

# Leptin and endothelin-1 mediated increased extracellular matrix protein production and cardiomyocyte hypertrophy in diabetic heart disease

Pijush Majumdar  
Shali Chen  
Biju George  
Subhrojit Sen  
Morris Karmazyn  
Subrata Chakrabarti\*

*Departments of Pathology and Physiology and Pharmacology, Schulich School of Medicine, University of Western Ontario, London, Ontario, Canada*

\*Correspondence to:

Subrata Chakrabarti, Department of Pathology, University of Western Ontario, London Health Science Centre, 339 Windermere Road, London, Ontario N6A 5A5, Canada.  
E-mail: subrata.chakrabarti@schulich.uwo.ca

## Abstract

**Background** We investigated the role of leptin and its interaction with endothelin 1 (ET-1) in fibronectin (FN) synthesis and cardiomyocyte hypertrophy, two characteristic features of diabetic cardiomyopathy.

**Methods** Endothelial cells [human umbilical vein endothelial cells (HUVECs)] were examined for FN production and neonatal rat cardiomyocytes for hypertrophy, following incubation with glucose, ET-1, leptin and specific blockers. FN, ET-1, leptin and leptin receptors mRNA expression and FN protein were measured. Myocytes were also morphometrically examined. Furthermore, hearts from streptozotocin-diabetic rats were analysed.

**Results** Glucose caused increased FN mRNA and protein expression in HUVECs and cardiomyocytes hypertrophy along with upregulation of ET-1 mRNA, leptin mRNA and protein. Glucose-mimetic effects were seen with leptin and ET-1. Leptin receptor antagonist (leptin quadruple mutant) and dual endothelin A endothelin B (ETA/ETB) receptor blocker bosentan normalized such abnormalities. Hearts from the diabetic animals showed hypertrophy and similar mRNA changes.

**Conclusion** These data indicate that in diabetes increased FN production and cardiomyocyte hypertrophy may be mediated through leptin with its interaction with ET-1. Copyright © 2009 John Wiley & Sons, Ltd.

**Keywords** diabetic cardiomyopathy; leptin; fibronectin; endothelin-1

## Introduction

With worldwide increasing incidence of diabetes, more diabetics face problems of morbidity and mortality because of chronic complications. Diabetic individuals are 2–4 times more likely to have heart disease compared with the normal population, and 75% of diabetes-related deaths are due to heart diseases [1,2].

Cardiac involvement in diabetes includes coronary atherosclerosis, diabetic cardiomyopathy and autonomic neuropathy [2]. Forty to fifty percent of diabetics may demonstrate features of diabetic cardiomyopathy and manifest ventricular diastolic dysfunction, without any known coronary disease. They present clinically as impaired relaxation. Cardiomyocyte hypertrophy, and subsequent cellular apoptosis, focal myocardial interstitial and perivascular

Received: 23 December 2008

Revised: 2 March 2009

Accepted: 10 March 2009

fibrosis are structural hallmarks of diabetic cardiomyopathy [2,3]. Pathological findings further include microangiopathic lesions such as capillary basement membrane (BM) thickening and focal fibrosis. Hyperglycemia is the most important factor leading to almost all chronic diabetic complications including diabetic cardiomyopathy [4]. Hyperglycemia causes several metabolic defects, including diacylglycerol-mediated protein kinase C (PKC) activation. Such biochemical changes may upregulate several growth factors, vasoactive and cardioactive factors [4,5]. One such molecule with extensive vasoactive and cardioactive properties is endothelin-1 (ET-1) [6]. Endothelins are a family of four 21 amino acid peptides known as ET-1, ET-2, ET-3 and vasoactive intestinal contractor. The primary isoform expressed in the heart is ET-1 [6]. ET-1 is produced by both endothelial cells and cardiomyocytes and has widespread effects on the heart [6,7]. We have previously demonstrated that diabetes upregulates ET-1 and ET receptor expression in the heart and that ET-1 may play a significant role in diabetes-induced cardiac hypertrophy as well as increased extracellular matrix (ECM) protein synthesis [7,8].

Recent evidences indicate that adipocyte-derived leptin may also regulate ET-1 production. Although, adipose tissue is the main source of leptin, its synthesis has been demonstrated in several other organs including the heart. Several endocrine, paracrine and autocrine actions of leptin have been demonstrated [9–11]. Leptin signals through six different alternately spliced receptors (Ob-Ra–Ob-Rf). Ob-Rb appears to be the main receptor for this peptide, through which leptin exerts its physiological action primarily via activation of Janus tyrosine kinase (JAK) and signal transducers and activators of transcription (STAT) family [12]. In addition, an increasing number of leptin transduction pathways such as mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, extracellular-signal-regulated kinase (ERK) are involved in the signalling events downstream of the leptin receptors [12]. Leptin and its receptors are expressed in smooth muscle cells, cardiac endothelial cells and in cardiomyocytes [13–15]. We and others have previously demonstrated that leptin and ET-1 play significant roles in chemically induced cardiomyocyte hypertrophy [15,16]. Leptin has also been shown to regulate diabetes-induced increased ECM protein production in renal mesangial cells [17].

However, evidence that leptin may be involved in hyperglycemia-induced cardiomyopathy is lacking. The purpose of this study was to investigate whether leptin is involved in hyperglycemia-induced cardiomyocyte hypertrophy and increased ECM protein production. We also wanted to find out whether such action of leptin may be mediated by ET-1. We focused on a key ECM protein, fibronectin (FN). We further investigated a splice variant of FN, that is, extradomain-B-containing FN (EDB<sup>+</sup>FN), which is supposed to be absent in mature healthy adult tissue [17,18]. We have previously demonstrated ET-1 and transforming growth factor  $\beta$  (TGF- $\beta$ ) dependent, FN as well as EDB<sup>+</sup>FN upregulation in endothelial

cells, one of the primary target of high glucose and in the heart of diabetic animals as well as in human diabetes [8,18]. This is functionally important as it produces outside-in signalling [18]. We used human umbilical vein endothelial cells (HUVECs) and neonatal rat cardiomyocytes for these studies. We have previously used HUVECs to study glucose-induced FN synthesis and a large number of diabetes-related changes in this cell model [18,19]. We have also used neonatal rat cardiomyocytes to study cardiomyocyte hypertrophy [20,21]. We further extended the studies to the hearts from streptozotocin (STZ) diabetic animals. This is a well-studied model of all chronic diabetic complications including diabetic cardiomyopathy [18,21,22]. This is well suited for the examination of early changes as used in this study.

## Materials and methods

### Endothelial cell culture

HUVECs were used following previously described methods [8,19]. Briefly, HUVECs, obtained from Clonetics (Rockville, MD, USA), were plated at approximately 2500 cells/cm<sup>2</sup> in endothelial cell growth medium (EGM) and were incubated at 37 °C in 5% CO<sub>2</sub>. EGM is supplied with 10  $\mu$ g/L human recombinant epidermal growth factor, 1.0 mg/L hydrocortisone, 50 mg/L gentamicin, 50  $\mu$ g/L amphotericin B, 12  $\mu$ g/L bovine brain extract, and 2% foetal bovine serum. No insulin was present in the media. Cells were grown in 25-cm<sup>2</sup> tissue culture flasks. The cells (80% confluent) were serum starved for 24 h before exposure to glucose or other reagents.

### Cell survival assay

HUVECs were seeded onto 96-well plates at a density of  $1.0 \times 10^4$  cells per well in 100- $\mu$ L medium. Following exposure to appropriate reagents for 24 h, cell viability assay were performed. For such assay 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt (WST-1) (Roche Diagnostic Canada, Laval, Quebec, Canada) was added to each well and incubated for 4 h at 37 °C. A colorimetric determination (at 450 nm), based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells were carried out on a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

### Neonatal cardiomyocytes culture

Neonatal ventricular cardiomyocytes were used to examine hypertrophic response as previously described [20,21]. Briefly, isolated hearts from one-day old Sprague–Dawley rats were (Charles River Inc. St. Constant, Quebec, Canada) placed into solution consisting

of Hank's balanced salt solution and 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The ventricles were minced into small pieces. Myocytes were isolated as previously described [20,21]. The cells were incubated for 24 h with serum-free media and then treated with appropriate reagents as necessary. On the basis of the percentage of cells demonstrating myosin staining, there was less than 5% contamination by noncardiomyocytes in our preparation [20,21].

