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Renal, retinal and cardiac changes in type 2 diabetes are attenuated by macitentan, a dual endothelin receptor antagonist

S. Sen [a,1], S. Chen [a,1], B. Feng [a], M. Iglarz [b], S. Chakrabarti [a,*]

[a] Dept. of Pathology, University of Western Ontario, Canada
[b] Drug discovery Dept. Actelion Pharmaceuticals Ltd., Switzerland

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ABSTRACT

Aims: Diabetes is known to cause alteration of the endothelin (ET) system. We have previously demonstrated that ETs regulate augmented production of extracellular matrix proteins causing structural alterations in type 1 diabetes. Here we investigated the effects of macitentan, an orally-active, tissue-targeting dual ET receptor antagonist on chronic complications in type 2 diabetes.
Main methods: db/db mice and their age- and sex-matched controls were examined after 2 and 4 months of diabetes. Groups of diabetic animals were treated with oral macitentan (25 mg/kg/day). The animals were monitored with respect to body weight and blood glucose. Urine analyses were performed for albumin. Cardiac hemodynamic studies were carried out. Renal, cardiac and retinal tissues were analyzed for ET-1, transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), fibronectin (FN), extradomain B containing FN (EDB^+FN) and collagen α-I (IV) mRNA. Cardiac atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were measured. Protein expressions were measured by ELISA and Western blot. Microscopic analyses were performed in the kidneys.
Key findings: Diabetic animals showed hyperglycemia, increased urinary albumin and augmented serum creatinine levels. Diabetes caused increased renal, cardiac and retinal ET-1, TGF-β1, VEGF, FN, EDB^+FN, collagen α-I(IV) mRNA expression along with increased FN and collagen protein and NF-κB activation. Diabetic mice also demonstrated mesangial expansion, cardiac dysfunction and increased expression of ANP and BNP. Treatment with macitentan attenuated such abnormalities.
Significance: These experiments confirmed that ET system plays a significant role in the pathogenesis of chronic complications in type 2 diabetes. Such diabetes induced changes can be reduced macitentan therapy.

Introduction

Chronic diabetic complications involving the kidneys, retina, heart and large blood vessels are major causes of mortality and morbidity in the diabetic population (Zimmet et al., 2001; UKPDS, 1998). Diabetes is the leading cause of end stage renal failure in the western world (UKPDS, 1998; Bell, 1995; Brownlee, 2001). Clinically patients develop microalbuminuria, glomerular hyperfiltration followed by heavy proteinuria and reduced glomerular filtration rate leading to renal failure (Breyer et al., 1996). Pathological features of diabetic nephropathy include thickening of glomerular capillary basement membrane (BM), mesangial matrix expansion, and tubulointerstitial fibrosis. Diabetic retinopathy is one of the leading causes of blindness, which causes retinal permeability alteration, macular edema and neovascularization (Cai and Boulton, 2002). In early retinal microangiopathy, increased production of extracellular matrix (ECM) protein is a characteristic feature (Brownlee, 2001; Cai and Boulton, 2002; Chen et al., 2003a). Similarly, patients with diabetic cardiomyopathy show alterations of cardiac contractile functions. Structurally, focal myocardial sclerosis and microvascular BM thickening due to increased ECM protein production are characteristic features (Bell, 1995; Chen et al., 2003a).

Endothelin (ET) plays a key role in several chronic diabetic complications by modulation of blood flow and ECM protein production. ETs are produced by several cell types (Yanagisawa et al., 1988; Levin, 1995; Houde et al., 2011; Gagliardini et al., 2011). The ET isoforms, ET-1, ET-2, and ET-3, are encoded by distinct genes. Several cytokines have been shown to regulate ET expression (Yanagisawa et al., 1988; Levin, 1995; Gagliardini et al., 2011; Malek et al., 1999; Benatti et al., 1994; Emori et al., 1992; Kurihara et al., 1989; Kohno et al., 1992). In chronic diabetic complications, regulatory interaction of ETs with other vasoactive factors has been demonstrated (Khan and Chakrabarti, 2003; Chen et al., 2000). In the diabetic rat kidneys, increased ET-1 mRNA and renal ET-1 clearance has been

* Corresponding author at: Department of Pathology, Rm 4033 Dental Sciences Building, University of Western Ontario, London, Ontario, Canada. Tel.: +1 519 685 8500x36350; fax: +1 519 661 3370.
E-mail address: Subrata.Chakrabarti@lhsc.on.ca (S. Chakrabarti).
[1] Both contributed equally in this research.

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demonstrated in association with proteinuria (Turner et al., 1997; Chen et al., 2002; Rabelink and Kohan, 2011). Long-term consequences of ET peptides involve cellular changes requiring differential gene expression (Levin, 1995; Nakamura et al., 1995; Rubanyi and Polokoff, 1994). It has been demonstrated that diabetes-induced increased expression of ECM proteins and growth factors in the kidneys can be prevented by treatment with an ET_A receptor antagonist (Nakamura et al., 1995). We have shown that diabetes leads to upregulation of ET-dependent ECM protein synthesis in the kidneys, retina and heart (Chen et al., 2000) and that ET-1 regulates ECM protein fibronectin (FN) expression through NF-κB activation. We have further showed that ET blockade prevents diabetes-induced increased ECM matrix production, retinal capillary and glomerular BM thickening, mesangial expansion, and focal fibrosis in the heart (Chen et al., 2003b; Evans et al., 2000). However, whether ET blockade is important in preventing chronic diabetic complications in type 2 diabetes needs exploration. Furthermore efficacy of newly developed compound macitentan has not been investigated in this scenario.

Macitentan, also called Actelion-1 or ACT-064992[*N*-[5-(4-bromophenyl)-6-(2-(5-bromopyrimidin-2-yloxy)ethoxy)-pyrimidin-4-yl]-*N*′-propylaminosulfamide], is a tissue targeting dual ET receptor antagonist. It has been demonstrated that macitentan and its metabolite antagonize specific binding of ET-1 on the cell membranes over expressing either ET_A or ET_B (Iglarz et al., 2008; Sidharta et al., 2011; Raja, 2010). Pharmacokinetic experiments have demonstrated that macitentan and its metabolites have a long half-life and increased binding to receptors than existing ET receptor antagonists (Iglarz et al., 2008; Kummer et al., 2009).

Here, we investigated the preventive effects of macitentan on the development of biochemical, functional and structural changes of diabetic nephropathy, retinopathy and cardiomyopathy in db/db mice, a model of type 2 diabetes. db/db mice have a point mutation in the cytoplasmic domain of the leptin receptor which is abundantly expressed in the hypothalamus and develop features characteristic of several chronic diabetic complications (Wang et al., 2011; Kanda et al., 2009).

Materials and methods

Animals

All animals were cared for according to the Guiding Principle in the Care and Use of Animals. All experiments were approved by the University of Western Ontario council on animal care committee.

