Adverse effects of high glucose and free fatty acid on cardiomyocytes are mediated by connective tissue growth factor

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Wang X, McLennan SV, Allen TJ, Tsoutsman T, Sensarian C, Twigg SM. Adverse effects of high glucose and free fatty acid on cardiomyocytes are mediated by connective tissue growth factor. Am J Physiol Cell Physiol 297: C1490–C1500, 2009. First published July 22, 2009; doi:10.1152/ajpcell.00049.2009.—Diabetic cardiomyopathy is characterized by interstitial fibrosis and cardiomyocyte hypertrophy and apoptosis. Also known as CCN2, connective tissue growth factor (CTGF) is implicated in the fibrosis; however, whether it contributes to cardiomyocyte changes and adverse effects of high glucose and lipids on these cells remains unknown. Hearts from streptozotocin-induced diabetic rats had elevated CTGF and changes of pathological myocardial hypertrophy, fibrosis, and cardiomyocyte apoptosis. Rat H9c2 cardiomyocytes were then treated with recombinant human (rh)CTGF, high glucose, or the saturated free fatty acid palmitate. Each reagent induced cell hypertrophy, as indicated by the ratio of total protein to cell number, cell size, and gene expression of cardiac hypertrophy marker genes atrial natriuretic peptide (ANP), and α-skeletal actin. Each treatment also caused apoptosis measured by increased caspase3/7 activity, apoptotic cells by transverse-mediated dUTP nick end labeling (TUNEL) assay, and lower viable cell number. Further studies showed CTGF mRNA was rapidly induced by high glucose and palmitate in H9c2 cells and in mouse neonatal cardiomyocyte primary cultures. Small interfering RNA against CTGF blocked the high glucose and palmitate induction of hypertrophy and apoptosis. In addition, these CTGF effects were through the tyrosine kinase A (TrkA) receptor with tyrosine kinase bioactivity of other cytokines, especially transforming growth factor-β (TGF-β) (2), indicating that TGF-β protein is required for CTGF to exert its effect. In addition, CTGF was reported to activate TGF-β1 signaling pathway through the cell surface receptor, but CTGF action to cause fibrosis in tissues with accumulation of ECM components has been the main focus of our and other’s work in diabetes (16, 33), in some cells, CTGF causes apoptosis via activated caspase 3 pathways (21). CTGF can also cause cell hypertrophy, as documented in cardiac myocytes (18). To date, apoptosis induction by CTGF has not been reported in diabetic cardiomyocytes. Thus CTGF may be a common factor in the two pathologies of cardiac apoptosis and hypertrophy.

In vitro, CTGF is upregulated by metabolic stimuli present in diabetes, including high glucose (33) and soluble advanced glycation end products (47, 48), and by hemodynamic factors implicated in myocardial pathology: angiotensin II (3, 45), aldosterone (30), endothelin-1 (25), static pressure (22), and cellular stretch (6). However, it has not been reported in any cell system, including cardiac myocytes, whether FA regulates CTGF nor whether CTGF might mediate adverse effects of FA in diabetic cardiomyocytes or in any other cell type.

Cell surface membrane signaling of CTGF via a defined receptor pathway has not been reported in cardiac myocytes. In some noncardiac cell systems, CTGF functions by modulating bioactivity of other cytokines, especially transforming growth factor-β (TGF-β) (2), indicating that TGF-β protein is required for CTGF to exert its effect. In addition, CTGF was reported to function through the cell surface receptor tyrosine kinase A (TrkA) (1, 50). CTGF action through TrkA then secondarily impacts on TGF-β pathway signaling (50). Whether CTGF mediates adverse effects on cardiac myocytes through activation of TrkA is unknown.

With this background, the aims of this study were to test 1) whether CTGF is a hypertrophic and apoptotic factor in cardiomyocytes, 2) whether CTGF mediates adverse effects of FA and elevated glucose on hypertrophy and apoptosis in these
cells, and 3) whether effects of CTGF on cardiomyocytes are mediated by TrkA.

