

Distinct actions of ET_A selective versus combined ET_{A/B} receptor antagonists in early diabetic kidney disease

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Abbreviations:

ET, endothelin
GBM, glomerular basement membrane
HG, hyperglycemia
MCP-1, monocyte chemoattractant protein-1
MMP, metalloprotease
NO, nitric oxide
P_{alb}, glomerular permeability
sICAM-1, soluble intercellular adhesion molecule-1
STZ, streptozotocin
TGF-β, transforming growth factor beta
ZO-1, zona occludens-1

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Abstract

Selective ET_A and combined ET_A and ET_B receptor antagonists are being investigated for use in treating diabetic nephropathy. However, the receptor specific mechanisms responsible for producing the potential benefits have not been discerned. Thus, we determined the actions of ET_A and ET_B receptors on measures of glomerular function and renal inflammation in the early stages of diabetic renal injury in rats through the use of selective and combined antagonists. Six weeks after streptozotocin (STZ)-induced hyperglycemia, rats were given ABT-627 (5 mg/kg/d), a selective ET_A antagonist; A-182086 (10 mg/kg/d), a combined ET_{A/B} antagonist; or vehicle for 1 week. Sham controls received STZ vehicle (saline). Hyperglycemia led to significant proteinuria, increased glomerular permeability to albumin (P_{alb}), nephrinuria, and an increase in total matrix metalloprotease (MMPs) and transforming growth factor-beta 1 (TGF-β1) activities in glomeruli. Plasma and glomerular soluble intercellular adhesion molecule-1 (sICAM-1) and monocyte chemoattractant protein-1 (MCP-1) were elevated after 7 weeks of hyperglycemia. Daily administration of both ABT-627 and A-182086 for 1 week significantly attenuated proteinuria, the increase in P_{alb}, nephrinuria, and total MMPs and TGF-β1 activity. However, glomerular sICAM-1 and MCP-1 expression was attenuated with ABT-627, but not A-182086 treatment. In summary, both selective ET_A and combined ET_{A/B} antagonists reduced proteinuria, glomerular permeability and restored glomerular filtration barrier components integrity, but only ET_A selective blockade had anti-inflammatory and anti-fibrotic effects. We conclude that selective ET_A antagonists are more likely to be preferred for treatment of diabetic kidney disease.

Introduction

Diabetic nephropathy is the most common cause of end-stage renal disease in patients with diabetes mellitus. An early marker for diabetic nephropathy is the occurrence of microalbuminuria (Rosenstock and Raskin, 1986). Defects in the glomerular filtration barrier including the podocytes result in both functional and histopathological changes observed in the glomerulus of diabetic kidneys. Podocytes attach to the glomerular basement membrane through adhesion proteins, mainly $\alpha 3\beta 1$ integrins and the dystroglycan complex. The filtration slit between adjacent podocytes includes a number of cell-surface proteins including nephrin, podocalyxin and P-cadherin, which ensure retaining of large macromolecules, such as serum albumin. In diabetic nephropathy, the etiology of podocyte injury and subsequent proteinuria is via two primary mechanisms: podocyte apoptosis and/or reduced podocyte adhesion to the glomerular basement membrane (GBM) (Shankland, 2006). Detached cells are shed in the urine as live podocytes (Petermann et al., 2003). Loss of cell anchorage to the GBM may result from downregulation of the $\alpha 3\beta 1$ integrins (Dessapt et al., 2009) and increased levels of TGF- $\beta 1$ (Chen et al., 2000).

The endothelin system plays an important role in the development of diabetic nephropathy. ET_A receptors account for the majority of the vasoconstrictor and proliferative effects of ET-1 (Benigni and Remuzzi, 1995) as well as promoting mononuclear cell infiltration (Suzuki et al., 2001) and production of matrix proteins (Gomez-Garre et al., 1996). In contrast, ET_B receptors mediate endothelial-dependent nitric oxide (NO) release (Tack et al., 1997), contribute to ET-1 clearance from plasma (Fukuroda et al., 1994), and regulate renal sodium (Na⁺) and water excretion in the collecting duct and other tubular segments (Kohan et al., 1992). Our laboratory recently reported that glomerular and plasma serum levels of interstitial adhesion molecule (ICAM)-1 and monocyte chemoattractant protein (MCP)-1 were increased after 6 weeks of STZ-induced diabetes (Saleh et al., 2010a) with subsequent increase in macrophage infiltration into renal cortices after 10 weeks (Sasser et al., 2007). These effects were shown to

be ET_A receptor dependent. Several studies have noted that ET_A selective or combined ET_{A/B} antagonists prevent the development of diabetic nephropathy (Hochoer et al., 2001). However, given the contrasting actions of these receptor subtypes in the vasculature and kidney, it remains obscure as to whether blockade of the ET_B receptor in conjunction with ET_A antagonism would be harmful, beneficial, or neutral for the treatment of patients with diabetic nephropathy.

Previous animal studies have all examined treatment with antagonists at the time of STZ administration, thus we wanted to elucidate the effects of ET antagonist treatment after establishing diabetic nephropathy. This study addressed two basic questions. First, does administration of an ET_A or combined ET_{A/B} antagonist reverse proteinuria and the glomerular permeability defect in STZ-induced hyperglycemic rats. And secondly, do ET_A selective antagonists have an advantage over combined ET_{A/B} antagonists with regard to attenuating glomerular injury and inflammatory mediators.

Methods

STZ-Induced Hyperglycemia. Experiments utilized male Sprague-Dawley rats (250-275 g) from Harlan Laboratories. All protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Georgia and followed the American Physiological Society Guidelines for the Care and Use of Laboratory Animals. Rats were housed under conditions of constant temperature and humidity and exposed to a 12:12-hr light-dark cycle. Hyperglycemia was induced by injection of STZ (65 mg/kg, Sigma-Aldrich, St. Louis, MO), dissolved in sterile saline in the penile vein (n=30). Onset of diabetes was confirmed by the presence of glycosuria 48 hr after injection. In order to check the hyperglycemic state of the rats, the glucose concentrations in tail blood samples were measured with Accu-Chek Glucometer. All rats with blood glucose levels 400 mg/dl or greater were considered hyperglycemic. A group of control rats (sham) were injected with the sterile saline (n=18) and kept under identical housing conditions with free access to water and food *ad libitum*. After induction of

hyperglycemia, both groups of rats were kept under observation for 6 weeks before the start of 1-week treatment with endothelin antagonists. During this period, body weight, non-fasting blood glucose and severity of diabetic-related symptoms were monitored once a week. In order to prevent hyperglycemic rats from dying during the observation period, these animals were treated with a low level of insulin by using time-release implants (Linplant®, Scarborough, ON, Canada), whereas sham rats received the implant excipient, palmitic acid. The implants were sterilized in 2% povidone-iodine solution and inserted by a 16-gauge hypodermic needle under the dorsal skin of the neck. Every implant gradually released the insulin at a dose of approximately 1 unit per day.

