Regulation of cardiomyocyte hypertrophy in diabetes at the transcriptional level

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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 294: E1119–E1126, 2008.—Diabetic cardiomyopathy, structurally characterized by cardiomyocyte hypertrophy and increased extracellular matrix (ECM) protein deposition, eventually leads to heart failure. We investigated the role of transcriptional coactivator p300 and its interaction with myocyte enhancer factor 2 (MEF2) in diabetes-induced cardiomyocyte hypertrophy. Neonatal rat cardiomyocytes were exposed to variable levels of glucose. Cardiomyocytes were analyzed with respect to their size, mRNA expression of p300, MEF2A, MEF2C, atrial natriuretic polypeptide (ANP), brain natriuretic polypeptide (BNP), angiotensinogen (ANG), cAMP-responsive element binding protein-binding protein (CBP), and protein analysis of MEF2 were done with or without p300 blockade. We investigated the hearts of STZ-induced diabetic rats and compared them with age- and sex-matched controls after 1 and 4 mo of followup with or without treatment with p300 blocker curcumin. The results were that cardiomyocytes, exposed to 25 mM glucose for 48 h, showed cellular hypertrophy and augmented mRNA expression of ANP, BNP, and ANG, molecular markers of cardiac hypertrophy. Glucose caused a duration-dependent increase of mRNA and protein expression in MEF2A and MEF2C and transcriptional coactivator p300. Curcumin, a p300 blocker, and p300 siRNA prevented these abnormalities. Similarly, ANP, BNP, and ANG mRNA expression was significantly higher in the hearts of diabetic rats compared with the controls, in association with increased p300, MEF2A, and MEF2C expression. Treatment with p300 blocker curcumin prevented diabetes-induced upregulation of these transcripts. We concluded that data from these studies demonstrate a novel glucose-induced epigenetic mechanism regulating gene expression and cardiomyocyte hypertrophy in diabetes.

Cardiomyocyte hypertrophy as well as subsequent apoptosis and focal myocardial fibrosis are structural hallmarks of diabetic cardiomyopathy and functionally manifest as defective cardiac contractility (10, 36, 45). Oxidative stress, due to glucose-mediated increase in mitochondrial superoxide production, has been suggested to be of importance in several chronic diabetic complications, including cardiomyopathy (6, 19). We have previously demonstrated that diabetes leads to myocardial hypertrophy in association with an upregulation of vasoactive factors such as endothelin-1 and activation of redox-sensitive transcription factors such as NF-κB and activating protein-1 (AP-1) (11, 19, 27). Transcription factors are regulated by transcriptional coactivators, especially those containing histone acetyltransferase (HAT) activity; p300 is the best known of such proteins (5, 9, 10). Histone-dependent packaging of genomic DNA is a key mechanism of gene regulation at the chromosomal level (38, 52). The remodeling of chromatin within the nucleus, controlled by the degree of acetylation/deacetylation of histone residues on the histone core around which DNA is coiled, is important in allowing access of transcription factors for DNA binding and gene transcription. Nuclear histone acetylation is a reversible process that is regulated by groups of HATs that promote acetylation and histone deacetylases (HDACs), which promote deacetylation. HATs and HDACs act in an opposing manner, controlling the access of transcriptional activators and gene induction (37, 38). Transcriptional coactivator p300 is thought to regulate several cellular processes via multiple transcription factors (25). p300 was first described as a protein associated with adenovirus E1, an oncoprotein (1, 2). However, p300 has also been shown to be involved in regulating a number of transcription factors, such as AP-1 and NF-κB (5, 9). Association of NF-κB with p300 is essential for their transcriptional activation; without such association, NF-κB remains transcriptionally silent even following nuclear translocation (56). Furthermore, p300 plays an important role in regulating both myocyte enhancer factor 2 (MEF2) and GATA-binding protein 4 (24). We have previously demonstrated that p300 activation may be a key event in mediating diabetes-induced increased extracellular matrix (ECM) protein expression (30). As previously mentioned, the histone acetylation activity of p300 and related proteins is balanced by HDACs (21, 38, 55). Three classes of HDACs have been identified thus far: class I, class II, and class III. Of the class II HDACs, both HDAC5 and HDAC9 knockout mice exhibit MEF2F activation and myocardial hypertrophy in response to the appropriate stimuli (7, 8, 47, 55). Detailed examinations as to the role of HATs, such as p300, and the downstream transcription factors in diabetic heart disease have not been carried out. MEF2 is an important transcription factor in myocyte hypertrophy (40). MEF2 is associated with class II HDACs. Translocation of HDAC to the cytoplasm frees up MEF2, which allows for its association with HATs, like p300, leading to the transcription of effector genes (40, 54). MEF2 controls the expression of many fetal cardiac genes. The normal adult heart has no MEF2-dependent gene expression (53). However, MEF2 gene expression is activated in cardiac hypertrophy. Moreover, blockade of MEF2-dependent gene expression completely inhibits cardiac hypertrophy caused by a variety of prohypertrophic stimuli (14).