MATERIALS AND METHODS

Reagents. Glucose and palmitate were purchased from (Sigma-Aldrich, Sydney, Australia). Palmitate for cell culture addition was prepared as previously described (7). In brief, palmitate was dissolved in 0.5 ml 50% ethanol heated at 70°C for 1–2 min and then added to 4.5 ml 10% FA-free bovine serum albumin (BSA, Sigma-Aldrich). The palmitate-BSA compound was gently rotated for 1 h at 37°C and further diluted to the required concentration in media for treatment. Recombinant human CTGF (rhCTGF) protein was produced using an adenoaviral expression system and purified with heparin-sephrose affinity chromatography using HitTrap Heparin HP columns (Amer sham Biosciences, Piscataway, NJ) and quantified as previously described (6). A chemical inhibitor of TrkA, k252a, purchased from Merck-Calbiochem (Melbourne, Australia) was prepared by dissolving in DMSO, with final DMSO concentration in cell culture at 0.001%. TGF-β-induced early gene (TIEG) antibody (sc-34544) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for detection of phospho-TrkA (Tyr490) and total TrkA were purchased from Cell Signaling Technology (Danvers, MA). The anti-CTGF polyclonal antiseraum (194) was generated in rabbit by using a NH₄₃ terminal peptide common to rat and human CTGF as the immunogen, as previously described (45).

Animals. The study was approved by the Animal Ethics Committee of Sydney South West Area Health Services (SSWAHS), Australia. Type 1 diabetes was induced by intraperitoneal injection of streptozotocin in citrate buffer (65 mg/kg) in 8 wk of age male Sprague-Dawley rats, whereas citrate buffer alone was injected in the control group (n = 6 per group). In animals rendered diabetic, long-dose, long-acting insulin (Protaphane, Novo Nordisk, Australia) was injected as 2–4 IU im every second day to help maintain body weight, avoid ketoadiposis, and maintain random blood glucose levels at ~20 mmol/l, as described previously (31). After 8 wk of diabetes, animals were anesthetized by ketamine and then euthanized by exsanguination. The rat heart was collected for paraffin embedding or stored frozen for total RNA extraction.

Cell culture. H9c2 cells derived from embryonic rat heart myocardium were purchased from ATCC (Rockville, MD). Cells were cultured in DMEM containing normal 5.5 mM glucose, 4.4 mM t-glutamine, and 1.7 g/l bicarbonate supplemented with 10% FBS, 50 U/ml of penicillin, and 50 µg/ml of streptomycin. Each experiment was performed at least three times independently in triplicate.

Cardiomyocytes from primary cultures were obtained from 1- to 3-day-old neonatal C57Bl/6 mice (46). In brief, cardiomyocytes were extracted from ventricles by digesting the tissue samples in ADS buffer (in mM: 116 NaCl, 19.7 HEPES, 0.8 Na₂HPO₄·2H₂O, 5.6 glucose, 5.4 KCl, and 0.8 MgSO₄·7H₂O) containing 0.8 mg/ml collagenase type 2 and 0.3 mg/ml pancreatin. Extracted cells were plated in prelaid medium containing 68% medium M199, 17% DMEM, 10% horse serum, 5% fetal calf serum, and 170 U/ml penicillin-streptomycin for 1 h. Subsequently, the enriched cell population for cardiomyocytes was plated on gelatin-coated plates at a density of 3.2–4.4 × 10⁵ cells/well in six-well plates or four 6 × 10⁵ cells/well of a 96-well plate. More than 90% of the cells were beating spontaneously after 2 h incubation, indicating cultures of viable high purity cardiac myocyte cultures. The plating medium was replaced with serum-free maintenance medium (80% DMEM and 20% medium M199) for 24 h. Cells were then used for subsequent 24-h treatments in serum-free media as described in the text.

Cell treatment. Cells were grown in 6- or 96-well plates or 150-cm² flasks until ~80% confluent and then treated with additions in serum-free fresh DMEM media, containing 0.1% BSA for H9c2 cells. Cells were harvested at 6, 24, 48, or 72 h depending on the requirements of the experiment. Medium was not changed after addition of treatments.

