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RESEARCH ARTICLE

Role of endothelin-1, sodium hydrogen exchanger-1 and mitogen activated protein kinase (MAPK) activation in glucose-induced cardiomyocyte hypertrophy

Shali Chen[1]
Zia A. Khan[1,4]
Morris Karmazyn[2]
Subrata Chakrabarti[1,3]*

[1]*Department of Pathology, University of Western Ontario, Ontario, Canada*

[2]*Department of Physiology and Pharmacology, University of Western Ontario, Ontario, Canada*

[3]*Department of Microbiology and Immunology, University of Western Ontario, Ontario, Canada*

[4]*Vascular Biology Program and Department of Surgery, Children's Hospital Boston, Harvard Medical School, MA, USA*

*Correspondence to:
Subrata Chakrabarti, Department of Pathology, University of Western Ontario, London, Ontario N6A 5C1, Canada.
E-mail:
subrata.chakrabrati@fmd.uwo.ca

Abstract

Background Cardiac hypertrophy is a key structural feature of diabetic cardiomyopathy. Previous studies have shown that diabetes-induced endothelin-1 (ET-1) and sodium hydrogen exchanger-1 (NHE-1) mediate structural and functional deficits in the heart. In order to gain a mechanistic understanding of the role of ET-1 and NHE-1 in cardiomyocyte hypertrophy, we have utilized an *in vitro* endothelial-myocyte co-culture system to reveal cellular interactions that may arbitrate cardiomyocyte deficits in diabetes.

Methods and Results Rat ventricular cardiomyocytes were cultured in high glucose levels, which caused cellular hypertrophy. Hypertrophic markers, atrial natruritic peptide (ANP) and angiotensinogen (Agt), as well as inducible nitric oxide synthase (iNOS) were upregulated by high glucose. Treatment of cells with ET antagonist bosentan and NHE-1 inhibitor cariporide prevented glucose-induced cardiomyocyte hypertrophy and expression of ANP, Agt, and iNOS. Bosentan and cariporide treatment of cardiomyocytes co-cultured with endothelial cells produced a more pronounced normalization of glucose-induced changes as compared to cardiomyocyte cultured alone. To further explore the signaling mechanisms involved, we investigated the mitogen activated protein kinase (MAPK) pathway and its cross-interaction with signaling proteins known to be altered in diabetes. Our results indicate that MAPK activation is associated with cardiomyocyte hypertrophy and is inhibited by bosentan, cariporide, as well as protein kinase C inhibiton. Furthermore, MAPK activation was found to be upstream of the transcription factors, nuclear factor-κB and activating protein-1.

Conclusion These results demonstrate that ET-1 and NHE-1 may mediate cardiomyocyte hypertrophy via MAPK activation and provide an insight into the pathogenesis of diabetic cardiomyopathy.

Keywords endothelin; diabetic cardiomyopathy; hypertrophy; NHE-1; MAPK

Introduction

Cardiac involvement in diabetes includes diabetic cardiomyopathy, which is manifested as a defective contractile function of cardiomyocytes [1,2]. The structural abnormalities comprise capillary basement membrane thickening, cardiac hypertrophy, focal ischemic changes, interstitial fibrosis, and reduced microvessel density [1,3–6]. These pathogenetic changes lead to cardiomyocyte "cell" loss and reactive cellular hypertrophy. The mechanisms leading to cardiomyocyte loss and hypertrophy in chronic diabetes are multifactorial and

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may involve metabolic disturbances as well as vascular endothelial dysfunction [7]. Studies have shown that endothelium-derived vasoactive factors, including endothelins (ETs), are altered in the heart and mediate the cardiac structural and functional changes [7,8]. These studies implicate an important role of endothelial cell-derived vasoactive factors in diabetic cardiomyopathy.

The primary ET isoform expressed in the heart is endothelin-1 (ET-1) [9,10]. The predominant source of ET-1 in adult cardiac tissues has been shown to be vascular endothelial cells as compared to cardiomyocytes [11,12]. These findings implicate the interaction between endothelial cells and cardiomyocytes as an important regulatory complex involved in cardiac deficits during chronic diabetes. We have previously shown an upregulation of both ET-1 and ET receptors in the heart of diabetic rats [8]. Diabetes-induced capillary basement membrane thickening, increased extracellular matrix (ECM) protein synthesis, and blood flow alterations in the heart are prevented by ET-receptor antagonism [8]. Studies have also suggested that the mechanism of ET-1 action in the heart may involve sodium hydrogen exchanger (NHE) [13]. Evidence from a variety of experimental models show that the cardiac effects of ET-1 may be mediated through the activation of Na^+/H^+ exchange [14,15]. Among the several known isoforms of NHE, sodium hydrogen exchanger-1 (NHE-1) is the major isoform expressed in the heart and plays an important role in tissue injury during ischemia and reperfusion [14,16].

A regulatory relationship between ET-1 and NHE-1 may represent a novel mechanism in diabetic cardiomyopathy. Using a well-established animal model of chronic diabetic complications, we have shown that both ET and NHE-1 inhibition prevents diabetes-induced structural and functional changes in the heart [13]. The inhibition of NHE-1 results in the upregulation of ET-1 mRNA levels, whereas the ET-receptor antagonist significantly reduces NHE-1 mRNA expression [13]. Furthermore, vascular hypertrophy in the streptozotocin-induced diabetic rats has been directly linked to NHE-1 overexpression [17,18]. Therefore, we hypothesize that the regulatory interaction between ET-1 and NHE-1 may mediate structural changes in the heart. To test this, we have investigated the mechanisms of glucose-induced and ET-1/NHE-1 mediated cardiomyocyte hypertrophy in an endothelial-cardiomyocyte co-culture system. In addition, we have elucidated the signaling pathways that may arbitrate cellular hypertrophic changes in the cardiomyocytes.

Materials and methods

Cell culture

Primary cultures of isolated neonatal cardiomyocytes

Primary cardiomyocyte cultures were prepared from neonatal Harlan Sprague–Dawley rat heart ventricles as described previously [19,20]. Non-myocytes were removed by differential attachment as described [20]. Furthermore, cells were characterized by light microscopy and immunostaining [20]. Isolated primary cardiomyocytes were plated onto 6 well culture plates (Primaria™ Falcon, Lincoln Park, NJ) at a density of 3.0×10^4 cells/cm^2 and were maintained for 48 h in Dulbecco's Modified Eagle's Medium/Ham's F-12 supplemented with 10% fetal bovine serum, 10 μg/mL transferrin, 10 μg/mL insulin, 10 ng/mL selenium, 50 units/mL penicillin, 50 μg/mL streptomycin, 2 mg/mL bovine serum albumin, 5 μg/mL linoleic acid, 3 mM pyruvic acid, 0.1 mM minimum essential medium (MEM) non-essential amino acids, 10% MEM vitamin, 0.1 mM bromodeoxyuridine, 100 μm L-ascorbic acid, and 30 mM HEPES (pH 7.1). Cells were serum-starved overnight prior to all experiments.

Endothelial cells

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (Walkersvile, Maryland). Cells were plated at a density of 1×10^5 cells in Endothelial Growth Medium (Clonetics, Walkersvile, Maryland) and incubated at 37 °C/5% CO_2 until confluent as described by us previously [21].