The purpose of this study was to investigate the mechanisms of cardiomyocyte hypertrophy in diabetes. More specifically, we investigated the role of p300 in cardiomyocyte hypertrophy in diabetes and determined whether p300 exerts its effects...
through MEF transcription factors. These mechanisms were investigated at multiple levels of complexities such as in neonatal rat cardiomyocytes as well as streptozotocin-induced rat hearts.

MATERIALS AND METHODS

Isolation and culture of neonatal cardiomyocytes and siRNA transfection. Neonatal rat myocytes were isolated from newborn Harlan Sprague-Dawley rat heart ventricles, as described previously (17, 22).

Nonmyocytes were removed by differential attachment as described (17). Furthermore, cells were characterized by light microscopy and immunostaining (17). Isolated primary cardiomyocytes were plated onto 6-cm cell culture dishes (Primaria Tissue Culture Dish; Becton Dickinson) at a density of \(3.0 \times 10^5\) 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 \(\mu\)g/ml transferrin, 10 \(\mu\)g/ml insulin, 10 ng/ml selenium, 50 units/ml penicillin, 50 \(\mu\)g/ml streptomycin, 2 mg/ml bovine serum albumin, 5 \(\mu\)g/ml linoleic acid, 3 mM pyruvic acid, 0.1 mM minimum essential medium (MEM) nonessential amino acids, 10% MEM vitamin, 0.1 mM bromodeoxyuridine, 100 \(\mu\)M L-ascorbic acid, and 30 mM HEPES (pH 7.1). The cells were serum starved overnight prior to all experiments. Unless otherwise stated, all chemicals were of reagent grade quality and were purchased from Sigma Chemical (Sigma, Oakville, ON, Canada). All experiments were carried out after 48 h of incubation unless otherwise indicated. Curcumin (20 \(\mu\)M) was added 1 h before the addition of D-glucose (glucose). Twenty-five mM L-glucose was used as osmotic control. This dose of curcumin is based on several previous studies (4, 13). To establish the role of p300, we also performed siRNA-mediated gene silencing in cardiomyocytes. siRNAs were constructed to target the p300 mRNA using the siRNA construction kit (Silencer; Ambion) as described previously (51). The potential sites in p300 were identified by scanning the domain for AA dinucleotide sequences. After identification of the target sequences, oligonucleotides were synthesized for in vitro transcription and siRNA generation as previously described by us (30). The cardiomyocytes were transfected with p300 siRNAs (100 nM) using TransMessenger transfection reagent (Qiagen, Mississauga, ON, Canada). Scrambled siRNA were used as controls. siRNA transfection efficiency was determined by real-time RT-PCR.

Cell viability. Cell viability was examined by trypan blue dye exclusion test. Trypan blue stained 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 microliters of cell suspension was added to 20 µl of trypsin blue solution, and 500 cells were microscopically counted in Burker cytometer. Cell viability was expressed as a percentage of the trypsin blue-negative cells in untreated controls.

