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Endothelin, Nitric Oxide, and Reactive Oxygen Species in Diabetic Kidney Disease

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Abstract

Mechanism(s) of the endothelin (ET) and reactive oxygen species (ROS) pathways in conjunction with the nitric oxide (NO) pathway that promote and/or blunt the progression of diabetic kidney disease have been the focus of many laboratories to reveal new therapeutic targets. In both animal models and patients with diabetic nephropathy, pharmacological blockade of ET receptors results in a significant reduction. However, edema has been documented as a persistent side effect. It is unclear whether selective ET_A antagonists or non-selective ET_{A/B} antagonists are preferred in diabetic conditions. We have proposed that ET_B activates the NO pathway to blunt the diabetes-induced nephropathy such that ET_A selectivity should be more efficacious. The NO pathway in diabetes facilitates vascular dysfunction while in the renal tubular system, NO serves to blunt disease progression. NO synthase isoform activity is also critically regulated in diabetic kidney disease within the renal vascular and tubular systems through a complex interaction with reactive oxygen species. We will examine the complexities of the ET and NO pathways in diabetic kidney disease to propose novel mechanisms for future investigation.

Several laboratories are beginning to dissect the mechanism(s) of the endothelin (ET) and nitric oxide (NO) pathways that promote and/or blunt the progression of diabetic kidney disease. We have organized the first part of this chapter to describe our understanding of the ET pathway in diabetic kidney disease followed by a discussion focusing on the NO pathway and how it interacts with the ET system. We conclude with a perspective on possible future directions that could further elucidate novel mechanisms and targets for diabetic kidney disease.

Activation of the ET pathway in diabetic kidney disease

Brownlee and coworkers [1] have proposed a “unifying hypothesis” that mitochondrial-derived superoxide (O₂^{•-}) promotes production of reactive oxygen species (ROS) from the other major pathways and is the critical component in diabetes-induced oxidative stress. Glucose is a fuel for the mitochondrial respiratory chain and oxidative phosphorylation and/or cytosolic glycolysis activating cellular oxidases and ROS. Many investigators have postulated that local intracellular sources of oxidative stress are critical in the development of diabetic nephropathy. Asaba et al [2] have recently reported that apocynin (antioxidant and/or NADPH oxidase inhibitor) reduces advanced oxidation protein product-induced perturbations in inflammatory measures, renal NADPH oxidase activity, and gp91phox expression in the diabetic kidney. Studies using a variety of cell types in culture have established that ROS, in particular H₂O₂ and O₂^{•-}, are important stimulators of ET-1 production [3, 4]. ET-1 can act in an autocrine or paracrine fashion, and urinary ET-1 reflects renal ET-1 production [5]. Our group has published that urinary ET-1 maximally increases within 2 weeks of induction of diabetes in the rat and remained elevated throughout the course of the experiment to 10 weeks post-induction [6]. Urinary measures of oxidative stress were also increased in a similar time course suggesting an interaction in these pathways. [6] We initiated a study to determine whether the enhanced diabetes-

induced oxidative stress stimulated renal ET-1 production. In vivo tempol (free radical scavenger) treatment of diabetic rats significantly blunted urinary ET-1 excretion as well as inner medullary mRNA ET-1 expression [7]. Tempol treatment also blocked the ROS production in renal tissues from the diabetic rats compared to non-treated diabetic rats. Thus, these in vivo data in concert with the in vitro data demonstrate that diabetes-induced ROS activate renal ET-1 production.

Several studies have also shown that ET_A receptors activate NADPH oxidase and other pathways leading to oxidative stress in the vasculature, mesangial cells, and renal tubules in a variety of non-diabetic renal diseases. ET_B receptors have been shown to also activate NADPH oxidase in neuronal cells and stimulate ROS production in models of hypertension. Sasser et al [6] demonstrated that indeed renal NADPH oxidase expression as well as plasma and urinary measures of oxidative stress were increased in the STZ-induced model of diabetes compared to the sham rat, although ET_A receptor antagonism did not ameliorate the oxidative stress nor the increased urinary ET-1 excretion. Moreover, ET_A receptor antagonism significantly reduced the diabetes-induced albuminuria [6], thus demonstrating that diabetes-induced ET_A receptor activation promotes renal injury but not via oxidative stress.

