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The Role of Nitric Oxide in the Development of Diabetic Angiopathy

Abstract

Diabetic angiopathy is the main cause of morbidity and mortality in patients with diabetes mellitus. Clinical manifestations and pathophysiological mechanisms of diabetic angiopathy can be traced back to the development of endothelial cell dysfunction with alterations in the eNOS/NO system production or availability as the *primum movens* in its natural history. Hyperglycemia *per se* or through the accumulation of AGEs, increased oxidative stress, leading to NOS uncoupling and NO-quenching by excess superoxide and peroxynitrite, and individual genetic background are thought to be responsible for this NO metabolism imbalance. The complex interplay of these mechanisms results in a perturbation of the physiological properties of NO in the maintenance of endothelial homeostasis, such as vasodilation, anticoagulation, leukocyte adhesion, smooth muscle cell proliferation, and antioxidant capacity. Hence, abnormality in NO availability results in generalized accelerated atherosclerosis, hyperfiltration, glomerulosclerosis, tubulointerstitial fibrosis and progressive decline in glomerular filtration rate, and apoptosis and neovascularization in the retina. Indeed, the parallel development of nephropathy, retinopathy, and macroangiopathy may be considered as manifestations of endothelial dysfunction at distinct vascular sites. Given this scenario, intervention targeting any of the pathways involved in the NOS/NO system cascade may prove potential therapeutic targets in the prevention of long-term diabetic complications.

Key words
Diabetes · Nephropathy · Retinopathy · Macroangiopathy · Endothelial dysfunction

Nitric Oxide and Endothelial Cell Dysfunction

Diabetic microangiopathy and macroangiopathy are the main causes of morbidity and mortality in patients with diabetes mellitus [1,2]. Microangiopathy is the hallmark of nephropathy, retinopathy and neuropathy, whereas macroangiopathy is manifested by accelerated atherosclerosis.

Development of endothelial cell dysfunction (ECD) is likely to represent a common pathophysiological pathway for diabetic complications [3].

This term was initially introduced to describe defective endothelium-dependent vasorelaxation in patients at risk for development of atherosclerosis even before angiographic or ultrasonographic evidence of the disease becomes detectable [4,5]; however, it has been broadened to encompass disturbances in vascular endothelium barrier function [6,7], impaired antithrombogenic properties, perturbed angiogenic capacity, inappropriate regulation of vascular smooth muscle tone, proliferative capacity, and migratory properties as well as deterrent of neutrophils and monocytes from diapedesis. Endothelium-dependent vasorelaxation is the gold standard in assessing endothelial function and dysfunction [8]. This demonstrated paradoxical vasocon-

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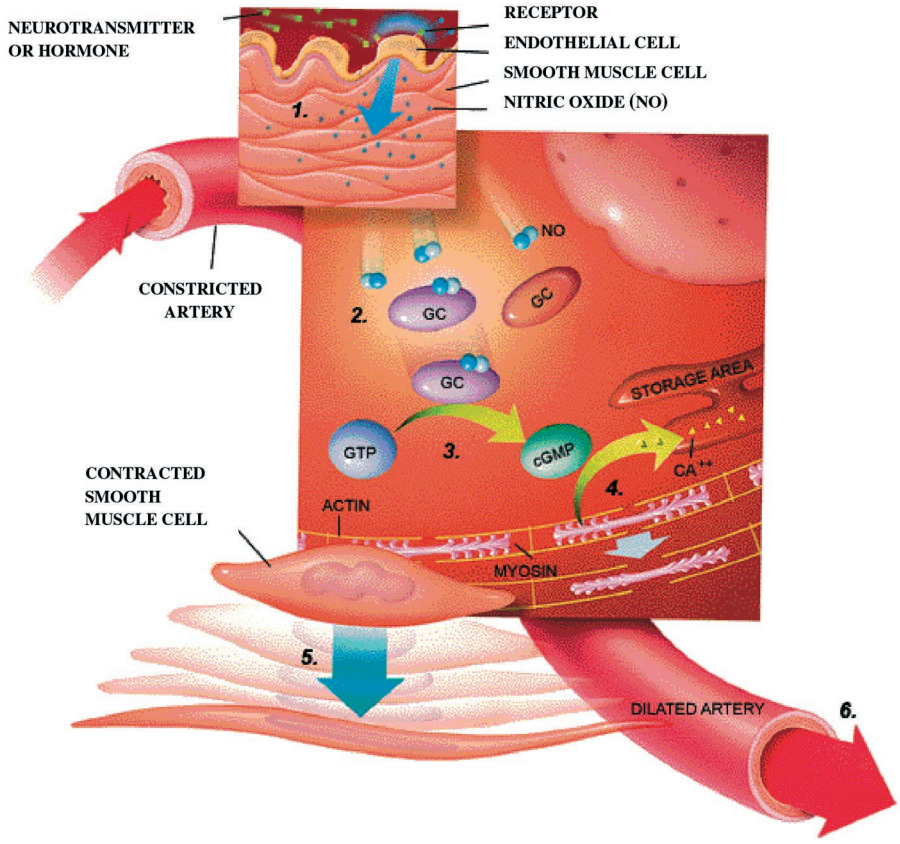

Fig. **1** Neurotransmitter or hormone binds with receptors on endothelial cells lining the artery, which releases nitric oxide in response. NO molecules from the endothelium travel into smooth muscle cells where they activate an enzyme, guanylyl cyclase (GC). GC converts guanosine triphosphate into cyclic guanosine monophosphate (cGMP). After a cascade of cellular reactions (yellow triangles), the smooth muscle cells relax (turquoise and white arrows) and the blood vessel dilates.

striction in atherosclerotic coronary arteries in response to infusion of acetylcholine (ACh) has indicated the pivotal role of endothelial nitric oxide synthase (eNOS) in the pathogenesis of ECD. Indeed, many of the above-mentioned aspects of ECD are intimately linked to the expression and function of this enzyme. In particular, nitric oxide (NO) generation inhibits platelet aggregation [9]; similarly, adhesion of leukocytes to the vascular endothelium is inhibited by NO [10–12]. Endothelial regulation of vascular smooth muscle relaxation, proliferation, and migration is in part governed by the integrity of the L-arginine-eNOS-NO system [13–15]. In addition, vascular/endothelial permeability and some synthetic functions of endothelial cells have been linked to the activity of eNOS [16]. Hence, NO interaction or availability can regulate diverse functions in endothelial cells *per se* and their interaction with circulating formed elements (both inflammatory and thrombogenic interactions) and vascular smooth muscle cells; finally, NO exerts antioxidant effects by direct scavenging of superoxide [17]. These effects are mediated largely through activation of guanylate cyclase leading to increases in cyclic guanosine monophosphate (cGMP) within platelets and smooth muscle cells [18] (Fig. **1**).

Animal studies clearly suggest that endothelium-dependent vasodilatation is altered in experimental diabetes, which is probably based on altered production or degradation of NO [19]. Human studies on type 1 diabetes mellitus (T1DM) are less consistent [20–22].

In support of an involvement of the eNOS/NO system dysfunction in the development of diabetic angiopathy, an association of a functional inducible NOS promoter variant that confers higher inducible NOS (iNOS) expression with complications (nephropathy, retinopathy, neuropathy) has been observed in type 2 diabetics [23].

Determinants of NO system impairment in diabetic angiopathy

One of the early alterations observed in diabetes mellitus that may lead to initiation of ECD and eventually diabetic angiopathy is the defect in the L-arginine: NO: cGMP pathway. The defect could occur at one or several stages of the pathway. There could be reduced basal or stimulated NO release or both, reduced vascular smooth muscle cell (VSMC) responsiveness, or decreased availability of NO. Experimental evidence suggests that all these defects are plausible in diabetes.

