review

Reactive Oxygen Comes of Age: Mechanism-Based Therapy of Diabetic End-Organ Damage

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Reactive oxygen species (ROS) have been mainly viewed as unwanted by-products of cellular metabolism, oxidative stress, a sign of a cellular redox imbalance, and potential disease mechanisms, such as in diabetes mellitus (DM). Antioxidant therapies, however, have failed to provide clinical benefit. This paradox can be explained by recent discoveries that ROS have mainly essential signaling and metabolic functions and evolutionally conserved physiological enzymatic sources. Disease can occur when ROS accumulate in nonphysiological concentrations, locations, or forms. By focusing on disease-relevant sources and targets of ROS, and leaving ROS physiology intact, precise therapeutic interventions are now possible and are entering clinical trials. Their outcomes are likely to profoundly change our concepts of ROS in DM and in medicine in general.

Highlights

Lack of comprehensive understanding of DM and its complications, together with its increasing prevalence, represent a major unmet medical need. There is quite a lot known about its mechanisms, but more is needed.

Reactive oxygen species (ROS) may hold the answer to this need, but long-standing misconceptions about oxidative stress and the inefficacy of antioxidants have prevented clinical breakthroughs.

A new understanding of ROS as both essential signaling molecules and pathomechanism have led to the identification, at different stages of diabetes, of different therapeutically relevant sources and targets of ROS.

Network pharmacology and precision diagnostics, currently in advanced stages of clinical development, in conjunction with essential lifestyle changes, will revolutionize the therapy and prevention of diabetes and its end-organ damage.

A New Approach to Diabetes Mellitus and Reactive Oxygen Species

Diabetes mellitus (DM) and its related end-organ damage, such as diabetic nephropathy, neuropathy, retinopathy, and cardiomyopathy, are major causes of death and long-term disability. Their underlying mechanisms are incompletely understood, which is why none of the current antidiabetic therapies target the underlying causes or are curative, but focus instead on normalizing surrogate parameters or risk factors such as blood glucose or hypertension [1]. Hence, our lack of mechanistic understanding of lifestyle change-resistant diabetic end-organ damage, together with the increasing prevalence of DM, represent a significant major unmet medical need.

One mechanism that has been suggested for decades to cause pancreatic β cell dysfunction and diabetic end-organ damage is '**oxidative stress**' (see Glossary), originally defined as an overproduction of **reactive oxygen species (ROS)**. Antioxidants were considered the obvious therapeutic countermeasure but, clinically, have consistently disappointed [2]. Even worse, meta-analyses of clinical trials show that antioxidants may not only be ineffective, but harmful, and even increase mortality [2].

Recently, however, important conceptual breakthroughs in our understanding of ROS in general and DM in particular explain the failure of antioxidants and point towards entirely different mechanism-based and possibly curative therapeutic approaches. Our new understanding of ROS requires that many long-held misconceptions, such as the '**redox balance hypothesis**' and the view that ROS are primarily stressors, disease triggers, and metabolic waste products, must be overcome. Instead, the many physiological roles of ROS and the existence of at least seven evolutionarily conserved ROS-producing enzymes (NOX1–5,

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DUOX1–2), in addition to their alternatively spliced variants, should be recognized. In particular, the discovery of **NADPH oxidase** isoforms, whose only known function is to produce ROS [i.e., NOX1, NOX2 (aka gp91^{phox}) NOX3, NOX4, and NOX5], made it clear that ROS are not merely waste or toxic by-products that need to be removed in order to prevent cellular damage (Box 1).

In contrast, ROS are part of signaling networks that include ROS targets, such as **nitric oxide synthase (NOS)** and **soluble guanylate cyclase (sGC)**, and ROS-metabolizing enzymes, in particular those genetically regulated by **nuclear factor (erythroid-derived 2)-like 2 (NRF2)** [3,4]. The differential expression of these players in different subcellular compartments [5] suggests that a homogenous cell-wide redox level or balance does not exist, but in fact points to there being differential asynchronous hot spots of ROS signaling within the cell. This then makes ROS quite similar to other classical signaling mechanisms [6], including those inducing post-translational protein modifications such as phosphorylation.

ROS signaling contributes to physiological functions and processes such as the oxidative burst of the innate immune response, cell proliferation and angiogenesis, vasodilation, hearing, hormone synthesis, insulin secretion, and insulin sensitivity [3,7,8]. Dysfunction in ROS signaling includes formation of excessive amounts of ROS, appearance of ROS at nonphysiological subcellular sites or in cell types that normally do not form relevant amounts of ROS, or shifting from a physiological to a nonphysiological type of ROS [e.g., from hydrogen peroxide (H_2O_2) to superoxide].

These mechanistic insights are now leading to new therapeutic concepts for which DM is one of best understood and most suitable pathologies. Moving forward, ROS-related drug development will have to focus on the delicate task of identifying the main disease-relevant sources of deleterious ROS while at the same time leaving essential physiological sources of ROS and their signaling pathways intact. One example is the selective inhibition of specific NOX isoforms or the use of NRF2 agonists that enhance the expression of endogenous antioxidant enzymes at their physiological sites, which is qualitatively different to exogenous scavenging antioxidants acting broadly, in all cells and all cellular compartments, and thus in a nonphysiological manner. As a complicating factor, during the course of disease, the enzymatic sources of ROS may change. One example of this is the triggering of vascular dysfunction by NOX1, leading to uncoupling of NOS3, which may become a secondary, yet quantitatively more relevant source of ROS [9]; another example is ROS-induced ROS release in mitochondria [10].

Box 1. NADPH Oxidase Family

Unlike several ROS sources (e.g., mitochondria, xanthine oxidase, cytochrome P450 enzymes, and uncoupled NOS), NADPH oxidases are the only enzyme family known to produce ROS as their primary and sole function [5]. NADPH oxidases are enzyme complexes with a membrane spanning catalytic NOX subunit in addition to other membrane and cytosolic proteins. There are seven identified members of the NADPH oxidase family, NOX1–5, and two dual oxidases (DUOX), DUOX1 and DUOX2. In humans, all seven enzymes are expressed and each NOX isoform has specific tissue expression, regulation, and type of ROS produced [3,5].

The main NOX isoforms to be considered in pathophysiology of DM are NOX1 and NOX5, which produce superoxide, and NOX4, which produces H_2O_2. NOX3 and the DUOXs have very limited roles in the inner ear and in the synthesis of thyroid hormone, respectively [87]. NOX2 is a key enzyme of the innate and inflammatory response and its inhibition or genetic defects are associated with immune deficiency and increased risk of infection, in particular in DM [13]. In addition, NOX2 has been suggested to be involved in an excessive and unlikely number of disease models [5], which indicates a possible positive publication bias, as shown by a meta-analysis of NOX2 studies in stroke [88], or an epiphenomena without therapeutic relevance.