For experiments using CTGF small interfering RNA (siRNA) and k252a, cells were either transiently transfected with CTGF siRNA for 16 h or pretreated with k252a for 1 h, respectively, and then medium was replaced with fresh serum-free media containing 0.1% BSA and the treatment additions. Cells cultured in 96-well plates were used for determination of caspase 3 and 7 levels. Cells in six-well plates were collected for gene and protein analysis and transferase-mediated dUTP nick end labeling (TUNEL) staining, as well as determination of cell viability by direct counting.

siRNA studies. Rat CTGF siRNA (SI01503285) was purchased from Qiagen (Melbourne, Australia). The targeted sequence for the CTGF siRNA, CACCAAGCTTGGACTATA, and the scrambled sequence, GAAUGUGUCAACUAUAUAUC, were designed according to the siRNA target designer program. HiPerFect (Qiagen, Valencia, CA) was used as transfection reagent according to the manufacturer’s protocol for transient transfection of the RNA at concentrations described in the text.

Total RNA isolation and analysis by quantitative real-time PCR. Total RNA from left ventricular tissue was isolated by using Fibrosis RNeasy Mini kit (Qiagen). Total RNA of each cell treatment was isolated from cells in six-well plates in triplicates using the RNeasy Mini kit (Qiagen). RNA amount was quantitated spectrophotometrically using the SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories, Richmond, CA), absorbance was measured at 260 and 280 nm, and the purity was determined from the A₂₆₀/A₂₈₀ ratio (at or above 1.80–2.00). RNA was then reverse transcribed to cDNA using Random Hexamer Primer (Invitrogen) and SuperScript III Reverse Transcriptase (Invitrogen). The resulting cDNA was analyzed by quantitative real-time PCR using Rotor Gene 6000 (Corbett Research), using SYBR green as fluorescence dye, as described previously (44). The following sequence-specific primers were used for rat CTGF, BNP, β-MHC, rat and mouse ANP, and α-SKA and 18s mRNA expression. Forward: 5'–GGAGGAAAACATTAA-GAGGGGCAA–3', reverse: 5'–CCGCAACAGGCTTTGATGA–3' for CTGF; forward: 5'–GGCGGTAAGATGAGGTCA–3', reverse: 5'–GGGCTCCAATCTTGCTAAC–3' for ANP; forward: 5'–ATCT-CAGTTCAGCTGTGCTAC–3', reverse: 5'–ACACCAGCGTCTG- GTTGAT–3' for α-SKA; forward: 5'–TCGTGCTCTGCTTTGCTAT–3', reverse: 5'–GGACTATGTGCCATCTTGGA–3' for BNP; forward: 5'–GCAACACATCCATGAGAG–3', reverse: 5'–TCCAAAG-GCCTTCCAGGTCTCAGGCC–3' for β-MHC, rat and mouse ANP, and collagen-III, fibronectin (FN), and tissue inhibitor of metalloproteinase (TIMP-1) were also measured, using primers as follows: collagen-III, forward 5'–GCGGTTCTGGCCGAGTA–3', reverse 5'–GTGTTCTTGGGAGTCTAA–3'; FN forward 5'–GAGTGGCAGCTG–3' and reverse 5'–GTCATCGGAAC–3'; and TIMP-1, forward and reverse primers as previously described (13). Each RNA sample was analyzed in duplicate by real-time PCR. Relative quantitation of rat 18s ribosomal RNA and rat CTGF, α-SKA, and ANP mRNA, and collagen-III, FN, and TIMP-1 were calculated using the comparative threshold cycle number for each sample fitted to a six-point standard curve. Standard curves were constructed using a serial dilution of the plasmid with the gene of interest inserted. In each case, the gene of interest expression level was normalized to 18s ribosomal RNA and related to the relevant control, as previously described (45, 46) and as indicated in the text.