Experimental Protocol. Six weeks after induction of hyperglycemia, rats were randomly allocated to the following six experimental groups: (i) untreated non-hyperglycemic sham rats (S; n=6), (ii) sham rats treated with ET_A antagonist; ABT-627(atrasentan); 5 mg/kg/d (S+ABT-627; n=6), (iii) sham rats treated with mixed ET_{A/B} receptor antagonist; A-182086; 10 mg/kg/d (S+A-182086; n=6), (iv) untreated hyperglycemic rats (HG; n=10), (v) hyperglycemic rats treated with ABT-627; 5 mg/kg/d (HG+ABT-627; n=10), (vi) hyperglycemic rats treated with A-182086; 10mg/kg/d (HG+A-182086; n=10). ABT-627 and A-182086 were kindly provided by Abbott Laboratories (Abbott Park, IL). Both drugs have been shown to produce near maximum inhibition of the pressor response to ET-1 or the pre-cursor Big ET-1 in conscious rats when administered at these doses (Wessale et al., 2002; Wu-Wong et al., 2002). Oral administration of A-182086 at 10 mg/kg completely abolished the vasodilator response to sarafotoxin 6c and significantly inhibited the pressor response as well (Wessale et al., 2002). ABT-627 and A-182086 have very high binding affinity (K_i) in Chinese hamster ovary cell preparations expressing ET_A receptors at 0.034 and 0.20 nM, respectively, while the K_i for ET_B binding are 63.3 and 1.23 nM, respectively (Wu-Wong et al., 2002). The Drugs were administered in the drinking fluid at concentrations calculated to deliver the above-mentioned doses. Because of poor water solubility of the antagonists, dilute solutions of sodium hydroxide were used to

prepare a concentrated stock solution (1 g/L of 0.1 M NaOH) before diluted into the drinking water. Daily food and water consumption were monitored and the concentrations of drugs in the drinking water were adjusted to maintain appropriate dosing. All rats were kept in metabolic cages during the one-week treatment for urine collection and proteinuria analysis. On the seventh day after treatment had been started, rats were anesthetized with sodium pentobarbital (50 mg/kg). Plasma samples were collected from arterial blood drawn from the abdominal aorta. The kidneys were removed for further evaluation.

Isolation of Glomeruli. Kidneys were decapsulated and placed in phosphate-buffered saline (PBS; pH 7.4, 4°C) containing phenylmethylsulfonylfluoride (PMSF, 1mM). Glomeruli were isolated as previously described (Saleh et al., 2010b; Saleh et al., 2010a). In brief, cortical tissue was minced and then passed through a 180 µm stainless steel sieve to separate glomeruli from tubular fragments and vasculature. The material was again filtered through a 200 µm micro-cellulose filter. The filtrate was passed through a smaller pore size micro-cellulose filter (70 µm) with glomeruli being retained on the top. Glomeruli were then washed with ice-cold PBS/PMSF and decapsulated glomeruli re-suspended in ice-cold PBS buffer. Tubular contamination was always confirmed at less than 5% of total tissue under light microscopy. The glomeruli were washed two more times and re-suspended in PBS before the final pellet was snap frozen in liquid nitrogen and stored at -80°C.

For immunoassays and western blotting, the glomeruli were re-suspended in lysis buffer (20 mM HEPES, pH 7.4, 10 mM NaCl, 5 mM EDTA, 0.2% Triton X-100, 10 mM sodium fluoride, 1 mM sodium ortho-vanadate, 1 mM PMSF, 1 µg/mL leupeptin and 1 µg/mL pepstatin) and homogenized by ultrasonic homogenizer (20 sec). This suspension was centrifuged at 10000 × g for 10 min and the supernatant used for immunoassays and western blotting as well as protein determination by the Bradford method (Bio-Rad Laboratories, Hercules, CA).

For analysis of mRNA expression, quantitative real time polymerase chain reaction (RT-PCR) was conducted using frozen glomeruli that were first processed for RNA extraction using

Qiagen RNeasy RNA isolation kit and QIAshredder homogenizer columns (Qiagen, Valencia, CA).

Measurements and Calculation of P_{alb} . P_{alb} was determined in isolated glomeruli without freezing. Glomeruli were re-suspended at room temperature in 5% BSA containing 115 mM NaCl, 5 mM KCl, 10 mM sodium acetate, 1.2 mM dibasic sodium phosphate, 25 mM sodium bicarbonate, 1.2 mM magnesium sulphate, 1 mM calcium chloride and 3.5 mM glucose, pH 7.4.

The theory and detailed method for determining albumin permeability has been described previously (Saleh et al., 2010b; Saleh et al., 2010a). In brief, images of individual glomeruli (usually 5-10 glomeruli per rat) were taken through an inverted microscope equipped with a digital camera before and after changing the standard media (described above) to one containing 1% BSA. The change in media creates an oncotic gradient across the filtration barrier to produce a change in glomerular volume ($\Delta V = (V_{final} - V_{initial}) / V_{initial}$) that was analyzed off-line (Digimizer, MedCalc Software, Belgium). The program calculated the average glomerular radius to then calculate glomerular volume ($V = 4/3\pi r^3$). The change in volume (ΔV) was used to calculate the albumin reflection coefficient (σ_{alb}) by the following equation:

$$(\sigma_{alb})_{\text{experimental}} = (\Delta V)_{\text{experimental}} / (\Delta V)_{\text{control}}$$

The σ_{alb} of the control glomeruli was set at 1.0. Albumin permeability (P_{alb}) is defined as $(1 - \sigma_{alb})$ and is an index of albumin movement across the filtration barrier relative to water. A σ_{alb} of zero indicates albumin movement at the same velocity as water such that P_{alb} is 1.0. When σ_{alb} is 1.0, albumin cannot cross the filtration barrier down its concentration gradient and so P_{alb} is zero.

Biochemical Analyses. Commercially available kits for sICAM-1 (Quantikine, sICAM-1 Immunoassay, R&D Systems, Minneapolis, MN) and MCP-1 (RayBioTech, Inc., Norcross, GA) were used for determining concentrations of these two cytokines in plasma and glomerular homogenates. Nephryn concentration was determined in urine *via* ELISA kit (Excoell,

Philadelphia, PA). Urinary protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA). Glomerular active TGF- β 1 concentrations were measured with the Quantikine mouse/rat/porcine/canine TGF- β 1 kit (R&D Systems, Minneapolis, MN). Total MMP activity was determined in the same supernatant as that for collagen assay using the SensoLyteTM 520 Generic MMP assay kit (Anaspec, San Jose, CA).