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In addition to preventing ROS synthesis and metabolism, ROS-induced damage can also be repaired at both the molecular and functional level. This is exemplified by recoupling of **uncoupled NOS**, allosteric sensitization of the nitric oxide (NO) receptor, sGC, for lowered NO levels, and the activation of oxidatively damaged, heme-free apo-sGC by heme-mimetics, respectively [3,5]. Collectively, these discoveries have revolutionized the field of ROS in general and, as indicated by one of first clinical applications, DM.

We here review, first, the physiological roles of ROS (e.g., NO, H_2O_2, superoxide, and peroxynitrite) in insulin secretion and signaling (Box 2), as well as in physiology and pathophysiology of organs affected by functional and structural damage due to type 2 DM (T2DM). Importantly, the sources and mechanisms of ROS differ in health, early and late stages of

Box 2. Insulin and ROS

Pancreatic β Cells

The secretion of insulin from pancreatic β cells is mainly regulated by plasma glucose levels. Glucose is taken up via the glucose transporter 2 (GLUT2) and thereafter oxidized to produce ATP. This leads to the closure of K_{ATP} channels, depolarization of the plasma membrane, opening of voltage-dependent Ca^{2+} channels, and a subsequent increase in intracellular Ca^{2+}, which enables exocytotic insulin release [89]. Two ROS species, NO and H_2O_2, increase intracellular Ca^{2+} and thereby facilitate insulin release. Low levels of NO from NOS1 do so by stimulating cGMP formation through sGC, and cGMP in turn activates cGMP-dependent protein kinase (PKG), which inhibits K_{ATP} channels [90]; mitochondrial glucose metabolism leaks small amounts of superoxide, which, upon dismutation to H_2O_2, stimulates ryanodine receptors to also increase intracellular Ca^{2+} [7,89]. Importantly, the use of ROS by pancreatic β cells comes at a risk. Compared with most other cells, pancreatic β cells have some of the lowest expression and activity levels of ROS-metabolizing (antioxidant) enzymes [7], making them more vulnerable than other cells to the potential cytotoxic effects of ROS, such as DNA and protein damage.

In early stages of T2DM, production of NO and ROS begins to exceed the antioxidant resistance of pancreatic β cells. Peripheral **insulin resistance** (see below) and high glucose and fatty acid levels trigger pancreatic β cells to compensate by releasing more and more insulin in a mitochondrial ROS-dependent manner [91]. In later stages, this mechanism is exhausted as superoxide anion induces a leak of protons across the mitochondrial inner membrane, decreases the mitochondrial membrane potential and ATP production, and eventually leads to a lower insulin secretion [92]. Moreover, upregulation of the renin-angiotensin system (RAS) and inflammatory cytokines further increase superoxide overproduction from additional sources (Figure 1) (i.e., NOX1 and NOX2) [87]. The additional induction of NOS2, which produces NO, toxifies superoxide to yield peroxynitrite which not only further reduces insulin secretion but also induces pancreatic β cell death [93].

Insulin-Sensitive Tissues

In peripheral insulin-sensitive tissues such as skeletal muscle, fat cells, and liver, insulin controls the switch from lipolysis/fatty oxidation during fasting to lipid storage/glucose oxidation following feeding [94]. Binding of insulin to the insulin receptor (IR) phosphorylates substrate proteins, IRS1 and IRS2, activating phosphatidylinositol 3-kinase (PI3K)-Akt (protein kinase B) signaling, which leads to the translocation and activation of glucose transporters (mainly GLUT4 in muscles and fat cells) and subsequent glucose uptake [94,95].

ROS come into play in insulin signaling through activation of PI3K and alternative protein kinase C (PKC) activation to increase NOX4 activity, forming H_2O_2 [95]. H_2O_2 augments insulin-IR-PI3K signaling twofold, by inhibiting protein tyrosine phosphatase 1B (PTP1B) and the phosphatase and tensin homologue, PTEN, which dephosphorylates IR and downregulates PI3K signaling [94,95], and by activating MAP kinase phosphatase-1, which dephosphorylates IRS1 [96].

Further increased ROS production is associated with peripheral insulin resistance, a main feature of T2DM [97]. In early stages of T2DM, NOX4 causes fat and liver cell apoptosis, fibrosis, and inflammation [97,98]; NOX2 decreases skeletal muscle insulin-induced Akt phosphorylation, GLUT4 expression and translocation, and thereby glucose uptake [99]. In later stages, mitochondrial ROS formation is induced in skeletal muscles, fat cells, and liver, activating serine kinases and further impeding insulin signaling [97,98]. In skeletal muscles, upregulation of the RAS system further activates NOX2 to aggravate insulin resistance [99].

Glossary

Atherosclerosis: the narrowing of arteries due to plaque build-up on the arterial wall, a chronic inflammatory condition of large- to medium-sized arteries and major cause of cardiovascular disease.
Glomerular hyperfiltration: a condition of abnormally high glomerular filtration rate in the kidney, which occurs as early manifestation of diabetic nephropathy.
Insulin resistance: a state when body cells do not respond sufficiently to insulin and a driving factor leading to T2DM.
NADPH oxidase: a membrane-bound enzyme family which consists of seven members, NOX1–5 and two dual oxidases (DUOX1 and 2). They are the only known dedicated source of ROS and have no other function.
Nitric oxide synthase (NOS): a family of enzymes that consists of three isoforms; NOS1–3, catalyzing the formation of NO, an important cellular signaling molecule, from L-arginine.
Nuclear factor (erythroid-derived 2)-like 2 (NRF2): a transcription factor that regulates the expression of cytoprotective and antioxidant proteins.
Oxidative stress: an outdated view of ROS as primarily toxic metabolic waste products that need to be controlled by antioxidant enzymes or antioxidant drugs.
Reactive oxygen species (ROS): a collective term for compounds such as superoxide, hydrogen peroxide, hydroxyl radical, peroxynitrite, and nitric oxide, although reactivity varies greatly between individual members.
Redox balance hypothesis: an outdated hypothesis that was frequently used to describe a supposedly neutral steady-state level of ROS (i.e., balance between ROS formation and cellular antioxidant defense).
Renin-angiotensin system (RAS): a hormone system which controls blood pressure and sodium homeostasis. Its effects are coordinated through integrated actions in the kidney, cardiovascular system, and the central nervous system.
Soluble guanylate cyclase (sGC): a heme-containing enzyme that acts as a receptor for NO to produce the

disease, and with respect to different cells and organs (Figure 1, Key Figure), sometimes even qualitatively. These differences need to be carefully dissected and defined for T2DM and its characteristic complications as well as other conditions in order to allow future precision antidiabetic interventions. With respect to ROS, the relevant therapeutic targets (see above) form a causal signaling network and in T2DM will be best targeted by mechanism-based network pharmacology, where multiple drugs are combined to synergistically correct a pathological into a near-physiological state [11]. These drugs include some new compound classes that act directly on ROS sources and targets but also some registered drugs, which act, at least in part, through indirectly modulating the ROS network. Finally, mechanism-based diagnostics may enrich these new therapeutic approaches with respect to ROS and DM in order to stratify patients for personalized and precision therapy.

second messenger cGMP, which activates protein kinases and causes vasodilation or regulates phosphodiesterases.
Tubuloglomerular feedback: an adaptive mechanism that links the rate of glomerular filtration to the concentration of salt in the distal tubules.
Uncoupled NOS: when NOS enzymatic activity is uncoupled to produce superoxide instead of NO. This state usually occurs under pathological conditions induced by ROS.