## Reagents

All reagents were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise specified. We used various concentrations of D-glucose ranging from 5 to 25 mmol/L and leptin peptide (human recombinant for HUVEC and rat recombinant for cardiomyocyte) ranging from 1 to 12.5 nmol/L. L-glucose was used at a concentration of 25 mmol/L as control. ET-1 peptide (Peninsula Laboratories, Belmont, California, USA) was used at two different concentrations; 5 and 10 nmol/L. Leptin quadruple mutant (Protein Laboratories Rehovot, Israel), which acts as a leptin receptor antagonist was used at a concentration of 200  $\mu$ mol/L for HUVECs and 100  $\mu$ mol/L for cardiomyocytes for leptin blockade [23,24]. To block ET-1 action, we used bosentan (BST), a dual (ETA/ETB) receptor blocker (Courtesy of Dr. M. Clozel, Actelion, Basel, Switzerland) at a concentration of 10  $\mu$ mol/L [7,19,22]. The reagent concentrations are based on the previous studies from our laboratory and by other laboratories [8,19]. All experiments were carried out after 24 h of incubation unless otherwise indicated. The inhibitors were added 30 min before addition of glucose. At least three different batches of cells, each in triplicate, were investigated.

## Morphometric analysis of cardiomyocytes

Cell surface area was analysed using a Leica inverted microscope equipped with a Polaroid digital camera at 10 $\times$  magnification. For each treatment, at least 10 pictures, each containing at least three cells were taken. Cell area was measured using Mocha software (SPSS Inc., Chicago, IL, USA).

## Animal experiments

The experiments were performed in accordance with regulations specified by the Canadian Council on Animal Care. University of Western Ontario Animal Care and Veterinary services approved all experiments. Male Sprague–Dawley rats (Charles River Canada Ltd., St. Constant, Quebec, Canada), weighing 200–250 g, were made diabetic by single intravenous injection of STZ (65 mg/kg, in citrate buffer, pH = 6.5). Age and sex

matched rats were used as controls and were given equal volume of citrate buffer.

The animals were maintained for 4 months with regular monitoring of their blood glucose, urine glucose, urine ketones and body weight. Diabetes was confirmed by increased blood glucose (blood glucose = >20 mmol/L for two consecutive days) and increased urine glucose levels. All diabetic rats were implanted with slow release insulin (~1 U/day) implants Linplant™ (Linshin Canada Inc., Scarborough, ON, Canada) to prevent ketosis. The animals were killed after prescribed time. Hearts were dissected out. A cross-section (2 mm) from 3 mm above the apex was fixed in formalin and embedded in paraffin. Five micrometer sections from paraffin-embedded tissues were stained with haematoxylin and eosin (H & E) and trichrome stain. Left ventricular thickness was measured using an orthogonal intercept approach [25]. Trichrome stains were used to assess focal fibrosis. Hearts were harvested and snap frozen in liquid nitrogen for RNA isolation and protein extraction. Blood collected at the time of killing were used for serum leptin assay by rat leptin TiterZyme EIA kit (Assay designs, Ann Arbor, MI, USA) using the protocol given by manufacturer.

## RNA isolation and cDNA synthesis

RNA from cells and rat heart tissues were isolated using TRIZOL™ (Invitrogen, Burlington, ON, Canada) reagent as previously described [19,21]. RNA concentration was assessed on a spectrophotometer (Gene Quant, Pharmacia Biotech, USA). First-strand cDNA was made by using Superscript-II (Invitrogen, Burlington, ON, Canada) system. The resulting products were stored at –200 °C [19,21].

## Real-time reverse transcriptase polymerized chain reaction (RT-PCR)

Real-time RT-PCR was performed in LightCycler™ (Roche Diagnostics Canada, Laval, Quebec, Canada) to quantify the mRNA expression of FN, EDB<sup>+</sup>FN, ET-1, leptin, atrial natriuretic peptide (ANP; for cardiomyocytes) [20–22]. For a final reaction volume of 20  $\mu$ L, the following reagents were added: 10  $\mu$ L of SYBR (Sigma-Aldrich), 1.6  $\mu$ L of MgCl<sub>2</sub>, 1  $\mu$ L forward/reverse primer (Table 1), 4.4  $\mu$ L of H<sub>2</sub>O, and 2  $\mu$ L of cDNA. To optimize the amplification of the genes, melting curve analysis (MCA) was used to determine the melting temperature (T<sub>m</sub>) of specific products and primer dimers. According to the T<sub>m</sub> value of specific products for respective genes, an additional step (signal acquisition step, 2–3 °C below T<sub>m</sub>) was added after the elongation phase of RT-PCR. This additional step in the PCR reactions allowed for signal acquisition from specific target products [19]. The data were normalized to  $\beta$ -actin to account for differences in reverse transcription efficiencies and amount of template in the reaction mixtures.

Table 1. Oligonucleotide sequences for real-time RT-PCR

Gene sequence (5'–3')	Temperatures
B-actin CCTCTATGCCAACACAGTGC	Denaturation 95 °C – 0 s
CATCGTACTCTGCTTGCTG	Annealing 55 °C – 5 s
	Extension 72 °C – 8 s
ET-1 AAGCCCTCCAGAGAGCGTTAT	Denaturation 95 °C – 0 s
(Human) CGAAGGTCTGTCACCAATGT	Annealing 55 °C – 5 s
6FAM-TGACCCACAACCGAG-GBNFQ	Extension 72 °C – 4 s
ET-1 GCTCCTGCTCCTCTTGATG	Denaturation 95 °C – 0 s
(Rat) CTCGCTCTATGTAAGTCATGG	Annealing 58 °C – 5 s
	Extension 72 °C – 20 s
FN GATAAATCAACAGTGGGAGC	Denaturation 95 °C – 0 s
(Human) CCCAGATCATGGAGTCTTTA	Annealing 58 °C – 5 s
	Extension 72 °C – 20 s
FN GATAAATCAACAGTGGGAGC	Denaturation 95 °C – 0 s
(Rat) CCCAGATCATGGAGTCTTTA	Annealing 60 °C – 0 s
	Extension 72 °C – 17 s
Leptin GGGATCACAAGGTCACTAGA	Denaturation 95 °C – 0 s
(Human) TTGGTCACTGTACTCTCC	Annealing 57 °C – 5 s
	Extension 72 °C – 7 s
Leptin GAGACCTCTCCATCTGCTG	Denaturation 95 °C – 0 s
(Rat) CATTCAAGGGCTAAGGTCCAA	Extension 72 °C – 7 s
	Extension 72 °C – 6 s
ANP CTGCTAGACCACCTGGAGGA	Denaturation 95 °C – 0 s
(Rat) AAGCTGTTGACGCTAGTCC	Annealing 55 °C – 5 s
	Extension 72 °C – 13 s
EDB <sup>+</sup> FN CCGCCATTAATGAGAGTGAT	Denaturation 95 °C – 0 s
(Human) AGTTAGTTGCGGCAGGAGAAG	Annealing 55 °C – 5 s
	Extension 72 °C – 8 s

ET-1 transcript was quantified by Taqman™ probe which was designed using primer express v2.0 (Applied Bios stems, Foster City, California, USA) [18]. The data were normalized to housekeeping gene  $\beta$ -actin to account for differences in reverse transcription efficiencies and amount of template in the reaction mixtures.