Male db/db ($Lepr^{db}$, DBA/J) mice and age and sex-matched controls (27–32 g) were purchased from Jackson Laboratories, USA. Randomly selected diabetic animals were monitored for either 2 months or for 4 months after onset of diabetes. Groups (n = 7/group, based on our previous studies) of the diabetic mice were subjected to oral macitentan treatment for the same period (25 mg/kg/day, food admix). The animals were monitored through assessment of body weight and blood glucose (Glucometer, Free Style Freedom Lite Inc.).

The animals were sacrificed after the follow-up period. Retina, portion of left ventricular myocardium and portion of renal cortical tissues were snap-frozen. The remaining renal cortical tissues were fixed in 10% neutral-buffered formalin for paraffin embedding and subsequent histological analysis.

Cardiac functional studies

In vivo hemodynamic measurements were performed under anesthesia with sodium pentobarbital (40–50 mg/kg, ip) immediately before sacrifice, as previously described (Feng et al., 2001; Peng et al., 2003; Radovits et al., 2009). Briefly, a catheter (3-Fr, Atom Medical) connected to a pressure amplifier (7P1G, Grass Medical Instruments) was inserted into the right carotid artery and advanced into the LV to measure simultaneous changes in pressure. A catheter (PE-50, Clay Adams) was also inserted into the femoral artery to measure systemic blood pressure. The first derivative of LV pressure was simultaneously monitored by using a Grass 7P20C differentiator amplifier. Heart rate was obtained from the LV pressure recordings by using a Grass 7P44B tachometer.

Measurement of renal parameters

Urinary albumin levels were measured after 2 and 4 months of the onset of diabetes by ELISA using microalbumin estimation kit (Albuwell, Philadelphia, USA) and expressed as μg/day excreted. Serum creatinine level was estimated by standard alkaline picrate method using the creatinine estimation kit (DetectX, Ann Arbor, MI, USA) and expressed as mg/dl of serum.

RNA extraction and cDNA synthesis

RNA was isolated from mice tissues as previously described (Nakamura et al., 1995; Malek, 1994). First-strand cDNA was made using Superscript-II (Invitrogen, Burlington, ON, Canada) system. The resulting products were stored at −20 °C.

Real time RT-PCR

Real time RT-PCR was performed in LightCycler™ (Roche Diagnostics Canada, Laval, Quebec, Canada) to quantify the mRNA expression of FN, extradomain B positive splice variant (EDB^+FN), collagen α-I(IV), vascular endothelial growth factor (VEGF), ET-1 and transforming growth factor-β1 (TGF-β1) as described previously (Chen et al., 2003a, 2003b). Primers were custom synthesized from Sigma-Genosys. To optimize the amplification of the genes, melting curve analysis (MCA) was used to determine the melting temperature (Tm) of specific products and primer dimers. According to the Tm value of specific products for respective genes, an additional step (signal acquisition step, 2–3 °C below Tm) was added after the elongation phase of RT-PCR. mRNAs were quantified with the standard curve method as previously described (Chen et al., 2003b). Standard curves for all transcripts were constructed using different amounts of standard template. The cycle number at the crossing point (C_p), which produced a significantly different fluorescence signal from baseline, was used to compute the relative concentration of target genes from the standard curves. The data were normalized to β-actin mRNA or 18s rRNA to account for differences in reverse transcription efficiencies and amount of template in the reaction mixtures and expressed relative to control groups.

Protein extraction and ELISA

Tissues were washed with cold phosphate buffered saline (PBS), homogenized and treated with lysis buffer (50 mmol/l HEPES, pH 7.6, 150 mmol/l NaCl, 50 μmol/l NaF, 2 mmol/l EDTA, 1 mmol/l sodium vanadate, 1% NP-40, and 2 mmol/l phenylmethylsulfonyl fluoride). The total protein concentration was measured using BCA™ protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The concentrations of all samples were adjusted to 500 ng/μl before performing ELISA. We performed FN (Kamiya Biomedicals, WA, USA) and VEGF (Invitrogen, Canada) protein measurement by ELISA following the manufacturer's protocols. FN and VEGF protein levels in the cell lysates are expressed as ng/500 ng of total protein and pg/μg of total protein respectively.

Western blotting

Twenty micrograms per lane of cellular proteins was resolved by 6–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Table 1
Clinical parameters of diabetic mice with or without macitentan treatment.

Groups	Body weight (g)	Blood glucose (mmol/l)	Urine volume range (ml)
Baseline	29.1 ± 1.65	7.88 ± 0.535	2.0 ± 0.16
2 months			
C	34.0 ± 2.5	8.86 ± 2.7	2.1 ± 0.15
D	44.4 ± 2.5*	29.1 ± 2.5*	5.72 ± 0.24*
DM	46.0 ± 2.6*	26.5 ± 1.7*	4.22 ± 0.24*
4 months			
C	36.0 ± 2.5	8.8 ± 2.7	2.12 ± 0.13
D	48.4 ± 2.1*	30.1 ± 2.5*	5.87 ± 0.24*
DM	47.2 ± 2.5*	32.2 ± 0.4*	3.9 ± 0.5*

C = control, D = diabetic, DM = macitentan treated diabetic.
* $P<0.05$ vs. C.

and analyzed by western blotting using collagen α-I(IV) and β-actin antibody (Santa Cruz Biotechnology). The signals were detected with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and developed with the chemiluminescent substrate (Amersham Pharmacia Biotechnology, Amersham, UK). The blots were analyzed by densitometry.

Nuclear protein extraction and NF-κB assay

Nuclear protein was isolated from the kidneys as described elsewhere, with some modifications (Chen et al., 2003a, 2003b). This was not performed in other tissues due to lack of available material. Tissues were suspended in 0.4 ml of cold *buffer A* [10 mmol/l HEPES, pH 7.9, 10 mmol/l KCl, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 1 mmol/l 1,4-dithiothreitol (DTT), and 0.5 mmol/l PMSF] by gentle pipetting. Twenty-five microliters of a 10% Igepal CA-630 was added, and homogenates after vortexing were centrifuged (10,000 *g* for 30 s). The nuclear pellet was resuspended in 50 μl of ice-cold buffer C (20 mmol/l HEPES, pH 7.9, 0.4 mol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DTT, and 1 mmol/l PMSF), and the tube was vigorously