Reduced NO release or cell responsiveness: Insulin has vasodilator actions *in vivo* in human arteries and veins that depend on endothelium-derived NO [24–26]. Indeed, insulin has been shown to induce NO release acutely from human umbilical vein endothelial cells (HUVECs) [27], while physiological concentrations of insulin induce a significant increase in NOS expression by human aortic endothelial cells (HAECs) [28]. A series of experiments has demonstrated that sensitivity to the vasodilatory action of insulin is positively correlated with insulin sensitivity with respect to glucose uptake in normal, obese and diabetic individuals [29–32]. By directly measuring NO in HUVECs, it has been shown that the insulin receptor tyrosine kinase, PI3K and Akt, which in adipose tissues participate in insulin-stimulated translocation of the glucose transporter GLUT4 [33], all play significant roles in insulin-stimulated NO production, whereas Ras is less important. Thus, it is possible that defects in insulin signal-

ing leading to insulin resistance may also lead to defects in insulin-stimulated production of NO. These data support the hypothesis that insulin signaling related to production of NO in vascular endothelium may play a role in coupling metabolic and hemodynamic hemostasis. Hence, NO release may be reduced in the presence of insulin deficiency in T1DM. Insulin NO synthesis enhancement impairment has also been demonstrated in insulin-resistant states. Using physiological insulin concentrations, insulin has been found to increase levels of eNOS mRNA, protein and its activity by 2-fold after 2 – 8 hours of endothelial cell incubation. Interestingly, this effect of glucose was seen in microvessels isolated from Zucker lean insulin sensitive rats, but not from insulin resistant Zucker fatty rats [34].

Even if NO release is normal, there could be defective VSMC response to NO as hyperglycemia interferes with NO-induced guanylate cyclase activation [35].

Reduced NO availability: However, the pivotal function that appears to be perturbed in the natural history of endothelial dysfunction and diabetic angiopathy is NO bioavailability. The triggers for this event may be hyperglycemia *per se* or through the production of advanced glycation end-products (AGEs), oxidative stress through the action of the reactive oxygen species (ROS), or alterations in the NO metabolism.

Hyperglycemia and reduced NO availability: A series of studies have shown that supraphysiological concentrations of D-glucose (30 mmol/l) are capable of scavenging NO [36]. Specifically, it has been demonstrated that acute exposure of human endothelial cells to glucose at levels found in plasma of diabetic patients results in a significant blunting of NO responses to eNOS agonists bradykinin and A23187. Monitoring NO generation by purified recombinant bovine eNOS *in vitro* using amperometric electrochemical detection and an NO-selective porphyrinic microelectrode showed that glucose causes a progressive and concentration-dependent attenuation of detectable NO. Addition of glucose to pure NO solutions similarly elicited a sharp decrease in NO concentration, indicating that glucose promotes NO loss. It can be hypothesized that each episode of hyperglycemia, as transient as it might be, will lead to the temporary decrease in the bioavailability of NO and the reversible impairment of NO-dependent functions of the endothelium. It should be emphasized that the glucose-NO adducts formed are unstable and gradually release bioactive NO, but this messenger molecule will then be delivered to inappropriate targets at the wrong time. It could be speculated that a consequence of hyperglycemic episodes, at the level of the vascular endothelium, is the transient decline in NO bioavailability with transient proatherogenic changes in the vascular wall.

AGEs and reduced NO availability: Another major consequence of the elevated plasma glucose levels, which, however, shows poor reversibility and in fact has a cumulative dependence on hyperglycemia, is the formation of AGEs. Their mode of action is linked to changes in physicochemical properties of matrix proteins leading to decreased protein solubility and susceptibility for enzymatic digestion. Recent data have demonstrated that AGEs consume endothelium-derived NO, thus compromising vasodilatory responses and diminishing the antiproliferative action of NO [37]. Accumulation of AGEs over time has been shown to reduce NO availability. Studies using a rat model of streptozotocin (STZ)-induced diabetes showed that reactive intermediates form which then react with and quench NO rapidly (< 5 s) early in the advanced glycation pathway as a result of direct reaction between NO radical and the AGEs both *in vitro* and *in vivo* [37].

These findings of NO quenching by AGEs provide the conceptual basis to link AGEs with the maintenance of ECD.

ROS and reduced NO availability: During the last 2 decades, a large body of evidence has suggested that endothelial dysfunction may be caused by accelerated inactivation of NO by ROS.

Superoxide production by vascular tissues and its interaction with NO play important roles in vascular pathophysiology [38,39]. The interaction between NO and superoxide occurs at an extremely rapid rate [40], three times faster than the reaction rate for superoxide with superoxide dysmuthase (SOD). Given this rapid reaction rate, there is likely always some superoxide reacting with NO within cells and in the extracellular space. Under physiological conditions, endogenous antioxidant defenses minimize this interaction and maintain what seems to be a tenuous balance between superoxide and NO. However, eNOS may be a source of superoxide production under certain conditions due to enzymatic "uncoupling" of L-arginine oxidation and oxygen reduction by the eNOS oxygenase and reductase domains, respectively. Recent studies suggest that reduced availability of the cofactor tetrahydrobiopterin (BH4) may result in eNOS uncoupling, which means that electrons flowing from the NOS III reductase domain to the oxygenase domain are diverted to molecular oxygen rather than to L-arginine, resulting in production of superoxide rather than NO. This mechanism may be an important contributor to the imbalance between production of NO and superoxide production in vascular disease. In particular, data from animal studies suggest a possible role for BH4 in mediating the eNOS dysfunction observed in diabetic vessels [41 – 43] and endothelial cells [44,45]. This suggests that increased superoxide production accounts for a significant proportion of the NO deficit in diabetic vessels.

Hyperglycemia increases NOS-dependent superoxide production in human endothelial cells [46]; both glucose challenge [47] and lipid and protein intakes [48] are followed by an increase in ROS generation by polymorphonuclear leukocytes (PMNs) and mononuclear cells (MNCs). It is possible that acute hyperglycemia and an acute fat and protein increase stimulate oxidative load, thus reducing NO availability.

These findings suggest the mechanisms underlying increased vascular superoxide production in human diabetes, and suggest clear associations with ECD characteristic of diabetic vessels, even in the absence of macroscopic atherosclerosis.

Altered NO metabolism and reduced NO availability: Finally, reduced NO bioavailability may result from the altered NO metabolism within diabetes mellitus. In fact, preferential binding of endogenous and exogenous NO to glycosylated deoxyhemoglobin and consequently an altered metabolic fate of NO in patients with T1DM has been demonstrated; this could influence micro-

vascular regulation and tissue perfusion [49]. It is possible that glycosylation of blood proteins alters their binding affinity for NO, thereby altering the metabolism of NO in T1DM patients. These findings might have a direct bearing on the bioactivity of NO in the microcirculation. The bioavailability of NO produced by the vascular endothelium might depend on its inactivation by glycosylated hemoglobin and other proteins, besides inactivation by ROS. Altered NO activity in people with diabetes mellitus is not satisfactorily explicable simply in terms of altered NO production or inactivation. The hyperglycemia-induced alteration in NO metabolism could affect NO bioavailability and limit peripheral NO release despite low pO_2, thus contributing to microvascular disease and impaired tissue perfusion.

Diabetic nephropathy

Diabetic nephropathy currently represents the leading cause of end-stage renal failure [50,51]. Clinical manifestations of diabetic nephropathy include microalbuminuria heralding incipient nephropathy, followed by albuminuria or nephritic range proteinuria, hypertension, development of glomerulosclerosis, interstitial fibrosis, and a relentless decline in glomerular filtration rate [50–53].

The pathophysiological origin of clinical manifestations of diabetic nephropathy can be traced back to microvasculopathy and macrovasculopathy with ECD as a common pathophysiological pathway. A considerable body of evidence ascribes clinical manifestations of both early and late diabetic nephropathy and their respective pathophysiological mechanisms to a dysfunction in the eNOS/NO system.