Preparation of cell lysates for protein quantitation. Cells in six-well plates treated in each case in triplicate were washed with cold phosphate-buffered saline (PBS) and then 150 µl of cold RIPA lysis buffer with a complete protease inhibitor cocktail at concentration recommended by the manufacturer (Roche Diagnostics, NSW, Australia) was added to each well. Plates were left on ice for 20 min, and lysates were collected after centrifugation at 10,000 g for 10 min at 4°C. Total protein concentration was measured using the DC Protein Assay (Bio-Rad Laboratories) and was used in determination as a hypertrophy marker (total protein/viable cell number at direct cell counting), as well as for sample loading for SDS-PAGE.
Western immunoblot analysis. Cell lysate samples were loaded at 30 μg of total protein per lane and separated on a 4–12% gradient SDS-PAGE gel (Invitrogen). Proteins were electrotransferred onto nitrocellulose, and membranes were blocked with 5% nonfat dry milk/TBS with 0.1% (vol/vol) Tween 20 for 1 h, followed by incubation in either in-house anti-CTGF IgG (1:1,000, termed 194) (44), anti-phospho-TrkA (1:150), anti-total TrkA (1:300), or anti-TIEG (1:150). After incubation of membranes with the appropriate horse-radish peroxidase-labeled secondary antibody (1:10,000, Vector Laboratories) for 1 h at 21°C. Immunoreactive protein bands were then detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). In each case, after stripping of nitrocellulose membranes with stripping buffer (48) was completed, anti-microtubulin (1:5,000) at 4°C overnight followed by the relevant secondary antibody then enhanced chemiluminescence as described above was used to detect α-tubulin as a loading control.

Cell viability measurement. The effect of various treatments on viable cell number was determined by direct cell counting by hemocytometer using the trypan blue exclusion method as previously described in these cells (27). Dead cells were shown as a distinctive blue color under a microscope while live cells counted did not take up trypan blue.

Quantitation of caspase activity. Total caspase-3 and -7 activities were measured by Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega). After treatments, 100 μl of Apo-ONE Caspase-3/7 reagent (substrate and buffer in the ratio of 1:100) were added to each well of a 96-well plate. After 1 h incubation in the dark at room temperature, the fluorescence of each well was measured at 485–520 nm (Fluroskan Ascent FL Labsystems).

Determination of apoptotic cells in rat heart tissue and cells in cultures by TUNEL staining. TUNEL staining was used to detect apoptosis in rat heart and H9c2 cells. In heart tissues, sections (5 μM) were deparaffinized by immersion in xylene, rehydrated, then incubated with proteinase K (20 μg/ml), and washed in PBS, and apoptosis was detected using the DeadEND Colorimetric TUNEL System (Promega, Sydney, Australia) according to the manufacturer’s instructions. This nonradioactive system is designed to provide simple, accurate, and rapid detection of apoptotic cells on the basis of DNA fragmentation in situ at the single cell level. With the use of this method, apoptotic nuclei are immunoperoxidase positive and stain dark brown. For cells, after various treatments, H9c2 cells were fixed with 10% formalin solution at 21°C for 30 min at room temperature. After a PBS rinse, cells were stained for apoptotic change using the DeadEND Colorimetric TUNEL System (Promega) according to the manufacturer’s instructions.

Cell size measurement. Cells following treatments were trypsinized, collected, and fixed in 10% formalin and subsequently embedded in paraffin. Cells were visualized by light microscopy after hematoxylin counter staining, and the Image J program was used to measure the area of each cell, as adapted from published methods (23, 39). In each case the average area of per cell following a treatment was determined by counting over 200 cells per well examined across triplicate wells.

Statistical analysis. Results are expressed as means ± SD. Cell size data are expressed as means ± SE. All numerical data were derived from three or more independent experiments. In some cases of imaging, representative data are given, as was seen in three independent experiments. Differences between groups were assessed using
one-way ANOVA, or Student’s two-tailed paired t-test, where only two groups were to be compared, as indicated in the text. $P < 0.05$ was considered statistically significant.

RESULTS

Apoptosis, pathological hypertrophy, and CTGF in the diabetic rat heart. To determine whether changes to be studied in vitro do occur in the diabetic rat heart in vivo, rats rendered diabetic were first examined. As an indicator of apoptosis, by TUNEL staining, more positively stained nuclei were consistently seen in diabetic hearts compared with control heart (Fig. 1A). After total RNA was isolated from the left ventricle (LV) of rat heart, the mRNA expression of four hypertrophic gene markers ANP, BNP, α-SKA, and β-MHC were measured by real-time RT-PCR. There were 13.9-, 2.9-, 2.2-, and 16.3-fold induction in diabetic hearts compared with the respective same gene control (Fig. 1B). CTGF expression was increased at both the mRNA (Fig. 1C) and protein level (Fig. 1D) in diabetic rat hearts, as has been reported previously (14, 52). Gene expression of cardiac fibrosis markers in the LV of collagen-III, FN, and TIMP-1 mRNA expression were also significantly increased (Fig. 1C).

