EVIDENCE INDICATES THAT long-standing diabetes mellitus (DM) may precipitate the development of heart failure (i.e., diabetic cardiomyopathy) even in the apparent absence of underlying ischemic events (11, 46). The mechanism(s) by which high glucose (HG) levels may induce the development of diabetic cardiomyopathy is unknown. Several possibilities have been proposed including 1) metabolic factors (i.e., oxidative stress); 2) intramyocardial microangiopathy; and 3) cardiac fibrosis (6).

Cardiac fibrosis can be an important component of a remodeled, failing heart: a large percentage of the cardiac mass of explanted hearts can be composed of extracellular matrix (ECM) proteins, which are mostly collagens (12, 15, 17). When isolated, cultured fibroblasts from human and other animal species are treated with HG concentrations (simulating DM conditions), increases in ECM protein synthesis can occur. Profibrotic effects have been observed in cultures of human (24, 25) and rat (42) kidney cells and human skin (7) and peritoneal fibroblasts (37) exposed to HG conditions. However, little is known about the effects of HG conditions on cardiac fibroblast (CF) functions including collagen and protein synthesis.

ANG II has been demonstrated to favor the in vitro and in vivo development of cardiac fibrosis (8, 19, 33, 51). These effects are mediated through the activation of ANG II type 1 (AT1) receptors. Results indicate that HG-level CFs synthesized more protein and collagen, and these effects were not due to changes in osmotic pressure. The addition of ANG II stimulated protein and collagen synthesis in NG-concentration but not HG-concentration CFs. Interestingly, losartan pretreatment blocked the HG- or ANG II-induced increases in both protein and collagen synthesis. HG or ANG II decreased total MMP activity. Decreases in MMP activity were blocked by losartan. AT1 mRNA levels were upregulated with HG concentration. Vitamin E pretreatment blocked the effects of HG on total protein synthesis and stimulated MMP activity. Results suggest that HG levels may promote fibrosis by increasing CF protein and collagen synthesis and decreasing MMP activity. HG levels may cause these effects via the upregulation of AT1 receptors, which can be blocked by losartan. However, vitamin E can alter HG concentration-induced changes in CF functions independently of AT1 mRNA levels.

Profibrotic influence of high glucose concentration on cardiac fibroblast functions: effects of losartan and vitamin E

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Asbun, Juan, Ana Maria Manso, and Francisco J. Villarreal. Profibrotic influence of high glucose concentration on cardiac fibroblast functions: effects of losartan and vitamin E. Am J Physiol Heart Circ Physiol 288: H227–H234, 2005. First published September 2, 2004; doi:10.1152/ajpheart.00340.2004.—Long-standing diabetes can result in the development of cardiomyopathy, which can be accompanied by myocardial fibrosis. Although exposure of cultured kidney and skin fibroblasts to high glucose (HG) concentration is known to increase collagen synthesis, little is known about cardiac fibroblasts (CFs). Therefore, we determined the influence of HG conditions on CF functions and the effects of losartan and vitamin E in these responses. We cultured rat CFs in either normal glucose (NG; 5.5 mM) or HG (25 mM) media and assessed changes in protein and collagen synthesis, matrix metalloproteinase (MMP) activity, and levels of mRNA for ANG II type 1 (AT1) receptors. Results indicate that HG-level CFs synthesized more protein and collagen, and these effects were not due to changes in osmotic pressure. The addition of ANG II stimulated protein and collagen synthesis in NG-concentration but not HG-concentration CFs. Interestingly, losartan pretreatment blocked the HG- or ANG II-induced increases in both protein and collagen synthesis. HG or ANG II decreased total MMP activity. Decreases in MMP activity were blocked by losartan. AT1 mRNA levels were upregulated with HG concentration. Vitamin E pretreatment blocked the effects of HG on total protein synthesis and stimulated MMP activity. Results suggest that HG levels may promote fibrosis by increasing CF protein and collagen synthesis and decreasing MMP activity. HG levels may cause these effects via the upregulation of AT1 receptors, which can be blocked by losartan. However, vitamin E can alter HG concentration-induced changes in CF functions independently of AT1 mRNA levels.

Profibrotic effects have been observed in cultures of human (24, 25) and rat (42) kidney cells and human skin (7) and peritoneal fibroblasts (37) exposed to HG conditions. However, little is known about the effects of HG conditions on cardiac fibroblast (CF) functions including collagen and protein synthesis.

The purpose of this study was to assess the effects of HG levels on CF protein and collagen synthesis and global MMP activity. Furthermore, we wished to determine potential additive and/or synergistic effects of HG concentration with ANG II, identify the involvement of AT1 receptors, and test for the effects of vitamin E on the above-listed parameters.