Western Blotting. Glomeruli were homogenized as described above with total protein determined by the Bradford method (Bio-Rad). Samples (20 μ g) were assessed by standard SDS-PAGE followed by blotting to PVDF membranes as previously described (Foster et al., 2009). All blots were incubated overnight with the following primary antibodies: integrin α_3 antibody (goat polyclonal IgG) or integrin β_1 antibody (rabbit polyclonal IgG). Both antibodies were purchased from Santa Cruz Biotechnology (catalog numbers sc-6592 and sc-8978, Santa Cruz, CA).. Blots were then developed for one hour using a secondary antibody tagged with infrared dye 680 (AlexaFluor 680 anti-rabbit or anti-mouse IgG; Invitrogen/Molecular Probes). To normalize all proteins, blots were then double-labeled by overnight incubation with monoclonal anti- β -actin antibody (Sigma, St. Louis, MO) and redeveloped for one hour with the secondary antibody tagged with infrared dye 800 (Rockland, Gilbertsville, PA). Densitometry was performed on the Odyssey Infrared Imaging System v3.0 (Li-Cor Biosciences, Lincoln, NE).

qRT-PCR. Total RNA concentration and purity were determined using NanoDrop ND-1000 Spectrophotometer (Thermo scientific, West Palm Beach, FL) *via* measuring absorbance at 260 nm (A_{260}) and the ratio of A_{260} to A_{280} , respectively. RNA (1 μ g) was reverse transcribed using the QuantiTect RT kit (Qiagen, Valencia, CA). A dilution of the resulting cDNA was used to quantify the relative content of mRNA by real-time PCR (StepOnePlusTM Real-Time PCR System, Applied Biosystems, Foster City, CA) using commercially available QuantiTect primer assays (Qiagen) to detect rat GAPDH, prepro-ET-1, ET_A, ET_B, nephrin, zonula occludens-1 (ZO-1) and podocin (catalog numbers. QT00199633, QT00371308, QT00182546, QT01084454, QT00189805, QT01615320 and QT00191233, respectively) with SYBR green as the fluorescent

probe. Fluorescence data were acquired at the end of extension. A melt analysis was run for all products to determine the specificity of the amplification. The cycle threshold (C_T) values for each gene were measured and calculated automatically by Applied Biosystems software. Expression of each target gene mRNA relative to GAPDH was calculated on the basis of the change in C_T , in which $\Delta C_T = C_{T,\text{target}} - C_{T,\text{GAPDH}}$, and normalized between the control group and corresponding treatment group and expressed as $-(\Delta\Delta C_T)$. With use of this method, an mRNA that is expressed at a greater level in the experimental than in the control group will have a negative $\Delta\Delta C_T$ value and a positive $-(\Delta\Delta C_T)$ value. The relative fold expression was calculated as $2^{-(\Delta\Delta C_T)}$.

Statistical Analyses. All data are presented as mean \pm SE. Differences between data obtained from sham, sham+ABT-627, sham +A-182086, HG, HG+ABT-627 and HG+A-182086 are compared using two-way ANOVA followed by Bonferroni post hoc tests. A $p < 0.05$ was considered statistically significant. Analyses were performed using GraphPad Prism Version 5.0 software (GraphPad Software Inc, La Jolla, CA).

Results

Metabolic Characteristics. As depicted in Table 1, after 6 weeks of hyperglycemia (referred to as pre-treatment), rats had significant lower body weights and elevated non-fasting glucose levels, food consumption, water intake and urine flow when compared with sham groups. One-week treatment with either ABT-627 or A-182086 did not change of any of these characteristics.

Proteinuria. In order to investigate the pathophysiological relevance of endothelin in incipient diabetic nephropathy, we examined the effects of relatively short-term (1-week) treatment with ET antagonists on urinary protein excretion in rats with established hyperglycemia. As illustrated in Figure 1A, after 6 weeks of hyperglycemia, rats had very high protein excretion rates (≈ 530 mg/day) when compared with sham groups (≈ 15 mg/day). Figure

1A depicts protein excretion over the course of the 1-week treatment period (week 6-7) in untreated/treated sham and hyperglycemic groups. ET antagonists had no effect on proteinuria in sham groups. After only one day of treatment, ABT-627 produced a significant decrease in proteinuria when compared with the corresponding values in untreated hyperglycemic rats (426 ± 13 versus 512 ± 15 mg/day). In contrast, A-182086 had no effect on proteinuria at day 1 (521 ± 15 versus 512 ± 15 mg/day). During the 1-week treatment, ABT-627 did not produce any additional decrease in proteinuria compared with day 1. However, A-182086 produced a gradual decrease in protein excretion level in hyperglycemic rats. On day 7, both ABT-627 and A-182086 treated hyperglycemic groups had similar decreases in proteinuria when compared with the hyperglycemic untreated group (HG+ABT-627; 384 ± 12 , HG+A-182086 416 ± 20 versus HG; 546 ± 22 mg/day).

P_{alb} ex vivo and in vitro. P_{alb} was increased to 0.83 after 7 weeks of hyperglycemia (Figure 1B). One-week treatment with both ABT-627 and A-182086 markedly reduced P_{alb} in hyperglycemic rats. Neither antagonist had any effect on P_{alb} in sham-treated rats. P_{alb} was closely correlated to proteinuria ($r=0.9959$, $p<0.0001$).

Podocyte markers. Nephlin, ZO-1 and podocin gene expression in isolated glomeruli, were significantly decreased at 7 weeks in hyperglycemic rats compared to sham, as quantified by real-time PCR. Treatment of hyperglycemic rats with ABT-627 significantly increased the expression of all these podocyte-marker genes. Surprisingly, ABT-627 also increased the gene expression of nephlin in sham rats. A-182086 significantly increased ZO-1 gene in HG+A-182086 group without any effect on the other two podocyte-marker genes (Figure 2A). Figure 2B illustrates the 24-hr nephlin excretion rate at the 7-week time point and was significantly increased in hyperglycemic rats compared to shams (61.7 ± 9.7 versus 11.6 ± 6.0 ng/day; $p<0.05$). Rats treated one-week with either ABT-627 or A-182086 restored urinary nephlin levels to sham levels (19.8 ± 5.0 and 18.6 ± 5.6 ng/day, respectively).

$\alpha_3\beta_1$ integrin expression. Under baseline conditions, a single band (132 kDa) was detected for both α_3 and β_1 integrins (Figure 3). Both α_3 and β_1 integrins expression was significantly down-regulated as a result of hyperglycemia ($p<0.05$). Treatment with ABT-627 or A-182086 resulted in a significant increase in the α_3 integrin when compared to hyperglycemic controls. However, neither ET antagonist affected the glomerular expression of β_1 integrin.

MMP activity and active TGF- β_1 content. Hyperglycemic rats displayed 122% and 207% increases in total glomerular MMPs (Figure 4A) and active TGF- β_1 (Figure 4B) content when compared with sham rats. Both ABT-627 and A-182086 significantly reduced total MMPs activity and restored them to sham levels. ABT-627, but not A-182086, prevented the increase in active TGF- β_1 content; however, the levels of active TGF- β_1 remained significantly higher in HG+A182086 when compared with levels from both sham and HG+ABT-627 rats.

Glomerular and systemic inflammation. ICAM-1 and MCP-1 are an important adhesion molecule and inflammatory chemokine, respectively, which have been documented to participate in the onset and development of diabetic nephropathy (Wu et al., 2006). Glomerular content (Figure 5A) and plasma concentrations (Figure 5B) of ICAM-1 and MCP-1 were increased significantly after 7 weeks of established hyperglycemia. Treatment of hyperglycemic rats for one week with ABT-627 completely abolished the increase in glomerular sICAM-1 and MCP-1 and plasma sICAM-1 concentration. A-182086 had no effect on the elevated levels of glomerular sICAM-1 and MCP-1 and plasma sICAM-1 concentration in hyperglycemic rats. Neither antagonist produced any change in plasma MCP-1 concentrations.