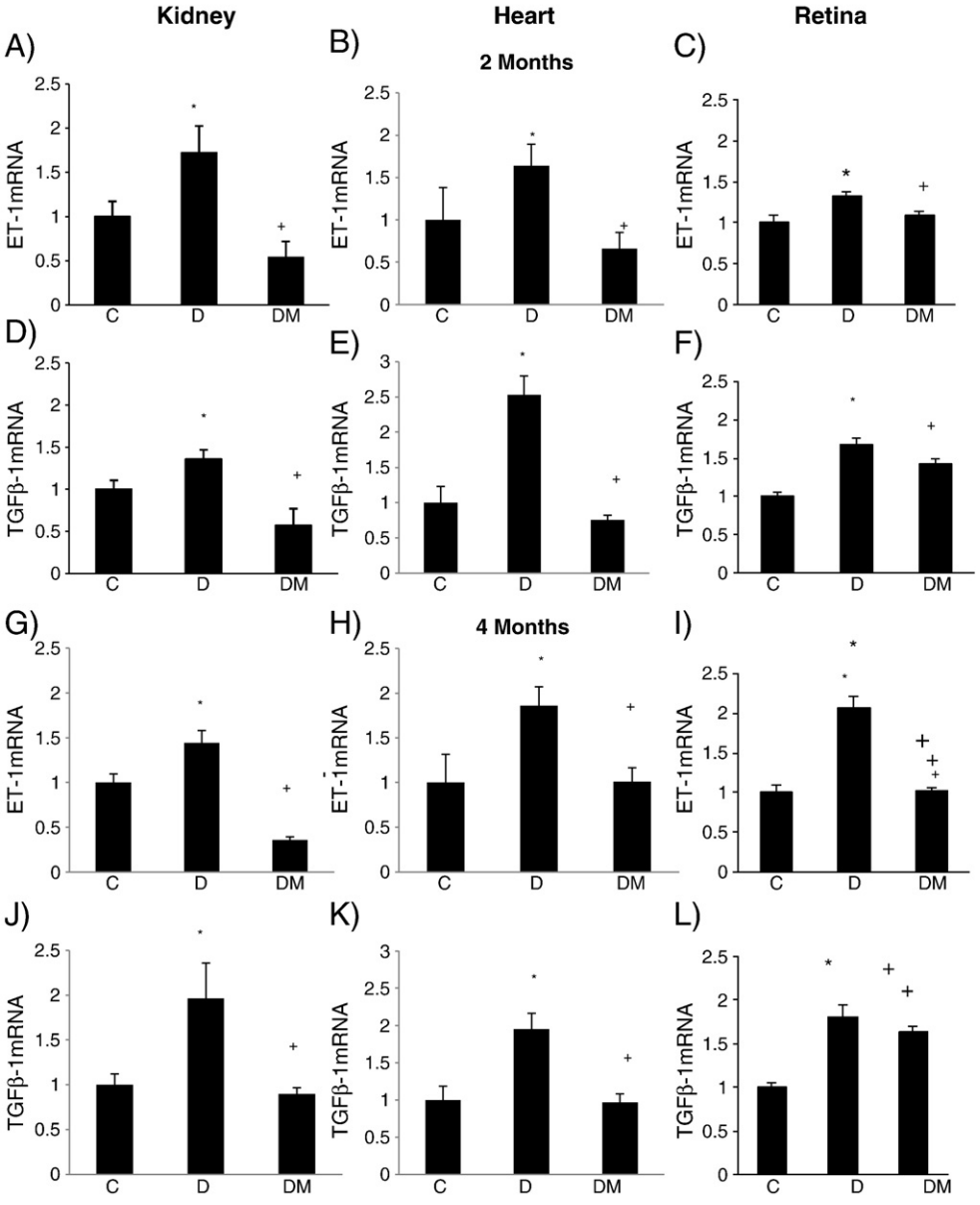

Fig. 1. qRT-PCR analysis of ET-1 (A–C, G–I) and TGF-β1 (D–F and J–L) mRNAs expression in the kidneys (A, D, G, J), hearts (B, E, H, K) and retinas (C, F, I, L) demonstrated diabetes-induced upregulation of these transcripts in all examined organs, both after 2 and 4 months of diabetes. Furthermore such upregulations were prevented by macitentan treatment. [C = control, D = diabetic, DM = diabetic on macitentan treatment. mRNA levels are expressed as a ratio to β-actin mRNA, and normalized to controls, * = significantly different from C, + = significantly different from D].

rocked at 4 °C for 15 min on a shaking platform. The nuclear extract was centrifuged at 4 °C (15,000 *g* for 5 min), and the supernatant was frozen at −70 °C. The protein concentrations were measured using the BCA protein assay, with bovine serum albumin as a standard (Pierce, IL). NF-κB (p65) protein was estimated in the nuclear extracts of all by ELISA following the standard manufacturer's protocol (TransAM transcription assay kit, CA, USA).

Histological analysis

Formalin-fixed tissues embedded in paraffin were sectioned at 5 μm thickness on positively charged slides. The sections were stained with hematoxylin and eosin and periodic acid-Schiff (PAS) stain.

Statistical analysis

The data are expressed as mean ± standard error of the mean. Statistical significance was determined by analysis of variance (ANOVA) followed by the Bonferroni–Dunn test. Differences were considered to be statistically significant at values of $P<0.05$.

Results

Clinical monitoring

Diabetic dysmetabolism was monitored through evaluating body weight gain and reducing sugar levels in the blood. The diabetic db/db mice showed significantly increased body weight gain and hyperglycemia compared to the control mice. Diabetic animals further showed polyuria. No effects of macitentan treatment were seen on these parameters (Table 1).

Macitentan treatment prevented increased production of vasoactive and fibrogenic factors in type 2 diabetes

Increased production of vasoactive factors and fibrogenic factors is characteristic features of all chronic diabetic complications. Previous studies from our and from other laboratories have shown increase in ET-1 and TGF-β1 are two important mediators of such process. Hence in our first set of studies we focused on these factors. Diabetic animals exhibited a significant increase in ET-1 mRNA expression