Early diabetic nephropathy: NO overproduction

A large body of evidence in humans indicates that microalbuminuria may be considered as the hallmark of a generalized endothelial vascular dysfunction [54–56]. In this regard, an abnormality in NO production and action has become an attractive hypothesis for the pathogenesis of diabetic nephropathy [57,58]. Several lines of evidence suggest that NO generation and action may be the same or even increased early in diabetes. Early hemodynamic alterations are characterized by the development of hyperfiltration in the diabetic kidney. NO may increase both blood flow and vascular permeability and thus is a candidate mediator of the vascular abnormalities described in early diabetic nephropathy [59]. Some observations have suggested an increased generation or action of NO as a mediator of hyperfiltration in early experimental diabetes. In fact, vasodilation due to increased NO generation or action has been implicated in the pathogenesis of glomerular hyperfiltration [60–62] and in the enhanced permeability to macromolecules leading to microalbuminuria [60–62]. In streptozotocin-induced diabetic rats, plasma and urinary excretion levels of stable products of NO oxidation were significantly higher than in normal rats, suggesting a generalized increase in NO synthesis [62].

NO has been reported to be important in the modulation of renal hemodynamics and, in particular, in maintaining the normal state of vascular tone [60,63].

Endothelial and neuronal NOS are expressed constitutively and produce small (pM) transient bursts of NO in a calcium-dependent manner. The inducible isoform, iNOS, mediates the synthesis of large amounts of NO (nM) for extended periods of time following stimulation by inflammatory cytokines or disturbances in the cellular milieu [64]. All three isoforms of NOS are expressed in the kidney, and iNOS is most conspicuously present in the thick medullary ascending limb and the inner medullary collecting duct [65]. In this regard, an increased renal expression of eNOS and iNOS proteins has been found after one week in STZ-induced diabetic rats [66]. This increased expression could be in part responsible for the attenuated tubuloglomerular feedback and the glomerular hyperfiltration observed in early diabetes. With the exception of this study, iNOS cortical protein and mRNA expression have been found to be unchanged [67,68] or barely detectable [69,70] in the hyperfiltering stage.

One of these studies investigated the renal functional effects of L-iminoethyl-lysine (L-NIL), which is 30 times more potent as an inhibitor of iNOS than of ecNOS, to test the hypothesis that induction of iNOS in glomeruli or preglomerular vessels contributes to glomerular hyperfiltration in STZ-diabetic rats [70]. The results support the notion that increased NO availability due to greater abundance of ecNOS contributes to the pathogenesis of glomerular hyperfiltration in early experimental diabetic nephropathy. In contrast, no functional or molecular evidence for increased glomerular expression and activity of iNOS was found in diabetic rats [70].

Lack of pharmacological agents that selectively inhibit a specific NOS isoform and that can be safely administered chronically in animal models of disease states makes it difficult to ascertain the distinct contribution of the different NOS isoforms in the pathogenesis of diabetic nephropathy with certainty. Strains of mice with homozygous deletion of each of the three NOS isoforms have recently been developed. One experiment was conducted in knockout (KO) mice with homozygous deletion of the iNOS gene to test the hypothesis that the absence of NO synthesis via this isoform of the enzyme would exacerbate chronic diabetic kidney disease [71]. The investigators concluded that iNOS-derived NO modulates glomerulosclerosis and tubulointerstitial fibrosis in chronic STZ nephropathy, probably as a result of the direct actions of NO on the synthesis and degradation of extracellular matrix proteins [72]. Conversely, at 1–2 weeks, iNOS activity exerted no influence on glomerular perfusion or single nephron glomerular filtration rate, nor did they detect iNOS protein in immunoblots prepared from kidney tissue of iNOS KO mice at 16 or 40 weeks of STZ diabetes, whereas eNOS protein expression was significantly higher at this stage [71]. It may be hypothesized that each of the three NOS isoforms plays a more prominent role in the pathophysiology of nephropathy at different phases of the disease.

Taken together, these data argue against the assumption that the inducible form of NO synthase is of importance in the glomerular hyperfiltration observed in the experimental model of diabetes mellitus.

Further studies have shown enhanced NO synthesis by ecNOS in afferent arterioles and glomerular endothelial cells in STZ-induced diabetic rats, suggesting a pivotal role of NO in preferential afferent arteriolar dilatation, glomerular enlargement, and functional glomerular hyperfiltration in the early stages of diabetic nephropathy [73]. Acute systemic infusion of NO synthase blockers caused a more pronounced decrease in glomerular filtration rate and renal plasma flow in diabetic rats, largely eliminating the difference between diabetic and control animals [57, 74 – 76], and a 8-week treatment with an experimental NO scavenger seems to prevent diabetes-induced endothelial dysfunction [77]. Similarly, chronic oral administration of L-arginine analog N-nitro-L-arginine methyl ester (L-NAME) prevents hyperfiltration development in diabetic animals [70]. Using the *in vivo* hydronephrotic kidney technique to investigate the renal microcirculation, diabetic rats demonstrated a significant basal vasodilation of all preglomerular and postglomerular vessels *vs.* control rats. In addition, staining for eNOS was present in the endothelium of all preglomerular and postglomerular vessels and in glomerular capillaries, and was more pronounced in diabetic kidneys, suggesting eNOS upregulation in the renal microvasculature [78].

Immunohistochemical eNOS staining intensities were significantly increased in endothelial cells from microalbuminuric type 2 diabetic patients as compared with the control subjects, suggesting that NO may contribute to the pathogenesis of glomerular hyperfiltration in Japanese type 2 diabetic patients [79].

NO is known to decompose into nitrite (NO_2) and nitrate (NO_3) very rapidly in biological solutions [80]; these two stable compounds can be analyzed in serum, and are now considered as indicators of NO activity *in vivo* [81]. Our group showed that NO_2 + NO_3 serum content as well as GFR were significantly higher in microalbuminuric than in normoalbuminuric diabetic subjects, supporting the hypothesis that glomerular hyperfiltration due to high circulating NO level may be operative in young patients with T1DM and persistent microalbuminuria [82].

The etiology of NO system upregulation in diabetes is unknown, but a number of potential candidate mechanisms may be proposed. Recently, a glucose-dependent increase in NO production and action has become an attractive hypothesis for the pathogenesis of early diabetic nephropathy [41, 57, 58, 66]. High glucose enhances cytokine (IFN-γ)-induced NO production by rat mesangial cells. The activation of protein kinase C (PKC) and aldose reductase pathway may play a role in this enhancement. In addition, high glucose-induced NO production by the eNOS pathway may promote extracellular matrix accumulation by mesangial cells [83].

A significant correlation was observed between Hb_{A1c} and serum NO_2 + NO_3 content, confirming that poor glycemic control may directly influence NO synthesis. This correlation was observed in both normoalbuminuric and microalbuminuric adolescents, suggesting that NO production is increased early in the natural course of diabetes and independently of the presence of microvascular complications [82].

In endothelial cell cultures, high glucose concentrations directly induce eNOS expression by increasing the intracellular calcium concentration. Vascular endothelial growth factor (VEGF) is known to stimulate NO production in endothelial cells [84]. In experimental diabetes, the expression of VEGF in visceral epithelial cells and VEGF receptors in endothelial cells of glomerular capillaries and preglomerular and postglomerular vessels is upregulated [85]. VEGF blockade was found to prevent renal dysfunction as well as eNOS upregulation in diabetic kidneys, thus indicating VEGF as an alternative mediator of the increased eNOS expression in the diabetic kidney [86].

Late diabetic nephropathy: reduced NO availability

If the early stages of diabetes are characterized by increased NO generation and action, by contrast, stability and action may progressively decline later in diabetes due to increases in the production of ROS, oxidized low density lipoproteins (LDL) and AGEs combined with impaired antioxidant defense systems.

The NO-dependent cGMP response to cholinergic agonists is rapidly and selectively reduced in glomeruli isolated from diabetic rats and in vascular tissue and endothelial cells exposed to high media glucose, possibly due to PKC-dependent Gi protein phosphorylation [87].

The cGMP response to exogenous NO donors is progressively reduced in glomeruli isolated from diabetic rats and endothelial cells cultured in high glucose, also suggesting that NO stability is reduced later in diabetes [88].

Endothelial-dependent vasodilatory response has been reported to be impaired in patients with Type 1 and 2 diabetes [21, 89] and in several vascular beds in animals with experimental diabetes [90], supporting a progressive decrease in NO activity. Impaired vasodilatory response in experimental models of diabetes can be partially or completely restored by administration of antioxidants both *in vitro* and *in vivo*, further suggesting that NO stability rather than generation is impaired in diabetes [90].