Glomerular expression of ET pathway genes. Seven weeks of hyperglycemia did not evoke any change in glomerular prepro-ET-1, ET_A, or ET_B mRNA levels when compared with their corresponding shams. However, 1-week treatment with ET_A or ET_{A/B} antagonist produced a significant reduction in glomerular expression of all ET pathway genes in hyperglycemic rats. In sham groups, treatment with A-182086, but not ABT-627, resulted in a significant decrease in prepro-ET-1 and ET_A gene expression, with no effect on ET_B expression (Figure 6).

DISCUSSION

This study provides further evidence that the endothelin pathway initiates a functional glomerular defect and injury in a type 1 diabetes animal model. An important new finding is that ET_A selective or mixed ET_{A/B} antagonists can produce rapid reversal of established hyperglycemia-induced changes in the glomerular filtration barrier. Our experimental protocol was designed to provide clinical relevance by determining whether proteinuria and increased glomerular permeability to albumin (P_{alb}) could be reversed even after diabetes was established. Using an *ex vivo* method that eliminates the influence of glomerular capillary pressure and flow dynamics, we demonstrated that the ability of ET_A or ET_{A/B} antagonists to improve glomerular permeability to albumin following hyperglycemia were similar. These data suggest that the ET_A receptor contributes to the glomerular defect seen in diabetes, while the ET_B receptor has little role, if any. These findings are consistent with direct *in vitro* measurements that ET_A, but not ET_B receptor antagonists to improve P_{alb} in this model (Saleh et al., 2010a). We also observed that ET_A selective antagonism had a profound anti-inflammatory effect in the diabetic kidney in contrast to the non-selective ET_{A/B} antagonist. Together, these findings support the potential therapeutic advantage of selective ET_A *versus* combined ET_{A/B} antagonists.

We observed a difference in the rate of proteinuria reduction between the two antagonists. In a preclinical study conducted by our group, Sasser *et al.* showed that albuminuria increases during the first ten weeks of STZ-induced hyperglycemia and the ET_A antagonist, ABT-627, completely ameliorated albuminuria (Sasser et al., 2007). These data are consistent with those of Hocher and colleagues who investigated both a selective ET_A antagonist (LU 135252) and a mixed ET_{A/B} (LU 224332) antagonist (Hocher et al., 2001). In contrast, Gross *et al.* observed that the ET_A selective antagonist, LU 135252 (darusentan), prevented renal histological alterations in STZ-diabetic rats, and significantly decreased urinary ET-1 excretion, but had no effect on albuminuria (Gross et al., 2003) although the reason for

these different findings is unclear. Nonetheless, our current findings demonstrating a differential effect of the selective versus combined antagonists would suggest that a lack of selectivity could reduce efficacy in terms of reducing proteinuria.

In previous studies using selective ET_A antagonists, blood pressure was significantly reduced along with albuminuria (Sasser et al., 2007; Gagliardini et al., 2009) and so it has been impossible to distinguish the benefits of reduced glomerular capillary pressure versus direct effects on permeability. Preclinical studies in subjects with non-diabetic chronic kidney disease showed a blood pressure-independent reduction in proteinuria in response to acute selective ET_A antagonist administration (Dhaun et al., 2009). These studies support the hypothesis that ET_A antagonism can reverse renal dysfunction independent of blood pressure and renal hemodynamics and suggest a direct effect on the glomerular filtration barrier structure.

The specific independent role of ET_B receptors in modulating proteinuria and glomerular permeability cannot be identified using ET_B selective antagonists *in vivo* because pharmacological blockade of ET_B receptors results in hypertension via increased ET_A activity by reduced ET-1 clearance (Pollock and Pollock, 2001). The current study demonstrated a more rapid and efficient level of reduction in proteinuria with the ET_A selective blocker compared to the mixed antagonist. This is consistent with the hypothesis that the ET_B receptor functions to oppose the direct role of the ET_A receptor in terms of influencing the integrity of glomerular permeability and proteinuria.

The significant improvement of proteinuria progression produced by both types of antagonists was accompanied by marked restoration of glomerular filtration barrier components and function. One of the molecular mechanisms that lead to proteinuria in diabetic nephropathy is the down-regulation of podocyte- and filtration-slit molecule expression. We observed that hyperglycemia was associated with reduced expression of podocyte foot-process proteins, namely nephrin, zonula occludens-1 (ZO-1) and podocin in isolated glomeruli, with an increase in nephrin urinary excretion rate. We have previously shown that chronic ET-1 infusion in

normoglycemic rats resulted in an ET_A-dependent increase in nephrin excretion rate (Saleh et al., 2010b). In addition, serum from pre-eclamptic women contains a factor or a group of factors that stimulate the production of ET-1 from glomerular endothelial cells triggering nephrin loss from podocytes (Collino et al., 2008). Loss of podocyte attachment to glomerular basement membrane and subsequent podocyturia may result from down-regulation of the glomerular basement membrane integrin, $\alpha 3\beta 1$ (Korhonen et al., 1990; Adler, 1992). Our data showed that both ABT-627 and A-182086 restore integrin $\alpha 3$ protein but not the $\beta 1$ subunit.

We observed that both ABT-627 and A-182086 significantly reduced the diabetes-induced increases in active glomerular TGF- β , but the mixed antagonist was less effective. We have previously reported that this model of hyperglycemia does not have any overt fibrosis as detected by histological staining (Sasser et al., 2007) and so we have relied on early markers of fibrosis such as TGF- β . Inhibition of TGF- β prevents fibrosis under experimental diabetic conditions (Sharma et al., 1996; Chen et al., 2003). Several previous studies reported anti-fibrotic properties of long-term treatment with either selective ET_A or non-selective ET_{A/B} antagonists (Nakamura et al., 1995; Hocher et al., 2001). We previously reported that ABT-627 prevents increases in TGF β in this model (Sasser et al., 2007). Prior evidence also suggests that ET_B receptors may possess anti-fibrotic action *via* endothelial NO synthase derived NO signaling following inhibition of TGF- β (Dreieicher et al., 2009) and so the observation that the combined ET_{A/B} antagonist was less effective was perhaps predictable. However, comparison of the two types of antagonists on measures of glomerular fibrosis in our studies was somewhat inconclusive since both ET_A and ET_{A/B} antagonists normalized activity of glomerular metalloproteinases (MMPs). Over-expression of glomerular TGF- $\beta 1$ in diabetes is associated with increased activity of MMPs, mainly gelatinases (MMP-2 and MMP-9) that contribute to glomerular basement membrane thickening (Krag et al., 2007).