### Protein extraction and enzyme-linked-immunosorbent serologic assay (ELISA)

After 24 h of treatment with glucose or other reagents, HUVEC's/cardiomyocytes were washed twice with cold

phosphate-buffered saline (PBS) and lysed in lysis buffer (50 mmol/L HEPES, pH 7.6, 150 mmol/L NaCl, 50  $\mu$ mol/L NaF, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L sodium vanadate, 1% NP-40, and 2 mmol/L phenylmethylsulphonyl fluoride). Rat heart tissues were homogenized, washed with PBS and pelleted by centrifugation (1500 g for 5 min). These were lysed for protein extraction using lysis buffer. The total protein concentration was measured using BCA™ protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. We performed FN protein measurement in the conditioned media and leptin protein assessment in the cell protein extracts. The mean  $\pm$  SD of cellular protein concentration of all samples were 605.5  $\pm$  63.4 ng/ $\mu$ L. Protein concentrations of individual samples were within 1 SD of the mean. The concentrations of all samples were adjusted to 500 ng/ $\mu$ L before performing the ELISA. As demonstrated by others, FN being a secreted protein, its measurement in the conditioned media gives accurate assessment of its protein level [26]. Conditioned media was used to measure the FN protein level using a human FN ELISA kit (Millipore Upstate, Temecula, CA, USA) according to the manufacture's instructions. Leptin EIA kits (Assay designs, Ann Arbor, MI, USA) were used to measure the leptin protein levels according to the manufacturer's instructions. The developed colour was measured at 450 nm wavelength with the Bio-Rad micro-plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

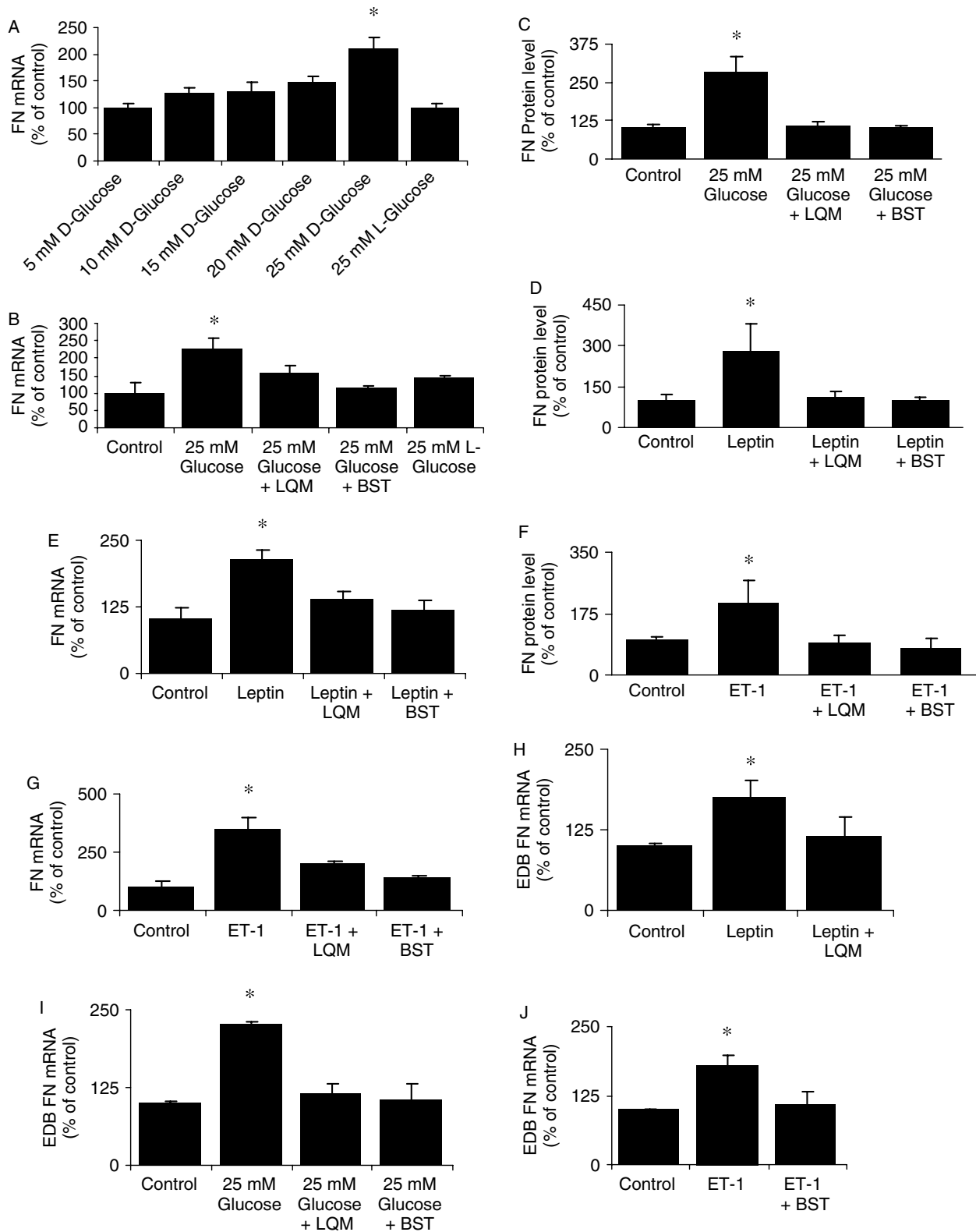
### Statistical analysis

All experimental data are expressed as means  $\pm$  SEM and were analysed with either Student's *t*-test or by analysis of variance (ANOVA) followed by *t*-test with bonferroni corrections wherever necessary. Differences were considered significant at values of  $p < 0.05$ .

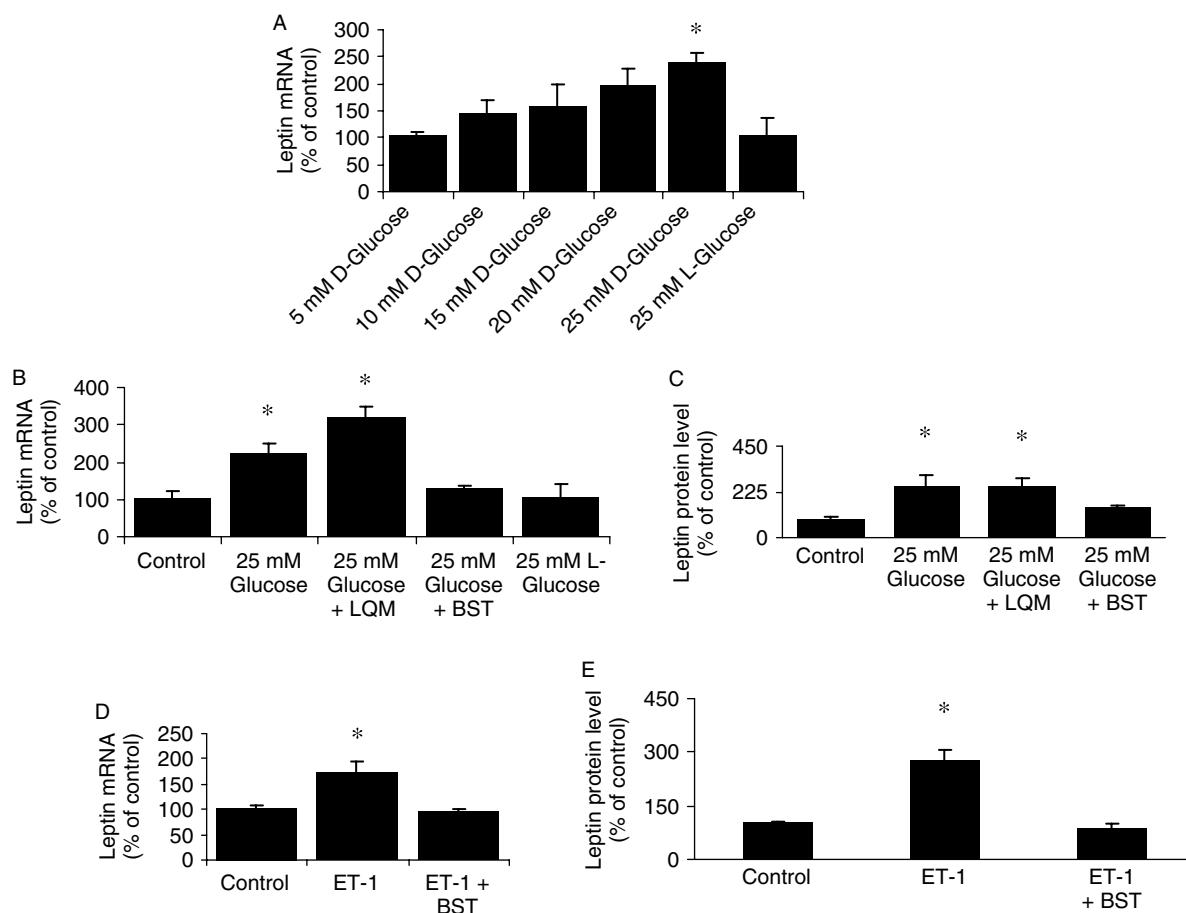
## Results

### Glucose causes increased FN, leptin and ET-1 in HUVECs

We first investigated the effects of glucose with respect to specific mRNA and protein production. In keeping with our previous findings [19], HUVECs showed dose-dependent upregulation of FN (Figure 1A) and ET-1 (Figure 3A) mRNA exposed to various D-glucose levels peaking at 25 mmol/L. Leptin mRNA also showed similar pattern (Figure 2A). Hence, in the subsequent experiments, 25 mmol/L D-glucose was used. No effect on mRNA expression was seen in 25 mmol/L of L-glucose with respect to any of these transcripts (Figures 1A, 2A and 3A), suggesting that the change in FN mRNA expressions were not due to change of osmolality. ELISA of FN (Figure 1C) and leptin (Figure 2C) also showed significant increase in these proteins in the HUVECs