ROS in Diabetic End-Organ Damage/Injury

The most patient-relevant end-organ complications in DM include chronic kidney disease (CKD) (27.8% of patients with DM), retinopathies (18.9%), heart attack (9.8%), and stroke (6.6%) [12]. In all of these, ROS have been suggested to play a causal role [13–15]. Collectively, these represent major causes of disability and death in diabetics and are only moderately prevented by most glucose-lowering antidiabetic drugs, in particular diabetic kidney disease [16]. Although ROS play detrimental roles in these complications, certain ROS forms fulfil important physiological functions in several organs, for example, NO, which is produced by constitutive NOS enzymes (i.e., NOS1 and NOS3) and H_2O_2, which is produced by NOX4. Some examples of these are discussed in the following sections.

Indeed, in T2DM, several events such as hyperglycemia, dyslipidemia, advanced glycation end-products (AGEs), and upregulation of the **renin-angiotensin system (RAS)** contribute to detrimental ROS production. Despite the fact that many enzymes are, in principle, capable of forming ROS, NOXs appear to often be the primary source and disease trigger [14]. Importantly, they represent the only enzyme family with no other known function than to produce ROS [5]. All other ROS sources have other primary functions and ROS production is a biochemical 'accident' often requiring a prior (often ROS-induced) damage or uncoupling before ROS formation is initiated. Therefore, we will focus here on the role of main NOX isoforms (Box 1) in diabetic end-organ damage. The roles played by other ROS sources, such as mitochondria and xanthine oxidase (XO), and the ROS-toxifying enzyme, myeloperoxidase (MPO) as well as NRF2 are briefly discussed in Box 3.

ROS in Blood Vessels

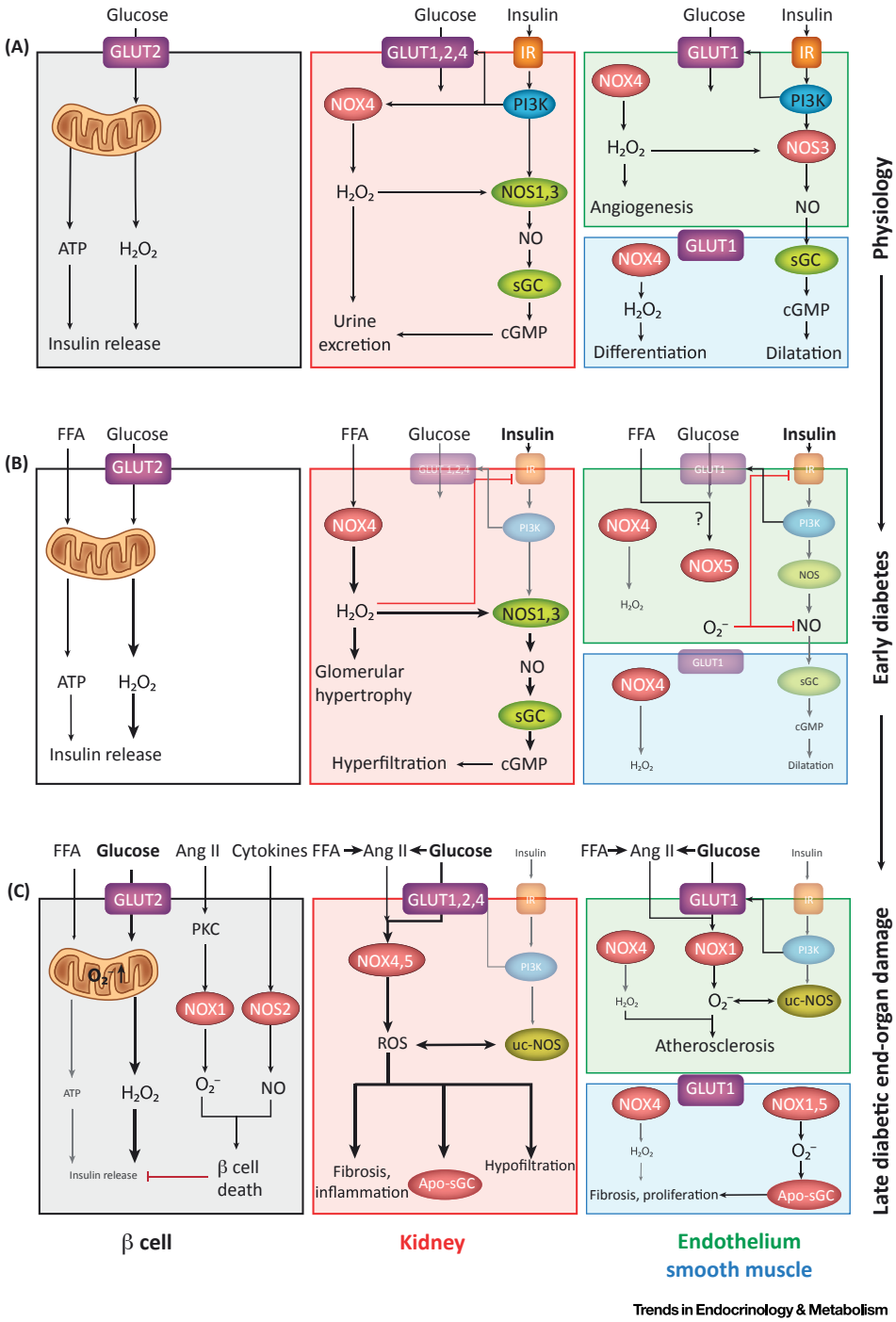
In a normal blood vessel wall, NO produced by NOS3 activates sGC, resulting in cGMP-dependent inhibition of smooth muscle contraction. In human vascular cells, NO shows atheroprotection by inhibiting leukocyte adhesion in endothelial cells and proliferation of smooth muscle cells [17,18]. NO also displays antithrombotic properties in human endothelial cells by inhibiting platelet adhesion and aggregation [18]. H_2O_2 produced by endothelial NOX4 enhances vasodilation in mice [8], both in a cGMP-dependent manner, by increasing the expression and activity of NOS3 [19,20], and in a cGMP-independent manner, by oxidative activation of protein kinase G I (PKGIα) [21]. Moreover, H_2O_2 is a key signal in angiogenesis in both human and animal vascular cells [22,23].

