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ET_A receptor specific stimulation of glomerular inflammation and injury in streptozotocin-induced diabetic rats

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Abstract

Aims/hypothesis—ET_A receptor activation increases glomerular permeability to albumin (P_{alb}) and elevates pre-inflammatory markers in hyperglycaemic (HG) rats.

Methods—Male Sprague-Dawley rats were given streptozotocin (STZ), n=32 or saline (sham,), n=32. Half of the animals in each group received the ET_A-selective antagonist, ABT-627 (atrasentan; p.o.), beginning immediately after hyperglycaemia was confirmed. Glomeruli were isolated by sieving techniques and P_{alb} determined from the change in glomerular volume induced by oncotic gradients of albumin. Glomerular nephrin expression was assessed by immunofluorescence, whereas urinary nephrin was measured by immunoassay.

Results—Three and six weeks after STZ injection, proteinuria was significantly increased compared to sham controls and was significantly reduced by ABT-627 treatment. P_{alb} was also increased at 3 and 6 wk post-STZ; ABT-627 had no effect on P_{alb} or protein excretion in sham rats. In glomeruli isolated from HG rats, incubation with BQ-123, a selective ET_A antagonist, reduced P_{alb}, whereas BQ-788, a selective ET_B antagonist had no effect (n=6 rats/group, 5-8 glomeruli/rat). Glomerular and plasma content of soluble inter-cellular adhesion molecule-1 (sICAM-1) and monocyte chemoattractant protein-1 (MCP-1) were significantly increased 6 wk after STZ (ELISA). ABT-627 attenuated these increases. After 6 weeks of hyperglycaemia, glomerular nephrin expression was decreased with a concurrent increase in urinary nephrin excretion; ABT-627 prevented glomerular nephrin loss in the HG rats (n=5-8 rats in the eight groups).

Conclusions/Interpretation—These observations support the hypothesis that ET-1, *via* the ET_A receptor, directly increases glomerular permeability to albumin, possibly *via* nephrin loss, as well as early inflammation in the HG rat.

Keywords

diabetic nephropathy; endothelin-1; sICAM-1; MCP-1; albuminuria; glomerular permeability; P_{alb}; nephrin

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Introduction

Diabetes is the leading cause of end-stage renal disease in the western world, affecting approximately 30% of type I diabetic patients [1]. Microalbuminuria is the earliest clinical marker of renal complications in diabetes [2]. Patients develop macro-proteinuria as renal injury progresses and suffer from glomerular filtration rate reduction, which subsequently leads to renal failure.

In addition to vascular endothelium, components of the ET-1 system are located in many cell types within the kidney including mesangial cells [3], podocytes [4], and tubular epithelium [5]. Aside from vasoconstriction [6], ET-1 is a potent proinflammatory and profibrotic mediator [7]. In diabetes, intrarenal ET-1 activity appears to be increased [8]. Glomerular ET-1 mRNA levels are elevated in rats with streptozotocin (STZ)-induced diabetes [9]. Kidney ET_A receptor expression is up-regulated in rabbits with alloxan-induced diabetes [10]. In concurrence with these observations is the finding that urinary ET-1 excretion (a marker of intrarenal ET-1 generation) is increased in diabetic patients with microalbuminuria [11]. Furthermore, our laboratory demonstrated that ET-1 plasma levels were not changed but ET-1 excretion was increased in rats after induction of STZ-induced hyperglycaemia [12].

Studies from several labs including our own have shown that chronic blockade of ET_A receptors reduces albuminuria and renal inflammation in the STZ diabetic rat along with a modest decrease [12] or no significant change [13] in arterial pressure. However, the mechanism for this effect is not known. Inflammation is considered to be a contributing factor in the development of diabetic nephropathy and may contribute to proteinuria as well as interstitial fibrosis and cellular damage. Pro-inflammatory chemokines monocyte chemoattractant protein-1 (MCP-1) and soluble inter-cellular adhesion molecule-1 (sICAM-1) are important in attracting infiltrating cells and their attachment to the endothelium, thus facilitating the early process of macrophage infiltration into the kidney [14]. Increased ICAM-1 expression leading to increased leukocyte infiltration has been demonstrated in experimental models of nephropathy [15].

Proteinuria represents an early sign of glomerular injury, and its presence predicts not only an elevated risk for nephropathy, but also cardiovascular disease in general [16]. The mechanistic pathways of proteinuria in diabetes have not been fully resolved. One of the glomerular filtration barrier defects that lead to glomerular injury and subsequent proteinuria is damage to the filtration-slit formed by glomerular podocytes. Nephrin, a 1241-residue transmembrane immunoglobulin protein, is an important filtration-slit molecule [17]. The expression and urinary excretion of nephrin shows characteristic changes in diabetes and in other acquired proteinuric diseases [18].

The present study was undertaken to test the hypothesis that ET_A receptors contribute to glomerular inflammation and permeability defects in a rat model of hyperglycaemia by direct impairment of glomerular permeability and promotion of glomerular inflammation. More specifically, we investigated whether ET-1 via the ET_A receptor has a direct effect on the glomerulus to induce nephrin shedding and reduce permeability to albumin, thus providing a mechanism for diabetic proteinuria. Furthermore, we investigated whether the anti-inflammatory actions of ET_A receptor blockade were due to changes in expression of early inflammatory response pathways such as MCP-1 and sICAM-1. We used an *in vivo* model to specifically address these questions in the STZ model of type I diabetes.