We recently reported that chronic ET-1 infusion in the rat increases glomerular and renal inflammation independent of hypertension (Saleh et al., 2010a). Furthermore, we have also

reported that selective ET_A blockade in the STZ-diabetic rat prevents the increase in early markers of inflammation, ICAM-1 and MCP-1 (Saleh et al., 2010b). The current study extends these findings to demonstrate that we could reverse the diabetes-induced increase in inflammation, but only with an ET_A selective antagonist and not the combined blocker. These findings suggest that the ET_B receptor functions as an anti-inflammatory factor and that targeting the endothelin pathway for treatment of diabetes should be restricted to ET_A selective compounds.

The general beneficial effects of both ET_A selective and combined receptor antagonism have been demonstrated in a wide variety of studies including our own. However, in terms of systemic and glomerular inflammation, ET_B receptor antagonism appears to counteract the benefit of ET_A blockade. ET_B receptors are involved in the synthesis of the vasodilator NO by endothelial cells, through activation by either ET-1 or ET-3 (Namiki et al., 1992). Inhibition of NO has also been associated with an increase in leukocyte adhesion to mesenteric venules, reflecting that the increases in monocyte/macrophage infiltration are due to decrease in NO production (Dubey et al., 1996). Another possible explanation for the non-beneficial effect of ET_{A/B} blockade is the involvement of the ET_B receptor in the clearance of ET (Fukuroda et al., 1994). The non-selective NOS inhibitor, L-NAME, exacerbates liver and kidney injury accompanied with increased leukocyte infiltration in animal models of endotoxemia (Saetre et al., 2001). Part of these effects has been attributed to increased adhesion molecule expression (ICAM-1, P-selectin) after inhibition of endogenous NO production. In addition, mice lacking endothelial NO synthase aggravates anti-GBM glomerulonephritis indicating a protective role of endogenous NO during renal inflammation (Heeringa et al., 2000).

We did not observe any changes in mRNA expression of glomerular ET-1, ET_A and ET_B receptors after 7 weeks of hyperglycemia; however, treatment with an ET_A or ET_{A/B} antagonist produced marked decreases in glomerular expression of each of these genes. A previous study from another lab reported that glomerular ET-1 mRNA levels were increased in kidneys from

STZ-treated rats, whereas the mRNA levels for ET_A and ET_B receptors remained unchanged (Fukui et al., 1993). Of course, mRNA levels do not always reflect protein expression and function. On the contrary, Shin et al. reported that moderate hyperglycemia in diabetic rats is associated with a reduction in renal ET-1 mRNA levels early after the induction of diabetes, however, plasma ET-1 levels were not affected (Shin et al., 1995). These data demonstrated that the intrarenal ET-1 system may be affected independently of the systemic ET-1 system.

Factors such as hyperglycemia (Yamauchi et al., 1990), shear stress (Hochoer et al., 1997) due to glomerular hyperfiltration, and urine flow (Hochoer et al., 1998) have been shown to stimulate ET-1 synthesis or release. Glucose stimulates also ET-1 synthesis *in vitro*. Studies examining systemic and intrarenal ET-1 in diabetes have yielded conflicting results. Plasma ET-1 levels have been described as either undetectable (Takahashi et al., 1991), unchanged (Shin et al., 1995), enhanced (Nakamura et al., 1995; Hochoer et al., 1998) or suppressed (Hu et al., 1993), and renal ET-1 levels have been shown to be unchanged (Takahashi et al., 1991), enhanced (Fukui et al., 1993), or reduced (Shin et al., 1995). Accordingly, it is suggested that these changes are caused by differences in the diabetic state, and these differences may be due to the degree of hyperglycemia, renal localization, or varying duration of diabetes. Glomerular ET-1 mRNA levels have been reported to increase with progression of diabetic nephropathy in STZ-diabetic rats, whereas the mRNA levels for ET_A and ET_B do not change in diabetes (Fukui et al., 1993). Of course, mRNA levels do not always reflect protein expression and function. On the contrary, early after the induction of diabetes, renal ET-1 mRNA and protein expression have been reported to be reduced and plasma ET-1 levels unchanged, implying that the intrarenal ET-1 system may be affected independently of the systemic ET-1 system (Shin et al., 1995).

From a clinical perspective, both ET_A and ET_{A/B} antagonist would appear to have beneficial effects for reducing proteinuria in the long-term. However, the ET_A selective antagonist was significantly more effective than the combined antagonist at reducing TGF-β

and inflammatory mediators in the model of type 1 diabetes. Our data suggest an anti-fibrotic and anti-inflammatory role for the ET_B receptor, thus providing rationale for use of ET_A selective rather than mixed $ET_{A/B}$ antagonists for treatment of diabetic nephropathy and perhaps other forms of proteinuric renal disease and inflammation.

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Authorship Contributions

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Footnote

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Figure Legends

Figure 1. (A and B) Proteinuria and glomerular permeability to albumin (P_{alb}) increase significantly after 6 week of hyperglycemic induction. Both ET receptor antagonists significantly reduced proteinuria and P_{alb} after 1 week treatment.

Figure 2. (A) After 7-week of hyperglycemia, gene expression of nephrin, ZO-1 and podocin in glomeruli were significantly attenuated. Both ET-1 antagonists significantly increased the gene expression of these proteins at different levels in HG treated groups. * $p < 0.05$ versus sham, † $p < 0.05$ versus HG and § $p < 0.05$ versus HG+A-182086. (B) Seven-week hyperglycemic rats exhibited significant increases in nephrin excretion when compared with sham rats. Both ET receptor antagonists reduced nephrinuria to its control non-hyperglycemic levels. * $p < 0.05$ versus sham and † $p < 0.05$ versus HG.

Figure 3. Seven-week hyperglycemia decreased the expression of both α_3 and β_1 integrins which attaches the podocytes to the glomerular basement membrane (GBM). Both ET receptor antagonists significantly increased the expression of α_3 integrin when compared with the control hyperglycemic glomerular levels, however, neither ET receptor antagonist had an effect on the expression of β_1 integrin. * $p < 0.05$ versus sham and † $p < 0.05$ versus HG.

Figure 4. Hyperglycemia induction increased both (A) total MMPs activity and (B) active TGF- β_1 in glomeruli after 7 weeks of established hyperglycemia. Both ET receptor antagonists significantly reduced these changes. However, the attenuation effect of the mixed antagonist on active TGF- β_1 remained significantly higher compared to ET_A receptor selective blockade. * $p < 0.05$ versus sham, † $p < 0.05$ versus HG and § $p < 0.05$ versus HG+ABT-627.

Figure 5. (A) Glomerular sICAM-1 and MCP-1 were significantly increased after seven weeks of established hyperglycemia. Selective ET_A receptor antagonism significantly reduced the elevated inflammatory protein levels. Non-selective ET receptor antagonists did not have any effect on glomerular sICAM-1 and MCP-1 indicating the counteracting effects of the ET_A receptor and ET_B receptor in hyperglycemia-induced inflammation. (B) Plasma concentrations of inflammatory molecules, sICAM-1 and MCP-1, were significantly increased after seven weeks of established hyperglycemia. Selective ET_A receptor antagonism significantly reduced the elevated sICAM-1 but not MCP-1 in HG rats. Non-selective ET receptor antagonists did not have any effect on plasma sICAM-1 (or MCP-1) suggesting a counteracting effect of the ET_A receptor and ET_B receptor in hyperglycemia-induced inflammation. * $p < 0.05$ versus sham, † $p < 0.05$ versus HG and § $p < 0.05$ versus HG+ABT-627.