Aberrant inflammatory responses in the kidney, such as infiltration of macrophages and the associated pathways have been discovered early in diabetes as well as in established diabetic nephropathy in both human and animal models and, subsequently, linked to glomerular and tubular damage as well as renal fibrosis [8-11]. Although these reports support the concept that inflammatory processes promote and/or perpetuate the progression of diabetic nephropathy, the mechanisms involved in diabetes-induced renal inflammation are not well understood. Is renal inflammation during diabetes a response to hyperglycemia or to other metabolic factor(s) that could play a role in driving this process? We sought to address this question by hypothesizing that the diabetes-induced activation of the ET pathway stimulates inflammation. We observed that blockade of the ET_A pathway in the STZ-induced model of diabetes disrupts the infiltration of macrophages and T cells in the renal cortex and medulla, and suppresses accumulation of pro-inflammatory mediators, especially MCP-1 and TGF- β [6]. MCP-1 expression increases at the site of tissue injury and functions to direct macrophage recruitment to the site [12]. Mechanistically, MCP-1 binds to the inducible CCR2 to promote chemotaxis to a specific area [13]. CCR2 regulates monocyte and macrophage recruitment and is necessary for macrophage-dependent inflammatory responses [14]. While there are a number of studies showing that ET-1 increases the expression of inflammatory molecules *in vitro*, we have recently obtained *in vivo* data indicating that ET-1 increases plasma MCP-1 and infiltration of macrophages and T cells in the kidney in the absence of hypertension [15]. Therefore, these data indicate that diabetes-induced ROS activates renal ET production and ET_A receptor mediated increased inflammation and albuminuria.

Activation of NO pathway in diabetic kidney disease

All three NO synthase (NOS) isoforms are expressed in the kidney under physiological and diabetic conditions. NOS1 (neuronal NOS or nNOS) was first described in the brain, and is expressed in the macula densa, renal tubules, arteriolar smooth muscle, and vasa recta. NOS2 (inducible NOS or iNOS) was initially found in inflammatory cells under conditions of cytokine induction. However, NOS2 is constitutively expressed in the mTAL as well as being induced in the rat STZ model of diabetes [16]. NOS3 (endothelial NOS or eNOS) was first described in the endothelium of the aorta, and is expressed in the endothelium of renal arteries and arterioles as well as renal tubules.

Most studies exploring the impact of diabetes on renal NOS activity have centered on the hypothesis that elevated NOS activity contributes to renal hyperfiltration or the eventual development of diabetic nephropathy with a focus only on the vasculature [17, 18]. Indeed STZ-induced diabetes in NOS3 knockout mice have exacerbated renal injury [19], although it is unknown whether this is a consequence of the elevated blood pressure and/or insulin resistance. Shankar et al [20] have also shown that NOS3 knockout mice are insulin resistant at the level of the liver and peripheral tissues, whereas the NOS1 knockout mice are insulin resistant only in peripheral tissues. It has been reported that insulin therapy reversed the renal functional and histological changes due to diabetes in the NOS knockout mice leading to the proposal that endothelial dysfunction is a surrogate marker for nephropathy [21]. As discussed below, this may be an overly simplistic view of NOS in diabetic kidney disease.

The diabetic kidney has high levels of $O_2^{\bullet-}$ leading to an overall reduced NO bioavailability. Strong evidence indicates that the reaction of $O_2^{\bullet-}$ with NO during diabetes has dual consequences: decreased NO bioavailability and generation of peroxynitrite ($ONOO^-$). Stadler et al [22] have recently reported that NOS2 is the major source of radical generation in the kidney of diabetic rats and implicated $ONOO^-$ as the reactive intermediate leading to lipid peroxidation observed in many models of diabetes as well as diabetic patients. Goligorsky's laboratory has shown that chronic ebselen treatment (anti-oxidant and $ONOO^-$ -scavenger) ameliorated proteinuria, inflammation, and prevented 3-nitrotyrosine modification of proteins in renal tissue from Zucker diabetic rats [23]. "NOS uncoupling" or NOS-dependent $O_2^{\bullet-}$ production represents a significant source of $O_2^{\bullet-}$ in vascular beds during hyperglycemia and is a major determinate in the endothelial dysfunction observed in diabetes [24-26]. It should be noted that in diabetes, endothelial $O_2^{\bullet-}$ production is NOS-dependent [24, 25] while mTAL $O_2^{\bullet-}$ production is NOS-independent [35]. Therefore, mechanisms regulating NOS function in the endothelium and mTAL during diabetes are vastly different. In particular, endothelial NOS uncoupling during diabetes has been attributed to the loss of NOS substrate, L-arginine, via diabetes-induced arginase activity and/or oxidation of the NOS co-factor, tetrahydrobiopterin. Very little is known about the regulation of arginase and tetrahydrobiopterin in the renal tubules especially in the mTAL during diabetes.