Whether CTGF can cause apoptosis and/or hypertrophy in cardiac myocytes was then tested in H9c2 cells under serum-free conditions. Apoptosis detected by caspase 3 and 7 activity was increased at 24, 48, and 72 h by 500 ng/ml CTGF treatment in a time-course study (Fig. 2A). TUNEL-positive staining was also shown in CTGF-treated cells compared with untreated cells at 48 h (Fig. 2B). CTGF treatment reduced viable cell numbers most clearly seen at 500 ng/ml dosage (Fig. 2C). Then a time and dose-response study of CTGF treatment in assessing hypertrophy showed that a single dose of 100 or 500 ng/ml of CTGF-induced hypertrophy as indicated by an increase in total protein per cell number at 24 and 48 h (Fig. 2D). The mRNA level of hypertrophic gene markers ANP and α-SKA were also induced by the addition of CTGF after 24 h of treatment (Fig. 2E). As further evidence of hypertrophy, cell size was increased by CTGF treatment at 48 h in

Fig. 2. CTGF increases apoptosis and hypertrophy in H9c2 cardiomyocytes. Cells were treated with CTGF in serum-free media with 0.1% BSA at 80% confluence. A: caspase 3/7 activity measurement in a time course of 24, 48, and 72 h. B: TUNEL staining of CTGF treatment at 48 h shown by immunoperoxidase staining (400× magnification). C and D: time course and dose-response effects of CTGF with detection of viable cell numbers and the hypertrophy marker total protein/cell number. E: ANP and α-SKA mRNA expression measured by real-time RT-PCR after 24 h CTGF treatment. F and G: cell size visualization (400× magnification) and quantitation after 24 h CTGF treatment. Data are shown as means ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control by t-test respectively in A, C, D, and E by ANOVA. In G, data are shown as means ± SE. $***P < 0.001$ vs. control by t-test.
visualized image and by cell size quantitation (Fig. 2, F and G, respectively).

Effects of elevated glucose and the saturated FA palmitate on apoptosis and hypertrophy in H9c2 cardiomyocytes, were then examined. Cells were treated with control media containing 5.5 mM glucose or with 30 mM glucose or 50 and 100 μM of palmitate. Caspase 3/7 activities were increased by high-glucose (2.3-fold) and palmitate (1.9- and 2.8-fold) treatments (Fig. 3A). There was no difference in caspase activity by the mannitol (25 mM) or ethanol vehicle (0.0625%) control treatments (Fig. 3A); data are shown at 24 or 72 h (not shown). Increased positive TUNEL staining by palmitate and glucose 48-h treatments were seen (Fig. 3B). There was also a significant 21%, 32%, and 62% reduction in viable cell numbers by glucose and palmitate treatments (Fig. 3C), and the higher dose of palmitate (100 μM) had the greatest effects on cell death. Hypertrophy determination showed that the total protein/cell numbers were increased to 150%, 165%, and 397% by high glucose and palmitate (Fig. 3D). The ANP mRNA level was increased by the glucose and palmitate treatments to 340%, 161%, and 143% and α-SKA mRNA to 201%, 155%, and 189% compared with the no addition control (Fig. 3E). BNP and β-MHC were not detectable in this cell line. No effect of combination treatments of glucose and palmitate on apoptosis or cell hypertrophy were observed (data not shown). Induction of increased cell size was observed by the glucose and palmitate treatments (Fig. 3F) also as analyzed by Image J software (Fig. 3G).

To then examine whether endogenous CTGF might contribute to the hypertrophy and apoptosis caused by glucose and palmitate, CTGF regulation was first studied. CTGF mRNA was upregulated by these treatments compared with control: over a 6-, 24-, and 48-h time course by glucose to 162%, 164%, and 219% of control; by 50 μM palmitate to 298%, 208%, 178% of control, and by 100 μM of palmitate to 321%, 289%, and 139% of control, at respective time points (Fig. 4A).