Morphometric analysis. Cardiomyocyte surface area was determined to assess cellular hypertrophy. Cells were visualized with a Leica inverted microscope, and images were captured at ×20 magnification. Cell area was determined using Mocha Software (SPSS). Cardiomyocyte surface area was determined from 50 randomly selected cells per petri dish and expressed as micrometers squared.

mRNA expression analysis. Rat myocytes were plated in a 6-cm dish 2 days before treatment. Cells were treated with fresh medium containing different agents for 48 h, and the total cellular RNA was extracted with TRIzol reagent (Invitrogen Canada) as previously described (13, 50, 51). Total RNA (2 µg) was used for cDNA synthesis with oligo(dT) primers (Invitrogen Canada). Reverse transcription was carried out by the addition of Superscript reverse transcriptase (Invitrogen Canada). The resulting cDNA products were stored at −20°C. Real-time quantitative RT-PCR was performed using the LightCycler (Roche Diagnostics Canada). For a final reaction volume of 20 µl, the following reagents were added: 10 µl of SYBR Green Tag ReadyMix (Sigma-Aldrich), 1.6 µl 25 mM MgCl₂, 1 µl each of forward and reverse primers (Table 1), 5.4 µl of H₂O₂; and 1 µl of cDNA. Melting curve analysis was used to determine melting temperature (Tm) of specific amplification products and primer dimers. For each gene, the specific Tm values were used for the signal acquisition step (2–3°C below Tm). The data was normalized to β-actin to account for differences in reverse-transcription efficiencies and amount of template in the reaction mixtures. All experiments were repeated at least twice in triplicate.

Protein expression analysis. After 48 h of treatment with high glucose and curcumin, rat myocytes were washed twice with cold PBS and lysed in lysis buffer (50 mmol/l HEPES, pH 7.6, 150 mmol/l glucose and curcumin, rat myocytes were washed twice with cold PBS and assayed for mRNA and protein expression. Total RNA (2 ug) was used for cDNA synthesis with oligo(dT) primers (Invitrogen Canada). For reverse transcription, the resulting cDNA products were stored at −20°C. Real-time quantitative RT-PCR was performed using the LightCycler (Roche Diagnostics Canada). The melting curve analysis was used to determine melting temperature (Tm) of specific amplification products and primer dimers. For each gene, the specific Tm values were used for the signal acquisition step (2–3°C below Tm). The data was normalized to β-actin to account for differences in reverse-transcription efficiencies and amount of template in the reaction mixtures. All experiments were repeated at least twice in triplicate.

RESULTS

Glucose causes hypertrophy of neonatal rat cardiomyocytes. We first determined whether we could reproduce our previous experimental results with respect to glucose-induced cardiomyocyte hypertrophy, since this is a key morphological feature in diabetic cardiomyopathy. We tried out exposing the cells to various glucose levels for various durations (data not shown). Treatment with 25 mM glucose for 48 h leads to a significant increase in the size of cardiomyocytes. Hence, subsequent analyses were carried out with 48-h treatments. To determine
whether cardiomyocyte hypertrophy was associated with cytotoxicity, we assayed for cell viability through a trypan blue assay. Our results indicate no significant cytotoxicity in cells cultured in high glucose (data not shown). No changes in gene expression or hypertrophy were seen when the cells were exposed to 25 mM-glucose (Figs. 1–4). Furthermore, real-time RT-PCR analyses revealed an upregulation of hypertrophic markers atrial natriuretic polypeptide (ANP), brain natriuretic polypeptide (BNP), and angiotensinogen (ANG) in association with glucose-mediated hypertrophy (Figs. 1 and 2).

Glucose-induced hypertrophy of neonatal rat cardiomyocytes is associated with p300 upregulation and activation of p300-dependent transcription factors. Next, we examined the epigenetic mechanisms and specific transcription factors in mediating glucose-induced cardiomyocyte hypertrophy. Twenty-five mM glucose caused upregulation of p300 mRNA, as evidenced by real-time PCR analysis, which was in association with hypertrophy. Immunofluorescence staining also showed increased nuclear immunoreactivity of p300 in cells treated with high glucose (Fig. 3). Such increase in p300 was also associated with augmented MEF2A and MEF2C mRNA production by cardiomyocytes when exposed to 25 mM glucose. Western blot analysis revealed increased MEF2 protein levels in cells exposed to 25 mM glucose (Fig. 4).