Methods

STZ induced-hyperglycaemia

Experiments utilized male Sprague-Dawley rats (250-275 g) from Harlan Laboratories (Indianapolis, IN, USA). All protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Georgia and followed the NIH Public Health Service Policy on Humane Care and Use of Laboratory Animals. Rats were housed under conditions of constant temperature and humidity and exposed to a 12:12-h light-dark cycle. Four groups of rats were studied: 1) sham, 2) hyperglycaemic, HG, 3) sham+ABT-627 (sham+ABT-627; 5 mg kg^{-1} day^{-1}, in drinking water), and 4) hyperglycaemic + ABT-627 (HG+ABT-627; n=8 in all groups). Hyperglycaemia was attained by injection of STZ (Sigma-Aldrich, St. Louis, MO, USA) at the dose of 65 mg kg^{-1} of the body weight intravenously through the penile vein under isoflurane anesthesia; sham animals received saline injection. ABT-627 is a selective ET_A blocker (≈1000 times greater affinity for ET_A *versus* ET_B receptors) and provides maximum ET_A blockade and selectivity at this dose *in vivo* [19]. Drug treatment was started one day after STZ injection and after confirming that all rats had blood glucose levels >20 mmol/l. At the same time, insulin or palmitic acid (blank) implants (Linshin, Scarborough, Canada) were inserted subcutaneously into the HG and sham rats, respectively. Insulin implants maintained the blood glucose levels at 20-25 mmol/l (Table 1). Glucose in whole blood (taken from a small incision on the tail) was measured by Accu-Chek Glucometer. Glycaemia was monitored twice a week throughout the study. During the final two days of treatment, rats were placed in metabolic cages in order to collect urine for determination of the excretion rates of protein. At the end of all experiments, rats were anesthetized using sodium pentobarbital (50 mg kg^{-1}; i.p.) and a blood sample immediately taken from the abdominal aorta and plasma stored at -80°C. Kidneys were removed and glomeruli isolated.

Glomerular isolation

Glomeruli were isolated by gradual sieving techniques [20]. Upon removal, kidneys were decapsulated and placed in ice-cold phosphate-buffered saline (PBS; pH 7.4) containing phenylmethylsulfonylfluoride (PMSF, 1 mmol/l). The cortex was dissected and minced into small pieces. The cortical tissue was then passed through a 180 μM stainless steel sieve to separate glomeruli from larger fragments of renal tubules and vasculature. The resulting tissue was then passed through a 200 μM micro-cellulose filter. The filtrate was re-circulated on a smaller pore size micro-cellulose filter (70 μM). The glomeruli retained on top of the 70 μm sieve were washed with ice-cold phosphate buffered saline (PBS)/PMSF into a 50 ml Eppendorf tube. The resulting decapsulated glomeruli devoid of afferent and efferent arterioles were re-suspended in ice-cold PBS buffer. Tubular contamination was verified to be less than 5% of the tissue as assessed under the light microscope. The glomerular suspension was then centrifuged (10000 × g, 10 min) and the pellet re-suspended in PBS. The glomeruli were washed one final time using the same centrifugation and the final pellet was re-suspended in 1 ml PBS.

For immunoassays, the final suspension of isolated glomeruli was snap frozen in liquid nitrogen and stored at -80°C. The frozen glomeruli were re-suspended in lysis buffer (20 mmol/l HEPES, pH 7.4, 10 mmol/l NaCl, 5 mmol/l EDTA, 0.2 vol/vol Triton X-100, 10 mmol/l sodium fluoride, 1 mmol/l sodium ortho-vanadate, 1mmol/l PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin) and homogenized by ultrasonic homogenizer (20 seconds). After centrifugation at 10000 × g for 10 min, the supernatant was used for analysis and protein determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

Measurement of P_{alb}

After isolation, glomeruli were re-suspended in 5 % wt/vol bovine serum albumin (BSA) containing (115 mmol/l NaCl, 5 mmol/l KCl, 10 mmol/l sodium acetate, 1.2 mmol/l dibasic sodium phosphate, 25 mmol/l sodium bicarbonate, 1.2 mmol/l magnesium sulfate, 1 mmol/l calcium chloride and 3.5 mmol/l glucose, pH 7.4) at room temperature.

The rationale and methodology for the determination of albumin permeability has been described in detail previously [21]. In brief, images of 10–15 glomeruli per kidney preparation (i.e., per rat) were captured using a digital camera through an inverted microscope before and after a medium change to one containing 1 % wt/vol BSA. The medium exchange created an oncotic gradient across the basement membrane resulting in capillary expansion and a glomerular volume increase ($\Delta V = (V_{final} - V_{initial})/V_{initial}$), which was measured off-line by an image analysis programme (Digimizer, MedCalc Software bvba, Mariakerke, Belgium). The software determined the average radius of the glomerulus in two-dimensional space, and the volume was then derived from the formula $V=4/3\pi r^3$. The magnitude of ΔV was related to the albumin reflection coefficient, σ_{alb}, by the following equation: $(\sigma_{alb})_{experimental} = (\Delta V)_{experimental}/(\Delta V)_{control}$; the σ_{alb} of the control glomeruli was assumed to be equal to 1. P_{alb} is defined as $1-\sigma_{alb}$, and describes the movement of albumin subsequent to water flux. When σ_{alb} is zero, albumin moves across the membrane with the same velocity as water, and P_{alb} is 1.0. Conversely, when σ_{alb} is 1.0, albumin cannot cross the membrane with water, and P_{alb} is zero.

In additional experiments, we examined the role of ET_A receptors and ET_B receptors on P_{alb} in glomeruli isolated from untreated HG rats (n=6) utilizing a selective ET_A antagonist (BQ123; Calbiochem, San Diego, CA, USA) and a selective ET_B antagonist (BQ788; Calbiochem, San Diego, CA, USA). Glomeruli were pre-incubated with these antagonists at concentrations of 10^{-9} to 10^{-5} mol/l for 15 minutes at 37°C, and the P_{alb} response was determined. These experiments utilized a minimum of 5 glomeruli from each rat.

Biochemical analyses

Commercially available kits for sICAM-1 (Quantikine, sICAM-1 Immunoassay, R&D Systems, Minneapolis, MN, USA) and MCP-1 (RayBioTech. Inc., Norcross, GA, USA) were used for determining concentrations in plasma and glomerular homogenates. Nephrin concentration was determined in urine *via* ELISA kit (Exocell Inc., Philadelphia, PA, USA). Urinary protein concentrations were determined using the Bradford method.