**Figure 1.** Glucose caused dose-dependent upregulation of FN mRNA (A) and protein expression in HUVECs. Glucose-induced FN mRNA (B) and protein expression (C) were prevented by 200  $\mu\text{mol/L}$  leptin quadruple mutant (LQM) and 10  $\mu\text{mol/L}$  bosentan (BST). A glucose-like effect with respect to FN mRNA and protein expression were seen when the cells were exposed to 5 nmol/L leptin (E) or ET-1 (G). Such upregulation was also prevented by 200  $\mu\text{mol/L}$  LQM and 10  $\mu\text{mol/L}$  BST (G). Protein analysis (D and F) reflected in FN mRNA levels. EDB<sup>+</sup>FN mRNA also followed similar pattern of change following exposure to leptin (H) glucose (I) ET-1 (J) leptin [mRNA is expressed as a ratio of target to  $\beta$ -actin (% relative to control); \* =  $p < 0.05$  as compared with 5 mmol/L of D-glucose (control),  $n \geq 10/\text{group}$ , except for G–H,  $n = 6$ ]



**Figure 2.** D-Glucose caused dose-dependent upregulation of leptin mRNA expression peaking at 25 mM in HUVECs. No effect of L-glucose was seen (A). Glucose-induced increased leptin mRNA (B) and protein expression (C) was prevented by 10  $\mu$ mol/L BST. No changes were seen with 25 mmol/L of L-glucose (A). Leptin mRNA (D) and protein expression (E) were also upregulated when the cells were exposed to 10 nmol/L ET-1. Such upregulations were also prevented by 10  $\mu$ mol/L BST [mRNA is expressed as a ratio of target to  $\beta$ -actin (% relative to control); \* =  $p < 0.05$  as compared with 5 mmol/L of D-glucose (control), # - leptin protein level is expressed as a ratio of leptin protein level in  $\mu$ g/mL to total protein in  $\mu$ g/mL;  $n \geq 10$  per group]

conditioned medium or in cellular proteins following exposure of the cells to 25 mmol/L of D-glucose for 24 h. Similar to FN, expression of its splice variant EDB<sup>+</sup>FN were also upregulated by 25 mmol/L of D-glucose (Figure 1I). Glucose exposure did not cause any alteration of cell viability by WST assay (data not shown) and no significant alteration of cell counts was seen in any of the flask.

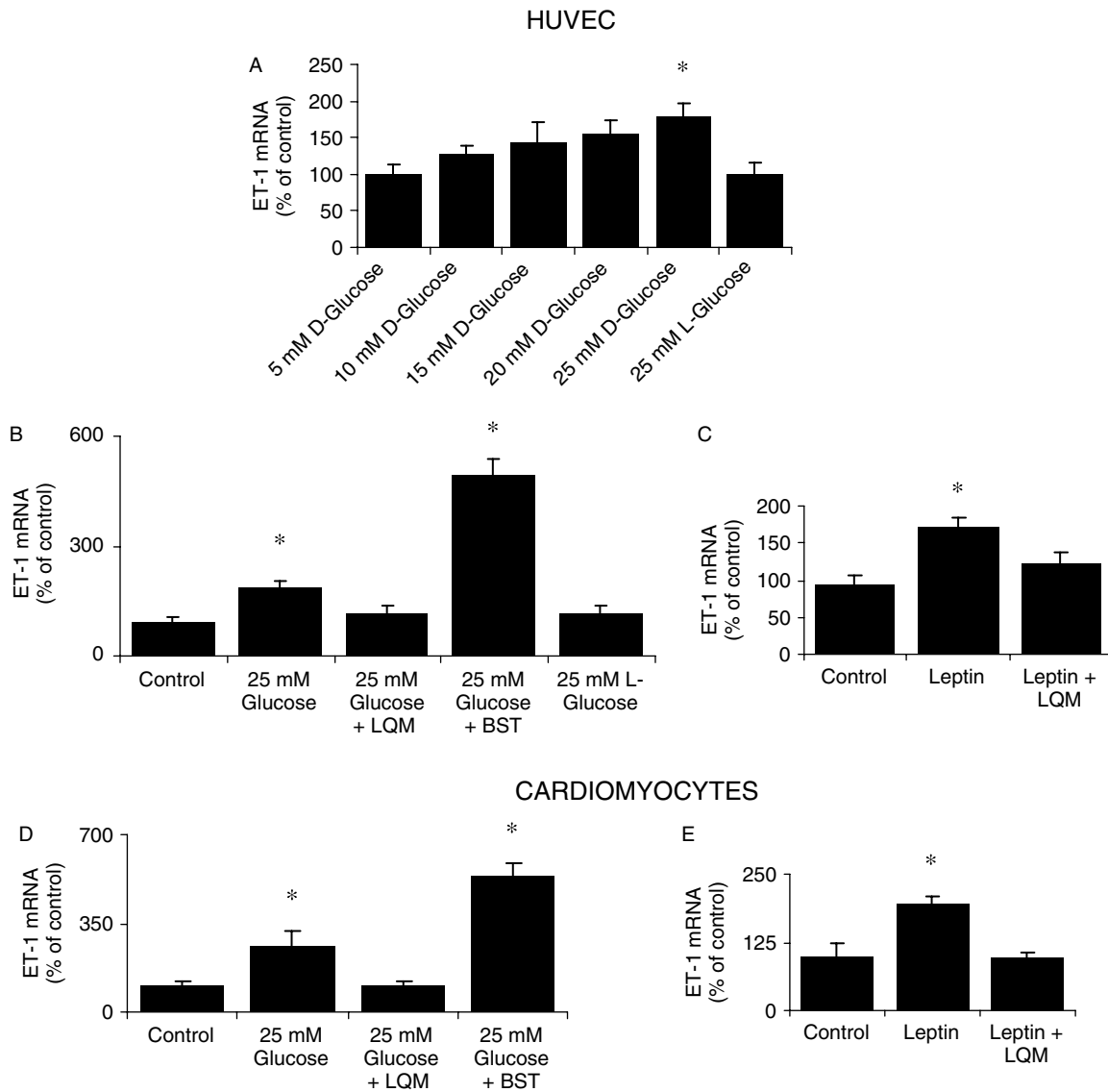
### Leptin and ET-1 may regulate glucose-induced FN production in HUVECs

To characterize the role of leptin and ET-1 in glucose-induced FN production, we used a leptin quadruple mutant (LQM) and a dual ETA/ETB receptor blocker - BST. mRNA expression analysis and protein analysis showed significant abrogation of glucose-induced FN and EDB<sup>+</sup>FN (Figure 1B-D, I) expression by LQM and BST, indicating both leptin and ET-1 play important roles in glucose-induced FN and EDB<sup>+</sup>FN production. To further explore these mechanisms, we incubated HUVECs

in various concentrations of leptin and ET-1 peptides. We found that 5 nmol/L leptin and 10 nmol/L ET-1 resulted in peak FN upregulation (data not shown). Of further interest is that similar to glucose, both leptin and ET-1-induced FN expression were blocked by ET receptor blocker BST and LQM (Figure 1E-G). In addition, both these peptides upregulated each others' mRNA and protein production (Figures 2D, E and 3C). Leptin at 5 nmol/L concentration by itself, upregulated ET-1 (Figure 2D) production and vice versa (Figure 3C). The blockers prevented such changes (Figures 2D, E and 3C). These data indicate that leptin and ET-1 regulate each other and play important role in glucose-induced augmented FN synthesis.