Vascular Disease/Atherosclerosis

In blood vessels, ROS have been suggested to cause hypertension, **atherosclerosis**, and a prothrombotic stage, either directly or by interfering with protective NO [13,24]. In large- and medium-sized arteries, ROS-induced atherosclerosis is thought to be further accelerated by DM [13]. Surprisingly, however, this does not account for all ROS; NOX4-derived H_2O_2 is

Key Figure

Differential Roles of ROS in Physiology and DM



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Box 3. ROS Sources and Metabolism

Mitochondria, XO, and MPO

In addition to NOXs and uncoupled NOS, mitochondria and XO as alternative ROS sources, and MPO as an important ROS-toxifying enzyme may also contribute to the pathogenesis of diabetic end-organ damage. XO produces ROS such as superoxide and H_2O_2 during oxidative conversion of xanthine to uric acid [5]. Mitochondria produce superoxide, by one electron reduction of O_2, which is then dismutated to H_2O_2 by SOD [100]. MPO is a heme peroxidase expressed in neutrophils and monocytes and is important for innate immune system. MPO converts NOX- or XO-derived H_2O_2 in conjunction with halides and nitrite to more reactive species such as hypochlorous acid [5] and, in conjunction with nitrite, represents an alternative source for peroxynitrite, besides the canonical NO and superoxide interaction [101]. Importantly, elevated XO activity and MPO levels correlate with the development of T2DM and diabetic end-organ damage [102,103] (e.g., in kidney fibrosis and proteinuria [104], neuropathy [105], and atherosclerosis [103]). Mitochondrial ROS also have been suggested as causative factors of insulin resistance and implicated in initiation and progression of diabetic complications [14]. Therefore, targeting mitochondria, XO, and MPO may be of additional benefit in DM and, indeed, mitochondrial-targeted antioxidants, XO, and MPO inhibitors are in clinical development (see below) (Table 1).

NRF2

Besides ROS-forming and -metabolizing enzymes, there appears to also be a therapeutic option via endogenous ROS-eliminating (antioxidant) enzymes, in particular those genetically regulated by the transcription factor NRF2. In vasculature, NRF2 is stimulated in response to steady laminar shear stress and ROS to promote atheroprotection. Conversely, oscillatory shear stress and endothelial dysfunction are associated with decreased NRF2 activity and atherogenesis [106]. In the diabetic milieu, NRF2 and its target genes increase in response to hyperglycemia and high fat, suggesting adaptive activation against increased ROS [107]. However, chronic hyperglycemia and prolonged ROS production inhibit NRF2 activation, which is associated with endothelial cell death and foam cell formation [108]. In the diabetic kidney, high glucose and increased ROS production are accompanied by NRF2 upregulation, which initially protects against injury via inhibition of TGF-β and accumulation of extracellular matrix [109]. Sustained hyperglycemia and formation of AGEs, however, downregulate NRF2 and decrease its activity in the kidney, which is associated with increased fibrosis and renal dysfunction [110]. Moreover, in CKD patients, a decrease in the NRF2 signature of peripheral blood mononuclear cells correlates with increased NF-κB related proinflammatory responses [111]. Retinal NRF2 activation is increased in DM, which is thought to protect against ROS overproduction and inflammatory cytokines as well as blood–retina barrier dysfunction, however, the DNA binding activity of NRF2 is decreased [112]. Similarly, in diabetic nerves, acute and chronic hyperglycemia are associated with increased and decreased levels of NRF2, respectively [113]. Thus, in addition to NOX, XO, MPO, NOS, and sGC, NRF2 also has to be considered for the treatment or prevention of diabetic end-organ damage and all of these targets may form a causal inter-related signaling network for mechanism-based network pharmacology (Figure 2).

antiatherosclerotic by reducing fibrosis and proliferation of smooth muscle cells [25]. Conversely, a different type and source of ROS (i.e., NOX1- [13] and possibly NOX5-derived [26] superoxide), may be proatherosclerotic in DM. These examples show the complexity of ROS pathobiology with different sources/types of ROS having qualitatively opposing effects, making precise targeting of the most disease-relevant isoform pertinent for any chronic therapy in T2DM. Nonspecific, chronic NOX inhibition may interfere with angiogenesis, collateral formation, capillarization, and may also be proatherosclerotic and immunocompromising.

Figure 1. Panels in (A) depict physiologic effects of ROS; (B), pathological changes in early DM; (C), late-stage DM end-organ damage. In each row, the black boxes on the left represent pancreatic β cells; red in the middle, renal cells; green and blue on the right, endothelial and vascular smooth muscle cells, respectively. Black arrows and red blocks represent stimulatory and inhibitory effects, respectively. ROS mediate both physiological and pathophysiological signaling. Mechanisms can be enhanced (bold black text and arrows) or diminished (small/transparent text and arrows) leading to pathological changes that are implicated in early and late stages of DM. Abbreviations: Ang II, angiotensin II; DM, diabetes mellitus; FFA, free fatty acids; GLUT, glucose transporter; H_2O_2, hydrogen peroxide; IR, insulin receptor; NO, nitric oxide; NOS, nitric oxide synthase; NOX, NADPH oxidase; $O_2^{\cdot-}$, superoxide; PI3K, phosphatidylinositide 3-kinase; PKC, protein kinase C; ROS, reactive oxygen species; sGC, soluble guanylate cyclase.

Superoxide appears to be the most disease-relevant type of ROS. It can decrease NO biophase levels by direct chemical scavenging, leading to intermediate peroxynitrite, protein tyrosine nitration, reducing endothelial insulin receptor expression, and inhibiting phosphatidylinositol 3-kinase (PI3K)-Akt-NOS3 signaling in the endothelium [24,27] (Figure 1). In addition, superoxide uncouples NOS3, which decreases NO production and simultaneously increases superoxide production from uncoupled NOS3 [9] (i.e., an example of ROS-induced ROS). This has also been coined as the 'kindling-bonfire-radical' hypothesis [28] (Figure 1), because the total amounts of ROS formed from uncoupled NOS can eventually exceed those of the initial trigger enzyme, NOX. Finally, superoxide and/or peroxynitrite can damage the NO receptor, sGC [29,30], leading to a collectively threefold interruption of NO-cGMP signaling by: (i) scavenging of NO, (ii) uncoupling NOS3, and (iii) damaging the NO receptor sGC.