Endothelial-cardiomyocyte co-culture

For co-culture studies, HUVECs (1×10^5 cells per insert) were seeded onto 13-μm thick, 25 mm diameter polyethylene terephthalate membrane inserts with 0.4 μm pores (Becton Dickinson, Lincoln Park, NJ). The inserts were placed onto 6 well culture plates on which cardiomyocytes were previously cultured. The integrity of the endothelial monolayers was assessed as previously described [22]. Such a co-culture system creates a model in which conditioned media is shared between the two cell types and the study of cell–cell interaction is allowed. The cells were incubated for up to 2–4 days.

Cells were cultured in 25 mmol/L D-glucose (high glucose, HG) to study the effects of high glucose on cardiomyocyte hypertrophy. L-glucose was used as a control. In all experiments, dual ET-receptor antagonist bosentan (courtesy of Dr M Clozel, Acetelion ltd, Allschwill, Switzerland) [23], NHE inhibitor cariporide (Courtesy of Dr M Karmazyn) [24], and mitogen activated protein kinase (MAPK) inhibitor U0126 (Promega Corporation, Pittsburgh, PA) [25], were used at 10 μmol/L. Protein kinase C (PKC) inhibitor chelerythrine was used at 1 μmol/L [25]. For ET peptide treatment, cells were exposed to 5 nmol/L ET-1 (Peninsula Laboratories, Belmont, CA) [23]. The specificity and concentrations of these agents have been previously established [23–25]. All experiments were carried out after 2–4 days of incubation unless otherwise indicated. The experiments were conducted in three different preparations of the cells and were analyzed in replicates.

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Cell viability

Cell viability was examined by trypan blue dye exclusion test. Trypan blue stain was prepared fresh as a 0.4% solution in 0.9% sodium chloride. The cells were washed in phosphate buffered saline (PBS), trypsinized, and centrifuged. Twenty microlitres of cell suspension were added to 20 μL of trypan blue solution and 500 cells were microscopically counted in Burker cytometer. Cell viability was expressed as a percentage of the trypan blue negative cells in untreated controls.

Morphometric analysis

Measurements of the cardiomyocyte cell surface area were performed to assess cellular hypertrophy. Cells were visualized with a Leica inverted microscope and images were captured at 20X magnification. Cell area was determined using Mocha™ Software (SPSS, Chicago, IL). Cardiomyocyte surface area was determined from 50 randomly selected cells per experiment, averaged to provide an *n* value of one, and expressed as μm^2.

RNA isolation and cDNA synthesis

TRIzol™ reagent (Invitrogen Inc., Burlington, ON, Canada) was used to isolate RNA as previously described [21,23,25]. Total RNA (2 μg) was used for cDNA synthesis with oligo(dT) primers (Invitrogen Inc.). Reverse transcription was carried out by the addition of Superscript™ reverse transcriptase (Invitrogen Inc.).

Real time RT-PCR

Real time RT-PCR for ET-1, NHE-1, angiotensinogen (Agt), atrial natruritic peptide (ANP), and inducible nitric oxide synthase (iNOS) was carried out in the LightCycler™ (Roche Diagnostics Canada, Laval, PQ, Canada) using the SYBR Green I detection platform [23]. The reaction mixture consisted of 10 μL SYBR® Green Taq ReadyMix (Sigma–Aldrich, Canada), 1.6 μL of 25 mM $MgCl_2$, 1 μL of each forward and reverse 10 μM primers (Table 1) [23], 4.4 μL H_2O, and 2 μL cDNA template. The PCR reaction mixture for ET-1 consisted of 2.5 μL 10X PCR Buffer (Invitrogen Inc.), 1.25 μL of 5 mM dNTP (Invitrogen Inc.), 1.2 μL 50 mM $MgCl_2$, 1 μL primers, 9.8 μL H_2O, 2 μL cDNA, and 0.75 μL 15 mM Taqman probe (Table 1) [23].The data was normalized to the housekeeping gene (β-actin) to account for differences in reverse transcription efficiencies and the amount of template in the reaction mixtures. The results of RT-PCR were confirmed with 18S rRNA [23] normalization.

Table 1. Oligonucleatide sequences for RT-PCR

Gene	Sequence 5′ → 3′	Temperature profile[a]	
Rat ET-1	CTCGCTCTATGTAAGTCATGG	Denaturation	95°–0 s
	GCTCCTGCTCCTCCTTGATG	Annealing	58°–5 s
		Extension	72°–20 s
		Signal	84°–1 s
Human ET-1	AAGCCCTCCAGAGAGCGTTAT	Denaturation	95°–0 s
	CCGAAGGTCTGTCACCAATGT	Annealing	55°–5 s
	6FAM-TGACCCACAACCGAG-MGBNFQ	Extension	72°–4 s
		Signal	72°–1 s
Rat NHE-1	TCTGTGGACCTGGTGAATGA	Denaturation	95°–0 s
	GTCACTGAGGCAGGGTTGTA	Annealing	57°–5 s
		Extension	72°–10
		Signal	82°–1 s
Human NHE-1	CTCCACCGTCTCCATGCAGAACATCC	Denaturation	95°–0 s
	CCTTCAGCTCCTCATTCACCAGGTCC	Annealing	57°–5 s
	Ref. 26	Extension	72°–10
		Signal	82°–1 s
ANP	CTGCTAGACCACCTGGAGGA	Denaturation	95°–0 s
	AAGCTGTTGCAGCCTAGTCC	Annealing	55°–5 s
		Extension	72°–13 s
		Signal	88°–2 s
Agt	CTGCTAGACCACCTGGAGGA	Denaturation	95°–0 s
	AAGCTGTTGCAGCCTAGTCC	Annealing	50°–5 s
		Extension	72°–10 s
		Signal	80°–1 s
iNOS	ATGGAACAGTATAAGCGAAACACC	Denaturation	95°–0 s
	GTTTCCGGTCGATGTCATGAGCAAAGG	Annealing	57°–5 s
		Extension	72°–10 s
		Signal	83°–1 s
β-actin	CATCGTACTCCTGCTTGCTG	Denaturation	95°–0 s
	CCTCTATGCCAACACAGTGC	Annealing	55°–5 s
		Extension	72°–10 s
		Signal	83°–1 s

[a]Initial denaturation was carried out at 95 °C–1 min. Ramp rate for all PCR phases was 20 °C/s.

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Western blotting

Cells were lysed in lysis buffer (HEPES 50 mmol/L, pH 7.6, NaCl 150 mmol/L, NaF 50 μmol/L, EDTA 2 mmol/L, sodium vanadate 1 mmol/L, 1% NP-40, and phenylmethylsulphonyl fluoride 2 mmol/L). The protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Pierce, IL). Ten micrograms of protein was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were analyzed by western blotting using the rabbit anti-phospho-ERK44/42 antibody (1 : 1000; New England Biolabs, ON, Canada). Horseradish peroxidase-conjugated anti-rabbit antibody (1 : 5000; Santa Cruz Biotechnology, CA) was used for detection. The blots were stripped with Western Blot Stripping Buffer (Pearce Biotechnology Inc., Rockford, IL) and re-probed with anti-ERK (1 : 1000; Upstate Biotechnology, Charlottesville, VA). The blots were then quantified by densitometry using Mocha™ Software (SPSS, Chicago, IL) and the data expressed as a ratio of phospho-ERK44/42 to total ERK.