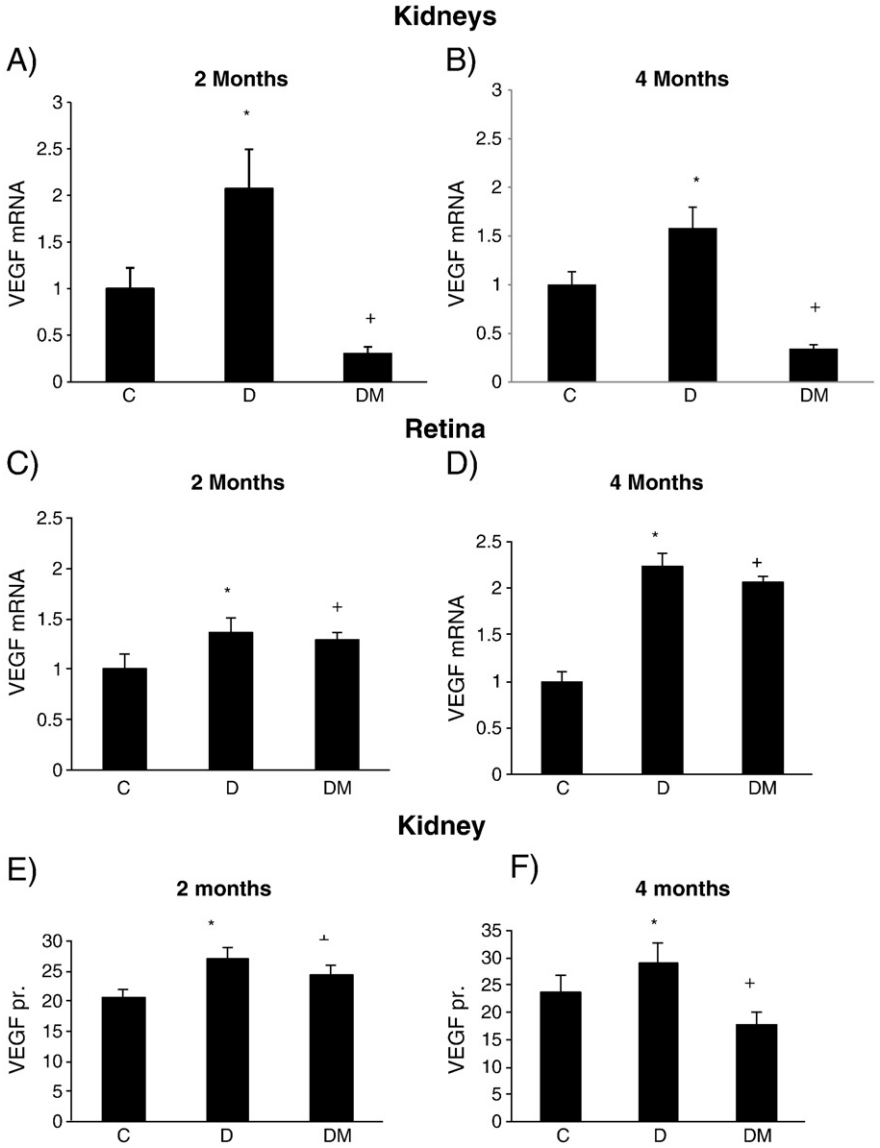

Fig. 2. qRT-PCR analysis of VEGF mRNAs expression in the kidneys (A, B), and retinas (C, D) and ELISA of VEGF protein levels in the kidneys (E, F) demonstrated diabetes-induced upregulation of these transcripts, both after 2 and 4 months of diabetes. Furthermore such upregulations were prevented by macitentan treatment. VEGF protein is expressed as pg/μg of total protein of the cell lysate [C = control, D = diabetic, DM = diabetic on macitentan treatment. mRNA levels are expressed as a ratio to β-actin mRNA, and normalized to controls, * = significantly different from C, + = significantly different from D].

compared to the control group in all examined organs, namely kidney, retina and heart (Fig. 1A–C, G–I). Such increases were seen after 2 months of follow-up and were sustained after 4 months. Along with ET-1 similar changes were seen in the mRNA expression of TGF-β1 (Fig. 1D–F, J–L). Treatment of diabetic animals with macitentan showed significant inhibition of these transcripts. We further examined VEGF in this scenario. Augmented VEGF expressions have been demonstrated in organs affected by diabetic complications. In addition, interdependency of ET with VEGF has been demonstrated in chronic diabetic complications. We focused on retina and kidney as role of VEGF upregulation is well established in these organs. Diabetes caused augmented VEGF mRNA expression in the retina and kidneys after 2- and after 4-months of diabetes. Macitentan treatment prevented diabetes-induced VEGF upregulation in these organs; the effects of macitentan were most pronounced in the kidneys (Fig. 2A–D). Increased VEGF protein production in the kidney tissues of diabetic mice was also significantly inhibited by macitentan treatment (Fig. 2E, F).

Macitentan treatment prevented NF-κB activation and increased extracellular matrix protein production in type 2 diabetes

It has been previously demonstrated that NF-κB activation is one of the main mechanisms mediating ET-dependent augmented ECM protein production in chronic diabetic complications. Hence, we examined such mechanisms and increased ECM protein production in this model. We focused NF-κB analysis in the kidneys as more tissues were available. As expected, diabetes caused increased nuclear NF-κB (p65) expression indicating NF-κB activation. Macitentan treatment prevented NF-κB activation both after 2 and 4 months of follow-up (Fig. 3).

We have previously demonstrated that FN is one of the key ECM proteins which is upregulated in diabetes through an ET-dependent mechanism. Such process also affects a splice variant of FN, namely EDB^+FN. This variant is especially interesting as this is not expressed in normal adults. Upon analysis, diabetic groups had significantly upregulated levels of FN mRNA in all examined organs (Fig. 4A–C, G–I). However, such abnormalities were prevented with treatment of macitentan (Fig. 4A–C, G–I). In addition to FN, we analyzed the mRNA expression of collagen α-I(IV) in the kidneys, retinas and hearts of the mice. Diabetic mice had significantly increased expression of collagen compared to their control counterparts in all organs (Fig. 4D–F, J–L). When treated with macitentan, they exhibited significantly lowered expression of this transcript (Fig. 4D–F, J–L) and no significant differences were found between the control animals and the diabetic animals treated with macitentan in any organs. Furthermore, examination of EDB^+FN transcripts, (performed after 2 months of follow-up) showed that diabetes induced upregulations of EDB^+FN were also prevented by macitentan (Fig. 4M–O).

We further expanded these investigations in an attempt to identify whether changes seen at the mRNA level are also reflected at the protein level. Hence, we examined FN levels in the kidneys and hearts and collagen I-α(IV) levels in the kidneys as more tissues were available in these. In parallel with the mRNA alterations, diabetes caused increased collagen I-α(IV) and FN protein expression. Such changes were also prevented by macitentan treatment (Fig. 5).

Macitentan treatment prevented structural and functional changes in type 2 diabetes

We then proceeded to determine, whether these molecular changes produce any structural changes at the level of the whole organ. We focused on kidneys for such analyses. The tissues were stained with PAS stain to visualize mesangial expansion in the glomeruli. The kidneys from the diabetic mice showed glomerular mesangial expansion whereas the kidneys from the diabetic mice treated with macitentan were reminiscent of the kidneys from the control animals (Fig. 6A).

We further examined the effects of macitentan treatment on the functional parameters. To this extent, we measured urinary albumin and serum creatinine levels. We also performed hemodynamic studies to assess cardiac function.

Diabetes caused albuminuria and increased serum creatinine levels. Such changes were pronounced after 4 months of diabetes and corrected by macitentan (Fig. 6B–E). Similarly, in the heart, we investigated functional alteration. Hemodynamic studies demonstrated that both after 2 and 4 months of diabetes these animals develop changes indicative of cardiac contractile dysfunction and cardiac failure (Fig. 7A–H). In keeping with such changes, their myocardium showed increased mRNA expression of atrial and brain natriuretic peptides. Treatment with macitentan prevented such abnormalities (Fig. 7I–L).