ROS in the Kidney

In normal kidney, ROS regulate urine excretion and blood pressure, and H_2O_2 stimulates prorenin-induced sodium reabsorption in mouse kidney [31] and vasodilates intrarenal arteries from human and rat through indirect NO-dependent mechanisms [19,32]. NO promotes natriuresis in animals by inhibiting tubular sodium reabsorption [33], promotes diuresis by increasing both total and regional renal blood flow through functional antagonism of the vasoconstrictor tone induced by renal sympathetic neurons [33,34], and blunts the **tubuloglomerular feedback** response by afferent arteriolar dilatation [35].

Diabetic Kidney Disease

Besides ROS, multiple inflammatory, fibrotic, and apoptotic signaling pathways are also implicated in the different stages of diabetic kidney disease. ROS, however, appear to integrate these and thereby play a crucial role in the initiation and progression of diabetic end-organ damage and thus represent an ideal target [14]. For this, diabetic nephropathy represents the clinically most advanced therapeutic approach (Table 1), where disease-relevant (not protective!) H_2O_2 is produced from NOX4, and superoxide, from NOX5. Despite being chemically distinct, both ROS seem to trigger kidney fibrosis, renal hypertrophy, and albuminuria [36,37]. H_2O_2 also increases the expression of vascular endothelial growth factor (VEGF) and profibrotic markers [36] and induces **glomerular hyperfiltration**, possibly by increasing intrarenal NOS expression [38]. While the disease progresses, several other renal mediators, such as angiotensin II, AGEs, transforming growth factor (TGF)-β, and protein kinase C (PKC), further increase the activity of NOX enzymes, resulting in a further aggravated renal damage [14]. Impaired cGMP signaling by ROS uncoupling of NOS3 and oxidizing sGC (Figure 1) may further aggravate tubulointerstitial damage and fibrosis [39].

ROS in Other Organs

In the retina, NO is necessary for visual function by increasing retinal blood flow, to maintain adequate nourishment and meet the high metabolic demand of the retina. In animal retina, NO modulates synaptic transmission from photoreceptors and activates sGC to produce cGMP, which is a key intermediate in the visual transduction cascade (reviewed in [40]). H_2O_2 may also act as an intracellular messenger in the human retina. Its physiological roles, however, are unclear [41].

In the brain of animals, NO functions as a neurotransmitter and neuromodulator, regulating synaptic plasticity, which is involved in cognitive functions such as memory formation and mediates neurovascular coupling [42]. H_2O_2 contributes to neurogenesis and differentiation, and neuronal plasticity [43]. In the peripheral nervous system of animals, NO also serves as a neurotransmitter of so-called nonadrenergic-noncholinergic nerves, which induce smooth

Table 1. Mechanism-Based Therapeutics and Their Clinical Status

Compound	Mechanism of action	Pathology	Clinical status
GKT137831	NOX1, NOX4, NOX5 inhibitor	T2DM nephropathy	Safe in Phase I clinical trial Failed to reduce albuminuria in a short-term Phase II trial (NCT02010242)
		T1DM nephropathy	Clinical trial ongoing (U1111-1187-2609)
		Primary biliary cirrhosis	Phase II clinical trial ongoing (NCT03226067)
Ronopterin (VAS203)	NOS2 inhibitor	Traumatic brain injury	Phase III clinical trial (NCT02794168)
		Renal function in healthy volunteers	Safe in Phase I clinical trial (NCT02992236)
Allopurinol	XO inhibitor	T1DM nephropathy	Phase IV (NCT02829177)
AZD3241	MPO inhibitor	Multiple system atrophy	Phase II (NCT02388295)
AZD4831	MPO inhibitor	Heart failure	Phase II (NCT03756285)
MitoQ	Mitochondrial-targeted antioxidant	Chronic kidney disease	Declared as dietary supplement without indication or application in Phase IV (NCT02364648)
SkQ1	Mitochondrial-targeted antioxidant	Dry-eye syndrome	Phase III (NCT03764735)
Folic acid	NOS recoupling agent	T2DM nephropathy	Failed to improve renal endothelial function or reduce albuminuria in a Phase III clinical trial (NCT00136188)
L-citrulline	NOS recoupling agent	Vascular dysfunction in T2DM	Clinical trial ongoing (NCT03358264)
		Peripheral artery disease	Phase III clinical trial ongoing (NCT02521220)
		Sickle cell disease	Phase I trial ongoing (NCT02697240)
Riociguat	sGC stimulator	Pulmonary hypertension	In the clinic
		Scleroderma	Phase II trial ongoing (NCT02915835)
		Sickle cell disease	Phase II trial ongoing (NCT02633397)
Vericiguat	sGC stimulator	Heart failure	Phase III clinical trial (NCT02861534)
Olinciguat (IW-1701)	sGC stimulator	Type I or II achalasia	Phase II clinical trial ongoing (NCT02931565)
		Sickle cell disease	Phase II clinical trial ongoing (NCT03285178)
Praliciguat (IW-1973)	sGC stimulator	Heart failure with preserved ejection fraction	Phase II trial ongoing (NCT03254485)
		T2DM and hypertension	Phase II trial completed (NCT03091920)
Nelociguat (BAY60-4552)	sGC stimulator	Chronic heart failure	Phase I completed (NCT00565565)
Ataciguat	sGC activator	Aortic valve calcification	Phase II clinical trial (NCT02481258)
Bardoxolone methyl	NRF2 activator	T2DM and stage 4 CKD	Increased estimated GFR, but did not improve proteinuria and was associated with cardiovascular toxicity in Phase III clinical trial (NCT01351675).
		T2DM and CKD	Increased measured GFR with no heart failure events due to fluid retention in Phase II clinical trial (NCT02316821)
		CKD associated with T1DM	Phase II clinical trial (NCT03366337)
CXA-10	NRF2 activator	Pulmonary arterial hypertension	Phase II clinical trial ongoing (NCT03449524)
		Primary focal segmental glomerulosclerosis	Phase II clinical trial ongoing (NCT03422510)

muscle relaxation [42]. H_2O_2 stimulates axon growth and nerve regeneration [43,44]. The human cell culture data [17,18,23,32,41] as well as the results in animals described above, if translatable to humans, would suggest that ROS contribute significantly and in multiple manners to human physiology.

Other End-Organ Complications

In diabetic retinopathy, H_2O_2 production is increased from an upregulated NOX4, resulting in increased VEGF expression and blood–retina barrier damage [45]. Moreover, NOX1 is activated to produce superoxide, which promotes cell death [46]. In chronic diabetic neuropathy, ROS from a yet undefined isoform of NADPH oxidase are associated with neuronal apoptosis [47]. In acute ischemic stroke [48], NOX4 is the main source of deleterious ROS and induces blood–brain barrier (BBB) damage and neuronal cell death [15,49]. In the setting of DM, additional isoforms (e.g., NOX1 and NOX5) may come into play.