Nuclear protein extraction

Nuclear extracts of cells were prepared as described elsewhere [21,27]. Briefly, the cells were homogenized, washed with PBS, and pelleted. The pellet was then resuspended in 0.4 mL 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 DTT, and 0.5 mmol/L phenylmethanesulfonyl fluoride (PMSF)). After 15 minincubation on ice, 25 μL 10% IGEPAL CA-630 was added to the samples. The homogenate was centrifuged and the nuclear pellet was resuspended in 50 μL ice-cold buffer C (20 mmol/L HEPES, pH 7.9, 0.4 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and 1 mmol/L PMSF). The nuclear extracts were centrifuged and stored at −70 °C.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay (EMSA) was performed following an established methodology and as described by us previously [21,27]. Briefly, nuclear factor-κB (NFκB) and activating protein-1 (AP-1) consensus oligonucleotide (Promega, WI) DNA probes (Table 2) were prepared by end labeling with [γ-^{32}P] ATP (Amersham, QC, Canada) using T4 polynucleotide kinase. Five micrograms of nuclear proteins were incubated with 100 000 cpm of 32P-labeled consensus oligonucleotides for 30 min at room temperature. Protein-DNA complexes were resolved on a standard 6% (NFκB) and 4% (AP-1) nondenaturing polyacrylamide gel in 0.5X Tris-boric acid-EDTA running buffer. Gels were dried under heated vacuum onto Whartman paper and subjected to autoradiography overnight upto 3 days. Anti-NFκB (p65) monoclonal antibody and anti-AP-1 (c-Jun) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used for the supershift assay. The specificity of binding was further confirmed by incubation with excess unlabeled oligonucleotides. The blots were quantified by densitometry.

Table 2. Oligonucleotide sequences for EMSA

Transcription factor	Consensus oligonucleotide sequences
NFκB	5′-AGTTGAGGGGACTTTCCCAGGC-3″ 3′-TCAACTCCCCTGAAAGGGTCCG-5′
AP-1	5′-CGCTTGATGAGTCAGCCGGAA-3′ 3′-GCGAACTACTCAGTCGGCCTT-5′

Statistical analysis

The data are expressed as mean ± SEM and analyzed by ANOVA followed by Student's *t*-test with bonferoni correction. Differences were considered significant at values of $p < 0.05$.

Results

Glucose induces cardiomyocyte hypertrophy and upregulation of hypertrophy-associated genes

Cardiomyocyte hypertrophy and dysfunction is a key deficit in diabetic cardiomyopathy. We investigated whether high levels of glucose induced cardiomyocyte hypertrophy. Exposure of cardiomyocytes to high glucose levels increased the cell surface area following 48 h (Figure 1) but not 24 h (data not shown) of incubation. Hence, subsequent analyses were carried out with 48-h treatments. In order to determine whether cardiomyocyte hypertrophy was associated with cytotoxicity, we assayed for cell viability with trypan blue assay. Our results indicated no significant cytotoxicity in cells cultured in high glucose (data not shown). Furthermore, real time RT-PCR analyses revealed upregulation of hypertrophic markers, ANP and Agt, in association with glucose-mediated hypertrophy (Figure 2).

Glucose-induced cardiomyocyte hypertrophy is mediated by vasoactive factor alteration

We have previously demonstrated that diabetes-induced changes in the heart are prevented by ET and NHE-1 inhibition [13]. Treatment of cultured cardiomyocytes with dual ET-receptor antagonist and NHE-1 inhibitor prevented glucose-induced hypertrophy of the cardiomyocytes (Figure 1). The reduction of cellular hypertrophy was also associated with the significant reduction of mRNA expression of ANP and Agt (Figure 2). Next, we

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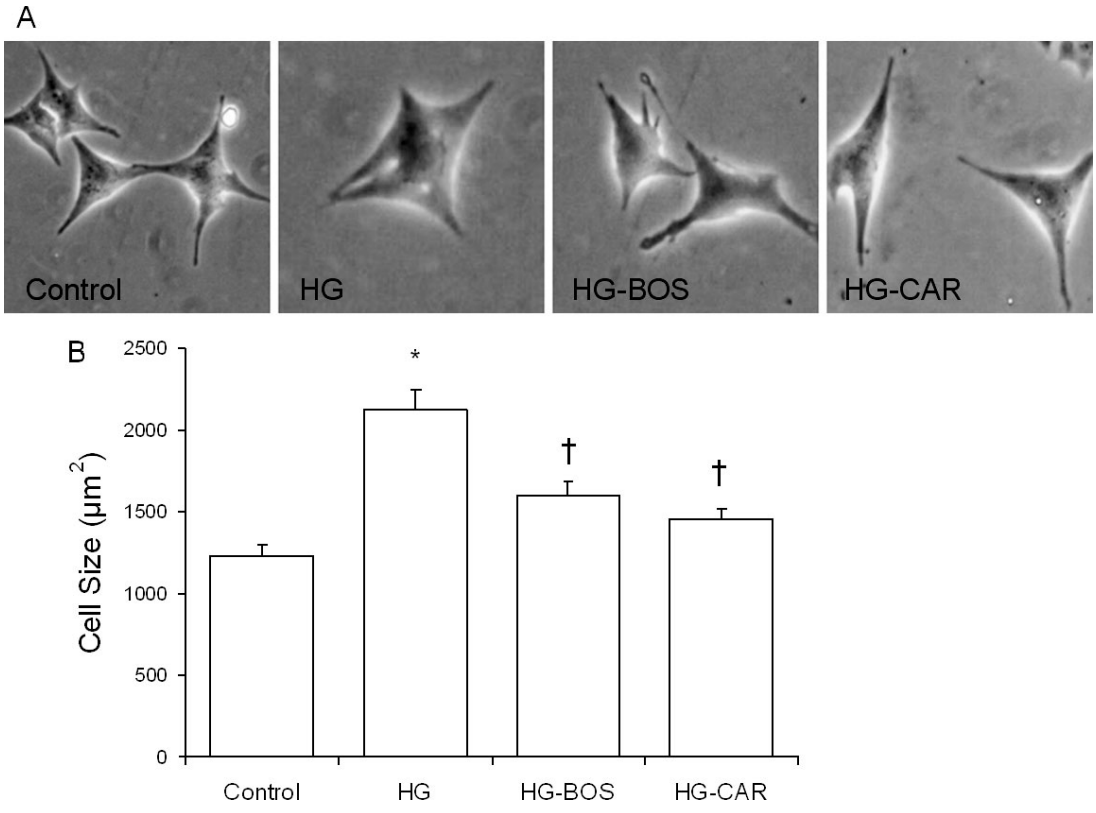

Figure 1. (A)Representative photographs of cardiomyocytes exposed to 5 mM of glucose (control), 25 mM of glucose (HG), 25 mM of glucose with bosentan (HG-BOS), and 25 mM of glucose with cariporide (HG-CAR). (B) cardiomyocyte size after 48 h of treatment [*$p < 0.05$ compared to control; †$p < 0.05$ compared to HG; $n = 6$/group, magnification same for (A-D)]