These findings have led to the hypothesis that even if NO synthesis is increased in diabetes, the oxygen radical superoxide interacts with NO and thus limits its bioavailability (Fig. **2**). NO action expression is largely dependent on the relative levels of NO and superoxide and on the ability of cellular antioxidant mechanisms to protect NO from conversion to damaging reactive nitrogen species such as peroxynitrite. The affinity of NO for superoxide is so high that its rate of reaction is limited only by diffusion [40]. Because superoxide effectively degrades NO to peroxynitrite, the biological activity of NO may be determined by the availability of superoxide. This superoxide-mediated quenching of NO-dependent action appears to play a physiological role in the renal microvasculature. NO modulates the renal vasoconstriction caused by agonists such as angiotensin II (Ang II), thromboxane A_2 (TXA_2), and endothelin 1 (ET-1) [91]. Many studies indicate that these agents stimulate NO production, which then act to buffer vasoconstriction. Some observations indicate that the tonic influence of NO in the renal microvasculature is suppressed and contributes to the endothelial dysfunction in T1DM [62]. Because superoxide rapidly scavenges NO, one possible explanation for the lack of NO influence under basal

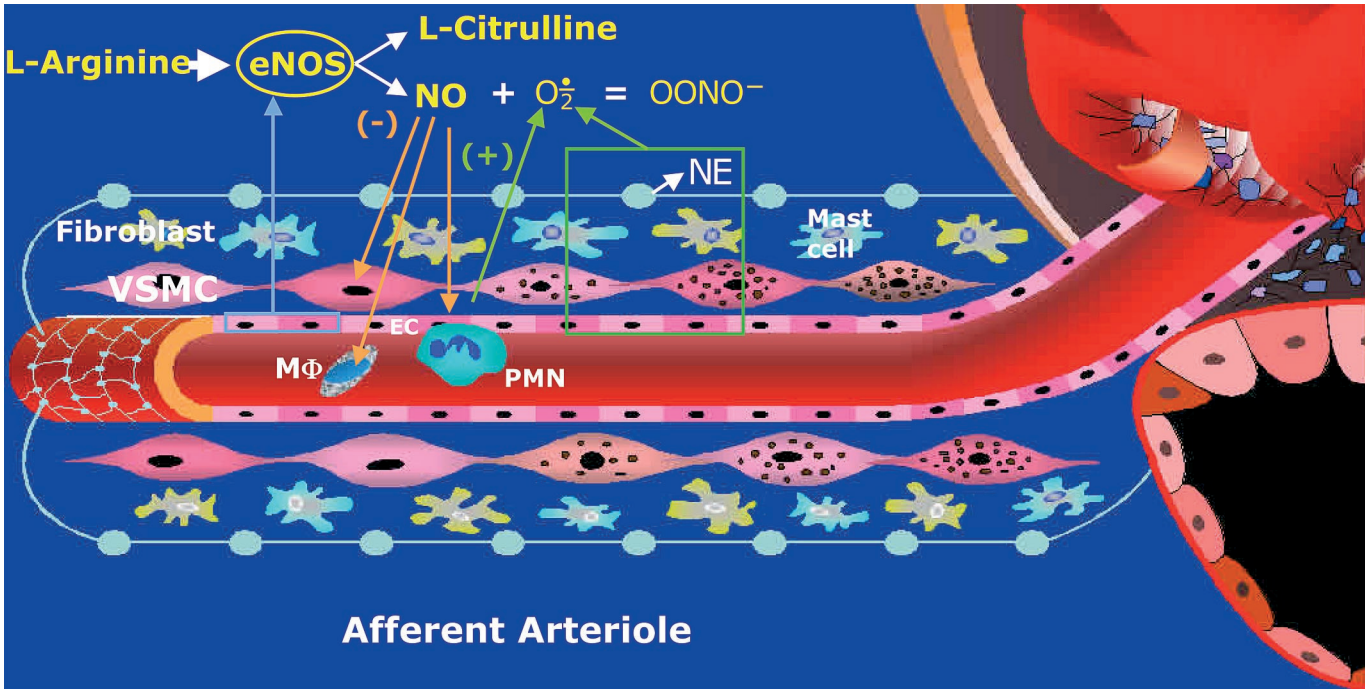

Fig. **2** ROS reduce the biological effects of NO. The potent vasodilator molecule, nitric oxide (NO), is produced by constitutively expressed endothelial cell (EC) nitric oxide synthase (eNOS) from l-arginine via a 5 electron redox reaction [cell source illustrated by blue box and arrows]. NO also inhibits vascular smooth muscle cell (VSMC) proliferation and migration, mononuclear (MF) and polymorphonuclear (PMN) leukocyte adhesion molecule expression [illustrated by orange arrows], and platelet aggregation. Injured vascular smooth muscle cells or endothelial cells, and activated vascular wall mast cells, fibroblasts, macrophages, and leukocytes, as well as oxidation of norepinephrine (NE) from renal sympathetic nerves, produce increased amounts of reactive oxygen species (ROS) [illustrated by green box and arrows] that then interact with NO to form the potent cytotoxic peroxynitrite radical (OONO-). This radical interacts with proteins in the kidney that are important for normal glomerular and tubular function to reduce their activity. Modified from Hypertension Online (www.hypertensiononline.org). Baylor College of Medicine. Copyright © 2001.

conditions in the diabetic renal microvasculature is excessive superoxide.

Indeed, renal cortical tissue from diabetic rats has increased superoxide production [92]. Some investigators [93] have demonstrated that the afferent and efferent arteriolar vasoconstrictor response to the NOS inhibitor N-nitro-L-arginine (L-NNA) is impaired in juxtamedullary nephrons of STZ-diabetic rats. Treatment with SOD restored the vasoconstrictor response to L-NNA. Similarly, vasodilatory responses of isolated renal arteries to SOD in STZ-diabetic rats were greater than in control rats [67]. These studies indicate that increased superoxide reduces modulation of basal tone through NO in renal microvessels in diabetes.

In addition to an impaired basal NO influence, the stimulation of NO-dependent vasodilation by a number of agonists is also impaired in diabetic kidneys [94] and may be due to elevations in oxygen radicals. Since superoxide limits the buffering capability of NO during agonist-induced vasoconstriction in the renal cortical microcirculation under normal conditions, it seems possible that the buffering capability of NO during agonist-induced vasoconstriction is decreased under conditions of oxidative stress such as diabetes mellitus (Fig. **3**). Indeed, Schoonmaker et al. [94] showed that the renal afferent arteriolar responsiveness to Ang II was enhanced in juxtamedullary nephrons from diabetic rats and that L-NNA did not alter the response. However, treatment with SOD restored the ability of L-NNA to enhance the vascular response to Ang II. These data suggest that excess superoxide is responsible for the lack of NO buffering of Ang II-induced vasoconstriction of afferent arterioles in diabetes.

In addition to the responsibility of ROS, some evidence exists that AGEs scavenge NO in the diabetic kidney [37]. These findings may explain the observed deficiency of angiogenic responses that require basal NO production at interstitial fibrosis sites. In addition, this functional NO deficiency may complement other factors in stimulating proliferation of fibroblasts and accumulation of the extracellular matrix, both of which are important contributors to the progression of diabetic nephropathy [95].