Nephrin immunofluorescence

Isolated glomeruli taken from kidneys perfused with PBS were placed on engraved glass slides and allowed to dry at room temperature before freezing at -80°C. Glomeruli were then fixed using paraformaldehyde (2 % vol/vol) in a slide chamber (Antibody Amplifier, IHC WORLD, LLC, Woodstock, MD, USA). Slides were then washed with PBS and incubated with normal goat serum (5 % vol/vol) in PBS and Triton-X (0.3% vol/vol) for 1 hr before being incubated overnight on a shaker at 4°C with goat anti-human nephrin primary antibody (1:500 vol/vol, sc-19000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). On the second day, washing and blocking procedures were repeated before incubating slides with a mixture of Alexa Fluor 488 chicken anti-goat IgG fluorescent-tagged secondary antibody (1:5000 vol/vol) and rhodamine phalloidin for F-actin staining (5 μM) for 1 h in the antibody amplifier chamber. Both antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Washing was repeated and slides mounted with glass coverslips. Images were acquired with the Olympus FV-1000 MPE confocal/multiphoton system (Olympus, Center Valley, PA, USA) available at the Indiana Center for Biological Microscopy (Indianapolis, IN). Confocal image stacks of whole glomeruli were collected with 60×W NA1.2 objective

at 512×512 frame size and sequential mode using 488 and 559 nm laser lines. Nephrin and actin signals were collected with the emission filter 520 and 612 nm respectively. At least, five glomeruli per rat were imaged. Metamorph software v.7.5 (Molecular Devices Inc., Downingtown, PA, USA) was used for quantitative analysis. A sum of all planes intensities for both channels within a Z-stack was used to measure the total intensity values within the glomerulus. Background values from control stacks were subtracted from the total intensity values for both channels. Nephrin signals were then normalized to the corresponding actin signal by calculating the ratio from the background corrected total intensity values.

Statistical analyses

All data are presented as mean ± SEM. Differences between data obtained from sham, sham +ABT-627, HG and HG+ABT-627 are compared using two-way ANOVA followed by Bonferroni post hoc tests. Differences between 3- and 6-week groups were compared by unpaired Student *t*-test. A $p<0.05$ was considered statistically significant. Analyses were performed using GraphPad Prism Version 5.0 software (GraphPad Software Inc, La Jolla, CA, USA).

Results

As expected, blood glucose levels were elevated in HG rats with or without ET_A receptor blockade (Table 1). Similarly, blood glucose in sham rats was not changed by treatment with ABT-627. Relative to sham and sham+ABT-627 rats, rats in the HG and HG+ABT-627 groups had lower body weights during the 3 and 6 periods of study despite having hyperphagia, polydipsia and polyuria. Treatment with ABT-627 did not change any of these parameters.

Glomerular permeability and proteinuria

P_{alb} significantly increased after three weeks of hyperglycaemia when compared with sham. Treating animals with ABT-627 significantly decreased the elevated P_{alb} value. P_{alb} further increased significantly after 6 weeks of hyperglycaemia. Again, ABT-627 significantly decreased P_{alb} after 6 weeks of treatment (Fig. 1a). Changes in urinary protein excretion followed a similar pattern as P_{alb}. As shown in Fig. 1b, rats with hyperglycaemia exhibited significant proteinuria at the 3 week time point compared to sham rats. ABT-627 reduced proteinuria to levels comparable to sham. Proteinuria was further increased after 6 weeks of hyperglycaemia. Again, ABT-627 prevented the increase in proteinuria at the 6 week time point.

In order to further determine the role of glomerular ET_A and ET_B receptors in regulating P_{alb} in HG rats, isolated glomeruli from HG rats were incubated for 15 min at 37°C with the soluble ET-1 antagonist peptides. The ET_A receptor antagonist, BQ123, at 10^{-7} mol/l significantly reduced the elevated P_{alb} (Fig. 2a). P_{alb} was further reduced at higher BQ123 concentrations. In contrast, the ET_B receptor antagonist, BQ788, had no effect on the elevated P_{alb} of HG glomeruli at any concentration of that antagonist (Fig 2b). Incubation of isolated glomeruli from HG rats with both BQ compounds reduced P_{alb} in a fashion similar to BQ123 alone (Fig. 2c).

Systemic and glomerular inflammation

Soluble ICAM-1 and MCP-1 are two early pro-inflammatory molecules implicated in the pathogenesis of diabetic nephropathy. After 3 weeks of hyperglycaemia, we did not observe any changes in plasma or glomerular levels of sICAM-1 and MCP-1 (Fig. 3a-3b and Fig. 4a-4b, respectively). After 6 weeks, however, plasma and glomerular sICAM-1 and MCP-1

were significantly elevated compared to those observed in sham animals. These increases were significantly attenuated by ABT-627.

Nephrinuria and glomerular nephrin expression

We monitored glomerular expression of nephrin by immunostaining using fluorescent microscopy. As depicted in Figure 5, glomeruli isolated from 6-week HG rats exhibited less nephrin expression when compared with sham levels. Glomeruli isolated from HG rats exhibited significantly less nephrin expression when compared to those of sham (Fig 6a). In addition, glomeruli isolated from ABT-627-treated HG rats had nephrin expression comparable to sham (Fig 6a). Nephrin also is excreted into urine in the early stages of diabetic nephropathy is an early sign of breakdown of the glomerular filtration barrier. Therefore, we determined whether nephrin loss in urine of HG rats is ET_A receptor dependent. As expected, urinary excretion of nephrin was significantly increased after 6 weeks of hyperglycaemia andABT-627 administration completely prevented the hyperglycaemia-induced increase in nephrin excretion (Fig. 6b).