### Glucose-induced cardiomyocyte hypertrophy is associated with leptin and ET-1 up regulation

In the cardiomyocytes, we first established the effect of glucose with respect to hypertrophy. Cardiomyocytes exposed to 25 mmol/L of glucose showed



**Figure 3.** Glucose caused dose-dependent upregulation of ET-1 mRNA expression peaking at 25 mM. No effect of L-glucose was seen (A). Glucose-induced increased ET-1 mRNA expression were prevented by 200  $\mu\text{mol/L}$  (HUVECs) (B) or 100  $\mu\text{mol/L}$  (cardiomyocytes) (D) leptin quadruple mutant (LQM) and 10  $\mu\text{mol/L}$  bosentan (BST). A glucose-like effect with respect to ET-1 mRNA expression were seen when the HUVECs (C) or cardiomyocytes (E) were exposed 5 nmol/L leptin. Such upregulations were also prevented by LQM [mRNA is expressed as a ratio of target to  $\beta$ -actin (% relative to control)]; \* =  $p < 0.05$  as compared with 5 mmol/L of D-glucose (control),  $n \geq 10$  per group]

significant hypertrophy, as measured morphometrically, in association with upregulation of ANP mRNA (Figures 4B and 5A, B). In addition, this concentration of glucose caused increased leptin and ET-1 mRNA expression (Figures 3D and 6C). Leptin protein levels also showed significant increase following exposure of the cells to 25 mmol/L D-glucose for 24 h (Figure 6D).

### Leptin and ET-1 may regulate glucose-induced cardiomyocyte hypertrophy

Similar to the HUVECs experiment, we used LQM and BST. Glucose-induced cardiomyocyte hypertrophy

and augmented ANP mRNA expression were completely abrogated by treatment with LQM and BST (Figures 4C, D and 5A, D). To further explore these mechanisms, we treated cardiomyocytes with leptin and ET-1 peptides. We found that 5 nmol/L leptin or 10 nmol/L ET-1 resulted in cardiomyocyte hypertrophy (Figures 4E, H and 5B, C). In addition, both these peptides upregulated each others mRNA and protein production, which, as expected were blocked by corresponding blockers (Figures 3D, E and 6C, D). Of further interest is that both leptin and ET-1 induced cardiomyocyte hypertrophy and ANP production were blocked by BST and LQM (Figures 4F, G, I, J and 5C, E, F). These data indicate that both these peptides may regulate each other and play important role in glucose-induced cardiomyocyte hypertrophy.

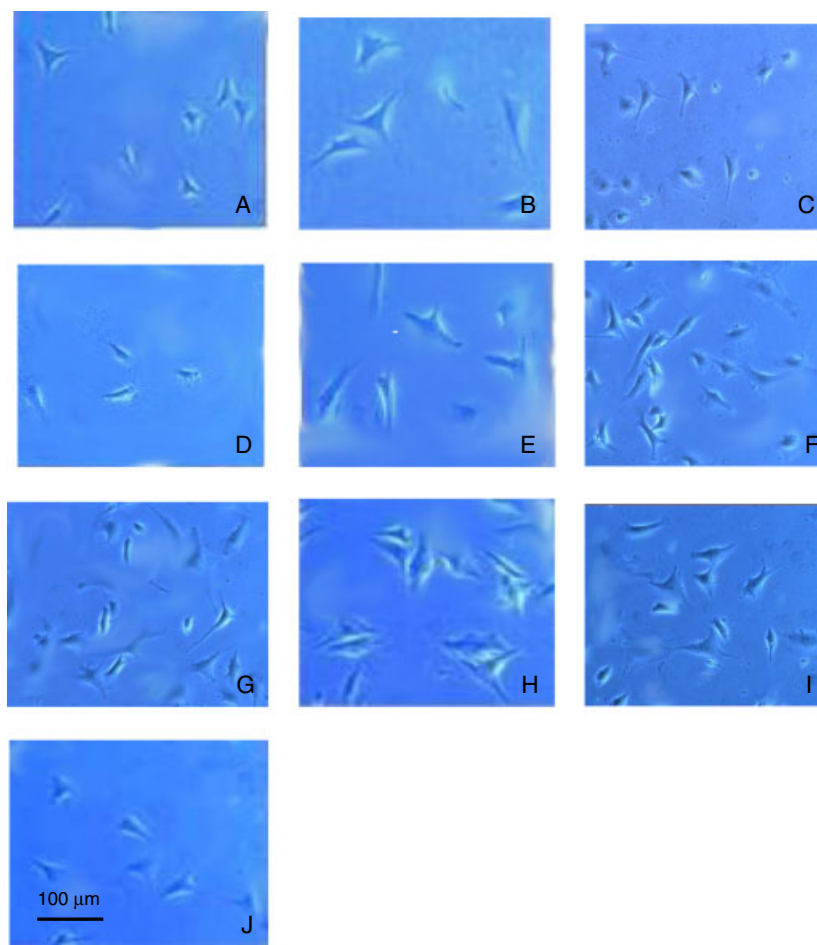


Figure 4. Representative photomicrographs of neonatal rat cardiomyocytes showing myocyte hypertrophy in 25 mmol/L of glucose (B) compared with 5 mmol/L of glucose (A). Such glucose-induced hypertrophy (B) was prevented by 100  $\mu$ mol/L leptin quadruple mutant (LQM) (C) or 10  $\mu$ mol/L endothelin receptor antagonist bosentan (BST) (D). Similar hypertrophy were noted when cells in 5 mmol/L of glucose were exposed to 5 nmol/L leptin (E) or 10 nmol/L endothelin-1 (H). Leptin-induced hypertrophy were prevented by LQM (F) or BST (G). Endothelin-1-induced hypertrophy was also prevented by LQM (I) or BST (J) [magnification same for all micrographs, Bar = 100  $\mu$ m]. This figure is available in colour online at [www.interscience.wiley.com/journal/dmrr](http://www.interscience.wiley.com/journal/dmrr)

### Heart from diabetic animals demonstrates changes similar to *in vitro* experiments

We further investigated whether these changes occur in the heart of diabetic animals. Diabetic animals showed hyperglycemia, reduced body weight, glucosuria and increased urine volume. The final body weights, blood glucose and glycated haemoglobin levels are illustrated in Table 2. Diabetic animals after 4 months showed increased heart weight (Table 2). Serum leptin level was lower in the diabetic animals (Table 2). Messenger RNA analysis demonstrated significant upregulation of ANP, a surrogate marker for hypertrophy and FN mRNA levels (Figure 7F). Furthermore, increased leptin and ET-1 mRNA were seen in the hearts of diabetic animals compared with controls (Figure 7C, E). Leptin protein levels were also increased in the heart of diabetic animals compared with control animals (Figure 7D). Also identified was focal myocardial fibrosis in diabetic animals (Figure 7A, B). Although fibrosis, as detected by trichrome stain is not a direct measurement of FN; in our

Table 2. Clinical and cardiac parameters of control and diabetic rats

Parameters	Controls	Diabetics
Blood glucose (mmol/L)	4.6 $\pm$ 0.2	19.0 $\pm$ 2.5 <sup>a</sup>
Body weight (g)	627 $\pm$ 21	482 $\pm$ 18 <sup>a</sup>
Glycated Hb (%)	5.6 $\pm$ 0.4	17.4 $\pm$ 0.6 <sup>a</sup>
Heart weight (g)	1.3 $\pm$ 0.04	1.5 $\pm$ 0.03 <sup>a</sup>
Left ventricular wall thickness ( $\mu$ m)	1.27 $\pm$ 0.02	1.66 $\pm$ 0.02 <sup>a</sup>
Plasma leptin level ( $\mu$ g/mL)	6941.71 $\pm$ 430.11	2755.14 $\pm$ 600.7 <sup>a</sup>

<sup>a</sup>Significantly different from controls,  $n = 6$  per group.

previous studies, we have found this simple measurement correlates with FN alteration in diabetic cardiomyopathy [25]. Such fibrosis was not seen in non-diabetic controls.

## Discussion

Diabetic heart disease accounts for 80% of deaths among the patients suffering from diabetes [1,27].