Discussion

In this study, we have shown that in type 2 diabetes, there is increased production of ET-1 in the heart, retina and kidneys. Such ET-1 upregulations are associated with augmented expression of vasoactive and fibrogenic factors such as TGF-β1 and VEGF, NF-κB activation and increased ECM protein production. Diabetic animals also developed functional and structural deficits in the organs. We also demonstrated that treatment of db/db mice with dual ET receptor blocker macitentan prevented such abnormalities.

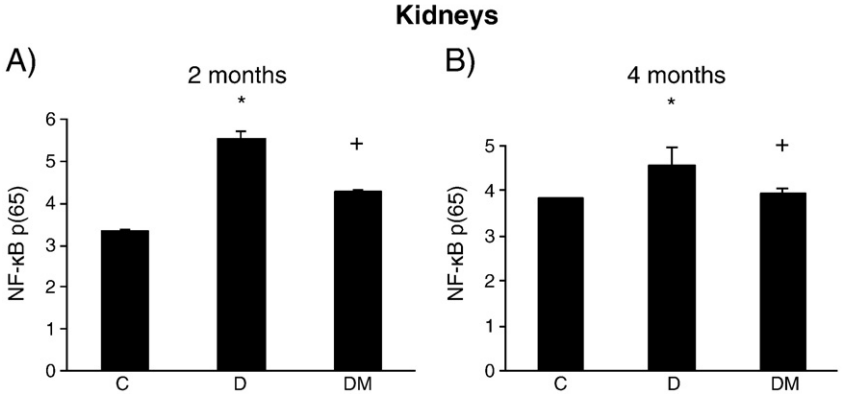

Fig. 3. Diabetes caused NF-κB (p65) activation in the kidneys after 2 and after 4 months of follow-up macitentan treatment resulted in significant prevention of NF-κB activation. NF-κB (p65) protein is expressed as ng/μg of total protein of the nuclear extract [C = control, D = diabetic, DM = diabetic on macitentan treatment, * = significantly different from C, + = significantly different from D].

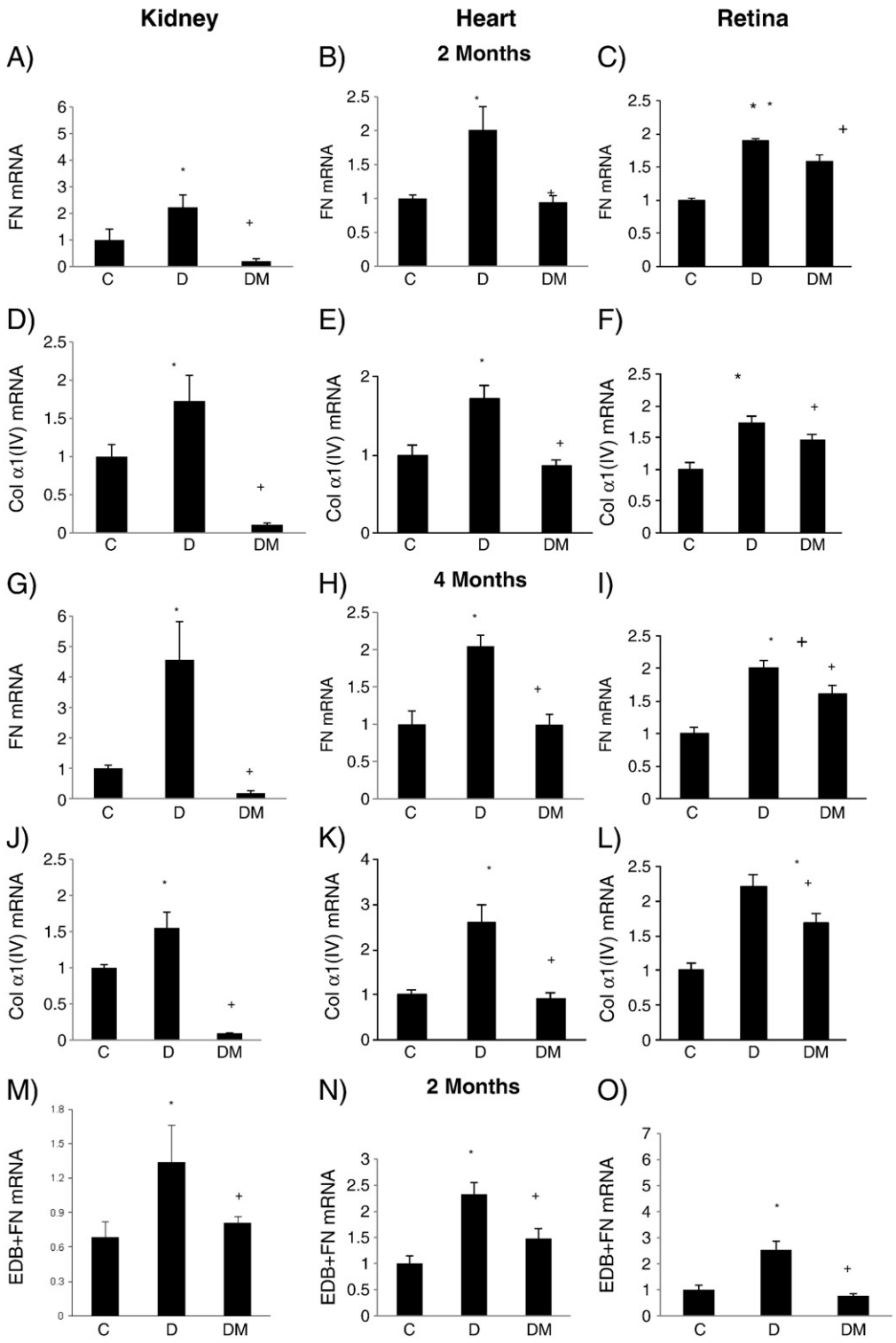

Fig. 4. qRT-PCR analysis of FN (A–C, G–I) and collagen α-I(IV) (D–F and J–L) mRNAs expression in the kidneys (A, D, G, J), hearts (B, E, H, K) and retinas (C, F, I, L) demonstrated diabetes-induced upregulation of these transcripts in all examined organs, both after 2 and 4 months of diabetes. Furthermore such upregulations were prevented by macitentan treatment. Furthermore diabetes-induced augmented EDB^{+}FN mRNA expression in the kidney (M), hearts (N) and retina (O) after 2 months were also prevented by macitentan therapy [C = control, D = diabetic, DM = diabetic on macitentan treatment, mRNA levels are expressed as a ratio to β-actin mRNA, and normalized to controls, * = significantly different from C, + = significantly different from D].