In these reduced NO bioavailability conditions, a series of functional and structural alterations occur in the diabetic kidney which may be mimicked by experiments of suppression of NO synthesis. In fact, chronic suppression of NO synthesis (2 months) in otherwise normal rats results in a sustained elevation of systemic and glomerular capillary pressure, proteinuria and glomerular sclerotic injury [96] as well as aggravation of glomerular injury in rats with subtotal nephrectomy [97]. Moreover, glomerular injury is accelerated by NO synthesis blockade in diabetic rats. L-NAME treatment significantly increased glomerular size and glomerular staining for TGFβ in diabetic rats, but not in controls [98]. Conversely, it has been demonstrated that NO, generated either exogenously by the NO donor SNAP or endogenously following exposure of mesangial cells to interleukin 1β (IL1β), suppresses bioactive transforming growth factor β (TGFβ) in mesangial cells cultured in 5.6 mM glucose, while suppressing or abolishing

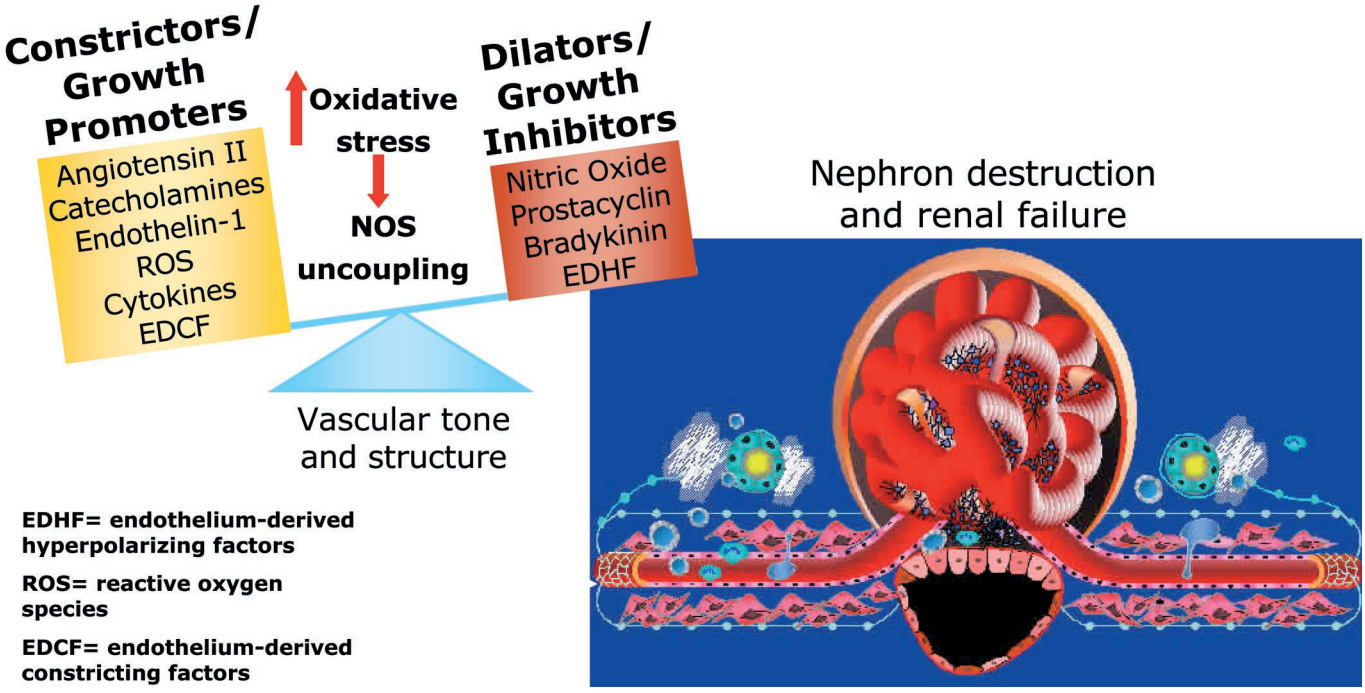

Fig. **3** Imbalance in factors affecting vascular tone and structure in diabetic nephropathy. Diabetes mellitus is associated with an altered balance in the elaboration or biological action of vasodilator and vasoconstrictor molecules. The production of vasodilators like prostacyclin and nitric oxide is diminished in diabetes while the production of catecholamines, reactive oxygen species (ROS), angiotensin II, endothelin-1 and other endothelium-derived constricting factors is either maintained or increased. Modified from Hypertension Online (www.hypertensiononline.org). Baylor College of Medicine. Copyright © 2001.

increases in TGFβ1 mRNA, TGFβ bioactivity and collagen synthesis induced by high glucose or phorbol 12,13-dubutyrate, through a mechanism involving PKC inhibition by NO [99].

All these studies support a protective role for NO in the glomeruli, a role progressively impaired in the natural history of diabetic nephropathy.

Summarizing, NO may exert both destructive and protective effects in diabetic nephropathy. Destructive roles include mediation of hyperfiltration and induction of tissue injury by generation of peroxynitrite and hydroxyl radical. Protective effects exhibited by NO include enhancement of capillary blood flow and antagonism of Ang II action [100]; inhibition of platelet aggregation [9]; suppression of mesangial cell proliferation and endothelial permeability to albumin [101]; inhibition of collagen I and III deposition [102]; reduction of oxidant stress in endothelial cells by inhibiting flow-induced O_2 production [17], reduction of oxidant stress in neutrophils by inhibiting NADPH oxidase activity [103], direct inhibition of LDL oxidation [104], activation of matrix metalloproteinases [102], suppression of PDGFB [105] and plasminogen activator inhibitor (PAI-1) expression [106], and suppression of TGFβ and matrix protein synthesis in glomerular mesangial and endothelial cells [99].

Therefore, eNOS/NO deficiency should lead to the impaired balance between the matrix deposition and degradation, result in activation of TGFβ and connective tissue growth factor, promote proatherosclerotic changes in the vascular wall, accelerate formation of AGEs, impair angiogenic remodeling of the vascular bed to the ischemic tissues, and interfere with insulin secretion and glucose utilization by skeletal muscles (both NO-dependent processes). All these sequelae of eNOS/NO deficiency have clear-cut relevance to the progression of diabetic nephropathy.

Is genetics involved in the altered NOS/NO system in diabetic nephropathy?

Despite maintenance of good metabolic control, 10–15% of patients develop diabetic nephropathy after twenty years of diabetes; conversely, several patients with good metabolic control develop complications, suggesting that additional factors may contribute to the risk of developing diabetic nephropathy [56]. The important role of NO in the regulation of the hemodynamic and metabolic milieu in the kidney suggests that an abnormal eNOS activity due to a genetic mutation could be implicated and may therefore aggravate renovascular injury in diabetic patients. Based on this rationale, a cross-sectional study investigated the association between ecNOS polymorphism with diabetic nephropathy and retinopathy in Japanese type 2 diabetic patients [107]. Analysis revealed that type 2 diabetic patients with nephropathy (not with microalbuminuria) were significantly different from type 2 diabetic patients without nephropathy and healthy control subjects in genotype distribution and frequency of the ecNOS4a allele, implying that this allele may be one of the risk factors for diabetic nephropathy. Although microalbuminuria is regarded as a risk factor for diabetic nephropathy, no association was found between the eNOS gene polymorphism and microalbuminuria in type 2 diabetic patients. Therefore, it is possible that this polymorphism could be an aggravating factor rather than an initiating factor for diabetic nephropathy.

The susceptibility to diabetic retinopathy was not linked to eNOS gene polymorphism. In this regard, a study performed on a Danish population with T1DM has showed a significant association between a 14-A allele of iNOS and low risk for diabetic nephropathy, but not retinopathy [108]. Since there are marked differences in temporal patterns of occurrence and incidence rates of diabetic retinopathy and nephropathy, it is possible to hypothesize that these two diabetic complications may evolve as two distinct processes influenced in different ways by some genetic or metabolic determinants.

Evidence for a possible relation of eNOS gene polymorphism and impaired function of eNOS was also revealed by a recent study of NO metabolite (NOx) levels in patients with different genotypes of the eNOS gene [109]. However, no significant association was shown between the angiotensin converting enzyme (ACE) or the eNOS gene polymorphism and intrarenal hemodynamic abnormalities (measured through resistive index) in patients with type 2 diabetes [110].

The eNOS gene is located on chromosome 7q35 [111]. Interestingly, the gene coding for aldose reductase [112], a candidate gene for genetic susceptibility to diabetic nephropathy in type 1 diabetic patients [113], and the gene of a muscarinic ACh receptor [108], which may also have influence on the vascular tone [114], have been localized on the same chromosomal region as the eNOS gene, so this may be a candidate region for genetic susceptibility to diabetic microvascular complications.