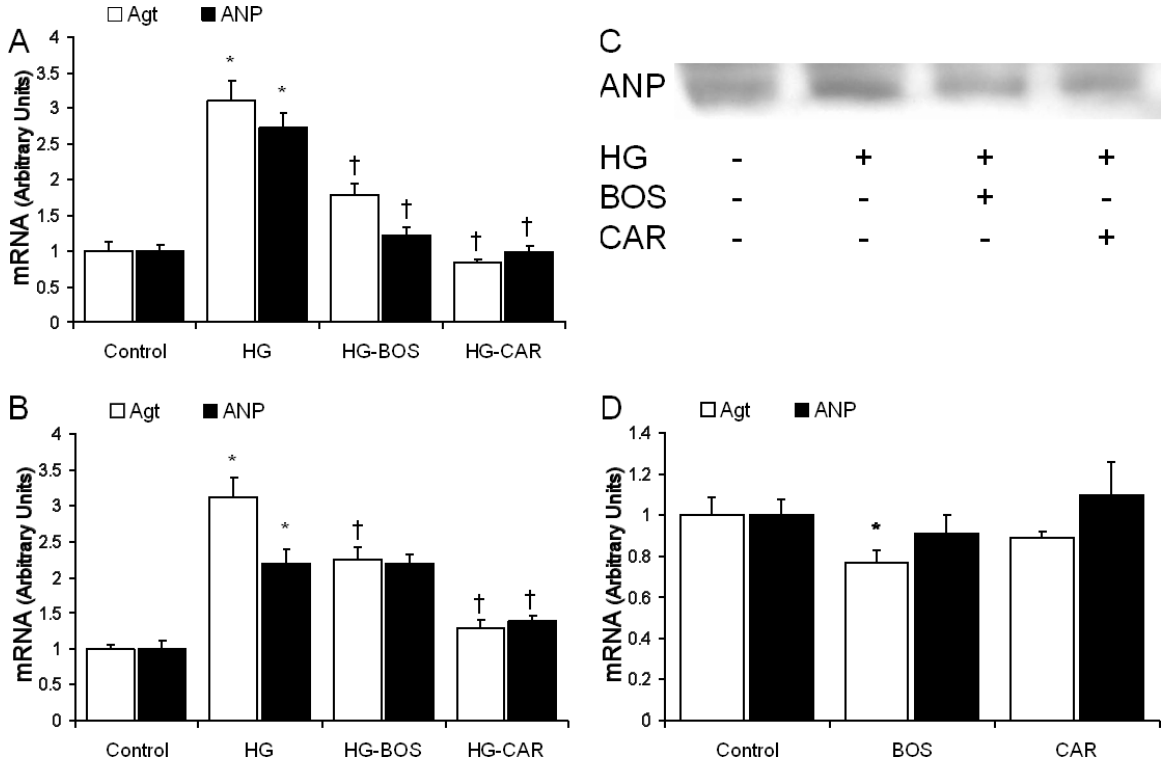

Figure 2. Real time RT-PCR and western blot analysis of cardiomyocyte hypertrophic markers showing, (A) Agt and ANP mRNA levels in cardiomyocyte-endothelial cell co-cultures, (B) Agt and ANP mRNA levels in cardiomyocyte monoculture, (C) ANP protein expression in co-cultures, and (D) effect of bosentan and cariporide (in 5 mmol/L glucose; control) on Agt and ANP mRNA in cardiomyocyte-endothelial co-cultures [mRNA is expressed as a ratio of target to β-actin (relative to control); *$p < 0.05$ compared to control; †$p < 0.05$ compared to HG; $n = 6$/group. HG = 25 mM glucose, BOS = Bosentan and CAR = Cariporide]

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determined whether co-culture of the cardiomyocytes with vascular endothelial cells would influence the effect of high glucose levels. Such a condition would provide a better assessment of the biochemical and structural changes in the hearts of diabetic patients. Interestingly, high levels of glucose increased Agt and ANP levels in cardiomyocytes with or without endothelial cell co-culture (Figure 2(A) and (B)). It is important to note that the relative levels of ANP were higher in the co-culture condition indicating an important perpetuating role of vascular endothelial cells. Furthermore, the effects of the inhibitors were more pronounced in the co-culture system (Figure 2(A)). Bosentan reduced cardiomyocyte hypertrophy but failed to normalize glucose-induced ANP levels in the cardiomyocyte monocultures. In the presence of endothelial cells, however, bosentan completely reversed the glucose-induced ANP upregulation suggesting a possible role of the endothelium in ANP regulation (Figure 2(A) and (C)). Treatment of co-cultures with bosentan or cariporide but without high glucose levels failed to show any significant changes in ANP (Figure 2(D)). These studies may also indicate parallel pathways of regulating cardiomyocyte hypertrophy.

Our next objective was to determine whether high levels of glucose cause alteration of specific vasoactive factors including ET-1 and nitric oxide (NO). Real time RT-PCR analyses showed increased mRNA expression of ET-1 and NHE-1 in cardiomyocytes cultured in high glucose concentration (Figure 3(A–C)). ET-1 levels, however, were significantly higher (>23%) in response to glucose in co-cultured cells as compared to cardiomyocytes cultured alone (Figure 3(A–C)). Treatment of cells with bosentan or cariporide reduced glucose-induced ET-1 and NHE-1 expression in both mono- and co-cultured cells. Cariporide treatment, however, reduced rather than increased ET-1 levels as previously shown in the whole heart [13].

Next, we assayed whether high glucose-induced changes in ET-1 mediate cardiomyocyte hypertrophy. Cardiomyocytes cultured with endothelial cells were exposed to ET-1 peptide and assayed for Agt and ANP expression as molecular markers of cardiomyocyte hypertrophic changes. Our results showed that ET-1 significantly increased ANP levels, which were significantly reduced with bosentan and only modestly with cariporide (Figure 4). Agt, however, exhibited slight changes in response to ET-1 and did not reach statistical significance.

Similar to ANP and Agt, iNOS mRNA levels were reduced to a greater extent in the co-cultured cells as compared to the cardiomyocyte monoculture (co-culture 81.5% *vs* monoculture 48% of HG; Figure 5). Furthermore, iNOS upregulation was evident at 24 h of treatment and sustained for 72 h (data not shown).