It has been previously reported that leptin receptor mutation is one of the causes of monogenetic obesity in humans (Farooqi and O'Rahilly, 2000). The genes affected in monogenic obesity encode ligands and receptors of the highly conserved leptin–melanocortin pathway, which is critical for the regulation of food intake and body weight (Farooqi, 2008; Farooqi and O'Rahilly, 2008). It is well-established that obesity is associated with cardiovascular disease, diabetes mellitus and certain cancers (Farooqi and O'Rahilly, 2008). Interestingly, leptin has been demonstrated to mediate obesity induced myocardial ET-1 upregulation (Adiarto et al., 2007). db/db mice, used in this study, have a point mutation in the leptin receptor and have been used for the study of type 2 diabetes and its associated

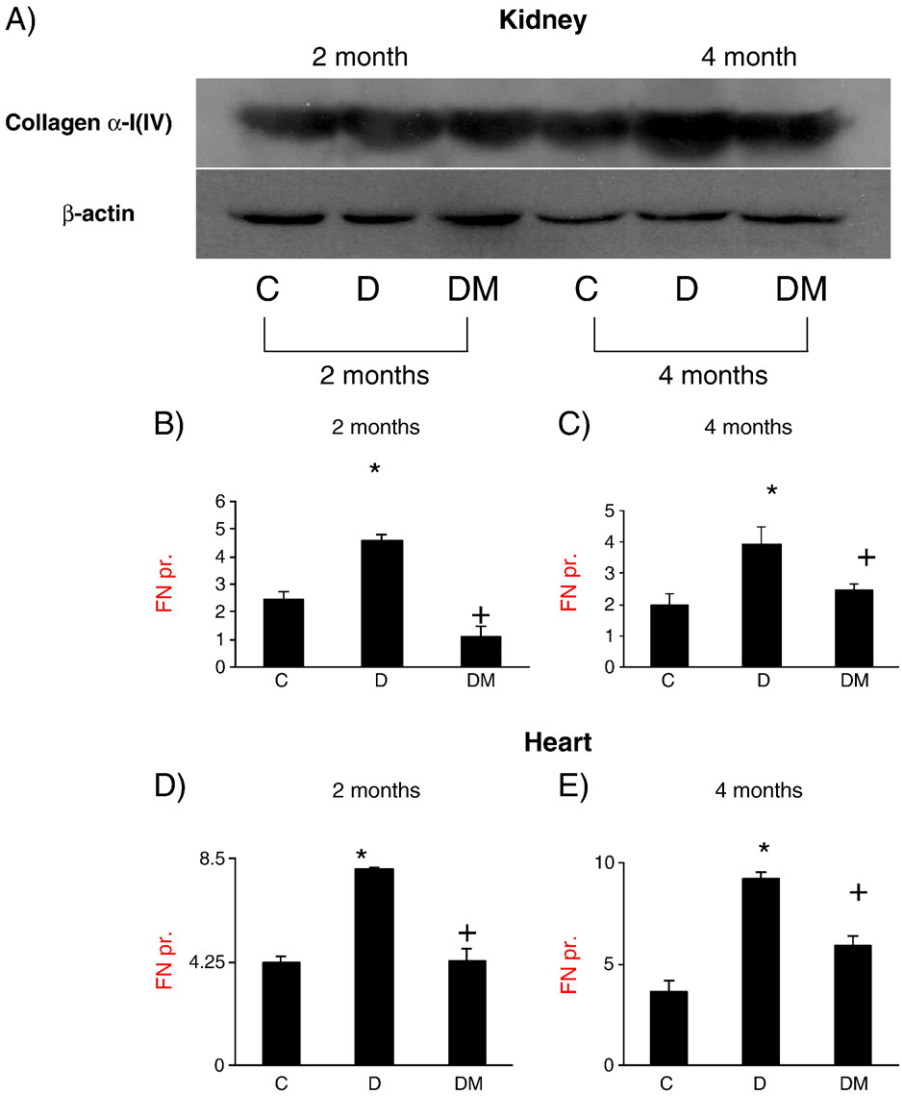

Fig. 5. Diabetes caused increased collagen α-I(IV) protein production in the kidneys (A, showing representative western blots) and augmented FN protein production (measured by ELISA) in the kidneys of (B,C) and heart (D,E) after 2 and 4 months of diabetes. FN protein is expressed as ng/500 ng of total protein content of the cell lysate [C = control, D = diabetic, DM = diabetic on macitentan treatment. * = significantly different from C, + = significantly different from D].

complications (Wang et al., 2011; Kanda et al., 2009). Adiarto et al. (2007) demonstrated the involvement of leptin in obesity induced upregulation of myocardial ET-1. Selected proteins, identified through studying renal transcriptome of db/db mice, were found to be altered in the type 2 diabetic patients with reduced GFR (Simonson et al., 2011). Although one previous study has shown an association between tubulointerstitial collagen deposition and ET-1 in the db/db mice, a direct cause–effect relationship was not studied (Mishra et al., 2006).

Macitentan is a highly potent, dual ETA/ETB receptor antagonist. In vivo, macitentan is metabolized into a pharmacologically active compound augmenting its activity (Iglarz et al., 2008; Sidharta et al., 2011; Raja, 2010; Kummer et al., 2009). We carried out studies in the db/db mice, a well-established model of type 2 diabetes, which develop pathologic changes, indicative of chronic diabetic complications in the retina, kidney and heart (Wang et al., 2011; Kanda et al., 2009; Li et al., 2010), unlike most of the previous endothelin receptor antagonist studies conducted on animals with type 1 diabetes. Although there are some investigations with regards diabetic nephropathy were performed in the type 2 models of diabetes, there are no studies that investigated the effects of ET blocker in diabetic retinopathy or cardiomyopathy. To establish a role of the ET system in the pathogenesis of such changes, we first confirmed that this model yields increased expression of ET-1 in these three organs. In diabetes, ET-1 contributes to blood flow alteration, increased permeability and increased ECM protein production (Evans et al., 2000; Deng et al., 1999). ET-1, in diabetes, plays interactive roles with other vasoactive and fibrogenic factors such as TGF-β1 and VEGF (Khan and Chakrabarti, 2003; Chen et al., 2000; Khan et al., 2004). In keeping with our previous data, we observed ET-1-dependent upregulation of structural proteins, such as collagen, FN and EDB + FN and vasoactive factors such as VEGF and TGF-β1. Association of ET-1 and collagen deposition in kidneys of db/db mice has been also reported by others (Mishra et al., 2006). Furthermore, we have shown that such changes are associated with alteration of specific transcription factors NF-κB (Chen et al., 2003a; Rubanyi and Polokoff, 1994; Chen et al., 2003b). Such prevention of biochemical changes by macitentan translated into amelioration of functional and structural changes. Prevention of diabetic nephropathy by ET-blockade has been demonstrated by several previous studies (Saleh et al., 2011a, 2011b; Simonson et al., 2011). It was also reported that macitentan prevented renal vasoconstriction, increased renal blood flow and glomerular filtration rate, vascular, tubulointerstitial lesions and glomerular damage and proteinuria in type 1 diabetic