In primary cultures of mouse neonatal cardiomyocytes, CTGF and palmitate treatments each significantly induced CTGF mRNA, as well as markers of hypertrophy and apoptosis (Table 1). These findings are consistent with the
effects of CTGF and palmitate seen in the H9c2 cells (Figs. 2 and 3).

To more definitively study whether CTGF may mediate hypertrophy and apoptosis caused by glucose and palmitate, specific CTGF siRNA was used to block CTGF expression. The efficiency of CTGF siRNA was first tested by time course and dose-response experiments. Treatments included three controls: media with no addition, transfect reagents only (HiPerFect alone), and CTGF siRNA scrambled sequence together with four doses of CTGF siRNA 2.5, 5.0, 10, and 20 nM under serum-free conditions with 0.1% BSA. Cell lysates were collected after 6, 24, and 48 h, and the mRNA level was measured. Figure 4 shows all four doses of siRNA blocked CTGF mRNA expression, with 70% effectiveness. The 5 nM siRNA dose was selected in later studies. Data also show there is no significant difference across the CTGF scramble sequence presence in place of scramble, HiPerFect alone, or the media control. CTGF protein expression was detected by Western blots of cell lysates collected at 48 h after siRNA against CTGF or scramble. The characteristic intact glycosylated CTGF protein doublet was reduced by siRNA treatment compared with the scramble (Fig. 4C). Thus CTGF mRNA and protein levels were knocked down by the CTGF siRNA.

H9c2 cells were then pretreated by CTGF siRNA or scramble control for 16 h followed by treatment with high glucose or palmitate. When compared with respective glucose and palmitate treatments with scramble control, CTGF siRNA partially inhibited caspase 3/7 activity at 24 h (Fig. 5A; data were similar at 48 h; not shown) and it stopped the loss of viable cell number caused by high glucose or palmitate (Fig. 5B). The total protein/cell numbers at 48 h were inhibited to basal levels.

Table 1. Induction of CTGF and hypertrophy and apoptosis markers in primary cultures of cardiac myocytes

<table>
<thead>
<tr>
<th>Treatments</th>
<th>CTGF mRNA</th>
<th>ANP mRNA</th>
<th>α-SKA mRNA</th>
<th>Apoptosis With (Caspase 3/7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±23.8</td>
<td>100±22.9</td>
<td>100±15.6</td>
<td>100±16.5</td>
</tr>
<tr>
<td>CTGF (500 ng/ml)</td>
<td>192±10.9†</td>
<td>253±39.9†</td>
<td>301±123.3‡</td>
<td>135±6.4*</td>
</tr>
<tr>
<td>Palmitate (50 μM)</td>
<td>142±18.0*</td>
<td>156±19.2*</td>
<td>157±30.9†</td>
<td>215±5.1‡</td>
</tr>
</tbody>
</table>

Data are combined from two independent experiments and are shown as means ± SD, as % of the respective control in each case. CTGF, connective tissue growth factor; ANP, atrial natriuretic peptide; α-SKA, α-skeletal actin.

*P < 0.05; †P < 0.01; ‡P < 0.001 vs. respective 100% control in each case.
by CTGF siRNA (Fig. 5C). The induced mRNA of ANP and α-SKA by glucose and palmitate were also blocked to basal levels (Fig. 5, D and E). CTGF mRNA induction by high glucose and palmitate was inhibited back to basal levels by the 5 nM siRNA (not shown). These data indicate that inhibition of CTGF can prevent the hypertrophy and apoptosis caused by high glucose and by palmitate.