Discussion

The current study provides new information regarding the mechanisms of endothelin effects in the glomerulus of the hyperglycaemic kidney and the potential use of ET receptor antagonists in proteinuric renal disease. This includes evidence of a direct, ET_A dependent increase in glomerular permeability and nephrin loss that occurs in the hyperglycaemic kidney. In addition, ET_A receptor blockade provides anti-inflammatory actions by reducing hyperglycaemia dependent increases in early inflammatory markers such as MCP-1 and sICAM-1. With the increased incidence of chronic diabetic complications, strategies that can improve the prevention of end-stage renal disease (ESRD) are urgently needed. Experimental data suggest that ET receptor blockade could be a novel therapeutic approach to ESRD [12] and results from clinical trials would support further use of these drugs in conditions such as diabetic nephropathy [22]. The protective effects of ET receptor antagonists on renal injury appear to be independent of blood pressure lowering.

The potential role of direct glomerular actions of ET receptors has not been defined. We observed that treatment with ABT-627, administered orally at 5 mg kg^{-1} day^{-1} to HG rats, prevented the increase in P_{alb} in conjunction with the reduction in proteinuria. The use of isolated glomeruli allowed us to measure the glomerular capillary P_{alb} independent of the potential confounding effects of changes in mean arterial blood pressure and or renal hemodynamics during measurement. Thus, our findings demonstrate that a reduction in MAP or GFR is not a prerequisite for the anti-proteinuric effect of ET_A receptor blocker in the early phases of diabetic nephropathy. Collectively, these data are consistent with a non-hemodynamic effect of ET-1 on glomerular filtration barrier function.

In subjects with diabetic nephropathy, Wenzel *et al* showed a reduction in macroalbuminuria after 12-weeks of treatment with the moderately selective ET_A receptor antagonist avosentan [22]. This effect was observed without a change in BP suggesting a pressure-independent anti-proteinuric effect of this antagonist [22]. Furthermore, most of those subjects were already on a combination of treatment with angiotensin receptor blockers and angiotensin converting enzyme inhibitors providing evidence that the anti-proteinuric effect of endothelin antagonism was independent any effect on the renin-angiotensin system. Opocenský *et al.* observed that late-onset ET_A receptor blockade reduces proteinuria in homozygous Ren-2 rats despite severe hypertension [23]. Hocher and his colleagues reported that the ET_A receptor antagonist, LU 135252, reduced proteinuria and completely normalized glomerular matrix protein expression in STZ-induced HG rats [24].

In vitro experiments utilizing isolated glomeruli from HG rats incubated with the water soluble ET peptide antagonists (BQ compounds) were conducted in order to investigate the specific role of glomerular ET_A and ET_B receptors on permeability, independent of hemodynamic influence. ET_A antagonism with BQ-123 reduced the P_{alb} defect of glomeruli from hyperglycemic rats. The rapid effect of the antagonist on the P_{alb} can be explained by the role of ET_A receptors in rearrangement of the actin cytoskeleton in the podocytes. Morigi *et al.*, has reported that shigatoxin stimulates the synthesis of ET-1, which may regulate cytoskeleton remodeling and thus glomerular permeability in an autocrine manner. Cytoskeleton rearrangement is associated with an increase in albumin permeability detected by transepithelial passage of fluorescent albumin to the basolateral compartment of podocytes [25]. It has been postulated that ET-1 released from either glomerular endothelial cells or podocytes might interact with signalling to filtration-slit components and directly influence podocyte function [26]. Selective blockade of ET_B receptors *via* BQ-788 had no effect on P_{alb} of isolated glomeruli from hyperglycemic rats. These results clearly suggest that ET_B receptors may not play a role in the regulation of glomerular permeability in hyperglycemic glomeruli. We recently observed that exogenous ET-1 added to cultured podocytes, increases P_{alb} directly, an effect that can be blocked by an ET_A but not ET_B antagonist [29]. Thus, we speculate that any effect of ET_B receptors to modulate proteinuria is *via* hemodynamic changes rather than direct effects on the filtration barrier.

Further evidence for a direct effect of ET-1 to influence glomerular filtration barrier function comes from our observations that ABT-627 reduced both nephrin excretion and glomerular nephrin expression in treated HG rats. In several animal models of diabetes, nephrin excretion is increased while glomerular levels are decreased consistent with the current study [27]. Additional evidence that ET-1 is an important factor in contributing to nephrin loss comes from a report that media taken from cultured endothelial cells conditioned with sera from pre-eclampsia patients produces nephrin loss from human cultured podocytes, an effect that can be blocked by ET_A receptor blockade [28]. Gagliardini *et al.* showed that avosentan prevented glomerular nephrin loss in the STZ diabetic model detected *via* immunohistochemistry [13]. More recently, we reported that chronic ET-1 infusion in normoglycaemic rats for 2 wk increases P_{alb} to albumin without any change in BP and that incubation of isolated glomeruli with ET-1 for a little as 15 min significantly increases P_{alb} [29]. Taken together, these studies all provide strong evidence that ET-1 directly facilitates increased P_{alb} within the glomerulus independent of hemodynamic effects.

We previously observed that ET_A receptor blockade with ABT-627 reduces infiltration of macrophages and T cells in kidneys of HG rats after 10 weeks of treatment [12]. The current study used the same model to profile the relative time course of early markers of inflammation, MCP-1 and sICAM-1. After 6 weeks of hyperglycaemia induction, plasma and glomerular sICAM-1 and MCP-1 concentrations were significantly increased; this increase was largely prevented by ABT-627. There was no significant increase in inflammatory markers at the 3 week time point. As we know from previous studies, the infiltration of macrophages in the glomeruli and interstitium is one of the primary pathological features in models of diabetic nephropathy [30]. Leukocyte infiltration into inflammatory sites is mediated by coordinated actions of both of cell adhesion molecules and chemokines. Therefore, our results support the hypothesis that ET-1, through the ET_A receptor, functions as an early pro-inflammatory signal. This hypothesis is further supported by previous studies in other model systems showing that ET-1 can increase pro-inflammatory signalling pathways, such as MAP kinase and NFκB, that are known to increase the production of chemoattractants and adhesion molecules such as MCP-1 and ICAM-1 [31].