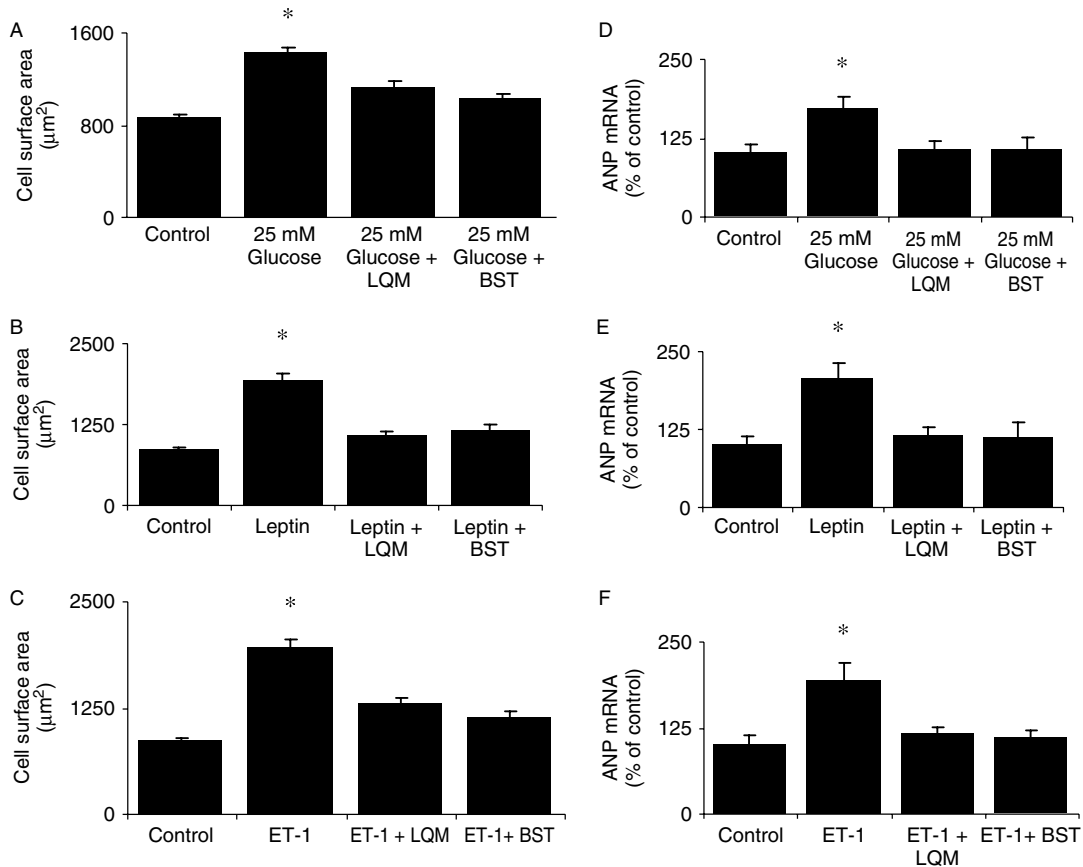


Figure 5. Twenty-five mmol/L of glucose caused increased cardiomyocyte surface area (A) and atrial natriuretic peptide (ANP) mRNA (D) expression as determined by real-time RT-PCR compared with 5 mmol/L of glucose (control). Such changes were prevented by 100  $\mu\text{mol/L}$  of leptin quadruple mutant (LQM) and 10  $\mu\text{mol/L}$  bosentan (BST) (A, D). A glucose-like effect were seen when the cells were exposed to 5 nmol/L leptin (B, E) or 10 nmol/L ET-1 (C, F) which were also prevented by LQM and BST [mRNA is expressed as a ratio of target to  $\beta$ -actin (% relative to control); \* =  $p < 0.05$  as compared with 5 mmol/L of D-glucose (control),  $n \geq 10$  per group]

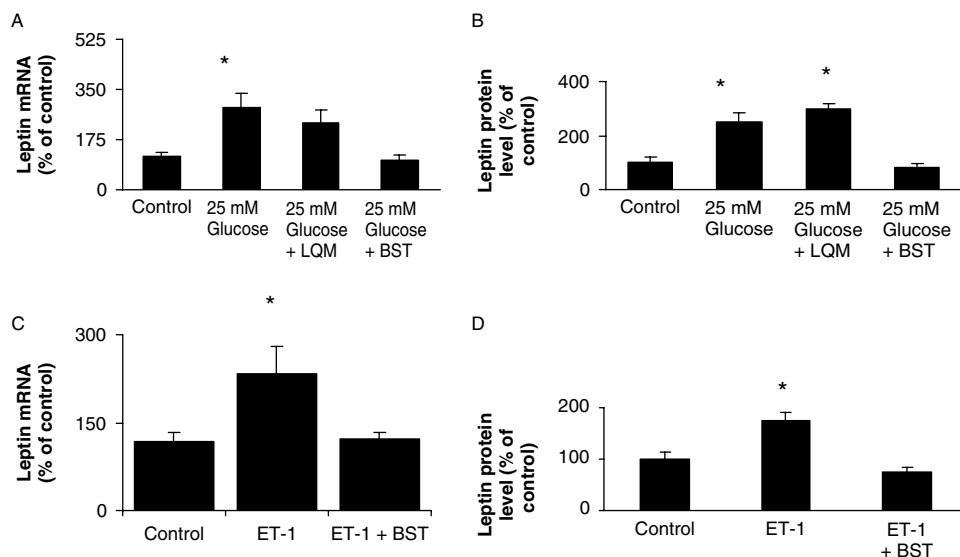
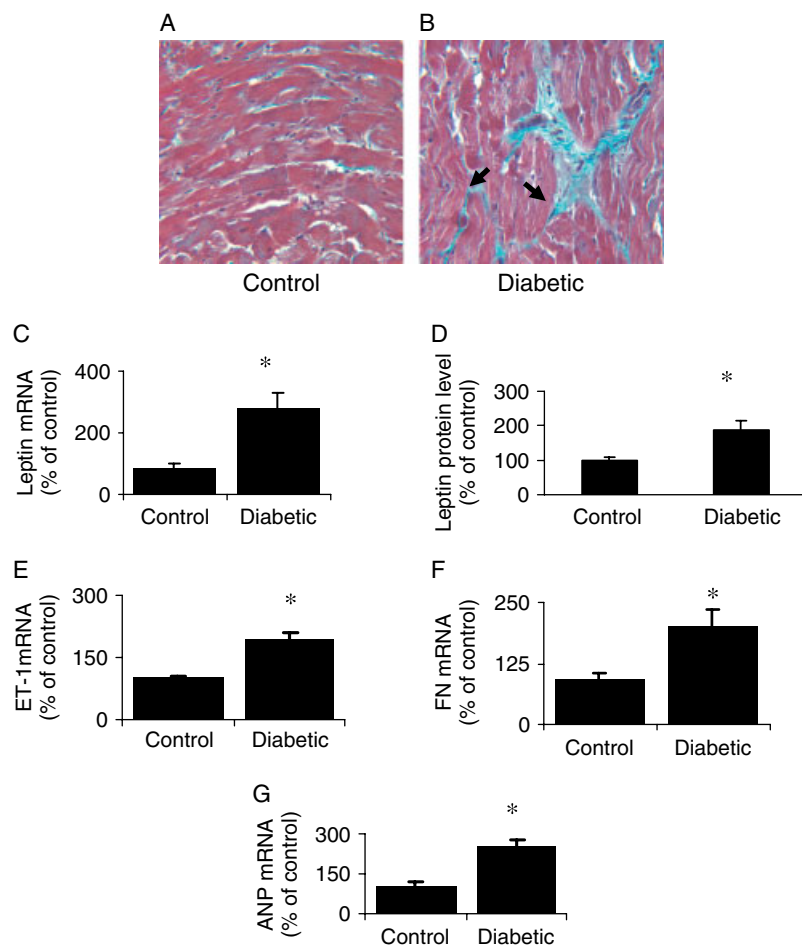


Figure 6. Twenty-five mmol/L of glucose caused increase in leptin mRNA (A) and protein (B) expression in cardiomyocytes as determined by real-time RT-PCR and ELISA, respectively, compared with 5 mmol/L of glucose (control). Glucose-induced increased leptin mRNA and protein expression were prevented by 10  $\mu\text{mol/L}$  bosentan (BST). Leptin mRNA (C) and protein expression (D) were upregulated when the cells were exposed to ET-1. Such upregulations were also prevented by BST [mRNA is expressed as a ratio of target to  $\beta$ -actin (% relative to control); \* =  $p < 0.05$  as compared with 5 mmol/L of D-glucose (control),  $n \geq 10$  per group]



**Figure 7.** Heart from diabetic rats after 4 months (B) showed focal myocardial fibrosis in trichrome staining (arrows) compared with age-matched control rats (A). Hearts from diabetic rats further showed significant upregulation of leptin mRNA (C) and protein (D) after 4 months of follow-up. ET-1 (E), FN (F) and ANP (G) transcripts were also upregulated [mRNA is expressed as a ratio of target to  $\beta$ -actin (% relative to control); \* =  $p < 0.05$  as compared with control,  $n = 6$  per group]. [Original magnification 50 $\times$  for both micrographs, \* =  $p < 0.05$  as compared with control, \*\* =  $p < 0.01$  as compared with control,  $n = 6$  per group]. This figure is available in colour online at [www.interscience.wiley.com/journal/dmrr](http://www.interscience.wiley.com/journal/dmrr)