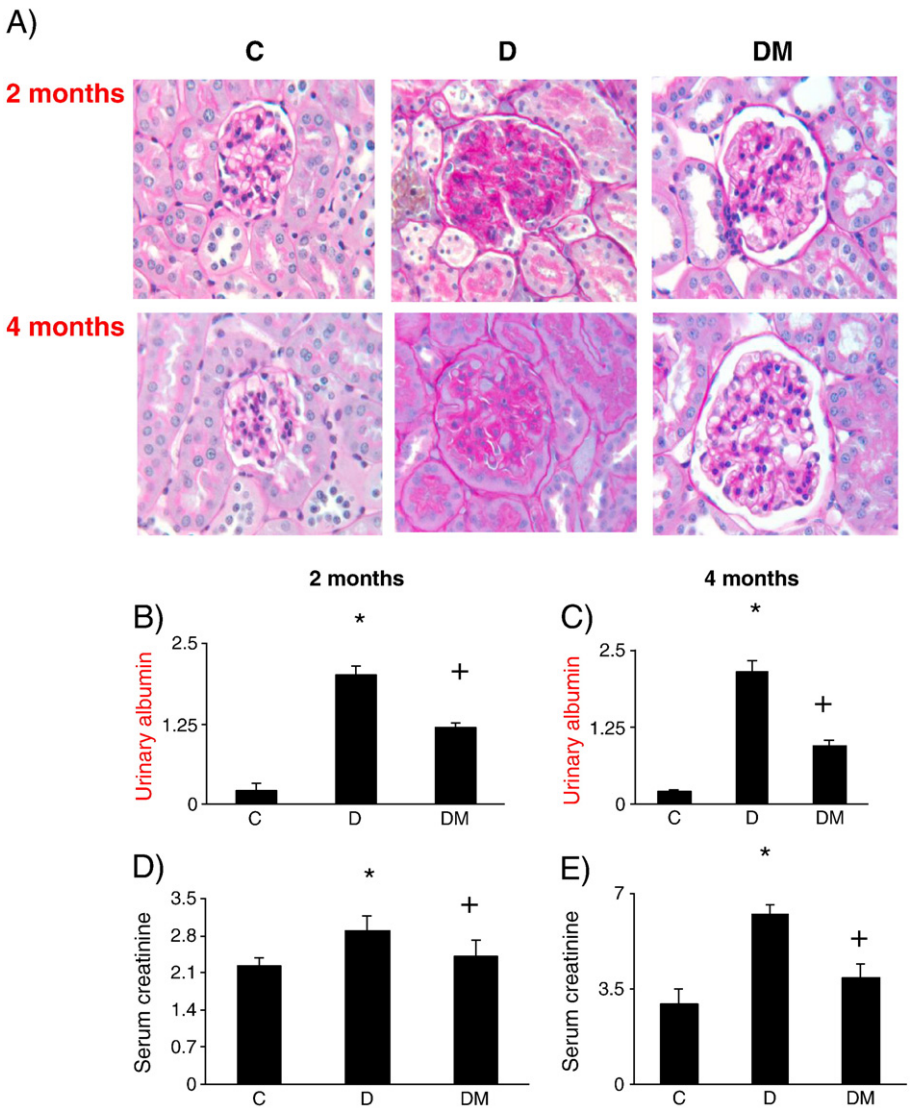

Fig. 6. A) Representative photomicrographs (PAS stain) showing diabetes-induced mesangial expansion is prevented by macitentan treatment. (B, C), increased urinary albumin excretion (μg/day) and (D, E) increased serum creatinine (mg/dl) levels in the diabetic animals were also prevented by macitentan treatment [C = control, D = diabetic, DM = diabetic on macitentan treatment, * = significantly different from C, + = significantly different from D, original magnification 40× for all micrographs].

rats (Iglarz et al., 2008). ETA receptor blocker has been reported to be more beneficial than combined ETA/ETB blockade in diabetic nephropathy (Saleh et al., 2011b). Interestingly, Benigni et al. (1998) have demonstrated that non-selective ET blockade is effective in preventing type 1 diabetes induced renal injury to a similar degree to that of ACE inhibition, without reducing the blood pressure to a level similar to the later. Same group has also demonstrated that in ZDF rat, a model of type 2 diabetes, combined ACE inhibition and ETA receptor antagonist therapy provide renoprotection through ACE inhibition and cardioprotection through ETA blockade (Zoja et al., 2011). On the other hand, some authors have reported ACE inhibition is more effective than ETA blockade in prevention of renal and cardiac dysfunctions in type 2 diabetes (Gross et al., 2004, 2003a, 2003b). Similarly in the L-NAME induced hypertensive model, although bosentan reversed renal fibrosis, such effects were found to be less compared to angiotensin II blockade (Chatziantoniou and Dussaule, 2005; Dussaule and Chatziantoniou, 2007). Interestingly these two groups of drugs demonstrated synergistic effects in the prevention of advanced structural changes such as tubulointerstitial fibrosis, podocyte loss in the kidney of rats with type 1 diabetes as demonstrated using a selective ETA blocker and ACE blocker (Gagliardini et al., 2009). Hence it appears that multiple mechanisms and cell types are involved in the process of renal fibrosis in chronic nephropathies (Remuzzi et al., 2006). Nevertheless, there is significant evidence that ETs are involved in the pathogenesis of such process. In several studies, along with other therapeutic modalities, ETA blockade has shown efficacy both in human and in the rodents (Barton, 2008).

Diabetes-induced increased vasoconstriction and impaired vasodilation is well documented as an early functional alteration. The most potent vasoconstrictor ET-1 and vasodilator NO have been shown to exhibit a state of imbalance in all target organs of diabetic complications (Deng et al., 1999; Dogra et al., 2001; Lambert et al., 1996; Johnstone et al., 1993). On the other hand, treatment with bosentan showed no effect on the phenylephrine induced contractility of the large vessels from the leptin deficient ob/ob mice (Okon et al., 2003). ETs are implicated in the regulation of other endothelial parameters (Yanagisawa et al., 1988). Inhibition of ET-receptor signaling prevents glucose-induced permeability and expression of ECM proteins, collagen and FN (Chen et al., 2003a; Rubanyi and Polokoff, 1994; Chen et al., 2003b). The mechanisms of ET action may entail activation of PKC via G protein-coupled ET receptor type B (ET_B) (Levin, 1995; Chen et al., 2000). We also previously demonstrated that, in endothelial cells and animal models of type 1 diabetes ET-1