Mechanism and Network-Based Redox Therapies

Considering the failure of classic antioxidants in clinical trials focusing on diabetic complications [50,51], new therapeutic approaches (i.e., mechanism-based redox therapies) are now state-of-the-art for future trials. These approaches include targeting specific ROS sources using pharmacological inhibitors, repairing ROS-induced damage, or upregulating endogenous antioxidant enzymes (Figure 2). The clinical status of these therapies is listed in Table 1. Importantly, targeting the ROS signaling network at multiple sites in a synergistic combinatorial manner enables a shift from single target approaches to network pharmacology, facilitating lower therapeutic doses with fewer side effects.

Drugs Targeting ROS Sources

NOX Inhibitors

Several NOX inhibitors exist [3]. However, none of them are isoform specific. The NOX1/4 inhibitors, GKT137831 and GKT136901, have shown beneficial effects in several preclinical studies addressing diabetic complications [13,36]. GKT137831 is currently the only NOX inhibitor in clinical trials focusing on diabetic nephropathy. It was safe in a Phase I trial, however failed to reduce albuminuria in a short-term Phase II trial in patients with T2DM and advanced nephropathy on maximal RAS blockade. Yet, there were positive signals on reduction of systemic inflammatory markers. Another trial is currently investigating the antialbuminuric effect of GKT137831 in type 1 diabetic nephropathy. This compound is also being tested in a clinical trial for primary biliary cirrhosis (Table 1).

In addition to GKT137831, other NOX inhibitors showed promising results in preclinical studies well, the pan-NOX inhibitor, APX-115, protects against nephropathy in a mouse T2DM model and was superior to GKT137831 in preserving renal function [52]. VAS2870, another pan-NOX inhibitor, reduces aortic contractility in diabetic rats by improving endothelial function [53] and, in stroke, stabilized the BBB, provided neuroprotection, improved neurological scores in mice [15], and inhibited reperfusion-induced hemorrhagic transformation in hyperglycemic rats [54]. Clearly, isoform-specific NOX inhibitors together with further testing in clinical trials focusing on diabetic complications are needed.

NOS Inhibitors

Despite its many beneficial actions, NO may also have detrimental effects when overproduced. After ischemic stroke, for example, overproduction of NO by NOS1 induces cell death and BBB hyperpermeability and in preclinical studies both nonselective NOS inhibitors or selective NOS1/2 inhibitors improved post-stroke outcome [55]. Thus far, however, no clinical trial