We hypothesized that CTGF mediates hypertrophic and apoptotic effects in cardiomyocytes through the cell surface receptor TrkA. To address this, cells were pretreated with the TrkA inhibitor k252a at 200 nM for 1 h before the addition of glucose and palmitate. Caspase 3/7 activity and mRNA of ANP and α-SKA were measured after 24 h treatments. Caspase activities induced by glucose and palmitate were blocked by k252a (Fig. 6A). Addition of k252a alone did not have an effect (Fig. 6A). For the gene expression markers, a differential profile was observed: α-SKA mRNA was fully blocked by TrkA combination treatments, whereas ANP results were in the opposite direction and ANP mRNA was induced under k252a treatments (Fig. 6, B and C). As a negative control for k252a effects, TNF-α cell treatment, which induced caspase 3/7 activity, was not inhibited by the TrkA inhibitor (Fig. 6D).

The TrkA blocker k252a was then studied during CTGF treatment. The k252a reagent blocked the CTGF-induced caspase 3 and 7 activity at 200 nM (Fig. 7A). The mRNA of ANP and α-SKA was measured at 24 h treatment of CTGF. As before in the glucose and palmitate treatments, mRNA of ANP was not blocked by k252a (Fig. 7B), whereas in contrast, α-SKA-induced expression by CTGF was fully blocked by k252a at 200 nM (Fig. 7C). These data suggest that CTGF through TrkA mediates the hypertrophy gene α-SKA and caspase 3 and 7 activity. In contrast, ANP gene expression appears to be under tonic inhibitory control by TrkA activity.

To determine whether CTGF regulates TrkA in cardiomyocytes, we tested whether CTGF activates phospho-TrkA and TIEG. H9 c2 cells were treated by 500 ng/ml CTGF, and protein was measured from cell lysates at 0, 5, 15, and 60 min (Fig. 7D). Phospho-TrkA in whole cell lysates was increased at 15 and 60 min; with no observed change for total TrkA; TIEG was increased at 5, 15, and 60 min.

**DISCUSSION**

This study demonstrates that CTGF and palmitate each induce hypertrophy and apoptosis in cultured H9 c2 cardiac myocytes as well as in primary cultures of neonatal cardiomyocytes. Inhibition of CTGF expression by CTGF siRNA prevents adverse effects of apoptosis and hypertrophy caused by high glucose and palmitate in H9 c2 cells. The study also shows that CTGF can activate the cell surface receptor TrkA and TIEG in cardiac myocytes. CTGF, glucose, and palmitate effects were blocked by the TrkA inhibitor k252a, suggesting that CTGF activates TrkA to mediate hypertrophic and apoptotic effects.
by glucose and palmitate. A schematic diagram (Fig. 8) summarizes these findings.

Cardiac hypertrophy in vivo involves both matrix and cellular components (24). It may be physiological as occurs following exercise training or may be pathological following abnormal stimuli (31). Our data demonstrate hypertrophy on the basis of increase in cell size and increased protein synthesis. The expression profile of genes involved in fetalization, ANP, and α-skeletal actin indicates that the hypertrophy induced was pathological. Interestingly, both pathological hypertrophy and apoptosis were induced by the stimuli of elevated glucose and FA, which confirms work by others in cardiac myocytes (26). We now add CTGF to this list as a mediator of this dual effect. That both changes can occur in cultured cells after a single stimulus at a time point suggests that cells either have a number of different cellular phenotypic pathways they may follow or at least they are variably sensitive to the effects of stimuli. Whether a particular cell travels through a hypertrophic path ultimately to apoptosis will require future studies with individual tracking of cells.

Cardiomyocytes require high-energy substrate to function optimally (12). However, as shown in the current study, an excess of substrate, whether that be high glucose or fatty acid, can lead to cell dysfunction. The CTGF induced could potentially be a pathological mediator or a counterregulatory protective agent. Our work clearly demonstrates that despite its reputation as a weak mitogen in some mesenchymal and endothelial cells (9), CTGF contributes to cell death. Whereas this is the first work to show that CTGF causes apoptosis in cardiomyocytes, similar apoptotic effects have been shown in other cells, such as aortic smooth muscle cells (21).