ICAM-1 is a cell surface glycoprotein primarily involved in promoting leukocyte attachment to the endothelium and transmigration through its expression on the vascular endothelium (diapedesis) and binding to β2 leukocyte integrins [32]. ICAM-1 expression can be induced by hyperglycaemia, advanced glycation end-products, oxidative stress, hyperlipidemia and hyperinsulinemia [33]. Previous studies have shown that the accumulation of macrophages resulted from increased expression of cell adhesion molecules, such as ICAM-1 and selectins, in kidneys of patients with diabetic nephropathy [34]. Several experimental models of diabetic nephropathy have investigated the correlation between ICAM-1 expression and disease activity. Sugimoto *et al*. reported that the up-regulation of ICAM-1 associated with macrophage infiltration in early diabetic renal injury, and was maintained during the period of study. The mechanism of ICAM-1 up-regulation was mainly attributed to endothelial shear stress associated with elevated GFR in the hyperfiltration phase in HG rats [15]; their studies suggested that monoclonal antibodies against ICAM-1 abrogated the infiltration of macrophages in glomeruli in these HG rats. Similarly in ICAM-1 deficient *db/db* mice, Chow *et al*. showed that ICAM-1 deficiency reduced glomerular macrophage infiltration with subsequent amelioration of glomerular hypertrophy and interstitial fibrosis [35] indicating a deteriorating mechanism for ICAM-1 in experimental diabetic nephropathy.

MCP-1, also termed CCL2, is a potent chemoattractant for monocyte/macrophage [36]. Numerous cell types, including tubular epithelial cells and mesangial cells, are known to be capable of expressing MCP-1. As reported previously, MCP-1 expression was up-regulated in association with renal macrophage infiltration in diabetic patients [37]. The mechanisms leading to the up-regulation of MCP-1 in various types of renal injury, including diabetic nephropathy, are not fully understood. However, human and rodent mesangial cells can synthesize MCP-1 in response to several factors that are thought to be involved in glomerular injury. These include interleukin-1, TNF-α, and low-density lipoprotein [38]. In the case of human mesangial cells, a high concentration of glucose and glycated albumin have been reported to promote MCP-1 production [39]. An additional influence that may induce synthesis of MCP-1 is the generation of reactive oxygen species [40]. MCP-1 deficient mice markedly reduced kidney macrophage accumulation and prevented the development of diabetic nephropathy [14]. Furthermore, blocking the MCP-1/CCR2 pathway ameliorated glomerulosclerosis, indicating that the MCP-1/CCR2 pathway has an important role in the progression of diabetic nephropathy [41].

We have recently demonstrated a relationship between ET-1, sICAM-1, and MCP-1 in the kidney using a chronic infusion of ET-1 in the rat at doses that do not produce hypertension [29]. Furthermore, ET-1 can increase expression of sICAM-1 and MCP-1 in other tissue or cell types. *In vitro* studies suggest that ET-1 induces neutrophil adhesion to cardiac myocytes by increasing ICAM-1 expression, which is mediated *via* ET_A receptor on cardiac myocytes. ICAM-1 expression induced by activation of ET_A receptors appears to be mediated through the PKC pathway [42]. Further studies, using human brain-derived endothelial cells, showed that *in vitro*, ET-1 can directly stimulate MCP-1 mRNA expression and MCP-1 protein; and this ET-1-induced MCP-1 production is mediated by the ET_A receptor [43].

Perspectives

Our results support the hypothesis that ET_A receptors contribute to glomerular permeability defects and inflammation in HG rats. In a model of diabetic nephropathy, we now show that ET-1 induces nephrin loss, which now provides a mechanism for how ET_A antagonists may produce changes in glomerular permeability to albumin. These findings provide more thorough mechanistic support for the therapeutic potential of ET_A receptor antagonists in

proteinuric renal disease as well as renal inflammation. Additional studies are needed to discern whether the beneficial effects of ET receptor blockade can reverse established changes in glomerular permeability, proteinuria, and renal inflammation. Available ET-related antagonists include both ET_A selective and combined ET_A and ET_B inhibitors. Given the often opposing actions of these receptor subtypes, it will also be important to distinguish receptor specific effects, if any, on these clinically relevant variables.

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Abbreviations

BP	Blood pressure
BSA	Bovine serum albumin
CCL2	Chemokine C-C motif ligand 2
CCR2	Chemokine C-C motif receptor 2
ESRD	End-Stage Renal Disease
ET-1	Endothelin
ET_A	Endothelin receptor-A
ET_B	Endothelin receptor-B
EDTA	Ethylenediamine tetra-acetic acid
GFR	Glomerular filtration rate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HG	Hyperglycaemic
MAP	Mean arterial blood pressure
MCP-1	monocyte chemoattractant protein-1
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor-κB
P_{alb}	Glomerular permeability to albumin
PBS	Phosphate-buffered saline
PMSF	Phenylmethylsulfonylfluoride
sICAM-1	soluble inter-cellular adhesion molecule-1

STZ	Streptozotocin
TNF-α	Tumor necrosis factor-α

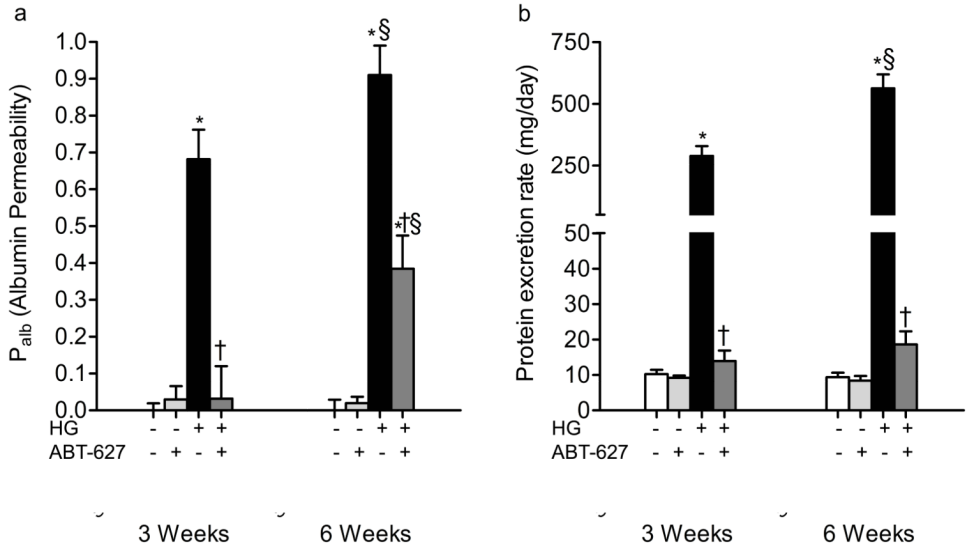

Figure 1.