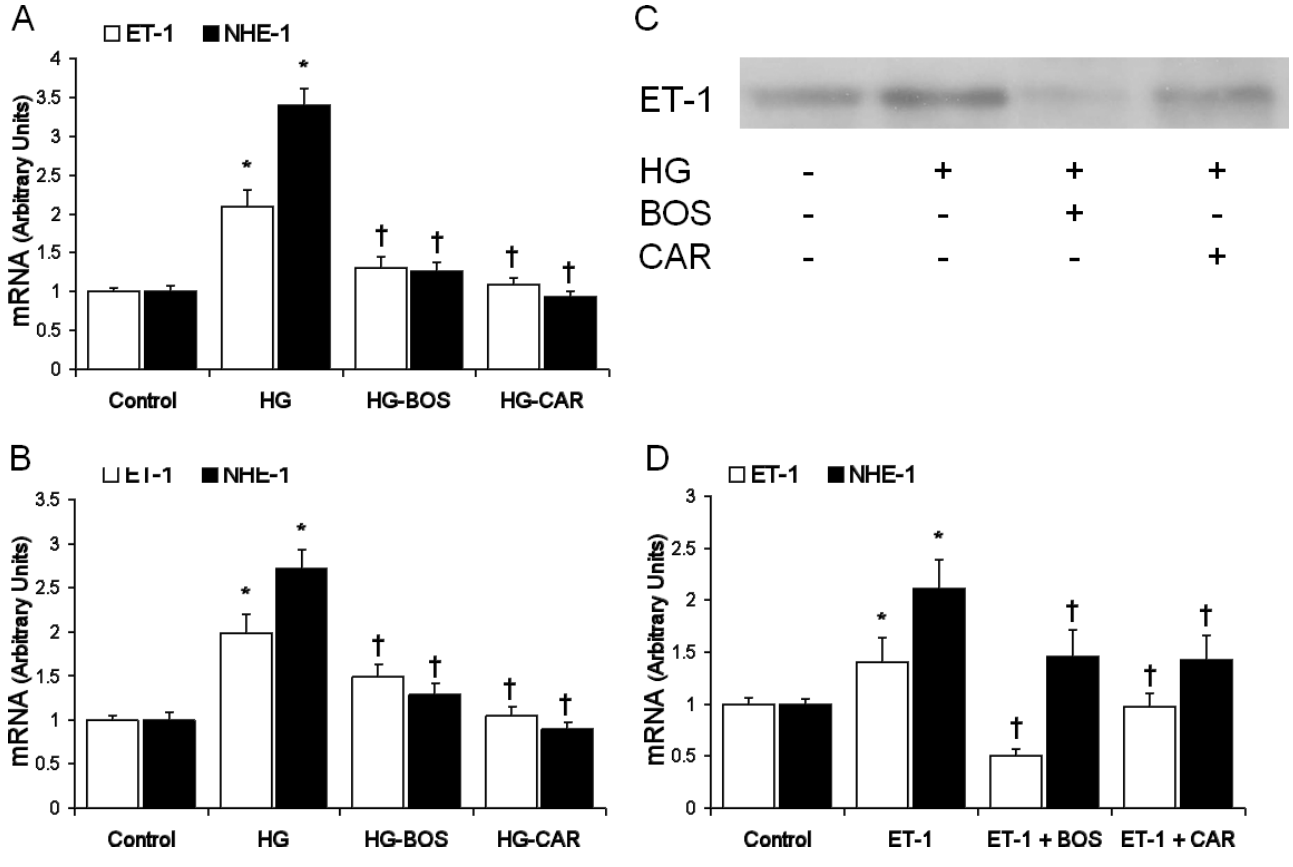

Figure 3. Vasoactive factor alteration in cardiomyocytes as assessed by real time RT-PCR and western blotting showing, (A) ET-1 and NHE-1 mRNA levels in cardiomyocyte-endothelial co-cultures, (B) ET-1 and NHE-1 mRNA levels in cardiomyocyte monocultures, (C) ET-1 protein levels in co-cultures, and (D) mRNA analysis of vasoactive factors in cardiomyocyte-endothelial co-cultures upon treatment with ET-1 peptide (5 nmol/L ET-1) [mRNA is expressed as a ratio of target to β-actin (relative to control); *$p < 0.05$ compared to control; †$p < 0.05$ compared to HG or ET-1; $n = 6$/group. ET-1 = 5 nM ET-1, BOS = bosentan and CAR = cariporide]

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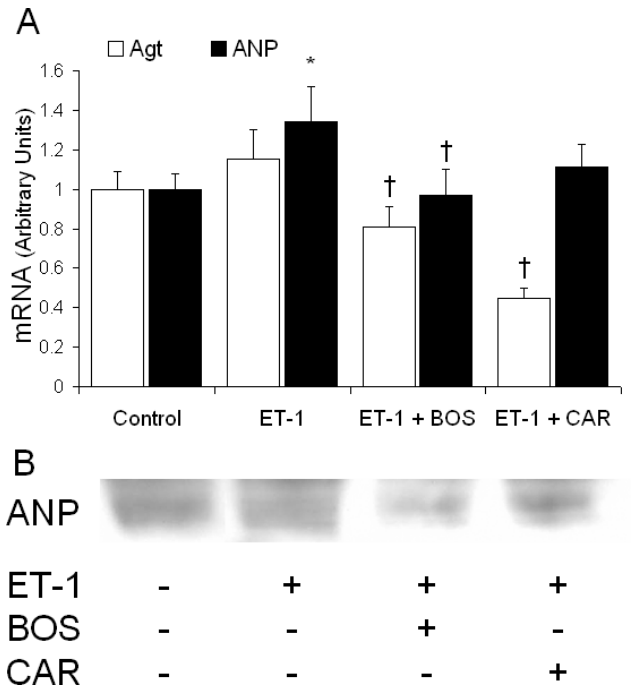

Figure 4. ET-1 mediated regulation of Agt and ANP expression showing, (A) mRNA analysis of Agt and ANP in cardiomyocyte-endothelial co-cultures, and (B) western blot analysis of ANP protein expression [mRNA is expressed as a ratio of target to β-actin (relative to control); *$p < 0.05$ compared to control; †$p < 0.05$ compared to ET-1; $n = 6$/group, BOS = bosentan and CAR = cariporide]

Treatment of co-cultured cells with ET-1 alone was sufficient to mimic high glucose effects in terms of NOS expression. These ET-1-induced iNOS levels were prevented with exposure of cells to both bosentan and cariporide (Figure 5(C)).

The presence of the endothelium resulted in a significantly better response of the cardiomyocytes to glucose-induced alterations, prompting investigation of the effect of high levels of glucose and ET/NHE inhibitors on endothelial cells. Our results indicate that high glucose levels increased expression of both ET-1 and NHE-1 in endothelial cells with or without cardiomyocyte co-culture (Figure 6). These results show that endothelial-derived vasoactive factors may be involved in perpetuating the hypertrophic signals for the cardiomyocytes.

Mechanism of glucose-induced vasoactive and hypertrophic factor expression

PKC activation has been demonstrated to be a key mechanism in several chronic diabetic complications [7]. We have also shown that MAPK, downstream of PKC, may contribute to increased ECM protein synthesis in the endothelial cells [25]. Such increased activity may also be mediated by transcription factors, NFκB and AP-1 [21,28]. Therefore, we investigated whether