Diabetic Retinopathy

The two fundamental abnormalities in diabetic retinopathy are increased retinal vascular permeability and progressive retinal vessel closure, which leads to tissue hypoxia and ischemia and neovascularization of the retina [115,116].

NO is a well-known potent vasodilator and has a role in ischemic processes [117]. Because factors such as ischemia and hypoxia induce iNOS, the microenvironmental changes in diabetic retinas may establish sustained and high NO production.

The neuronal NOS in the eye is thought to be responsible for producing NO in photoreceptors and bipolar cells [118], whereas eNOS is present in vascular endothelial cells [119]. However, iNOS, which is found in Muller cells and in retinal pigment epithelium, may be involved in phagocytosis of the photoreceptor outer segment [120], infectious [121], inflammatory [122] and ischemic processes, and in the pathogenesis of diabetic retinopathy [117].

A recent report has documented increased NOS activity in the retinas of diabetic rats compared to normal rats. Using immunohistochemical technique, it has been shown that expression of the inducible isoform of NOS is enhanced in the retina of subjects with diabetes mellitus [123]. These results agree with previous reports showing that levels of L-arginine-NO pathway-related metabolites are elevated in the aqueous humor of diabetic patients [124] and that vitreous levels of nitrite, the stable product of NO, are elevated in patients with proliferative diabetic retinopathy [125].

Several lines of evidence suggest that overproduction of NO into the retina directly or indirectly induces oxidative damage, ischemia and neoangiogenesis, suggesting its important pathogenic role in the development, and especially in the severity of diabetic retinopathy; *in vitro* as *in vivo*, it has been demonstrated that an overproduction of NO may induce oxidative stress in retinal cells. The loss of pericytes and the increased blood flow in the retinal beds that precede the development of diabetic retinopathy are in part due to overproduction of NO [126,127]; the risk of severe retinopathy has been associated with the presence of oxidative stress markers in human diabetes [128].

Elevated vitreous levels of cytokines and inflammatory mediators such as IL-1, tumor necrosis factor, and IFNγ have been reported to induce expression of the iNOS isoform in retinal pigment epithelium and Muller cells [129]. Expression of iNOS in response to cytokines is part of the inflammatory response and contributes to vasodilation, vascular leakage leading to tissue damage, migration and proliferation of endothelial cells, suggesting its possible role in the regulation of angiogenesis [130]. NO thus formed may also act as an angiogenic mediator by stimulating the growth-promoting effect of vasodilator peptides including VEGF, a molecule strongly implicated in the development of proliferative retinopathy [131]. Intravenous infusion of VEGF may acutely impair functional endothelial cell barrier integrity and relax resistance arterioles in ocular tissues through a mechanism involving activation of eNOS [132]. VEGF-induced angiogenesis and vascular permeability was abolished in eNOS knockout mice [133,134]; in a similar model of diabetic retinopathy, eNOS knockout mice and mice treated with L-NNA, a NOS inhibitor, showed an important reduction in vaso-obliteration and vitreous neovascularization [135]. In support of these findings, inhibition of iNOS expression, using iNOS knockout mice and the iNOS inhibitor 1400W, was shown to inhibit angiogenesis locally in the avascular retina. This phenomenon was mediated at least in part by downregulation of VEGF receptor; furthermore, pathological intravitreal neovascularization is considerably stronger in iNOS-expressing animals [136]. On the other hand, NO and VEGF increases without any interrelationship have been shown in the vitreous fluid of diabetic patients with proliferative diabetic retinopathy [137]. These results suggest that serum diffusion could play a significant role in explaining the increase of NO, whereas intraocular production seems to be the main factor responsible for the intravitreous enhancement of VEGF.

Atrophy in the inner nuclear layer and the ganglion cell layer in diabetic retinopathy has been described [138]. These changes appear clinically as progressive loss in visual function independent of the occurrence of neovascularization [139]. NO is known to influence apoptosis in a variety of models, and its effects can be proapoptotic or antiapoptotic [140].

Using the iNOS knock-out mice and a iNOS inhibitor, iNOS expression has been demonstrated *in vivo* to induce apoptosis locally in the inner nuclear layer of the avascular retina in ischemic proliferative retinopathy, and protein nitration may be involved

in this process [136]. This demonstrates that iNOS not only plays a crucial role in the retinal neovascular disease but also in retinal degeneration.

The mechanism involved in NO-induced apoptosis, nitrosative stress leading to protein nitration, is thought to be one of the major mechanisms responsible for NO-mediated neurotoxicity. The nitration of proteins may lead to the loss of functions of proteins as well as particular mitochondrial and antioxidant proteins [141 – 143], which can eventually lead to mitochondrial respiratory chain impairment resulting in apoptosis or necrosis depending on the energy status of the cell [144].

Summarizing, the consequences of increased levels of NO in retinas from subjects with diabetes could be twofold: neurotoxicity and angiogenesis. NO can be beneficial in its role as a vasodilator, but high concentrations of NO produced by iNOS are neurotoxic [145]. The toxicity of NO has been attributed to various mechanisms including DNA damage, peroxynitrate-mediated oxidative damage, and energy failure [146]. Moreover, NO produced by iNOS increases cyclooxygenase-2 activity after cerebral ischemia and increases the production of ROS and toxic prostanoid [147].

Chronic hyperglycemia is thought to be the initiating factor in the development of diabetic retinopathy in most patients of long duration. However, it is insufficient to produce severe disease in the majority of them, thus suggesting a potential additional role of genetic factors [148]. This hypothesis was embodied by a subanalysis of the Diabetes Control and Complications Trial (DCCT), which showed a strong familiar retinopathy transmission in patients with severe diabetic retinopathy, but not in those with non-severe disease [56]. Moreover, a 14-A allele of a CCTTT-repeat polymorphism of iNOS was associated with low risk of diabetic retinopathy in a mixed Type 1 and Type 2 diabetic Anglo-Saxon population [149]. More recently, another study on a Danish population with Type 1 diabetes has shown a significant association between a 14-A allele of iNOS and low risk for diabetic nephropathy, but not retinopathy [108]. To investigate the association between eNOS4 polymorphism and risk of severe diabetic retinopathy, a case control study was performed in a Caucasian population with T1DM, demonstrating that eNOS4a/a homozygous deletion and its 4a allele are associated with non-severe diabetic retinopathy (absent or background), and that eNOS4b/b and its 4b allele are associated with severe diabetic retinopathy (pre-proliferative or proliferative) [150]. Since there is evidence that plasma NO metabolite levels of subjects with the eNOS4a genotype are significantly lower when compared with those of individuals eNOS4b [151], it may be hypothesized that the first were not affected by severe diabetic retinopathy due to a reduction in NO. Similarly, in the Asian Indian population, allele 210 bp of the iNOS gene is a high-risk allele for developing retinopathy, and alleles 200 and 220 bp protect an individual from developing retinopathy [152]. Therefore, these reports strongly support the hypothesis that NO production may be a capital and permissive factor for the progression and severity of this complication.

In light of this considerable body of evidence, several experimental studies have shown the therapeutic value of downmodulating NO expression in different nosological entities including diabetic retinopathy, thus allowing recent molecular knowledge to translate into potential therapeutic progress [153 – 155]. In this regard, it has been reported that aminoguanidine treatment inhibits the development of diabetic retinopathy in rats [156]. Aminoguanidine selectively inhibits the cytokine inducible isoform of NOS, which appears to be responsible for the excess production of NO linked to disease status [157]. Furthermore, aminoguanidine has proven to ameliorate diabetes-induced vascular dysfunction in retinal and uveal vessels.

Macroangiopathy

Myocardial infarction and stroke constitute major causes of death in patients with diabetes mellitus. Established risk factors for coronary artery disease do not, however, fully explain the increased risk of diabetic patients [158]. Several human studies have shown that traditional risk factors for atherosclerosis predispose toward ECD. Impaired endothelium-dependent vasodilation in the coronary circulation of humans has profound prognostic implications in that it predicts adverse cardiovascular events and long-term outcome [159].