Effect of three and six week of STZ-induced hyperglycaemia (HG) with or without concurrent treatment with ABT-627 on glomerular permeability to albumin (P_{alb}) (a) and daily protein excretion rate (b). N=5-8 per group. * denotes differences against sham and † denotes differences against HG at the following *p* values: P_{alb}, 3 weeks, two-way ANOVA, p_{HG}=0.0001, p_{ABT}=0.0003, p_{HG*ABT}=0.0002 and 6 weeks, two-way ANOVA, p_{HG}<0.0001, p_{ABT}=0.0001, p_{HG*ABT}<0.0001. Proteinuria, 3 and 6 weeks, two-way ANOVA, p_{HG}<0.0001, p_{ABT}<0.0001, p_{HG*ABT}<0.0001). §p<0.05 *versus* 3-week treatment.

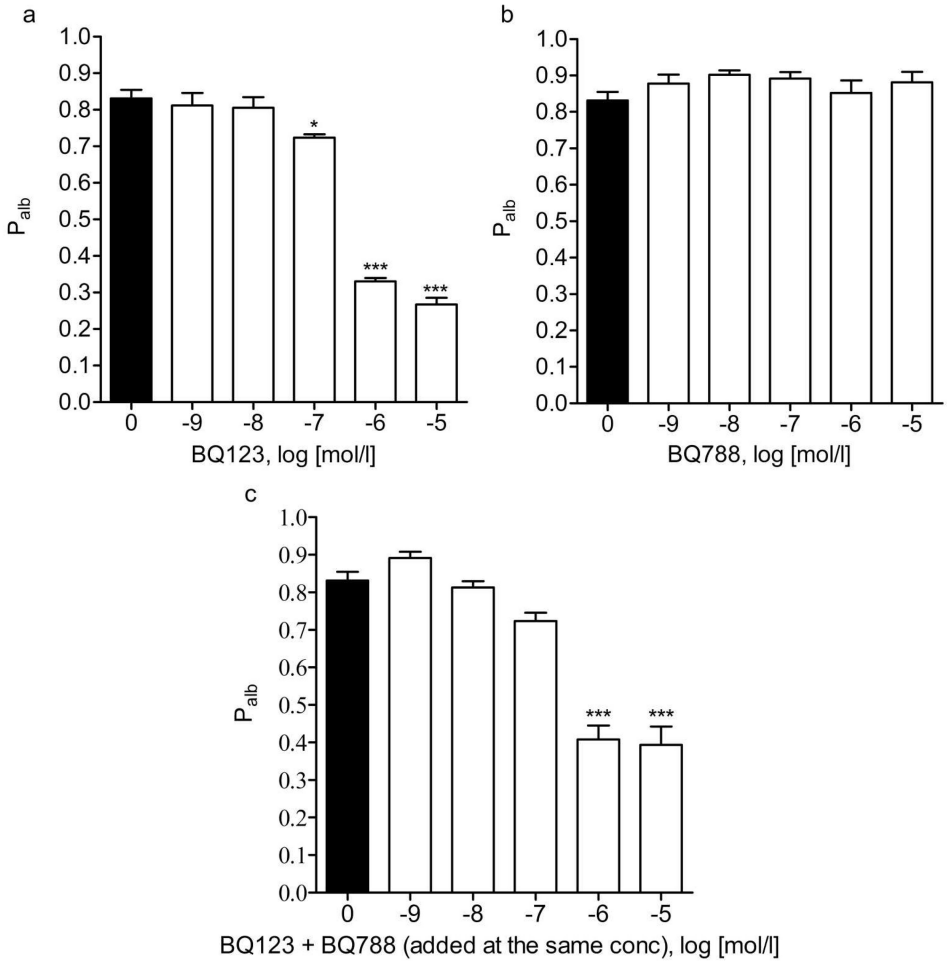

Figure 2.

In vitro effect of selective ET_A and ET_B receptor antagonists on P_{alb} of isolated glomeruli from untreated hyperglycemic (HG) rats after incubation with concentrations ranging from 10^{-9} to 10^{-5} mol for 15 min at 37°C. BQ123 (10^{-5} to 10^{-9} mol/l) significantly reduced the elevated P_{alb} of 6-wk hyperglycemic isolated glomeruli. BQ788 had no effect on elevated P_{alb} of HG glomeruli. Addition of both of them produced the same effect of BQ123 alone. *,**,***p<0.05, 0.01, 0.001 respectively *versus* zero concentration. Total 6 HG were used for treatment of glomeruli *in vitro*. At least, 5 glomeruli/rat were analysed.