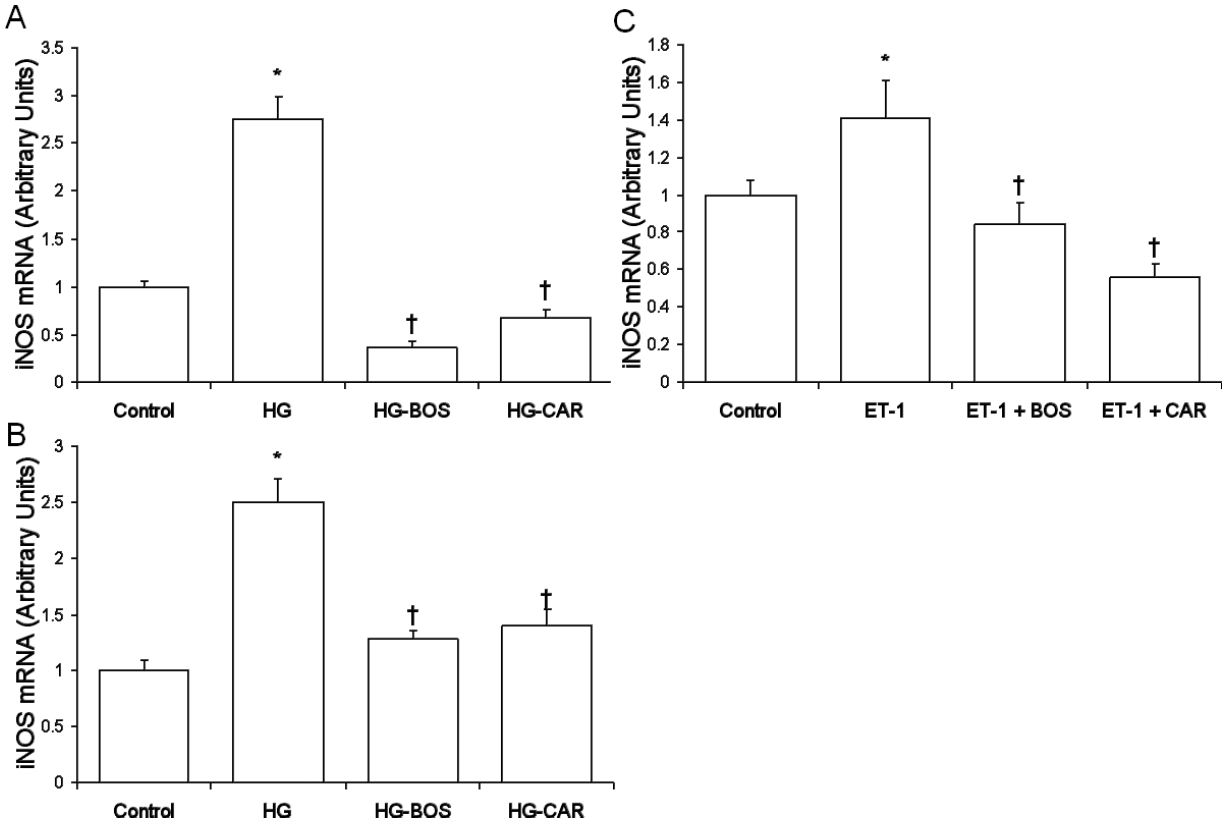

Figure 5. iNOS mRNA levels in cardiomyocyte-endothelial cell co-cultures (A), cardiomyocyte monocultures (B), and cardiomyocyte-endothelial co-cultures upon treatment with ET-1 peptide (C) [mRNA is expressed as a ratio of target to β-actin (relative to control); *$p < 0.05$ compared to control; †$p < 0.05$ compared to HG or ET-1; $n = 6$/group, BOS = bosentan and CAR = cariporide]

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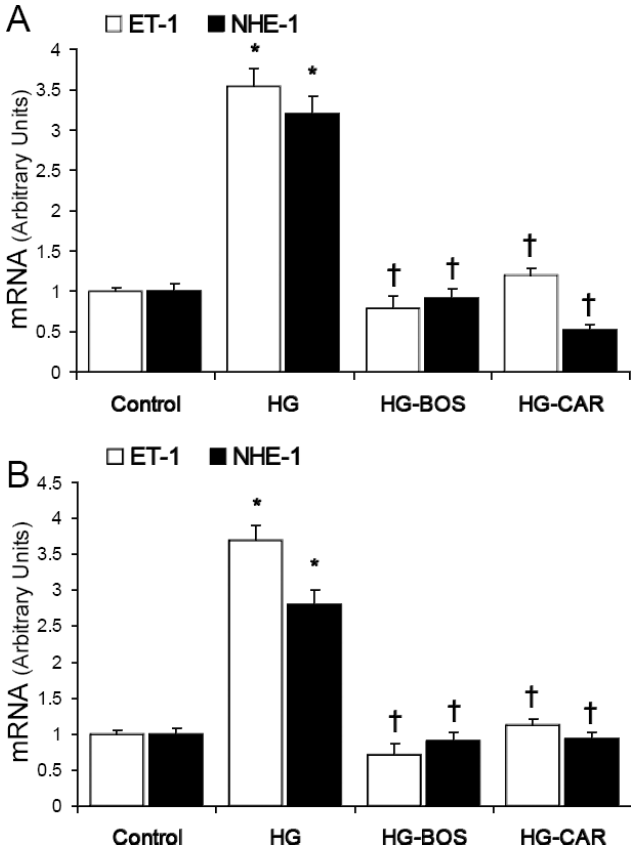

Figure 6. ET-1 and NHE-1 mRNA expression as assessed by RT-PCR in endothelial cells either cultured alone (A) or with cardiomyocytes (B) [mRNA is expressed as ratio of target to β-actin (relative to control); $^*p < 0.05$ compared to control; $\dagger p < 0.05$ compared to HG; $n = 6$/group, BOS = bosentan and CAR = cariporide]

such specific mechanistic pathways may play a role in glucose-induced upregulation of vasoactive factors and hypertrophic markers in the cardiomyocytes. Cardiomyocytes exposed to high glucose caused increased MAPK activity as assessed by immunoblot analysis of phospho-ERK44/42 (Figure 7(A) and (B)). Treatment of cells with bosentan and cariporide prevented glucose-induced MAPK activation. Our results further indicate an important cross-interaction between MAPK with the PKC pathway. Inhibition of PKC resulted in the complete normalization of glucose-induced MAPK activation. Exposure of the cells in low glucose levels (control) to the pharmacological inhibitors did not show any cytotoxic effects as assessed by trypan blue assay (data not shown). In order to determine whether MAPK may arbitrate glucose-induced cardiomyocyte hypertrophy and vasoactive factor alteration, we analyzed the cells following treatment with MAPK inhibitor U0126. Our results showed the complete attenuation of glucose-induced changes in ANP, ET-1 and NHE-1 mRNA levels following MAPK inhibitor treatment (Figure 7). Such normalization was also achieved with PKC inhibitor chelerythrine. Furthermore, Agt expression pattern paralleled to that of ET-1 and NHE-1.

We investigated the role of specific transcription factors, NFκB and AP-1, next as these transcription factors have been shown to be activated in various organs of chronic diabetic complications including the heart [21,28]. Exposure of cardiomyocytes to high glucose caused increased activation of NFκB and AP-1 (Figure 8). Such an activation of both transcription factors was prevented by PKC, MAPK, ET-1 and NHE-1 inhibition.

Discussion

Myocardial hypertrophy and subsequent cardiac failure are prominent features of diabetic cardiomyopathy [1,3–6]. The present study identifies signaling mechanisms leading to cardiomyocyte hypertrophy, which may be of importance in diabetic heart disease. The findings show that, (1) glucose-induced cardiomyocyte hypertrophy is mediated via both ET-1 and NHE-1, (2) pharmacological inhibition of hypertrophic factors was more effective in the presence of intact endothelium, (3) both PKC and MAPK play important roles in upstream and downstream effects of ET-1 and NHE-1, and (4) MAPK mediates changes possibly via the activation of transcription factors, NFκB and AP-1.

Over the past few decades, a wealth of knowledge accumulated suggests that the endothelial cells are critical in the proper function and maintenance of the cardiac tissue [29]. Cardiac endothelial cells, and in particular endocardium and myocardial capillary endothelial cells, directly interact with cardiomyocytes and may represent an important cell–cell junction for cardiac tissue integrity. It has been reported that the distance between a capillary endothelial cell and the most adjacent cardiomyocyte is approximately 1 μm [30,31]. This distance would suggest an efficient endothelial-cardiomyocyte signaling multiplex, even for endothelium-derived factors with a short half-life such as NO. It is, therefore, plausible that endothelial dysfunction together with metabolic disturbances in the myocytes may provide a microenvironment for further structural and functional changes including cellular hypertrophy. An interesting finding of the present study is that the effects of bosentan and cariporide on hypertrophic marker expression were more pronounced in the presence of intact endothelium. These results suggest that inhibiting endothelium-derived ET-1 and NHE-1 may influence other hypertrophy factors. For example, bosentan prevented cardiomyocyte hypertrophy, and protein kinase and transcription factor activation but failed to normalize glucose-induced ANP expression in cardiomyocyte monocultures. However, in endothelial-cardiomyocyte coculture, bosentan completely normalized glucose-induced ANP expression. It is to be noted that compared to the mRNA levels, ANP protein levels showed a modest increase, which may represent an early molecular change and/or a limitation of these experiments. These findings, however, suggest that endothelial cells may convey multiple signals leading to cardiomyocyte hypertrophy.