As the interface between circulation and the vascular wall, the endothelium interacts with both cellular and hormonal mediators from these two sources. In the presence of diabetes, a number of factors including hyperglycemia and oxidative stress contribute to impairment of the endothelium through mechanisms which ultimately affect NO bioavailability. Some observations show that long-term hyperglycemia by the formation of AGE, but also short-term hyperglycemic periods ("glucose spikes") damage cardiac endothelium in diabetes through a mechanism involving NO [160]. In addition to the above mechanism of NO-quenching, a novel mechanism has recently been described to explain hyperglycemia-mediated impairment of NO availability in coronary circulation. In fact, hyperglycemia, acting at least in part through the hexosamine pathway, may selectively impair the metabolic branch of insulin signaling that involves the phosphatidylinositol 3-kinase (PI3K)/Akt/eNOS pathway in human coronary endothelial cells, thus compromising insulin-dependent endothelial NOS activation [161].

In human aortic endothelial cells, prolonged exposure to high glucose concentrations increases eNOS gene expression, protein expression, NO_2 release and a marked concomitant production of superoxide [41]. The simultaneous generation of NO and superoxide anions enables the reaction of both species to form peroxynitrite which has been identified as an important mediator for the transformation of endothelium from an anticoagulant to a procoagulant state [162]. In addition, oxidized LDL *per se* downregulate endothelial NOS in human coronary cells [163]. Together with a functional loss of endothelium, these processes are assumed to impair the coronary perfusion and to provoke adaptive processes that finally lead to cardiac dysfunction and cardiac structure remodeling. In fact, mice lacking the endothelial-type NOS gene exhibit hypertension and enhanced vascular remodeling in response to injury [164,165]. The association of hypertension and diabetes mellitus mimics the alterations induced by L-NAME in rats, which suggests a role for NO in the pathophysiology of hypertensive–diabetic cardiomyopathy [166].

The contributions of NO in endothelium-dependent vasodilation may differ between large epicardial vessels and microvessels. Pretreatment with L-NAME blunts ACh-induced vasodilatation [167,168], indicating that ACh-induced endothelial vasodilatation is largely mediated by NO. Diabetic rats show impaired ACh-induced aortic relaxation and nitrate/nitrite production attributed to an abnormal metabolism of NO [169]. In contrast, ACh-induced endothelial dilation of the microcirculation seems to be only partly mediated by NO [167,168].

To investigate the involvement of a dysfunctional NOS/NO system in the pathogenesis of diabetic angiopathy further, one study evaluated urinary and serum nitrate/nitrite, lipid peroxidation, endothelial and *in vivo* platelet activation markers in type 1 and type 2 diabetic patients [170]. NO production was reduced in diabetes, and this reduction correlated with endothelial damage markers. In T1DM, the decreased nitrate/nitrite excretion was strongly associated with elevated plasmatic beta-thromboglobulin levels, suggesting that NO reduction may contribute to increased *in vivo* platelet activation in the setting of diabetes mellitus.

Importantly, improved endothelial function is the clinical marker of atherogenic risk factor modification. For example, ECD is improved by therapy with cholesterol-lowering statins [171]. It has been demonstrated that very short-term treatment with statins (3 days) with no effect on serum lipids improved endothelial function by increasing NO activity in patients with diabetes [172,173].

Several lines of evidence in clinical settings including diabetes have shown that endothelial dysfunction of the coronary microcirculation alone can cause or promote myocardial ischemia, even in the absence of flow-limiting stenosis of the large epicardial arteries, and may play a crucial role in acute coronary syndromes such as unstable angina pectoris or myocardial infarction [174], which are characterized by plaque rupture or erosion followed by platelet aggregation and local thrombus formation [174]. As previously stated, NO displays antiaggregant properties; in particular, the insulin-induced platelet anti-aggregating effect is NO-mediated through a mechanism involving both cGMP and cAMP in human platelets [175]. Hence, it is likely that insulin deficiency or resistance coupled with NO decreased availability in the setting of diabetes would play a crucial role in modulating these events, eventually triggering plaque rupture.

Endothelium-derived NO is now recognized as a well-established anti-inflammatory and anti-atherosclerotic molecule. A deficiency of endothelium-derived NO in atherosclerotic vessels might cause vasoconstriction, platelet aggregation, thrombus formation, increased VSMC proliferation, and enhanced leukocyte adhesion/invasion to the endothelium, all of which are pathophysiological and pathobiological features observed during the course of atherosclerotic and vascular disease [176,177].

In this regard, a considerable body of evidence *in vitro* and *in vivo* supports the hypothesis that insulin exerts anti-inflammatory actions through the stimulation of NO synthesis [178]. Indeed, it has been demonstrated that insulin at physiologically relevant concentrations inhibits the expression of intracellular adhesion molecule-1 (ICAM-1) [179] and monocyte chemoattractant protein-1 (MCP-1) [180], two major proinflammatory mediators, by HAECs and the expression of proinflammatory mediator nuclear factor (NF-kappa B) in the nucleus in parallel with an increase in eNOS expression [180]. *In vivo* studies have demonstrated that insulin and the insulin-sensitizer troglitazone inhibit NF-kB in obese subjects [181,182]. In addition, TNFα, a proinflammatory cytokine increased in insulin-resistant states, inhibits insulin-induced increase in eNOS and reduces insulin receptor content and phosphorylation in HAECs [183]. Interestingly, thiazolidinediones reduce plasma TNFα concentrations.

Taken together, these observations suggest that in the presence of insulin resistance, a series of mediators including TNFα may antagonize the vasodilatory and anti-inflammatory action of insulin at adipocyte and endothelial cell levels through mechanisms involving NO metabolism.

Studies on the pharmacological inhibition of NO synthesis in healthy adult rats have improved our insight into the mechanisms of development of postnatal vascular disease, while suggesting a possible scenario in which NO mediates an inflammatory cascade leading to accelerated atherosclerosis (Fig. **4**). Long-term administration of L-NAME to healthy adult rats markedly reduced endothelial NO [184–186]. In the early stages (day 3–7) of long-term NO synthesis inhibition, there are marked inflammatory changes (adhesion and infiltration of monocytes) as well as proliferative changes (appearance of proliferating cell nuclear antigen-positive cells) in the blood vessels, especially the coronary arteries [187]. Simultaneously, there is an increase in tissue ACE and Ang type I receptor, resulting in an increase in Ang II activity [184,186]; increased gene and protein expression of chemokines such as MCP-1 [187], and cytokines such as TGFβ, tissue factor [188,189]; increased expression of matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9), which might contribute to the development of macrovascular disease [190]; and increased production of superoxide anions responsible for oxidative stress, and increased activities of transcription factors (NF-kB and AP-1) [191,192]. All of these pathophysiological changes are probably the result of NO synthesis inhibition. In the late stage (week 4–8), structural changes in the cardiovascular system such as arteriosclerosis (medial thickening and fibrosis), cardiac hypertrophy and fibrosis, renal damage, and stroke [184–186] have been observed. These findings suggest that NO synthesis inhibition actually causes functional and structural vascular abnormalities and support the hypothesis that endothelium-derived NO plays a central role in the maintenance of normal homeostasis in the blood vessel wall. The molecular and cellular mechanisms of vascular lesion formation induced by long-term NO synthesis inhibition have been further investigated; ACE inhibition or Ang II type 1 receptor blockade have been reported to prevent early vascular inflammation and later arteriosclerosis [185,186,191], indicating that increased local activity of Ang II plays a central role in the development of pathological changes. Anti-oxidant therapy [191] or NF-kB blockade [192] prevents, and MCP-1 blockade partly reduces vascular lesion formation [193], while TGFβ1 blockade [188,194] prevents vascular fibrosis. Taken together, these observations suggest that oxidative stress and subsequent activation of NF-kB mediate vascular inflammation through the expression of MCP-1, whereas vascular fibrosis