In the present study, we have tried to explore some of the mechanisms of structural changes of diabetic cardiomyopathy, namely, interstitial fibrosis and cardiomyocyte hypertrophy. The main findings of this study are (1) glucose-induced increased ECM protein FN production as well as cardiomyocyte hypertrophy may be regulated by locally produced obesity-associated peptide leptin, (2) there is interaction between leptin and ET-1 and (3) both these peptides may regulate each other and play important roles in the development of diabetic cardiomyopathy. We used two different cell culture systems for *in vitro* experiments, namely HUVECs and neonatal cardiomyocytes. As there were no changes in FN expression or cardiomyocyte hypertrophy by L-glucose, we further confirmed that such changes are indeed glucose and not osmolality induced. Although the exact mechanism of diabetic cardiomyopathy is unknown, some common mechanisms that are of important in all chronic diabetic complications are increase polyol pathway activity, PKC activation, advanced glycation end products (AGE) formation, increased mitochondrial oxidative stress [2,4,5]. In addition, some properties,

specific to myocytes, such as increased free fatty acid uptake and altered calcium uptake by myocytes are also considered pivotal roles in the pathogenesis of diabetic cardiomyopathy [28,29]. Endothelial cell dysfunction, increased ECM protein production and subsequent fibrosis and injury to cardiomyocytes have also been postulated to be key mechanisms in diabetic cardiomyopathy [2,19,20]. Adipocytokine leptin primarily works as a satiety factor and may produce several effects in the non-adipose tissue [30]. Leptin has been shown to regulate cardiomyocyte contractility [30,31]. We have previously demonstrated, in non-diabetic condition, that leptin mediates hypertrophic effects of angiotensin II and ET-1 [21]. Leptin has been considered as marker of cardiovascular diseases because of its angiogenic and other effects on the endothelial cells and vasculature [32,33]. In keeping with other investigators, the STZ diabetic rats showed lower blood leptin levels [34]. Hence, this study indicates a direct role of locally produced leptin in glucose-induced increased ECM protein synthesis and cardiomyocyte hypertrophy in context of diabetic cardiomyopathy. Our data indicate that, along with FN,

its splice variant EDBFN is also upregulated *in vitro* by glucose can be prevented by leptin blockade. We have previously demonstrated that EDBFN may produce outside in-signalling and causes vascular endothelial growth factor (VEGF) alteration [8,18]. There are no data available on glucose-induced leptin-mediated FN production in endothelial cells. However, in mesangial cells from db/db mice, it has been shown to increase type I collagen production [17]. Increased leptin level has also been demonstrated in the vitreous of proliferative diabetic retinopathy [35]. We also have shown that leptin interacts with ET-1 and they regulate each other. ET-1 has been shown to play important role in the structural changes in the heart of diabetic animals [22,25,36]. In diabetes, ET-1 causes increased ECM protein synthesis that leads to capillary BM thickening and myocardial fibrosis [22,36]. We have previously demonstrated the role of ET-1 in cardiomyocyte hypertrophy and that ET-1 mRNA level in the heart correlates with its protein level [7,20,25]. In this study, we have demonstrated that ET-1 and leptin may have regulatory interactions in the production of their effects in diabetic cardiomyopathy. To characterize the effect of leptin, we used an LQM. This compound acts as competitive antagonist. The dose used in the study has been effective to prevent leptin signal transduction [23,24]. We have also used similar compound previously and demonstrated its efficiency [37,38]. Downstream effects of leptin signaling may involve JAK2/STAT3. Several other kinases such as Rho/Rock, ERK1/ERK2, p38 MAPK, PI3 kinases and PKC have been shown to be activated by leptin [30,37,38]. Interestingly several of these pathways are also activated by ET-1 [6,7,39,40]. It is to be noted that although neonatal cardiomyocytes have been widely used for the study of hypertrophy, potentially they may behave differently than adult cells. Such problems are difficult to address. However parallel to *in vitro* data, our animal experiments have demonstrated upregulation of leptin, ET-1, FN and ANP in association with cardiac hypertrophy (as evidenced by increased heart weight, myocardial thickness and ANP upregulation) and focal myocardial fibrosis after 4 months of diabetes, further indicating the role of leptin in diabetic cardiomyopathy. We have previously demonstrated that BST can prevent diabetes-induced structural changes in the hearts [22,36]. Although there are several publications as to the role of leptin *in vitro*, the *in vivo* role of leptin remains controversial [32]. This research suggests that leptin may be of importance because of diabetic cardiomyopathy. More studies are needed to establish this. These studies, however, would have been more powerful if we were able to prevent diabetes-associated structural and functional changes in the animal models through leptin blocker. However, as no inhibitors, suitable for animal use is available, such experiments were not possible. Other approaches may also include specific models such as ob/ob mice. Although genetic animal models have definite advantage, potential problems may also arise from their use. However, further experiments

are needed to establish definitive pathogenic role of leptin in diabetic cardiomyopathy.

Exact mechanism of diabetes-induced leptin and ET-1 upregulation is/are not clear. However, several mechanisms may be in place. PKC and MAPK activation, as demonstrated in the organs of diabetic complications, may be responsible factors [4,5,39]. Both ET-1 and leptin have been demonstrated to cause PKC activation [6,41]. It has also been demonstrated that ET-1 and leptin, in some tissues, may be regulated by PKC [6,42]. PKC activation may also be directly responsible to cardiac hypertrophy. It has been demonstrated that targeted overexpression of PKC $\beta$  in the heart may cause hypertrophy [43]. In addition, tissue hypoxia in diabetes may also cause increased mRNA expression of both leptin and ET-1 [6,44]. It is of further interest to note that O-linked N-acetyl glucosamine, one of the mediators of glucotoxicity may also cause increased leptin gene transcription [45,46]. Hence, a complex mechanism involving PKC, MAPK, hexosamine formation may lead to upregulation of these transcript and their interaction.

In summary, we have demonstrated novel role of leptin in diabetic cardiomyopathy and showed that leptin and ET-1 may regulate each other and produce increased ECM protein synthesis and cardiomyocyte hypertrophy in diabetes. Identification of such pathway is important as they may lead to potential treatment strategies for diabetic complications.

## Acknowledgements

This study was supported by grants from the Heart and Stroke Foundation of Ontario (HSFO) program in heart failure and Canadian Institute of Health Research (CIHR).

## Conflict of interest

None declared.

## References

1. Raman M, Nesto RW. Heart disease in diabetes mellitus. *Endocrinol Metab Clin North Am* 1996; **25**: 425–438.
2. Hayat SA, Patel B, Khattar RS, Malik RA. Diabetic cardiomyopathy: mechanisms, diagnosis and treatment. *Clin Sci (Lond)* 2004; **107**: 539–557.
3. Bell DS. Diabetic cardiomyopathy: a unique entity or a complication of coronary artery disease?. *Diabetes Care* 1995; **18**: 708–714.
4. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001; **414**: 813–820.
5. Sheetz MJ, King GL. Molecular understanding of hyperglycemia's adverse effects for diabetic complications. *JAMA* 2002; **288**: 2579–2588.
6. Levin ER. Endothelins. *N Eng J Med* 1995; **333**: 356–363.
7. Chen S, Khan ZA, Karmazyn M, Chakrabarti S. Role of endothelin-1, sodium hydrogen exchanger-1 and mitogen activated protein kinase (MAPK) activation in glucose-induced cardiomyocyte hypertrophy. *Diabetes Metab Res Rev* 2007; **23**: 356–367.