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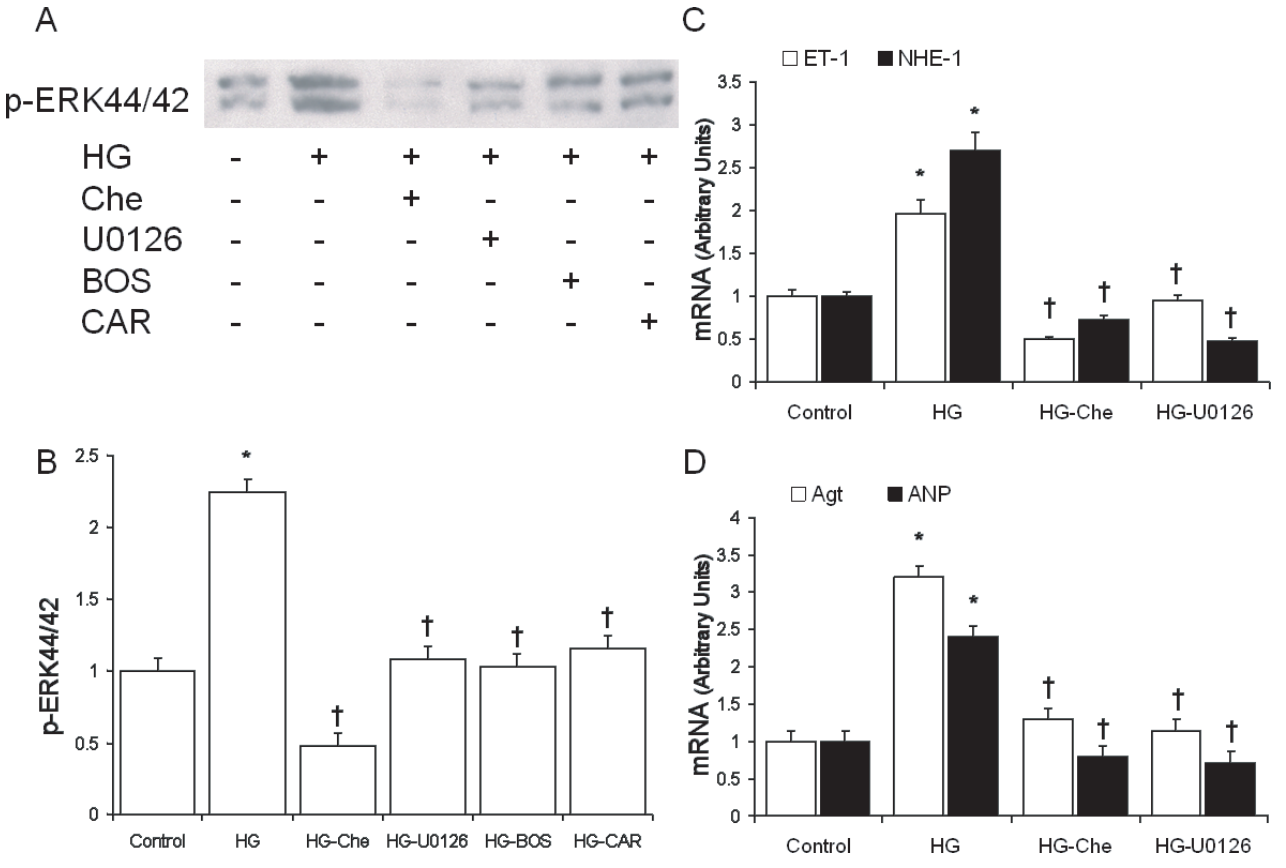

Figure 7. Glucose-mediated MAPK activation in cardiomyocytes showing, (A) representative immunoblot of phospho-ERK44/42, (B) densitometric analysis of MAPK activation, (C) ET-1 and NHE-1 mRNA levels following chelerythrine and MAPK inhibitor U0126 treatment, and (B) Agt and ANP mRNA levels following chelerythrine and U0126 treatment [densitometric analysis showing ratio of phospho-ERK44/42 to total ERK; *$p < 0.05$ compared to control; †$p < 0.05$ compared to HG; $n = 6$/group. HG = 25 mM glucose, BOS = bosentan, CAR = cariporide and Che = chelerythrine, U0126 is a MAPK inhibitor]

The exact mechanism, however, requires further investigation. It should be noted that a limitation of the co-culture system is species specificity. The exact relationship between endothelial cells and cardiomyocytes is difficult to interpret as not all of the factors released by the human endothelial cells may regulate rat cardiomyocyte function. Nonetheless, our studies do show that ET-1 expression in the cardiomyocytes is influenced by the presence of endothelial cells. In addition, inhibition of ET-1 and NHE-1 produced more pronounced effects in the co-culture system compared to the monoculture system.

Role of ET-1 and NHE-1 in cardiomyocyte and vascular hypertrophy has been demonstrated in other systems [17,18,32,33]. This study shows that they may play important roles in diabetic heart disease. ET and NHE-1 interact with each other to produce respective cellular effects [13]. This study further confirmed the regulatory relationships between the two factors in glucose-induced myocyte hypertrophy. This study further represents development of an *in vitro* model system to examine such mechanisms. Potential limitation of such a system, compared to *in vivo* studies, may include variation owing to the type of cells (adult *vs* neonatal) and species (rat *vs* human). We have shown that high levels of glucose increase the expression of ET-1 and NHE-1, which can be prevented by inhibiting either ET-1 or NHE-1. Determining the exact contribution of ET-1 and NHE-1 in the cardiomyocyte hypertrophic changes is hampered by the inability to specifically inhibit these factors in an endothelial- or cardiomyocyte-specific manner. In this study, we have used pharmacological inhibitors. These compounds, although specific, may have additional effects. Future studies using the gene knockdown approach may be of further help to delineate such mechanisms. It is, however, plausible that the interaction and cross-regulatory mechanism between ET-1 and NHE-1 may arbitrate cardiomyocyte structural and functional changes in the hearts of diabetic patients. Both ET-1 and NHE-1 have been shown to be augmented in diabetic patients [7,34]. In streptozotocin-induced diabetic rats, we have shown that ET-1 is upregulated in the heart and mediates increased ECM protein synthesis [28]. NHE-1, on the other hand, has been found to decrease in isolated cardiomyocytes [35]. However, increased mRNA expression as well as activity induction has been reported in mesenteric vasculature of 1-week streptozotocin-induced diabetic rats [18]. Such induction was associated with increased vascular hypertrophy and normalized by cariporide treatment. Our study parallels the aforementioned role of NHE-1. We have