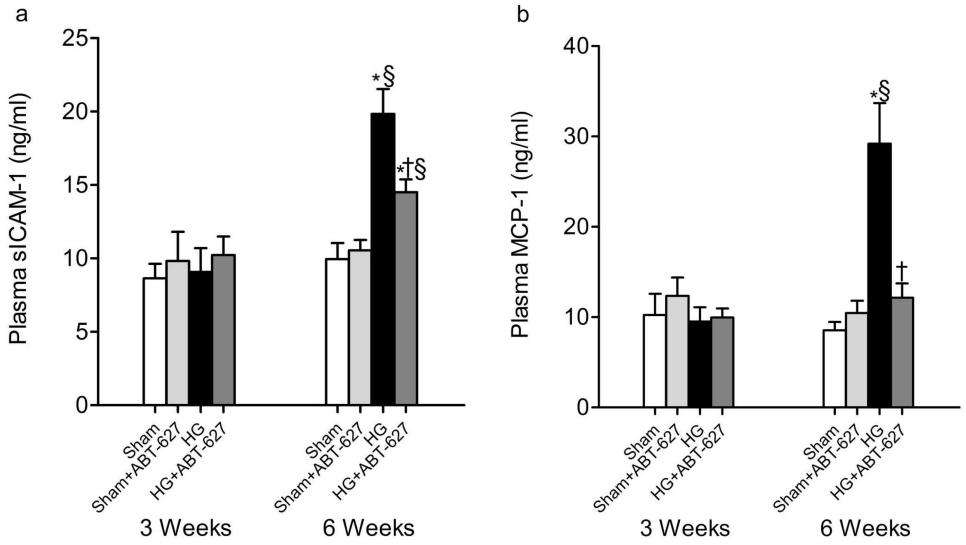

Figure 3.

Effect of 3 and 6 weeks of STZ-induced hyperglycaemia (HG) with or without concurrent treatment with ABT-627 on plasma sICAM-1 (a) and plasma MCP-1 concentrations (b). N=5-8 per group. * denotes differences against sham and † denotes differences against HG at the following *p* values: Plasma sICAM-1, 6 weeks, two-way ANOVA, p_{HG}=0.016, p_{ABT}=0.033, p_{HG*ABT}<0.0001. Plasma MCP-1, 6 weeks, two-way ANOVA, p_{HG}=0.0001, p_{ABT}=0.0056, p_{HG*ABT}=0.0008). §p<0.05 *versus* 3-week treatment.

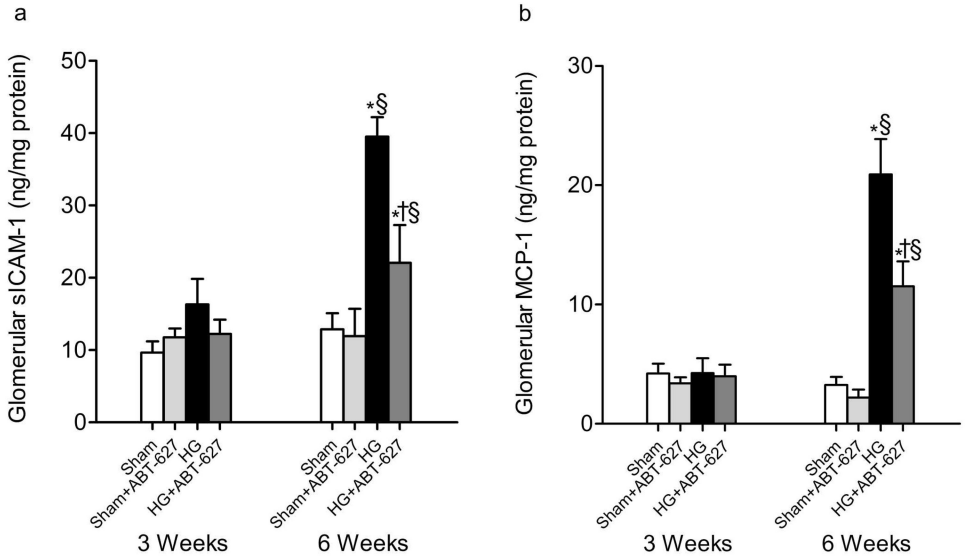

Figure 4.

Effect of 3 and 6 weeks of STZ-induced hyperglycaemia (HG) with or without concurrent treatment with ABT-627 on glomerular sICAM-1 (a) and glomerular MCP-1 concentrations (b). n=5-8 per group. * denotes differences against sham and † denotes differences against HG at the following *p* values: Glomerular sICAM-1, two-way ANOVA, 6 weeks, $p_{HG}<0.0001$, $p_{ABT}=0.0153$, $p_{HG*ABT}=0.0279$. Glomerular MCP-1, 6 weeks, two-way ANOVA, $p_{HG}<0.0001$, $p_{ABT}=0.0097$, $p_{HG*ABT}=0.0342$). §$p<0.05$ *versus* 3-week treatment.