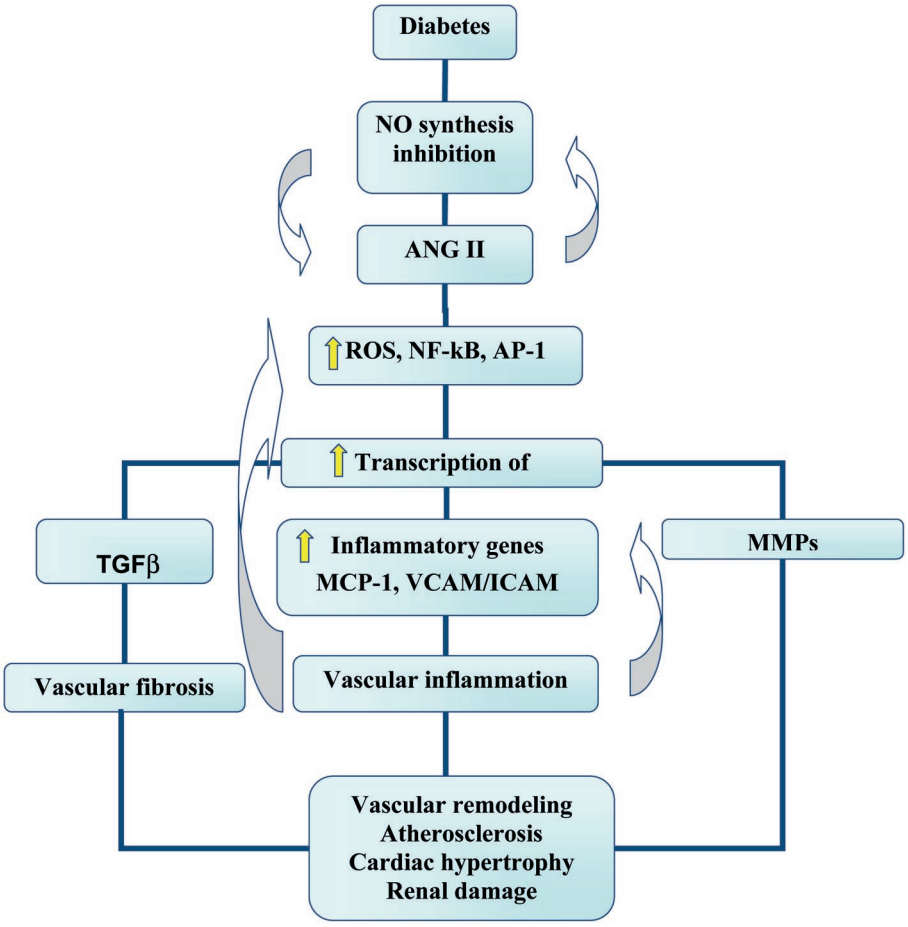

Fig. **4** Mechanism of vascular inflammation and subsequent arteriosclerosis induced by long-term nitric oxide synthesis inhibition. ROS, reactive oxygen species; MCP-1, monocyte chemoattractant protein-1; VCAM, vascular cell adhesion molecule-1; ICAM, intracellular adhesion molecule-1; MMPs, matrix metalloproteinases, TGFβ, transforming growth factor β.

is mediated independently by increased activity of TGFβ1. These data could be the basis for an *in vivo* model of how endothelium-derived NO has anti-inflammatory and anti-arteriosclerotic properties in the vascular endothelium. From the clinical point of view, therapies such as statins and ACE inhibitors that decrease the progression of arteriosclerosis and its complications all improve ECD in patients with risk factors.

Conclusions

The hypothesis presented here attributes the clinical manifestations and pathophysiological mechanisms in diabetic angiopathy to the development of endothelial cell dysfunction. The pivotal function of endothelial cells perturbed is eNOS/NO production or availability (Fig. **5**). Diabetes triggers mechanisms that simultaneously enhance and suppress NO bioavailability. These factors include hyperglycemia *per se* or through the accumulation of AGEs, relative hyperinsulinemia, increased expression of growth factors and cytokines, and increased oxidative stress, leading to NOS uncoupling and NO-quenching by excess superoxide and peroxynitrite, and involvement of the genetic background of each individual. It can be speculated with particular reference to nephropathy that the balance between these two opposing mechanisms is shifted towards NO overproduction during the early phases of disease, which in turn accounts for the hemodynamic abnormalities characterizing early diabetic nephropathy. As the duration of exposure to the diabetic milieu increases, factors suppressing NO availability gradually prevail, thus giving rise to the structural alterations leading to glomerulosclerosis, tubulointerstitial fibrosis and progressive decline in GFR.

On the other hand, in diabetic retinas the microenvironmental changes, characterized by ischemia and hypoxia, may establish sustained and high NO production**.** The main consequence of increased levels of NO in retinas from subjects with diabetes are neurotoxicity due to retinal cell apoptosis and angiogenesis.

NO is now considered as an anti-inflammatory and anti-atherosclerotic molecule. A deficiency of endothelium-derived NO in atherosclerotic vessels might cause a derangement of the physiological properties of NO in the maintenance of endothelial homeostasis, thus inducing vasoconstriction, platelet aggregation, thrombus formation, increased VSMC proliferation, and enhanced leukocyte adhesion/invasion to the endothelium, thus paving the way for accelerated atherosclerosis and generalized macroangiopathy.

Therefore, intervention studies targeting any component of the NOS/NO system cascade may help in further establishing a cause-and-effect relationship between NO dysfunction and diabetic angiopathy, and may prove potential therapeutic tools in the prevention of long-term diabetic complications.

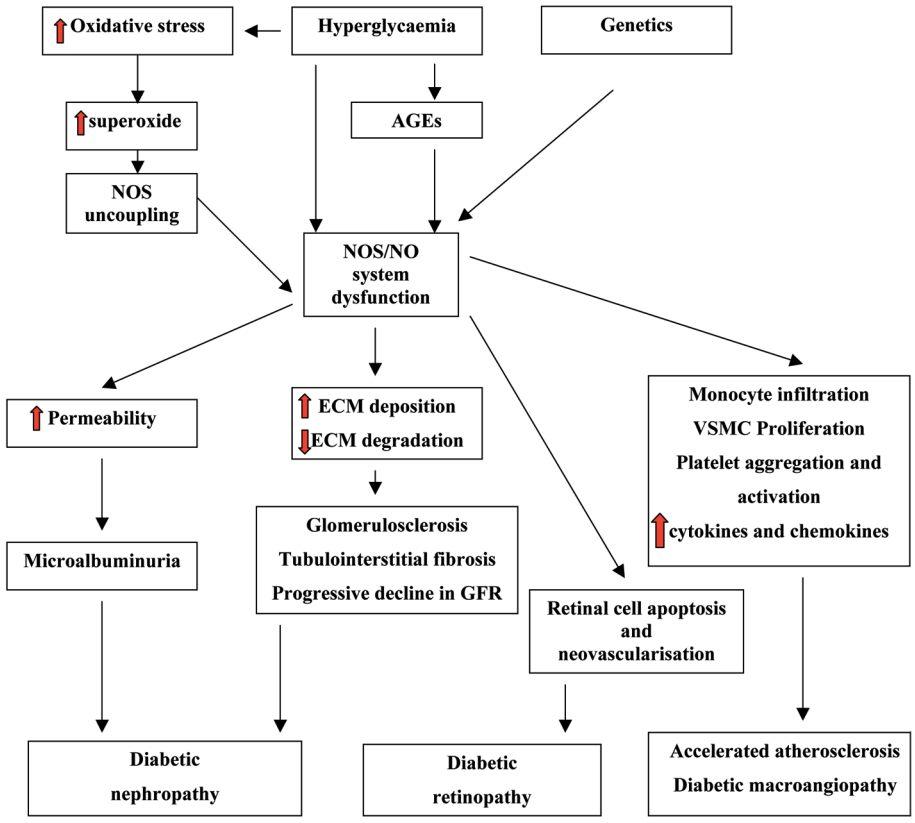

Fig. **5** Pivotal role of nitric oxide in the development of diabetic angiopathy.

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