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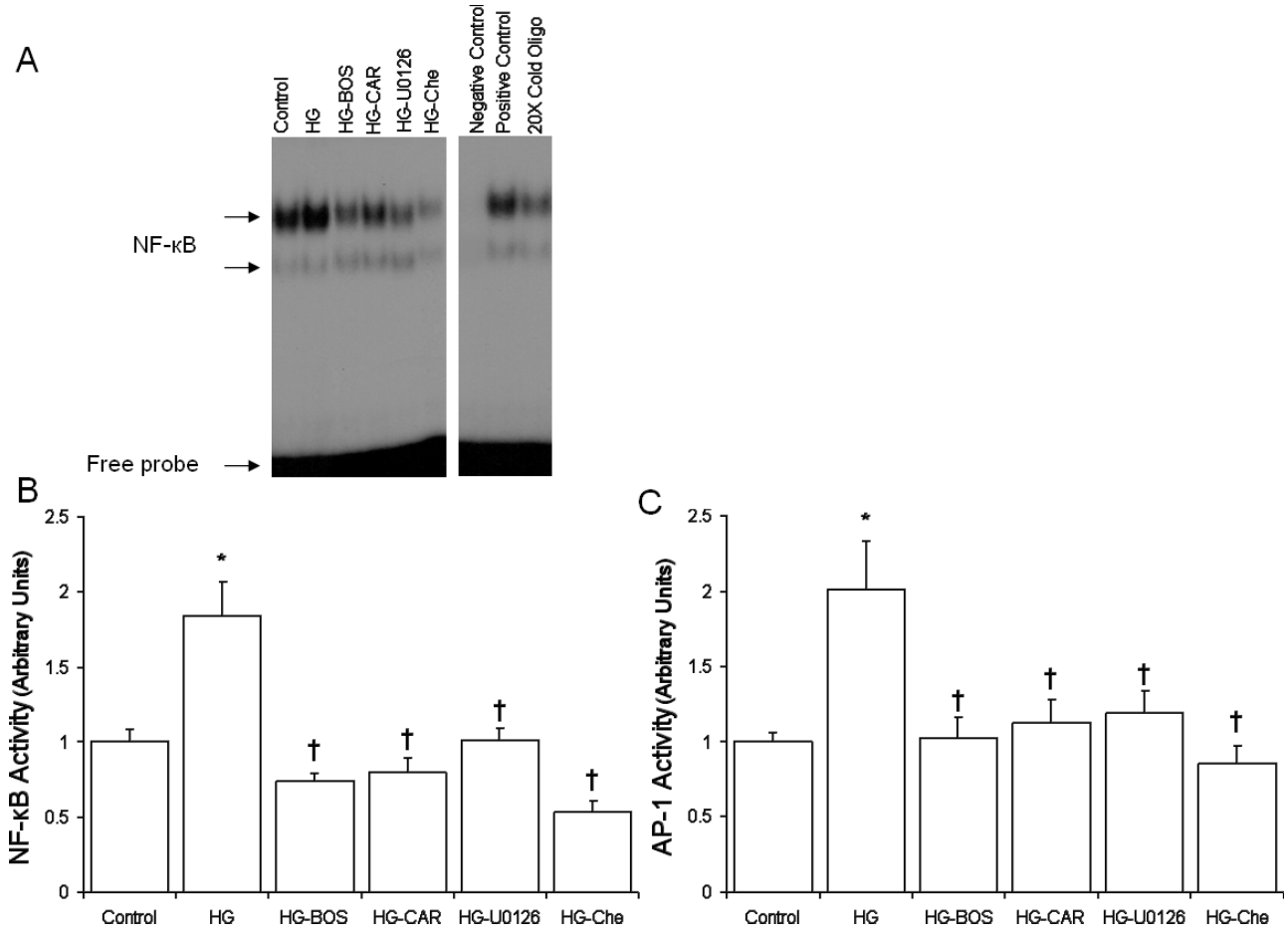

Figure 8. Transcription factor expression as assessed by electrophoretic mobility shift assay showing, (A) representative blot NF-κB binding, (B) densitometric analysis of NFκB activation, and (C) densitometric analysis of AP-1 activation [*$p < 0.05$ compared to control; †$p < 0.05$ compared to HG; $n = 6$/group BOS = bosentan, CAR = cariporide and Che = chelerythrine, U0126 is a MAPK inhibitor]

shown that both cardiomyocytes as well as endothelial cells cultured in high glucose increase expression of NHE-1. Furthermore, glucose-induced cardiomyocyte hypertrophy is prevented by treatment with cariporide.

The mechanism of ET-1 and NHE-1 induction in diabetes is not fully understood. Increased activity of PKC, which is well established in several organs affected by chronic diabetic complications including the heart, may mediate increased ET-1 and NHE-1. In fact, diacylglycerol-mediated PKC activation has been shown to upregulate ET-1 [7,36]. ET-1 has also been demonstrated to cause PKC activation [7,36], thus representing an important autoregulatory loop for the respective proteins. In addition, glucose-induced and PKC-mediated NHE-1 activation and increased mRNA expression has been demonstrated in the vascular myocytes [37,38]. NHE-1 may also be upregulated by ET-1 [13]. Results from our previous study suggest that NHE-1 may act downstream of ET-1. The current findings further support such a mechanism. Interestingly, NHE-1 may also regulate ET-1 expression as demonstrated in both cardiomyocytes and cultured endothelial cells. Hence, an inter-regulatory mechanism may control the availability of these molecules leading to the pathological changes in diabetes.

In an attempt to elucidate a common transducer of the adverse effects of high glucose, most studies have focused on identifying intracellular signaling molecules such as the MAPK pathway proteins. An important role of MAPK activation in diabetic complications is evident [25,39,40]. We have shown that glucose-induced ECM protein expression in endothelial cells is mediated, in part, by MAPK activation [25]. MAPK mRNA has also been demonstrated to correlate with glomerular lesions in diabetic nephropathy [41]. In addition, MAPK may regulate hypertrophy in renal tubular cells [42]. Also intriguing are findings which show reduced contractility of cardiomyocytes following MAPK activation [43]. Our present study shows that MAPK activation is associated with the augmentation of hypertrophic markers in the cardiomyocytes. As both cariporide and bosentan, partially inhibited MAPK activation, it appears that MAPK may be a downstream mediator by which both ET-1 and NHE-1 may exert their effects. The recent identification of p90 ribosomal S6 kinase (p90RSK) as the downstream mediator of oxidative stress and sustained acidosis may also implicate the p90RSK in the link between MAPK activation and NHE-1 induction [44,45]. Increased glucose-induced oxidative stress and ET-mediated MAPK

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activation may lead to increased p90RSK activity and subsequent expression of NHE-1. However, such a notion requires further investigation. Several other molecules may also play important roles in the intricate signaling pathways that regulate myocyte hypertrophy. Most significant MAPK inhibition following glucose exposure was seen with PKC inhibitor, chelerythrine, suggesting an important involvement of PKC. In addition, the results indicate an association between MAPK activation and the transcription factors, NFκB and AP-1. It is plausible that MAPK-mediated expression of hypertrophic genes is conveyed via these transcription factors. A current study indicates that NFκB and AP-1 may contribute to glucose-induced hypertophic factor production in myocytes. Moreover, hypertrophic genes assayed in the present study contain NFκB and AP-1 response element sites [44–48]. Hence, an intricate signaling mechanism may affect the myocyte abnormalities in diabetic patients. The findings of the present study show parallel activation of these inter-connected signaling pathways, which may provide an effective clue for combinatorial therapy.

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