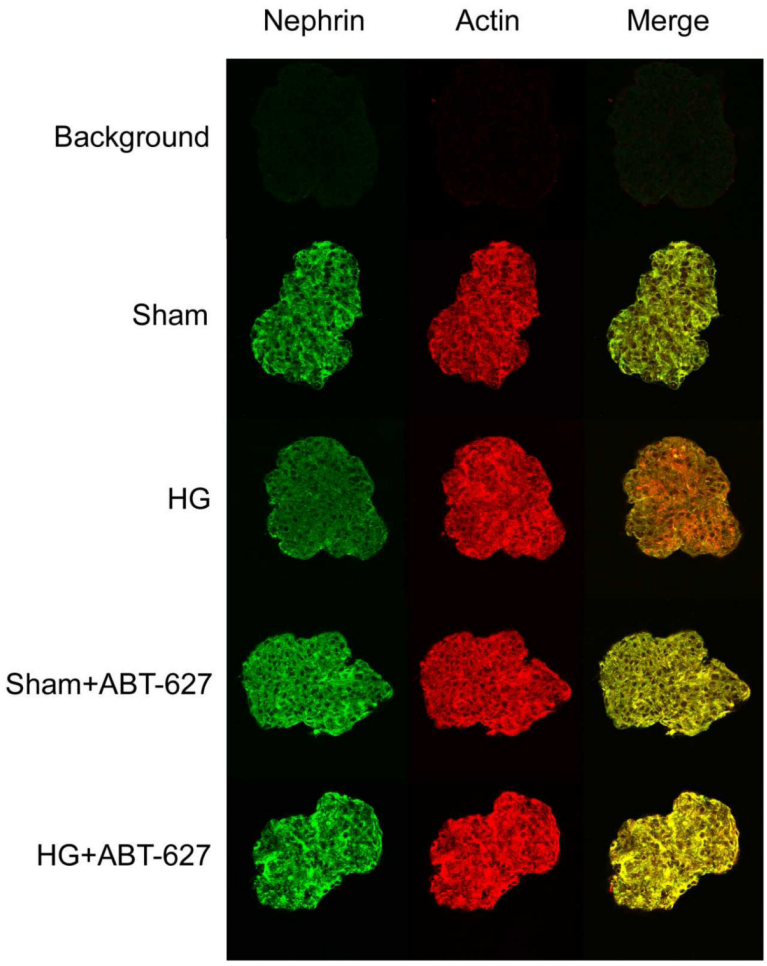

Figure 5.
Representative images of the localization of nephrin protein in the glomeruli of sham and hyperglycaemic (HG) untreated and ABT-627-treated rats determined by immunoflourescence. All photographs were taken under the same conditions for the laser confocal microscope using a 60× lens and quantified *via* calculating the ration to actin intensity using phalloidin staining. n=4-5 rats in each group. Negative controls for the primary nephrin antibody and the absence of phalloidin staining were evaluated as indicated and used to calculate the corrected intensity for both nephrin and actin staining.

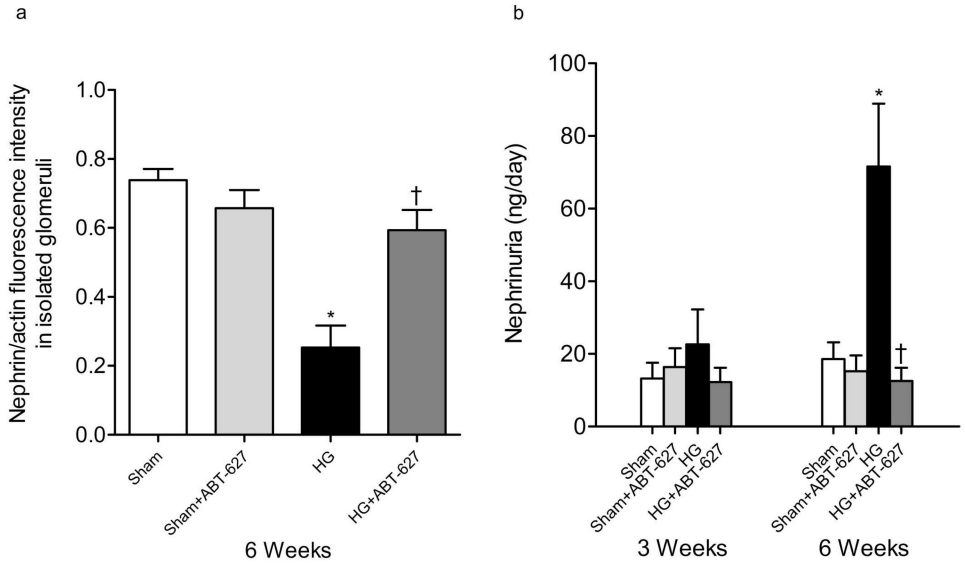

Figure 6.

(a) Effect of 3 and 6 weeks of STZ-induced hyperglycaemia (HG) with or without concurrent treatment with ABT-627 on glomerular nephrin expression evaluated by immunofluorescene. N=4-5 rats in each group (5-9 glomeruli/rat). * denotes differences against sham and † denotes differences against HG at the following *p* values: Nephrin to actin ratio, two-way ANOVA, 6 weeks, p_{HG}=0.0002, p_{ABT}=0.0328, p_{HG*ABT}=0.0017. (b) Effect of 3 and 6 weeks of STZ-induced hyperglycaemia (HG) with or without concurrent treatment with ABT-627 on nephrin excretion rate (nephrinuria). N=5-6 per group. * denotes differences against sham and † denotes differences against HG at the following *p* values: Nephrinuria, two-way ANOVA, 6 weeks, p_{HG}=0.0146, p_{ABT}=0.0035, p_{HG*ABT}=0.0078.

Table 1
Characteristics of the experimental rats after 3 and 6 weeks of treatment

3 WEEKS	Sham	Sham+ABT-627	HG	HG+ABT-627
Body weight (g)	311 ± 5	313 ± 11	280 ± 10*	282 ± 7*
Blood glucose (mmol/l)	5.6 ± 0.4	5.7 ± 0.6	22.2 ± 0.6*	23.1 ± 0.6*
Food intake (g/day)	23 ± 4	18 ± 2	42 ± 4*	42 ± 4*
Water intake (ml/day)	32 ± 2	30 ± 2	160 ± 21*	152 ± 11*
Urine flow (ml/day)	15 ± 2	18 ± 3	145 ± 12*	141 ± 10*
6 WEEKS	Sham	Sham+ABT-627	HG	HG+ABT-627
Body weight (g)	409 ± 13	412 ± 13	301 ± 6*	303 ± 9*
Blood glucose (mmol/l)	5.5 ± 0.5	5.7 ± 0.6	22.7 ± 0.7*	22.9 ± 0.9*
Food intake (g/day)	20 ± 3	18 ± 2	40 ± 2*	38 ± 3*
Water intake (ml/day)	29 ± 3	28 ± 3	195 ± 18*	187 ± 15*
Urine flow (ml/day)	15 ± 1	19 ± 2	170 ± 15*	163 ± 11*

Excretory data were derived from 24-h urine collections in metabolic cages. Data are means ± SEM (n = 8 in all groups). Sham+ABT-627, sham rats that were treated with ABT-627; HG, hyperglycaemic rats with partial insulin replacement to maintain limit the degree of hyperglycaemia; HG +ABT-627, HG rats that were treated with ABT-627.

* $p<0.05$ *versus* sham.