p300 inhibition prevents glucose-induced cardiomyocyte hypertrophy and MEF2 activation. To establish a cause-effect relationship, we blocked p300 using two different methods. We transfected the cells with p300 siRNA, and we also used a...
chemical compound curcumin. Curcumin is a known p300 blocker. Treatment with curcumin prevented glucose-induced cardiomyocyte hypertrophy and increased ANP mRNA expression. We also used p300 siRNA transfection to nullify effects of p300. Efficiency of siRNA transfection was assessed by real-time PCR, which showed significant inhibition of p300 mRNA production (Fig. 3). No effect of p300 siRNA or curcumin was seen on cAMP-responsive element-binding protein-binding protein (CBP) gene expression, indicating specificity of such inhibition. p300 siRNA showed limited effect on basal p300 mRNA expression. However, p300 siRNA as well as curcumin also prevented glucose-induced mRNA expression of the genes that are associated with cardiomyocyte hypertrophy, such as ANP and BNP (Fig. 2). p300 siRNA and curcumin also prevented glucose-induced MEF2A and MEF2C mRNA upregulation and MEF2 protein production. These data further indicate that MEF2 transcription factors are important in cardiomyocyte hypertrophy.

Hypertrophy of diabetic rat heart is associated with similar genetic alterations in vivo at 1 and 4 mo. We then expanded the study and investigated the diabetic rat hearts at 1 and 4 mo of diabetes. Diabetic animals showed reduced body weight, glucosuria, polyuria, and hyperglycemia. No effect of curcumin treatment was seen on these parameters (Table 2). Analysis of cardiac tissues in the diabetic animals showed significant upregulation of p300 as well as MEF2A and MEF2C mRNA. Diabetes was further associated with upregulation of hypertrophy-associated genes ANP, BNP, and ANG.

Table 2. Clinical monitoring

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>BW, kg</th>
<th>HW, g</th>
<th>HW/BW, g/kg</th>
<th>BG, mmol/l</th>
<th>BW, kg</th>
<th>HW, g</th>
<th>HW/BW, g/kg</th>
<th>BG, mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.51±0.02</td>
<td>1.25±0.07</td>
<td>2.5±0.2*</td>
<td>4.3±0.3*</td>
<td>0.62±0.02</td>
<td>1.44±0.18</td>
<td>2.3±0.2†</td>
<td>6.5±0.7†</td>
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<tr>
<td>Diabetic</td>
<td>0.43±0.03</td>
<td>1.20±0.10</td>
<td>2.8±0.2</td>
<td>20.9±2.3</td>
<td>0.48±0.04</td>
<td>1.47±0.21</td>
<td>3.1±0.2</td>
<td>21.4±3.5</td>
</tr>
<tr>
<td>Diabetic + Curcumin</td>
<td>0.41±0.01</td>
<td>1.12±0.08</td>
<td>2.7±0.1</td>
<td>19.9±1.4</td>
<td></td>
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</tbody>
</table>

Data are means ± SE; n = 6. BW, body weight; HW, heart weight; BG, blood glucose. *P < 0.05 compared with other groups; †P < 0.05 compared with diabetic group.

Fig. 5. mRNA analysis of cardiac tissues (ratio of target to β-actin, normalized to controls) from streptozotocin (STZ)-induced diabetic rats after 1 (1M) and 4 mo (4M) of diabetes showing diabetes-induced upregulation of p300 and markers for hypertrophy (ANP, ANG, and BNP, MEF2A, and MEF2C). One month of cur treatment prevented such upregulation (data are expressed as means ± SD; n = 6/group. *P < 0.05 compared with other 1M group; **P < 0.05 compared with other 4M group).
This upregulation was evident after 1 mo of diabetes and was sustained after 4 mo (Fig. 5). It is of interest to note that MEF2A activation was more pronounced at 1 mo, whereas MEF2C levels were similar at 1 and 4 mo of diabetes. The diabetic animals further showed increased heart weight and heart weight/body weight ratio (Table 2). Treatment of diabetic animals with curcumin prevented such abnormalities.

DISCUSSION

In this study, we have shown that diabetes-induced cardiomyocyte hypertrophy is regulated at the transcriptional level via p300-mediated regulation of transcription factor MEF2. We demonstrated this mechanism both in vivo and in vitro. This is the first demonstration of such epigenetic mechanisms leading to cardiomyocyte hypertrophy in diabetes.