Recently, Palm and colleagues found that NO regulates renal blood flow and glomerular filtration rate similarly in normal and diabetic kidneys [27]. Yet, specific NOS1 inhibition increased oxygen consumption and transport efficiency only in the diabetic kidney indicating a switch to NOS1 dependency specifically in the diabetic kidney for maintaining control of tissue oxygenation. Using the STZ model of diabetes, Palm et al [27] have proposed that NOS1 activity is critical as a "brake" to the increase in transport efficiency in the diabetic kidney. Direct effects of NO on sodium transport are evident in several nephron segments [28, 29]. The nephron segment for which there exists the strongest evidence of NO regulation of NaCl reabsorption is the mTAL [29], where NO decreases net NaCl reabsorption primarily via effects on apical transport mechanisms [30, 31]. While exogenous NO can inhibit Na transport in the normal mTAL, the extent to which chronic increases in NOS activity and endogenous NO production may reduce Na transport by the mTAL is not known. Furthermore, little is known about the status of the NO system in the renal tubules during diabetes, especially those in the medulla. Our laboratory has recently begun to explore these mechanisms. Indeed, we documented increases in NOS activity and a 2.5-fold increase in mTAL-derived NO production in the STZ model of type 1 diabetes [16]. Our recent studies revealed increased NOS1 and NOS2 activity in mTALs from diabetic rats with no change in expression of either of these isoforms indicating possible post-translational enzymatic activity. NOS3 expression and activity were unchanged in the mTAL under diabetic conditions [16]. These data in concert with the findings from Palm's laboratory support the importance of the NOS1 isoform in maintaining renal tubular

function during diabetes. However, Anderson's laboratory observed that long-term inhibition of NOS1 in diabetic rats had a modest nephroprotective effect in terms of proteinuria and glomerular injury rather than being deleterious [32]. Interpretation of pharmacological studies such as these is complex given the range of NOS1 function in the body and so more research is necessary to ascertain the differences in the functional role of NOS1 in the renal vasculature and tubular systems during diabetes.

Calcineurin (PP2B; CaN) activity and expression are elevated in the renal cortex [33] and medulla of STZ rats, specifically in the mTAL apical membrane [34]. We recently published that PP2B regulates NOS activity, specifically NOS1 and NOS2, and the subsequent increased NO production by the mTAL suggested that post-translational phosphorylation most likely stimulates NOS activity during diabetes [16]. These data indicate that increased PP2B activity in the mTAL during diabetes promotes NO production. Our preliminary data [35] show that total NOS inhibition increases ouabain-sensitive oxygen consumption by mTAL suspensions from STZ diabetic rats, suggesting that endogenously produced NO actively blunts the diabetes-induced increase in Na transport. Yet, chronic inhibition with cyclosporin A (CsA) abolishes the glomerular hypertrophy and extracellular matrix accumulation in STZ rats [33]. Further research is necessary to elucidate the details of the PP2B pathway in the renal vasculature and tubular systems.

A common feature of immunopathologies, such as diabetes, is the prominent role of type 1 (both CD4⁺ and CD8⁺) T cells [36]. Reciprocal regulatory mechanisms between T cell activation and NO production have been documented [37]. Type 1 T cells characteristically produce interferon γ and other pro-inflammatory cytokines, which can induce NOS2 in susceptible cells. NO has been shown to regulate T cell induction and activation, with low concentrations (5-10 μ M) of NO enhancing type 1 T cell proliferation and cytokine production, while high concentrations (100 μ M) of NO is immune suppressive [38]. T cell cGMP levels increase in concert with the enhanced type 1 proliferation independent of antigen-presenting cells [39]. These data indicate that the NO/cGMP pathway directly and selectively activates type 1 T cells. In addition, NO converts CD4⁺CD25⁻ T cells into CD4⁺CD25⁺ T_{reg} cells *in vitro* [37]. The NO-converted T_{reg} cells are as efficient as the natural T_{reg} cells in suppressing functional immune responses. NO-converted T_{reg} cells do not express Foxp3 distinct from natural T_{reg} cells [37]. We have postulated that the NO/cGMP pathway is an additional signal for the induction of T cell subpopulations by regulating T cell activation to dampen the potentially damaging autoimmune response in the kidney.

Interactions of the ET and NO pathways in diabetic kidney disease

Several laboratories have documented the ET_A receptor mediated vasoconstrictor actions as well as endothelial dysfunction in diabetes. Loomis et al reported that both ET_A and ET_B receptors are responsible for ET-induced O₂^{•-} in isolated endothelium-intact aortic rings *ex vivo*. Furthermore, this resulted in a significantly enhanced vasoconstriction that was blunted with NADPH oxidase inhibition or supplementation with tetrahydrobiopterin to relieve NOS uncoupling. We have postulated that increased oxidative stress and ET production during diabetes leads to activation of ET receptors in the vasculature enhancing vasoconstriction and O₂^{•-} production in a positive feedback mechanism.