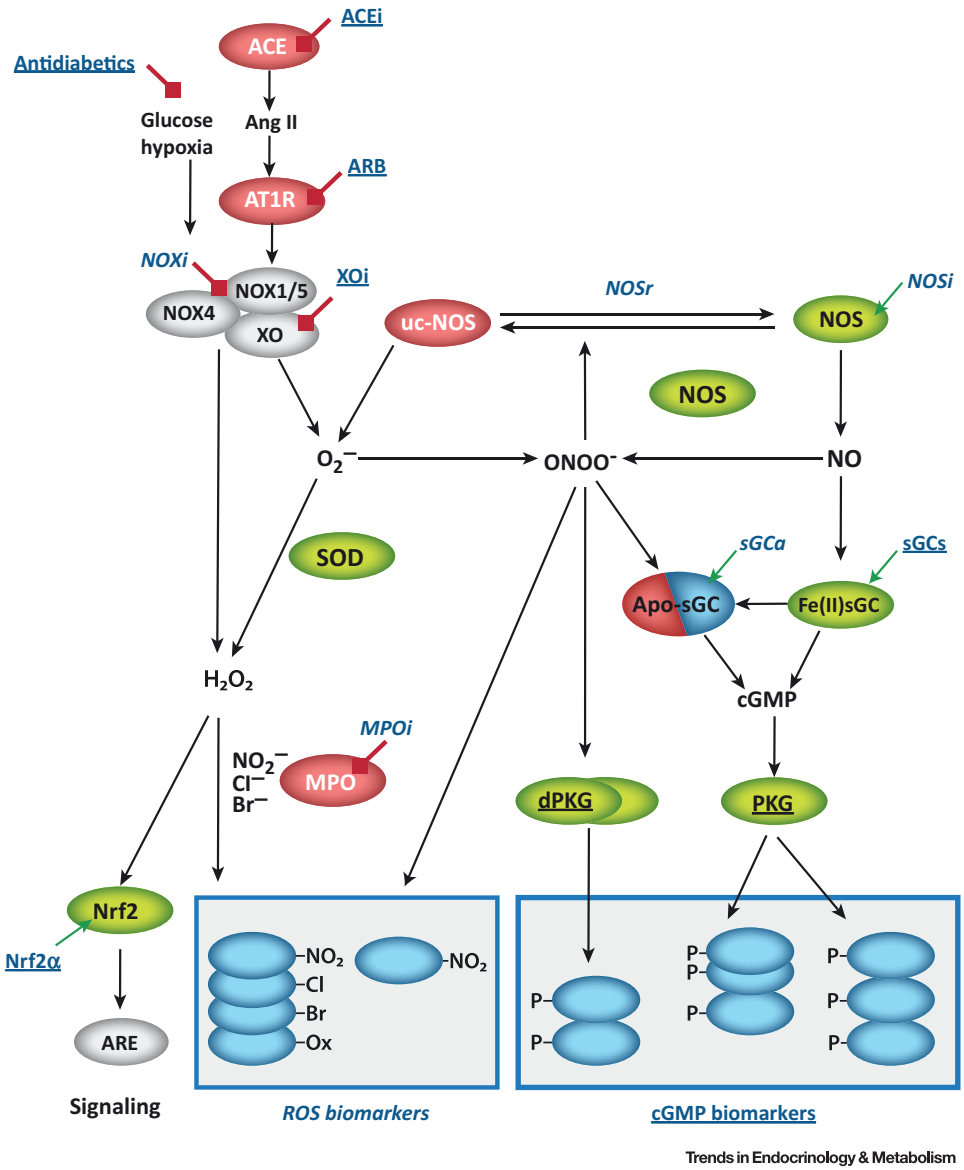

Figure 2. Network Pharmacology for ROS-Mediated Disease States. In DM, high glucose and activated renin-angiotensin system (ACE/Ang II/AT1R) lead to the production of superoxide from NOXs and XO, and H_2O_2 from NOX4. Superoxide interferes with NO/cGMP/PKG signaling by: (i) scavenging NO to form highly reactive $ONOO^-$, (ii) uncoupling of NOS, and (iii) oxidation of sGC to form heme-free and NO-unresponsive apo-sGC. Proteins can be nitrated by $ONOO^-$ or MPO/nitrite/H_2O_2. Via NRF2, ROS can activate ARE genes leading to the expression of antioxidant enzymes such as SOD, which dismutates superoxide to H_2O_2. Several drugs (in blue and underlined when already registered; in italic when in advanced clinical development) act within this network at different sites by either activating/stimulating (green arrow) or inhibiting their target (red blocks) to either activate physiological and inhibit pathophysiological signaling, respectively. At the bottom, proteins (in blue) indicate targets and biomarkers of ROS and cGMP signaling potentially suitable for diagnosing ROS-related disease states and patient stratification. Abbreviations: ACE, angiotensin converting enzyme; Ang II, angiotensin II, ARB, angiotensin receptor blockers; ARE, antioxidant responsive element; AT1R, angiotensin II receptor type 1; DM, diabetes mellitus; H_2O_2, hydrogen peroxide; MPO, myeloperoxidase; MPOi, MPO inhibitors; NO, nitric oxide; NOS, nitric oxide synthase; NOSi, NOS inhibitors; NOSr, NOS recoupling agents; NOX, NADPH oxidase, NOXi, NOX inhibitors; NRF2, nuclear factor (erythroid-derived 2)-like 2; NRF2a, NRF2 activators; O_2^-, superoxide; $ONOO^-$, peroxynitrite; PKG, protein kinase G; ROS, reactive oxygen species; sGC, soluble guanylyl cyclase; sGCa, sGC activators; sGCs, sGC stimulators; ucNOS, uncoupled endothelial NOS; SOD, superoxide dismutase; XO, xanthine oxidase; XOi, XO inhibitors.

has tested NOS inhibitors in a stroke setting. VAS203, a pan-NOS inhibitor, is currently in a Phase III clinical trial (NOSTRA-III) in a related, yet not diabetic indication (i.e., patients with traumatic brain injury).

Drugs Targeting Other ROS Sources

Selective inhibitors of XO and MPO and mitochondrial-targeted antioxidants represent promising drugs for treatment of DM-related organ injury. XO inhibitors such as allopurinol and febuxostat, which are used because of their uric acid-lowering effects for treatment of gout patients, are now also tested in clinical trials because of their ROS-lowering effects and are focused on DM [e.g., in diabetic nephropathy and diabetic coronary artery disease (ALLIENCE trial)]. In addition, MPO inhibitors, verdiperstat (AZD3241) and AZD4831, and mitochondrial-targeted antioxidants MitoQ and SkQ1 are being tested clinically, albeit in nondiabetic conditions (Table 1).

Drugs Targeting ROS Targets

NOS Recoupling

Uncoupling of homodimeric NOS enzymes occurs by multiple mechanisms, i.e., competition of the substrate arginine with asymmetric dimethyl-L-arginine (ADMA), reduced availability of the cofactor BH_4, or oxidative damage and monomerization [56]. All of these mechanisms can be triggered by ROS. Moreover, uncoupled NOS will produce superoxide instead of NO, leading to further ROS accumulation and cellular dysfunction and damage. Several NOS recoupling strategies reduce diabetic complications by improving endothelial dysfunction in humans and animals (reviewed in [56]). Recently however, high-dose folic acid, which increases BH_4 content, failed in a Phase III clinical trial to improve renal endothelial function or to reduce albuminuria in patients with DM (NCT00136188). Studies on L-arginine supplementation gave conflicting results, probably due to its low oral bioavailability (reviewed in [57]). L-citrulline supplementation could be a better alternative due to its ideal pharmacokinetics and is currently in a Phase II clinical trial (CIPER) for treatment of peripheral artery disease. Preclinically, 3 weeks of L-citrulline treatment protected from glomerular hyperfiltration and proteinuria in streptozotocin (STZ)-diabetic rats, whereas arginine did not [58].

sGC Stimulators and Activators

ROS can also damage the NO receptor, sGC [30], resulting in impaired responsiveness to NO in DM-associated organ injury [59]. This can be functionally repaired by two different classes of drugs, sGC stimulators and activators. The former can synergize with low levels of endogenous NO by allosterically binding to sGC, while the latter bind to oxidatively damaged, heme-free apo-sGC, which can no longer sense NO. sGC activators fully recover cGMP formation from apo-sGC and prevent its degradation [59,60]. Preclinical studies of diabetic complications using sGC stimulators and activators, respectively, show promising results. In diabetic NOS3-deficient mice (a model of late-stage diabetic nephropathy) the sGC stimulator, riociguat, combined with an angiotensin receptor blocker (ARB) significantly reduced albuminuria when compared with treatment with ARB alone [61]. The sGC activators, BI703704 and cinaciguat, showed beneficial outcomes on renal function and improved renal structure in diabetics, respectively [39,59]. HMR1766 (ataciguat), another sGC activator, improved NO/cGMP signaling and attenuated platelet activation in diabetic rats [62]. In addition, in a stroke model, treatment with the sGC activator, BAY 60-2770, decreased mortality, increased cerebral blood flow, decreased infarct size, attenuated BBB damage and protected from neuronal apoptosis [63]. Recently, riociguat has been approved for treatment of pulmonary hypertension and together with other sGC stimulators and activators is being tested in clinical trials, albeit so far only in nondiabetic disease conditions (Table 1).

NRF2 Activators

Induction of endogenous ROS metabolizing/eliminating enzymes by activating NRF2 boosts the endogenous control of physiological ROS signaling. Hence, NRF2 activation may be a promising approach to treat ROS-associated diseases, despite the failure of systemically applied antioxidants, which by definition interfere with any ROS, physiological or pathophysiological, leading to neutral or even detrimental outcomes. In a Chinese cohort of T2DM patients, the AA allele of the rs6721961 polymorphism in the *NRF2* gene is associated with lower total antioxidative capacity and pancreatic β cell function and increased risk of newly diagnosed T2DM [64]. Therefore, mild systemic activation of NRF2 may elicit protective effects against hyperglycemia and related end-organ damage. Several NRF2 activators, including sulforaphane and different synthetic triterpenoids, have promising effects in preclinical models of diabetic complications (reviewed in [4,65]). For instance, in ApoE-deficient mice rendered diabetic by STZ injections, a low dose of the potent triterpenoid, dh404, protected against atherosclerosis and improved both renal function and structure. The study also demonstrated target engagement of dh404 on NRF2 activation by reduced ROS and attenuated proinflammatory and profibrotic markers [66]. Another triterpenoid, RTA-405, improved renal function, serum glucose, and triglyceride levels in STZ-induced diabetic rats when compared with untreated diabetic rats [67]. Both dh404 and RTA-405 do not show adverse effects on kidney or liver in obese rats with T1DM [67]. The most developed triterpenoid for clinical use in DM is bardoxolone methyl. In a Phase II clinical trial (BEAM), bardoxolone methyl increased the estimated glomerular filtration rate (GFR) in patients with DM and moderate to severe CKD and this effect persisted for the whole treatment period of 52 weeks [68]. In a Phase III trial (BEACON), bardoxolone methyl increased estimated GFR in patients with T2DM and stage 4 CKD [69]. The trial was terminated prematurely after preliminary analyses showed that patients randomized to bardoxolone methyl experienced significantly higher rates of heart failure events in the first 4 weeks of the trial. Elevated brain natriuretic peptide and a history of congestive heart failure were identified as risk factors that led to fluid overload events in *post-hoc* analyses and have been used to mitigate risk in further clinical trials with bardoxolone methyl. The cardiovascular adverse effects of bardoxolone methyl may be associated with modulation of the endothelin pathway, leading to sodium and volume retention and to blood pressure elevation in this subset of at-risk individuals [70]. In fact, in the Phase 2 TSUBAKI trial assessing bardoxolone methyl in Japanese patients with stage 3 and 4 CKD associated with T2DM, no fluid retention-related adverse events were observed, indicating that the risk-mitigation criteria could be applied to future studies as well [71]. A new Phase 3 trial (AYAME) is being conducted in Japan to assess the efficacy of multiple doses of RTA 402, using time-to-onset of a ≥30% decrease in estimated GFR from baseline or end-stage renal disease.