Histone residues may be modified by a variety of processes, including acetylation/deacetylation, methylation, phosphorylation, etc. Such epigenetic modification may lead to a stable alteration (e.g., methylation) or a highly dynamic alteration in the case of acetylation (46). Histone acetylation at the lysine residues causes chromatin relaxation and augments gene expression (3, 28). p300 is one of the most well-characterized HATs. The role of transcriptional coactivator p300 has been demonstrated in various disease processes (16, 20). The findings in this study also support these notions. We have previously demonstrated that p300 activation through NF-κB and AP-1 is instrumental in the production of increased ECM protein production (30). Deposition of ECM proteins leads to focal fibrosis in the heart in diabetes. The current study indicates that p300 activation is also important in causing cardiomyocyte hypertrophy, the other characteristic structural alteration in the heart. Interaction of p300 with multiple transcription factors simultaneously may indicate transcriptional synergy, a recognized mechanism of action (15, 41, 42). This mechanism could underlie the ability of p300 to act as a bridge, as a scaffold, or by acetylation of core histone tails, which leads to increased DNA access, weakening of internucleosomal interaction, and destabilization of the chromatin structure. As mentioned previously, the activity of such acetylators are balanced by HDACs, which suppresses transcription (26). Association of p300 with the transcription factors is important for gene transcription (48). In this study, we demonstrated that transcription factors MEF2A and MEF2C are involved in mediating the hypertrophic action of glucose on cardiomyocytes. MEF2 is an important transcription factor in myocyte hypertrophy (33, 44) and is associated with class II HDACs (55). The transcription of effector genes is initiated once HDAC has been translocated to the cytoplasm, thereby allowing MEF2 to associate with HATs such as p300 (40, 54). p300 acts as an adaptor protein for MEF2 action (39). MEF2 controls the expression of many fetal cardiac genes. The normal adult heart has no MEF2-dependent gene expression (3, 43). However, MEF2 gene expression is reactivated in cardiac hypertrophy. Moreover, blockade of MEF2-dependent gene expression completely prevents cardiac hypertrophy caused by a variety of prohypertrophic stimuli (14). We have previously shown the reexpression of fetal genes such as extra domain B containing fibronectin in the retina and heart in diabetes (31, 32). However, this study clearly demonstrates the role of MEF2 in diabetic cardiomyopathy. Interestingly, slight duration-dependent differences in the activation pattern of MEF2A and MEF2C were seen. The exact significance of such differences is not clear and may require further investigation. Two p300 blockers were used in vitro. In vivo, we used curcumin as a p300 blocker. Curcumin, the active component in tumeric rhizomes (Curcuma longa L), has long been used in traditional Asian and Indian medicine. Several studies have indicated a beneficial role of curcumin in terms of antioxidant, antitumourigenic, and anti-inflammatory property (34, 35). Treatment with curcumin has been shown to reduce glucose-induced reactive oxygen species levels in human red blood cells isolated from patients (31). It has been shown that curcumin may mediate its effects through the inhibition of p300’s interaction with transcription factors (4). However, it is possible, as with any chemical inhibitor, that other mechanisms of action may exist. Nevertheless, our in vitro experiments utilizing a specific blocker, p300 siRNA, yielded similar results. Furthermore, no significant effect of p300 siRNA or curcumin on CBP, another HAT, was seen. It appears that p300 inhibition may be the main mechanism of action of curcumin in these experiments. Several signaling mechanisms activated by hyperglycemia may stimulate the activation of p300. We have previously demonstrated that, in endothelial cells, hyperglycemia-induced protein kinase C and mitogen-activated protein kinase, as well as protein kinase B activation, may enhance the activity of p300 (30). All such pathways have been demonstrated in the heart of diabetic animals and may potentially contribute to p300 activation (12, 23, 49). This study supports the notion that p300 may represent a common final pathway upon which several signaling mechanisms may converge. Hence, p300 may also lend itself as a potential therapeutic target. The capacity of naturally occurring nontoxic substances, such as curcumin, to prevent such activation is also interesting from the standpoint of adjuvant therapy development.

In summary, we have demonstrated that p300 activation, through transcription factor MEF2, may mediate cardiomyocyte hypertrophy in diabetes. p300 potentially may be a therapeutic target for prevention of such abnormalities.

GRANTS

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REFERENCES