Figure 6. Glomerular gene expression of ET-1, ET_A receptor and ET_B receptor did not change after seven weeks of hyperglycemia. Treatment with either antagonist, ABT-627 or A-182086, significantly suppressed gene expression of all ET-1 system components: ET-1, ET_A receptor and ET_B receptor. However, the one exception was that A-182086 significantly decreased ET-1 and ET_A receptor gene expression when compared to the sham. * $p < 0.05$ versus sham and † $p < 0.05$ versus HG.

Table 1. Characteristics of experimental animals after 6 weeks of STZ-induced diabetes (pre-treatment) and after 1-week of treatment with ET receptor antagonists (post-treatment).

	Body weight (g)		Glycemia (mg/dL)		Food intake (g)		Water intake (mL)		Urine flow (mL)	
Measurement	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Sham	369±1	380±1	102±8	109±5	25±2	33±2	32±1	28±3	14±1	18±1
Sham+ ABT-672	369±1	382±2	95±4	106±3	27±1	30±2	34±3	32±2	15±1	20±1
Sham+ A-182086	370±2	386±2	98±1	108±2	25±1	28±2	31±3	29±3	14±1	20±1
HG	320±4*	326±3*	569±9*	566±16*	48±2*	54±2*	240±3*	235±3*	220±3*	222±4*
HG+ ABT-627	319±3	323±2	567±11	594±4	52±2	53±2	247±3	237±3	221±4	213±2
HG+ A-182086	312±2	317±2	577±6	589±3	56±2	51±2	246±4	234±3	205±4	213±4

Data are means ± SEM (n=6 in Sham and Sham-treated groups and n=10 in HG (hyperglycemic) and HG-treated groups)