This is the first time that FA has been shown to regulate CTGF in any cell type. Such effects were seen in both H9c2 cells and primary cultures of cardiac myocytes. The finding is significant as FAs are thought to be a cause of tissue pathology in a number of organs other than the heart that develop tissue pathology by 10.220.33.6 on June 25, 2017 http://ajpcell.physiology.org/ Downloaded from

Fig. 6. Effects of apoptosis and hypertrophy by glucose and palmitate are inhibited by a tyrosine kinase A (TrkA) blocker. H9c2 cells were pretreated 200 nM k252a for 1 h at 80% confluence, and then control (5.5 mM glucose), high glucose (30 mM), TNF-α, or palmitate at 50 or 100 μM were incubated with or without k252a for 24 or 48 h. All treatments were under serum-free conditions with 0.1% BSA. A: caspase 3/7 activity measurement at 48 h. B and C: mRNA expression of ANP and α-SKA after treatments of glucose and palmitate with and without k252a at 24 h. D: caspase 3/7 activity measurement at 24 h following TNF-α treatment. *P < 0.05, **P < 0.01, ***P < 0.001 for respective treatments compared with control. #P < 0.05, ##P < 0.01, ###P < 0.001, for treatments with and without k252a addition.
damage in diabetes (49). CTGF is most extensively studied in diabetes as a mediator of renal pathology (17, 47), and it will be useful to determine whether FA induces CTGF in renal tissue in diabetes and by this means whether CTGF mediates metabolic substrate dysfunction in the kidney. CTGF is also thought to be a mediator of liver damage, including nonalcoholic fatty liver disease, which is common to obesity and to diabetes (37). Hepatocytes take up FA and regulation of CTGF in these cells in response to such a stimulus will need to be examined.

CTGF has been reported to be regulated by other components in lipid metabolism, especially LDL cholesterol (41, 42). Presumably the cellular mechanism of the effect differs from that used by FA, although this remains to be determined. As a potential indicator of the cell mechanism through which FA increases CTGF, we have generated data showing that CTGF expression in H9c2 cells is much less sensitive to unsaturated FA such as linolenic acid compared with that of the more potent palmitate (not shown). Further study is required to determine how FA regulates CTGF in cardiac myocytes. Activation of the sphingolipid (SPL) metabolic pathway is one possible cellular mechanism. Palmitate is converted to two intermediate SPL products that cause apoptosis: ceramide (5) and sphingosine (26). In contrast, for cell hypertrophy, the metabolite sphingosylphosphorylcholine (SPC) has been shown to induce CTGF and cell hypertrophy in renal mesangial and dermal cells (54, 55). Whether

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**Fig. 7.** CTGF mediates apoptosis and hypertrophy through activation of TrkA and TIEG. H9c2 cells were pretreated with 200 nM k252a for 1 h, followed by addition of 500 ng/ml CTGF to cells with or without k252a. A: caspase 3/7 measurement of CTGF with the addition and no addition of k252a. B and C: ANP and α-SKA mRNA expression by CTGF treatment with or without added k252a. D: detection of phospho-TrkA, total TrkA and TIEG protein by Western immunoblot; α-tubulin is a protein loading control. A time course after rhCTGF (500 ng/ml) treatment is shown. For each immunoblot species detected, the immunoreactive bands are derived from the same Western immunoblot. The internal dividing lines in some timecourses indicate that irrelevant sections were removed from the respective immunoblots in assembling the data. The entire immunoblot data were independently verified in a separate experiment (not shown). In each figure the control (no addition) had the same DMSO added (0.001%) as for the 200 nM k252a. *P < 0.05, **P < 0.01, difference compared with control. #P < 0.05, ###P < 0.001, difference in each treatment with and without k252a addition, respectively.

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**Fig. 8.** Schematic diagram indicating how CTGF mediates adverse effects of high glucose and fatty acid (FA) effects on cardiac myocytes. CTGF induced by FA and high glucose exerts its effects through TrkA (autocrine effect). CTGF may also be derived from other sources such as cardiac fibroblasts (paracrine effect).
**NOTE ADDED IN PROOF**

In an earlier online version of this article, the representative Western immunoblot in Fig. 7D was not correctly represented by the authors. The corrected Fig. 7D shows internal dividing lines in some time courses, which indicate that in the assembling of the data, irrelevant sections were removed from the respective immunoblots. For each immunoblot species detected we confirm that immunoreactive bands are derived from the same Western immunoblot. The entire immunoblot data was independently verified in a separate experiment (not shown).

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