Indirect ROS Modulation

The current standard treatment for DM and its complications involves normalizing glucose, blood pressure, and dyslipidemia. These interventions delay microvascular complications and are used for chronic treatment of the macrovascular complications of DM. Some of these treatment principles possess redox components, which may contribute to their beneficial effects on diabetic complications.

RAS

Drugs that block the RAS, such as angiotensin-converting enzyme inhibitors and ARB, are currently used to delay the progression of diabetic nephropathy. These drugs inhibit ROS production in different DM-associated conditions by indirectly lowering NOX expression and/or activity [48,72]. RAS blockers also improve endothelial dysfunction and repair the damage

Clinician's Corner

Apart from lifestyle changes, none of the current antidiabetic therapies are curative as they do not target a disease mechanism, but rather focus on normalizing surrogate parameters or risk factors such as blood glucose or hypertension.

One proposed causal disease mechanism involves reactive oxygen species (ROS), but exogenous antioxidants have failed in clinical outcome trials possibly due to the fact that ROS also have physiological functions.

New approaches, now in clinical development, include the activation of endogenous antioxidant responses, inhibition of disease-relevant enzymatic sources of ROS, or the (functional) repair of damage induced by ROS. This includes NRF2 activators, NADPH oxidase (NOX) inhibitors, and soluble guanyl ate cyclase (sGC) stimulators and activators, respectively.

Moreover, targeting the ROS signaling network with several drugs at different sites concurrently (network pharmacology) may enable usage of lower doses of each drug and possible fewer adverse side effects.

Ideally, new mechanism-based biomarkers will stratify patients that suffer from a dysfunction of ROS signaling to enable precision medicine.

induced by ROS in DM via recoupling of NOS [72,73]. In addition, ARB increase the endogenous antioxidant, superoxide dismutase (SOD), via inhibition of NOX and activation of NRF2 in kidneys of diabetic mice [74].

Statins

Statins as cholesterol-lowering and anti-inflammatory [75] drugs are frequently used as part of DM care to lower the risk of cardiovascular complications [76]. With respect to ROS, statins inhibit the activation of RAC1, which is required for the activation of NOX, and in DM improve endothelial dysfunction by also inducing recoupling of NOS3 [76,77].

Glucose-Lowering Drugs

Antihyperglycemic medications are commonly used to normalize glucose levels and include biguanides (metformin), glitazones, dipeptidyl peptidase-4 (DDP-4) inhibitors, and glucagon-like peptide-1 (GLP-1) agonists. With respect to ROS, NOX inhibition, NOS recoupling, and activation of endogenous antioxidant enzymes are all suggested mechanisms of action of these drugs [78–81]. Metformin is the first-line therapy for T2DM patients and is being tested in several clinical trials (more than 2000 on https://www.clinicaltrials.org) in diabetic and nondiabetic conditions, including cancer. Metformin indirectly inhibits NOX via activation of AMP-activated protein kinase (AMPK) and recouples NOS via upregulation of GTP cyclohydrolase 1 and thus BH_4 levels [82]. Metformin also activates the endogenous NRF2 antioxidant pathway via activation of AMPK [81]. Sodium-glucose cotransporter-2 (SGLT2) inhibitors, including empagliflozin, dapagliflozin, canagliflozin, and ipragliflozin, have been recently approved as antihyperglycemic therapies. These drugs possess several favorable effects in T2DM patients, such as improved insulin sensitivity, weight loss, uric acid lowering, and blood pressure reduction and show potentially direct cardiovascular and kidney benefits [83]. In addition to their glycosuric and natriuretic effects, several mechanisms have been suggested to explain these benefits, such as indirect inhibition of ROS production by NOX, recoupling of NOS, and activation of cGMP signaling [84–86].

Concluding Remarks and Future Perspectives

Theoretical and experimental evidence suggests a fundamentally new approach towards the contribution of ROS to T2DM and its pharmacological targeting. Instead of viewing ROS primarily as a metabolic by-product or waste, they are in fact essential signaling molecules with physiological and, upon dysregulation, also pathological roles. This dual quality of ROS depends on: (i) the type of ROS, (ii) the ROS sources, (iii) the ROS targets, and (iv) different functional consequences over time. Importantly, given the failure of classic antioxidant regimes in clinical trials, the field of ROS needs to evolve towards mechanism-based, precision medicine. Given the apparent safety of NOX inhibitors, the high efficacy of NRF2 activators in Phase III clinical trials, and the recent discovery of sGC stimulators and activators, clinical approaches of these targets in DM are ongoing or foreseeable in the very near future. These may also include their combination in a mechanism-based network pharmacology approach. Moreover, a better understanding of the causative mechanisms related to ROS formation in the development and progression of DM and its complications will allow identification of susceptible patients and more precise prevention and treatment (see Clinician's Corner). Indeed, development of mechanism-based diagnostics will allow early disease detection before the development of complications. Existing ROS biomarkers need to be validated in larger sample sizes and compared with current clinical diagnostic tools to establish them clinically (see Outstanding Questions). In any case, lifestyle changes need to be the primary intervention; pharmacotherapy only the *ultima ratio*.

Outstanding Questions

Will network pharmacology of specific ROS sources and targets in different stages of diabetes improve patient-relevant outcomes?

Are there additional sources and targets of ROS in diabetes for mechanism-based therapy?

Is there clinical synergy between direct and indirect modulators of ROS in diabetes?

Will most of the current glucose-lowering, symptomatic but not mechanism-based antidiabetics become obsolete in the not too far future?

Would effective lifestyle management programs of diabetes make pharmacotherapy obsolete in 90% of all diabetes patients? Is pharmacotherapy effective and safe in patients who do not succeed in lifestyle changes?

How can mechanism-based diagnostics help to stratify diabetes patients for early diagnosis, prevention, and curative therapy?

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