Data derived from 24 h urine collections in metabolic cages

* $p < 0.05$ versus Sham group

Figure 1

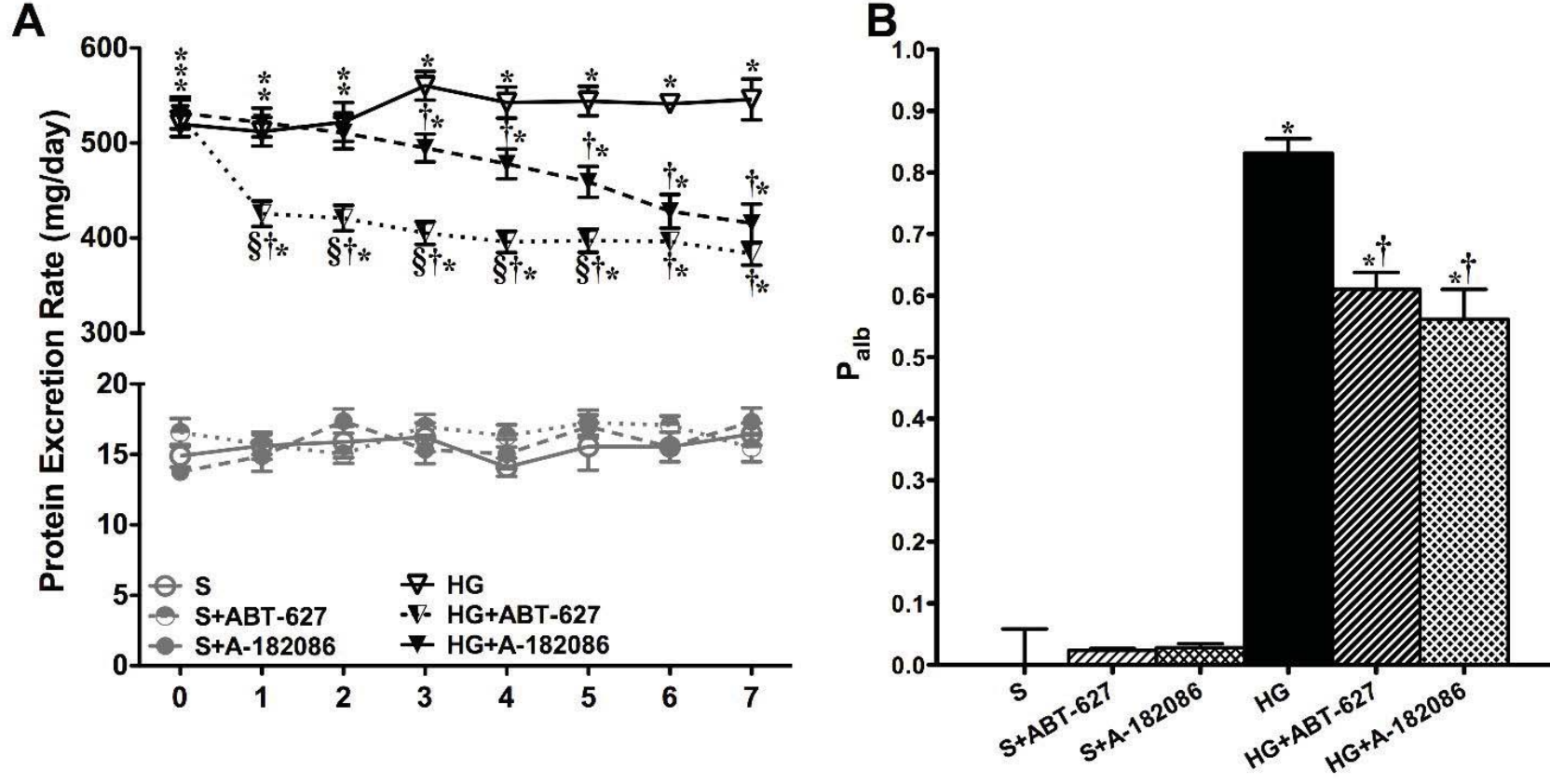


Figure 2

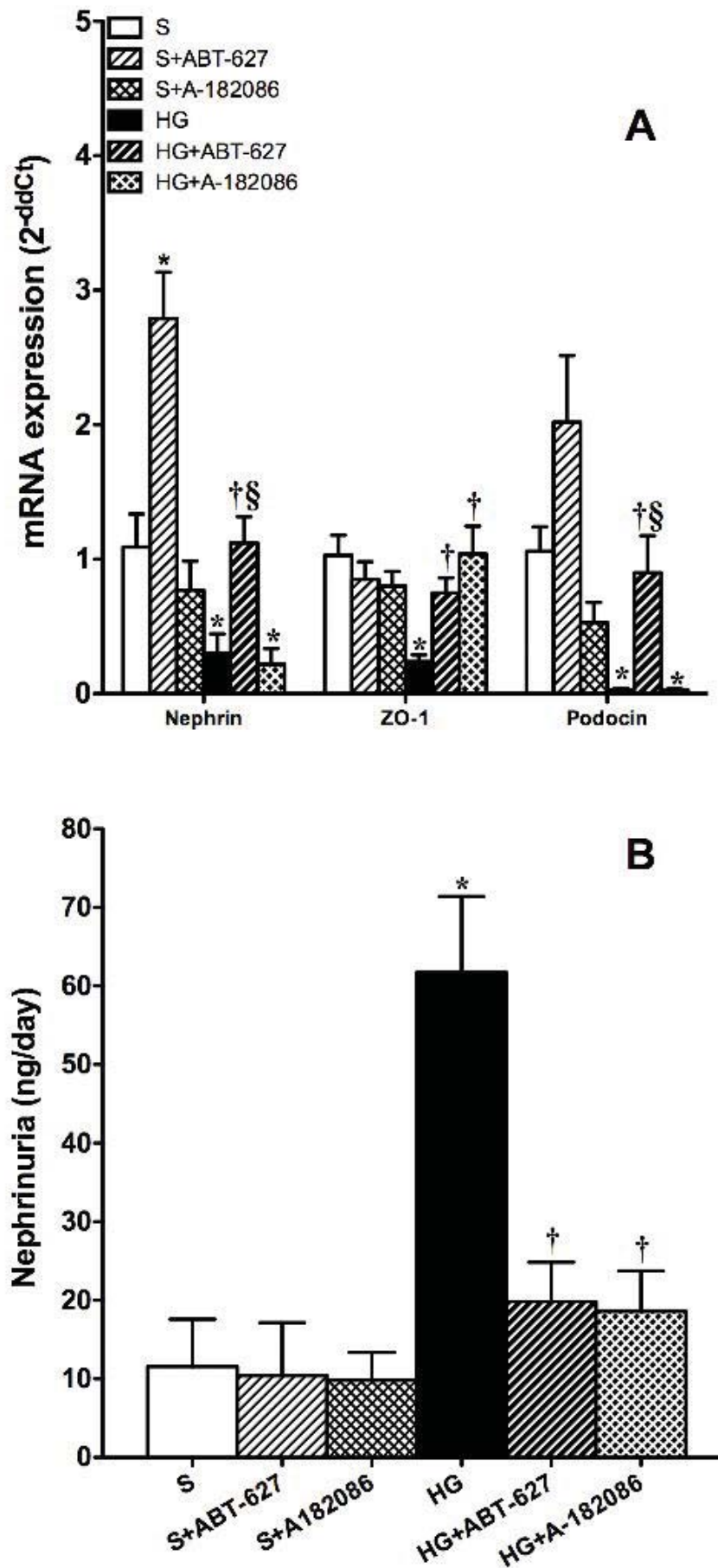