We have reasoned that the diabetes-induced increase in NO production and activation of the NO/cGMP pathway, at least in renal tubules, represents a potential means of relieving the burden of diabetes-induced renal complications. Thus, we have proposed that ET_B receptor actions in the kidney during diabetes are functioning to counter the actions of the ET_A receptor. Despite the availability of potent and selective receptor antagonists, results are

often contradictory and complicated because of possible vasoconstrictor actions of ET_B receptors and the autocrine and paracrine nature of ET-1 making it difficult to assess *in vivo* activity. Pfaff et al [38] reported that diabetes induced renal ET_B receptor expression, without a change in ET_A receptor expression. Furthermore, these authors have shown that the lack of ET_B receptor signaling greatly exaggerates diabetes-induced renal injury and hypertension. Future studies are necessary to differentiate the cellular and receptor sub-type dependent activation of ROS or NO in diabetes. Figure 1 depicts the interactions of the ET and NO pathways in diabetic kidney disease as described in this chapter.

The clinical utility of ET_A-selective versus dual ET_{A/B} receptor antagonists remains controversial and will most likely not be resolved by the pharmaceutical companies any time soon as they have committed themselves to one side of the argument or the other [39]. Direct side-by-side comparison studies in patients with diabetic nephropathy are needed to truly resolve the question. In a recent phase III trial, the relatively mixed ET antagonist, avosentan (ET_A/ET_B affinity ratio of approximately 200 in binding studies), was shown to reduce proteinuria by ~50% in patients with diabetic nephropathy after 3 and 6 months of therapy, even though these patients were already being treated with an angiotensin converting enzyme inhibitor or an angiotensin receptor blocker [40]. Unfortunately, the trial was ended early due to the development of edema, but new phase IIb trials have been initiated to manage this side effect. It is unclear at this point whether the development of edema is related to the relatively non-selective characteristics of avosentan or are mechanism-based on ET_A blockade. Another clinical trial is expected to investigate the impact of the non-selective ET antagonist, bosentan, on the excretion of TNF α as a marker of inflammation in patients with diabetic nephropathy [41], but the state of this trial is not known. Recently, Kohan and colleagues [42] reported that atrasentan, a fairly selective ET_A antagonist (ET_A/ET_B affinity ratio of roughly 1800), significantly reduces diabetes-induced albuminuria after 8 weeks of treatment in the presence of angiotensin enzyme inhibitors or angiotensin II blockers in type II diabetic patients and appeared to be well tolerated at low, but efficacious doses. These clinical findings argue for the use of ET_A selective antagonists rather than mixed receptor blockers and are consistent with pre-clinical studies in models of diabetes [51].

We hypothesize that ET_A inhibition will moderate diabetes mediated vasoconstriction, stimulation of Na transport, as well as blunt the diabetes-induced infiltration and activation of inflammatory cells. Renal cross-transplantation studies have revealed that high dietary Na-induced renal injury and blood pressure responses of ET_B receptor deficient (*sl/sl*) rats reflect extra-renal effects of ET_B deficiency or exaggerated ET_A receptor activation [43]. These authors did not explore the role of inflammatory cells in their experimental paradigm, nor did they investigate the role of the exacerbated hypertension observed in *sl/sl* rats with diabetes. Future studies utilizing renal cross-transplantation paradigms to determine if the exaggerated hypertensive response to diabetes in *sl/sl* rats is mediated via extra-renal or renal-derived mechanisms are currently being planned.

Perspectives

Severe uncontrolled diabetes can deplete both intracellular and extracellular fluid volumes; however, this situation is relatively rare in patients receiving routine insulin therapy, as it arises primarily during severe hyperglycemia with massive osmotic diuresis. More commonly observed in patients is a situation of sodium retention. Animal models and humans with moderate hyperglycemia in diabetes exhibit increased extracellular fluid volume, increased exchangeable sodium, and an impaired ability to excrete sodium in response to water immersion or isotonic saline infusion [44-46]. The etiology of sodium retention in diabetes is evident in the observations of DiPetrillo et al [47], who housed rats

with STZ-induced diabetes in metabolic cages with free access to food (normal sodium content) and water. While these animals exhibited increases in both sodium excretion and sodium intake, the hyperphagia accompanying diabetes elicited an increase in dietary sodium intake that exceeded the rise in sodium excretion. Thus, a state of positive sodium balance developed even in the face of increased sodium excretion. Despite considerable investigation, the mechanisms causing dysregulation of renal sodium reabsorption and volume homeostasis in diabetes remain poorly understood [48, 49]. It will be important to include studies investigating whether the ET system is involved in the extra-renal, macrophage-dependent regulation of sodium balance and inflammation as suggested by Titze et al [50]. Novel mechanistic targets for diabetic renal disease should include investigations of these extra-renal pathways as well as the renal ET/NO pathways.

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Figure 1.

Scheme depicting interactions of the ET and NO pathway as described in this chapter. On balance, ROS-induced ET-1 production results in elevated ET_A receptor activity that is not sufficiently counterbalanced by ET_B receptor function. The renal tubular ET_B receptor actions are mediated by NO. However, these mechanisms are unable to counteract ET_A-dependent vascular and inflammatory effects.