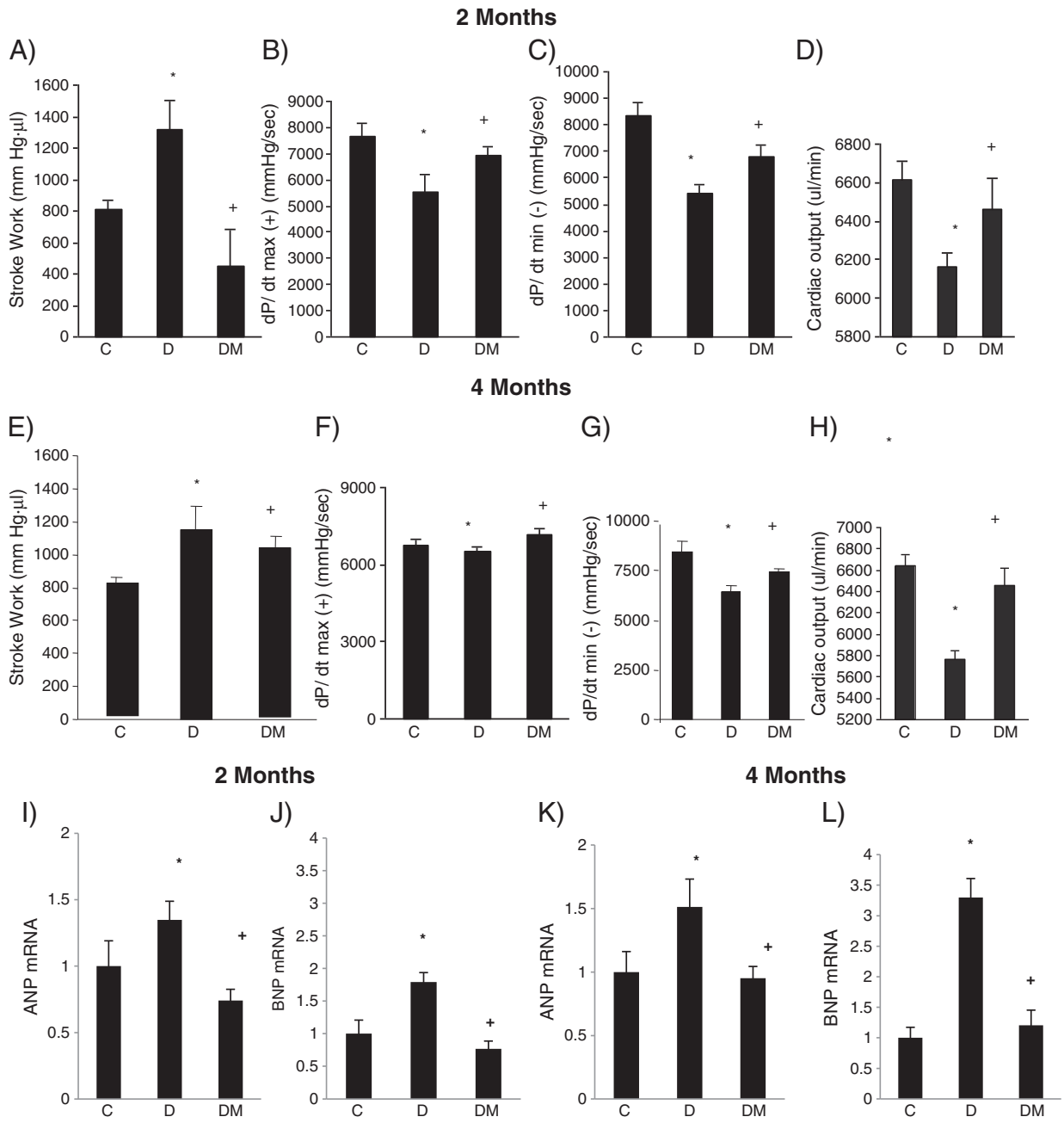

Fig. 7. Both after 2 months (A–D) and 4 months (E–H) hemodynamic analyses demonstrated changes consistent with diabetic cardiomyopathy as indicated in alteration of stroke work, dP/dt max (+/−) and cardiac output. Such changes were prevented by macitentan treatment. Diabetes also caused upregulations of ANP (I, J) and BNP (K, L) after 2 and 4 months of diabetes, which were prevented by macitentan therapy [C = control, D = diabetic, DM = diabetic on macitentan treatment, mRNA levels are expressed as a ratio to 18s rRNA, and normalized to controls, * = significantly different from C, + = significantly different from D].

overexpression leads to increased ECM protein expression, via activation of transcription factors NF-κB and activating protein-1 (AP-1) (Chen et al., 2003a; Rubanyi and Polokoff, 1994; Chen et al., 2003b). Interestingly, ET alteration in diabetes leads to alternative splicing of FN in the vitreous of patients with proliferative diabetic retinopathy and retinal tissues of diabetic animals. Such FN alternative splicing produces the embryonic variant of the ECM protein, $EDB^{+}FN$. $EDB^{+}FN$ was shown to cause endothelial cell proliferation and VEGF expression (Khan et al., 2004). We have further shown that in the heart, diabetes induced alterations are associated with ANP and BNP upregulations. However, other investigators failed to find such changes (Bartels et al., 2010; Magnusson et al., 2004; Yano et al., 1999; Nannipieri et al., 2002). Various diabetic models and/or duration of diabetes may be in part responsible.

Diabetes activates several pathways in the organs affected by chronic complications. These include the aldose reductase pathway, the advanced glycation end products pathway, the hexosamine pathway and the protein kinase C pathway. Increased oxidative stress may be a key mechanism leading to such activation (Brownlee, 2001). In chronic diabetes, protein kinase C and mitogen-activated protein kinase are known to upregulate ET-1 expression (Brownlee, 2001; Xin et al., 2004). Excessive amounts of oxidative stress also cause damage to the DNA which activates Poly ADP ribose polymerase (PARP) in an attempt to repair the damage (Brownlee, 2001; Virag and Szabo, 2002). Interestingly, we have previously demonstrated that PARP interacts with ET-1 (Chiu et al., 2008). Role of oxidative stress in such process has further been established as antioxidants such as curcumin and others are effective in preventing chronic diabetic complications

(Chen et al., 2003a; Kowluru and Kanwar, 2007; Farhangkhoee et al., 2006). Interestingly, ET-1 stimulated vascular reactive oxygen species/hydroxyl radical formation has been reported to be reduced in obesity (Mundy et al., 2007). It is however possible that, other factors may also cause ET-1 upregulation and other aforesaid abnormalities in diabetes. Further experiments are needed to determine the full extent of various lesions produced in diabetes and pathogenetic role of ET-1 activation in such process.

Conclusion

Data from this study demonstrate the role of ET-1 activation in the pathogenesis of chronic complications affecting multiple organs in type 2 diabetes. It further demonstrates that ET receptor blockade may emerge as a potential therapeutic modality to prevent such damage.

Conflict of interest statement

Dr. Marc Iglarz is employed by Actelion Pharmaceuticals Ltd. Actelion Pharmaceuticals Ltd. in part funded this research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.lfs.2012.03.032.

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