MATERIALS AND METHODS

Materials. Animals were obtained from Harlan Laboratories (Indianapolis, IN). All procedures were approved by our Institutional Animal Care and Use Committee and conform to published National Institutes of Health guidelines for animal research. The [3H]proline and [3H]leucine were purchased from PerkinElmer (Boston, MA). Collagenase was from Worthington Biomedical (Lakewood, NJ), and vitamin E was from Sigma (St. Louis, MO).

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pancreatin as well as vitamin E (dl-α-tocopherol) were acquired from ICN Biomedicals (Aurora, OH). Cell culture media, trypsin, and antibiotics were from GIBCO-BRL (Invitrogen; Carlsbad, CA), and FBS was from Omega Scientific (Tarzana, CA). ANG II and β- and γ-glucose were from Sigma (St. Louis, MO). All other reagents were cell culture and/or molecular biology grade. ANG II forward and reverse primers were a kind gift from Dr. D. Gurantz (19).

**Cell culture.** Primary adult rat CF cultures were generated from ventricular tissues of 6- to 8-wk-old male Sprague-Dawley rats (body wt, 250–275 g) as previously described (51). Briefly, rats were killed by CO2 asphyxia, and hearts were quickly removed under sterile conditions. Ventricular tissue was isolated, minced, and digested using an enzymatic solution of collagenase (100 U/ml) and pancreatin (0.6 mg/ml). Isolated CFs were pooled together, centrifuged, and resuspending in growth media that contained DMEM (pH 7.4), 5.5 mM β-glucose (NG), 10% FBS, and 1% penicillin, streptomycin, and fungizone (PSF). The CF suspension was plated onto tissue-culture dishes (13, 14). CFs were plated in 12-well tissue-culture dishes and allowed to reach 100% confluence. During the last 16 h of treatment, CFs were treated using the same glucose concentrations in serum-deprived (0.1% heat-inactivated FBS) medium. Drugs or their corresponding control media were added to each well during this time period. We used 1 μM ANG II, 10 μM losartan, and 45 μM vitamin E. Long-term treatment (72 h total culture time) was modeled as per previous publications on HG concentration-induced effects on cell functions (22).

**Incorporation assays for [3H]leucine and [3H]proline.** The [3H]leucine- and [3H]proline-incorporation assays were used as indirect indicators of total protein and collagen synthesis, respectively (13, 14). CFs were plated in 12-well tissue-culture dishes and allowed to reach 100% confluence. During the last 16 h of treatment, CFs were treated with [3H]leucine or [3H]proline (1 μCi/ml). To stop the experiments, each well was washed twice with cold PBS solution. Cold 10% trichloroacetic acid (TCA) was added for 30 min to lyse the cells and precipitate cellular proteins (500 μg/well). After the wells were washed three times with TCA, 250 μl of 1 N NaOH was added to each well to hydrolyze the proteins. Samples were neutralized with 250 μl of 1 N HCl for 30 min, and radioactivity was counted after the addition of Ecolite scintillation fluid (ICN Biomedicals).

**Global MMP activity.** Global MMP activity was assessed by reacting culture media or total cell lysate samples with 10 μmol/l Omnium-MP substrate (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg; Biomol Research Laboratories; Plymouth Meeting, PA) in assay buffer (see below). This probe is a highly quenched peptide that when cleaved by MMPs yields a fluorescent product that represents the sum of all MMP proteolytic activities and inhibitors present in the culture (e.g., tissue inhibitors of MMP (TIMPs)). Cells were washed with cold PBS and collected in fluorescence assay buffer that contained (in mM) 50 Tris, 150 NaCl, 5 CaCl2, and 0.2 Na2SO4, pH 7.6. Samples were centrifuged, and the supernatant was stored at –80°C until it was used. Total protein was determined by Bradford assay (Bio-Rad; Hercules, CA). Before assays were performed, fluorescence assay buffer and 1 μl of fluorescent MMP-specific substrate were added to each sample. Kinetic measurements were performed at 3-min intervals with an FLX800 Microplate Fluorescence Reader (Bio-Tek Instruments) using wavelengths of 340 nm (excitation) and 405 nm (emission). Results are expressed as relative fluorescence units per minute normalized to total protein content.

**Type I collagen immunoblotting.** Western blots were used to determine increases in type I collagen present in total cell lysate (i.e., deposition). Total type I collagen deposition reflects the balance between synthesis and MMP-mediated degradation. Briefly, equal amounts of total protein were separated by 10% SDS-polyacrylamide gels under reducing conditions and transferred onto polyvinylidene difluoride membranes. Membranes were blocked in 5% nonfat milk in Tris-buffered saline that contained 0.1% Tween 20 and were exposed to a mouse anti-type I collagen primary antibody (Calbiochem; San Diego, CA) used at a dilution of 1:500 in 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20. A monoclonal antibody against β-actin was used at a 1:5,000 dilution (Sigma) for normalization purposes. Membranes were incubated for 1 h at room temperature with their respective secondary horseradish peroxidase-labeled antibodies and then developed using an ECL Plus detection system (Amersham Pharmacia Biotech; Buckinghamshire, UK).