Figure 3

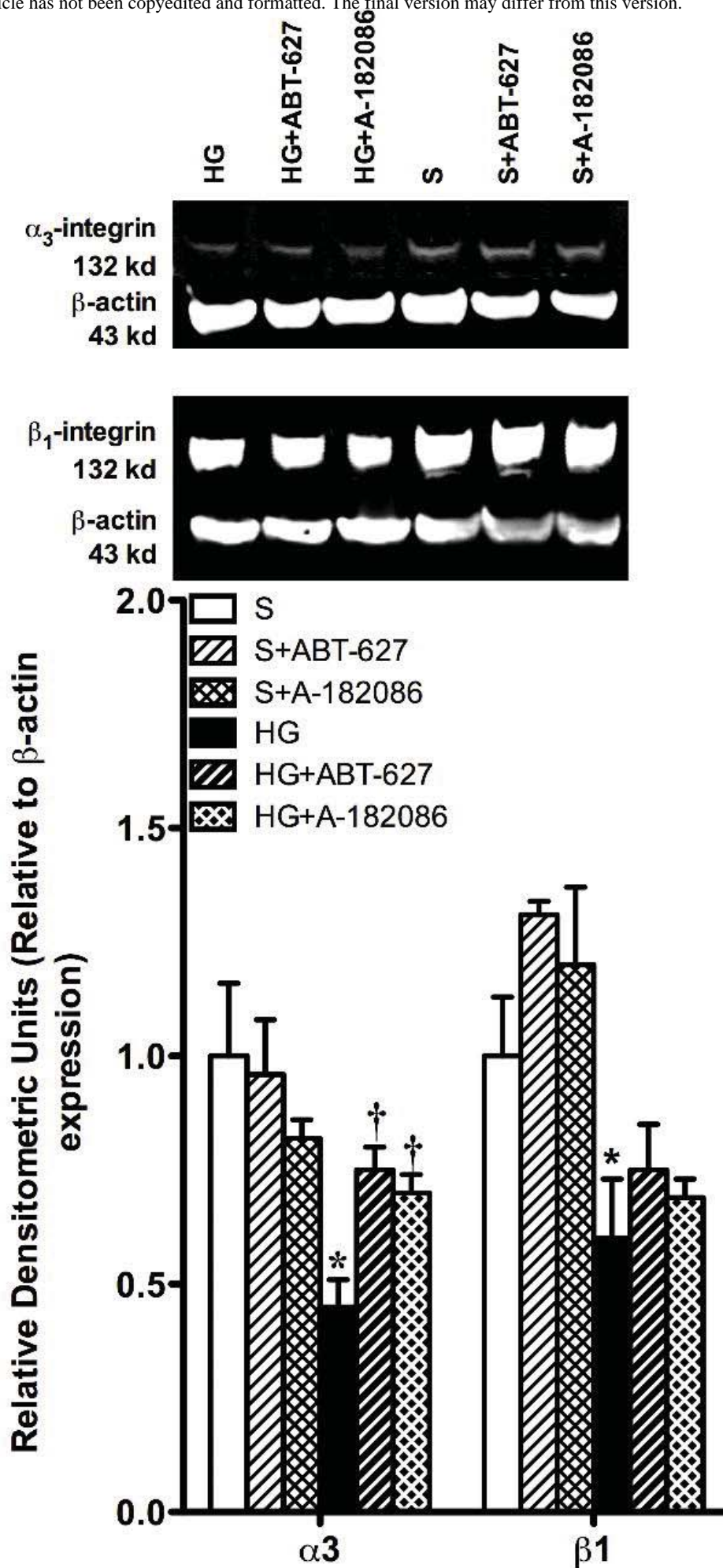


Figure 4

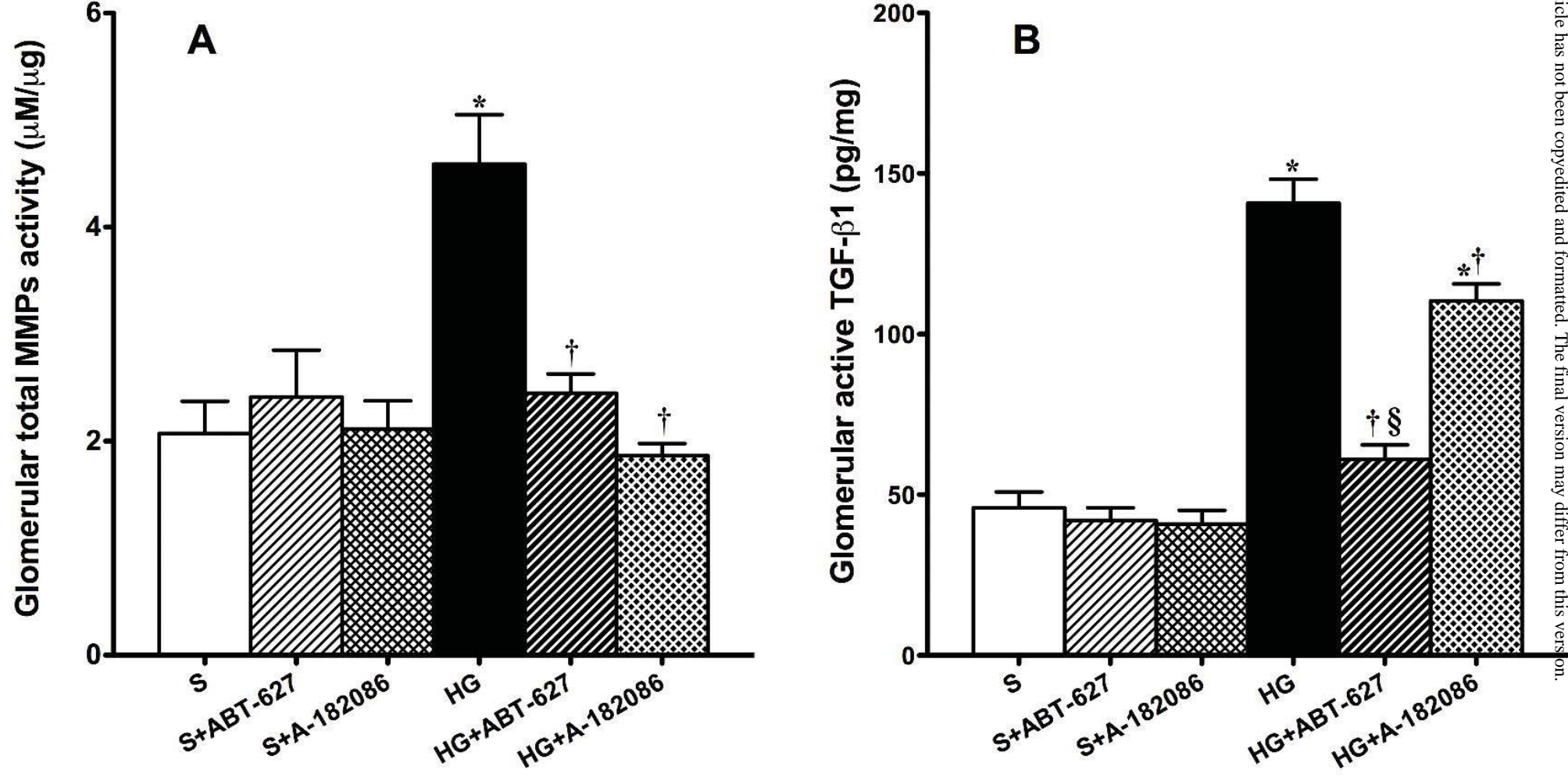


Figure 5

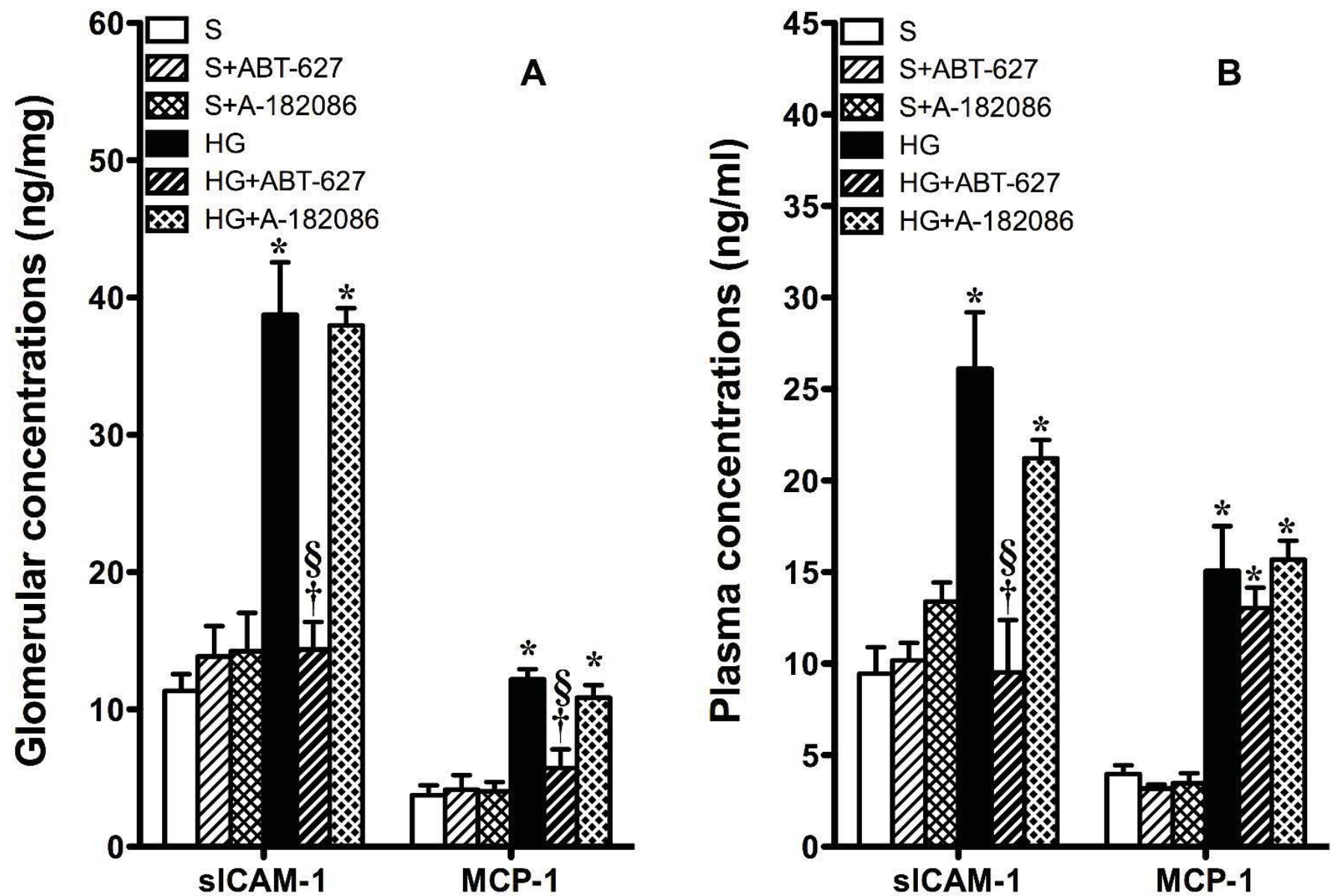


Figure 6