8. Khan ZA, Farhanghooe H, Mahon JL, *et al.* Endothelins: regulators of extracellular matrix protein production in diabetes. *Exp Biol Med* 2006; **231**: 1022–1029. Maywood).
9. Gulen S, Dincer S. Effects of leptin on oxidative stress in healthy and streptozotocin-induced diabetic rats. *Mol Cell Biochem* 2007; **302**: 59–65.
10. Kimura K, Tsuda K, Baba A, *et al.* Involvement of nitric oxide in endothelium-dependent arterial relaxation by leptin. *Biochem Biophys Res Commun* 2000; **273**: 745–749.
11. Purdham DM, Zou MX, Rajapurohitam V, Karmazyn M. Rat heart is a site of leptin production and action. *Am J Physiol* 2004; **287**: H2877–H2884.
12. Bjorbaek C, Uotani S, Da Silva B, Flier JS. Divergent signaling capacities of the long and short isoforms of the leptin receptor. *J Biol Chem* 1997; **272**: 32686–32695.
13. Bouloumie A, Marumo T, Lafontan M, Busse R. Leptin induces oxidative stress in human endothelial cells. *FASEB J* 1999; **13**: 1231–1238.
14. Lembo G, Vecchione C, Fratta L, *et al.* Leptin induces direct vasodilation through distinct endothelial mechanism. *Diabetes* 2000; **49**: 293–297.
15. Rajapurohitam V, Gan XT, Kirshenbaum LA, Karmazyn M. The obesity-associated peptide leptin induces hypertrophy in neonatal rat ventricular myocytes. *Circ Res* 2003; **93**: 277–279.
16. Xu F, Chen M, Wang Y, *et al.* Leptin induces hypertrophy via endothelin-1-reactive oxygen species pathway in cultured neonatal rat cardiomyocytes. *Circulation* 2004; **110**: 1269–1275.
17. Han DC, Isono M, Chen S, *et al.* Leptin stimulates type I collagen production in db/db mesangial cells: glucose uptake and TGFbeta type II receptor expression. *Kidney Int* 2001; **59**: 1315–1323.
18. Khan ZA, Caurterio J, Barbin YP, Chan BM, Uniyal S, Chakrabarti S. ED-B fibronectin in non-small cell lung carcinoma. *Exp Lung Res* 2005; **31**: 701–711.
19. Chen S, Khan Z, Cukiernik M, Chakrabarti S. Differential activation of NF-kappa B and AP-1 in increased fibronectin synthesis in target organs of diabetic complications. *Am J Physiol Endocrinol Metab* 2003; **284**: E1089–E1097.
20. Feng B, Chen S, Chiu J, George B, Chakrabarti S. Regulation of cardiomyocyte hypertrophy in diabetes at the transcriptional level. *Am J Physiol Endocrinol Metab* 2008; **294**: E1119–E1126.
21. Zeidan A, Purdham DM, Rajapurohitam V, Javadov S, Chakrabarti S, Karmazyn M. Leptin induces vascular smooth muscle cell hypertrophy through angiotensin II and endothelin-1-dependent mechanisms and mediates stretch-induced hypertrophy. *J Pharmacol Exp Ther* 2005; **315**: 1075–1084.
22. Evans T, Deng DX, Chen S, Chakrabarti S. Endothelin receptor blockade prevents extracellular matrix component mRNA expression and capillary basement thickening in the retina of diabetic and galactose-fed rats. *Diabetes* 2008; **49**: 662–666.
23. Peelman F, Van Beneden K, Zabeau L, *et al.* Mapping of the leptin binding sites and design of a leptin antagonist. *J Biol Chem* 2005; **279**: 41038–41046.
24. Niv-Spector L, Gonen-Berger D, Gourdou I, *et al.* Identification of the hydrophobic strand in the A-B loop of leptin as major binding site III: implications for large-scale preparation of potent recombinant human and ovine leptin antagonists. *Biochem J* 2005; **391**: 221–230.
25. Hileeto D, Cukiernik M, Mukherjee S, *et al.* Contributions of endothelin-1 and sodium hydrogen exchanger-1 in the diabetic myocardium. *Diabetes Metab Res Rev* 2002; **18**: 386–394.
26. Weigert C, Brodbeck K, Brosius III FC, *et al.* Evidence for a novel TGF-beta1-independent mechanism of fibronectin production in mesangial cells overexpressing glucose transporters. *Diabetes* 2003; **52**: 527–535.
27. Geiss LS, Herman WH, Smith PJ. Mortality in non-insulin-dependent diabetes. In *Diabetes in America*, Harris MI (ed). National Health Institute (Publication 95–1468): Bethesda, 1995; 233–257.
28. Feuvray D, Darmellah A. Diabetes-related metabolic perturbations in cardiac myocyte. *Diabetes Metab* 2008; **34**: S3–S9.
29. Fauconnier J, Andersson DC, Zhang SJ, *et al.* Effects of palmitate on Ca(2+) handling in adult control and ob/ob cardiomyocytes: impact of mitochondrial reactive oxygen species. *Diabetes* 2007; **56**: 1136–1142.
30. Karmazyn M, Purdham DM, Rajapurohitam V, Zeidan A. Signalling mechanisms underlying the metabolic and other effects of adipokines on the heart. *Cardiovasc Res* 2008; **79**: 279–286.
31. Dong F, Zhang X, Ren J. Leptin regulates cardiomyocyte contractile function through endothelin-1 receptor-NADPH oxidase pathway. *Hypertension* 2006; **47**: 222–229.
32. Yang R, Barouch LA. Leptin signaling and obesity: cardiovascular consequences. *Circ Res* 2007; **101**: 545–559.
33. Rahmouni K, Haynes WG. Endothelial effects of leptin: implications in health and diseases. *Curr Diab Rep* 2005; **5**: 260–266.
34. Sivitz WI, Walsh S, Morgan D, Donohoue P, Haynes W, Leibel RL. Plasma leptin in diabetic and insulin-treated diabetic and normal rats. *Metabolism* 1998; **47**: 584–591.
35. Gariono RF, Nath AK, D'Amico DJ, Lee T, Sierra-Honigmann MR. Elevation of vitreous leptin in diabetic retinopathy and retinal detachment. *Invest Ophthalmol Vis Sci* 2000; **41**: 3576–3581.
36. Chen S, Evans T, Mukherjee K, Karmazyn M, Chakrabarti S. Diabetes-induced myocardial structural changes: role of endothelin-1 and its receptors. *J Mol Cell Cardiol* 2000; **32**: 1621–1629.
37. Zeidan A, Paylor B, Steinhoff KJ, *et al.* Actin cytoskeleton dynamics promotes leptin-induced vascular smooth muscle hypertrophy via RhoA/ROCK- and phosphatidylinositol 3 kinase/protein kinase B-dependent pathways. *J Pharmacol Exp Ther* 2007; **322**: 1110–1116.
38. Zeidan A, Javadov S, Chakrabarti S, Karmazyn M. Leptin-induced cardiomyocyte hypertrophy involves selective caveolae and RhoA/ROCK-dependent p38 MAPK translocation to nuclei. *Cardiovasc Res* 2008; **77**: 64–72.
39. Xin X, Khan ZA, Chen S, Chakrabarti S. Extracellular signal-regulated kinase (ERK) in glucose-induced and endothelin-mediated fibronectin synthesis. *Lab Invest* 2004; **84**: 1451–1459.
40. Xin X, Khan ZA, Chen S, Chakrabarti S. Glucose-induced Akt1 activation mediates fibronectin synthesis in endothelial cells. *Diabetologia* 2005; **48**: 2428–2436.
41. Homs ME, Ducroc R, Claustre J, *et al.* Leptin modulates the expression of secreted and membrane-associated mucins in colonic epithelial cells by targeting PKC, PI3K, and MAPK pathways. *Am J Physiol Gastrointest Liver Physiol* 2007; **293**: 365–373.
42. Uchida Y, Ohba K, Ogawa A, Wada K, Yoshioka T, Muraki T. Protein kinase C mediates tumor necrosis factor- $\alpha$ -induced inhibition of obese gene expression and leptin secretion in brown adipocytes. *Naunyn-Schmiedeberg Arch Pharmacol* 1999; **360**: 691–698.
43. Wakasaki H, Koya D, Schoen FJ, *et al.* Targeted overexpression of protein kinase C beta 2 isoform in myocardium causes cardiomyopathy. *Proc Natl Acad Sci USA* 1997; **94**: 9320–9325.
44. Valerio A, Dossena M, Bertolotti P, *et al.* Leptin is induced in the ischemic cerebral cortex and exerts neuroprotection through NF-kappaB/c-Rel-dependent transcription. *Stroke* 2008; **40**: 610–617.
45. Issad T, Kuo M. O-GlcNAc modification of transcription factors, glucose sensing and glucotoxicity. *Endocrinol Metab* 2008; **19**: 380–389.
46. Fülöp N, Marchase RB, Chatham JC. Role of protein O-linked N-acetyl-glucosamine in mediating cell function and survival in the cardiovascular system. *Cardiovasc Res* 2007; **73**: 288–297.