**Molecular analysis for ANG II expression.** Total mRNA isolated from treated CFs was extracted using an RNeasy mini kit (Qiagen; Valencia, CA). Reverse transcription was performed using Invitrogen’s ThermoScript RT-PCR kit. From the cDNA obtained, 50 ng were allocated for real-time PCR using a QuantiTec SYBR Green PCR kit following the manufacturer’s instructions (Qiagen). Samples were run in duplicate, and primers for GAPDH and the AT1 receptor were loaded in different wells. The GAPDH forward-primer sequence was 5′-CTCATGACACACCTCATGC-3′, and the reverse-primer sequence was 5′-TTCAGCTCTCGATGAC-3′. The AT1 PCR forward and reverse primers used have been previously described (19), and the sequences for these were 5′-CACATTAGTAGATCGCTC-3′ and 5′-GCACAATCGCCATA-ATTACC-3′, respectively. PCR conditions included 15 min at 95°C to activate the HotStartTaq DNA polymerase and then 42 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 60 s. Real-time fluorescence detection was performed in a DNA Engine Option-2 instrument (MJ Research; Waltham, MA). Transcription efficiency was calculated for each sample, as suggested by Liu and Saint (29), and no differences between genes were observed (P = 0.32). Thus calculations were done using the difference in threshold cycle of PCR (∆Ct, at which the fluorescence reaches a given value in the log-linear range of amplification) between the housekeeping gene (GAPDH) and the target gene (AT1). Comparative Ct calculations for AT1 gene expression were all relative to NG-treated (calibrator) cultures. We obtained ∆ΔCt values by subtracting the average calibrator ∆Ct value from the ∆Ct value in each sample. AT1 gene expression relative to the calibrator with different treatments was evaluated using the expression 2−ΔΔCt. Specificity of the amplified products was determined using melting-curve analysis.

**Statistical analysis.** All groups were tested for normality and equal variance. ANOVA was used for statistical comparisons. Post hoc analysis was performed using Bonferroni’s t test if statistical significant differences were observed between groups (P < 0.05). Results shown are means ± SE; n, number of cultures.

**RESULTS**

Effects of HG concentration, ANG II, and losartan on leucine and proline incorporation. As shown in Fig. 1, when confluent CFs are treated for 72 h with HG concentration, significant increases in [3H]leucine and [3H]proline incorporation occur. On average, [3H]leucine increased 35% (P = 0.038), whereas [3H]proline increased by 40% (P < 0.011). These effects were not observed in high-osmolality-treated CFs, which rules out osmotic pressure changes as responsible for the results. We tested the ability of ANG II to generate additive and/or synergistic effects on CF leucine and proline incorporation. Results are expressed as relative fluorescence units per minute normalized to total protein content.
incorporation. Figure 2 shows the results of these experiments. Addition of ANG II to NG conditions yielded a statistically significant enhanced incorporation of leucine (28.8%; \( P < 0.001 \)) and proline (39.5%; \( P < 0.03 \)) compared with controls. However, the addition of micromolar ANG II to HG-treated CFs did not yield additional increases in leucine and proline incorporation vs. those noted for NG conditions. We also explored the capacity of the AT1 blocker losartan to reverse the effects of HG levels on leucine and proline incorporation. Results are illustrated in Fig. 3 and indicate that losartan is capable of reversing the effects of HG concentration on \([^{3}H]\)leucine and \([^{3}H]\)proline incorporation.

Effects of HG concentration, ANG II, and losartan on global MMP activity. We evaluated the effects of HG conditions on global MMP activity through fluorescent peptide probe assays. The results shown in Fig. 4 indicate that total MMP activity present in cell culture media was decreased in HG- vs. NG-treated CFs (\( P < 0.001 \)) as well as in the ANG II-treated groups regardless of the glucose concentration (\( P = 0.001 \) for both compared with NG concentration). Thus the addition of ANG II to HG-treated CFs did not further inhibit MMP activity. The addition of losartan by itself caused a nonsignificant (\( P = 0.065 \)) decrease on total MMP activity under NG conditions, but did further block the effects of HG concentration on total MMP activity (\( P = 0.057 \) vs. NG). In total cell lysate assays, no differences between treatments were observed for global MMP activity (data not shown).

Effects of HG concentration on AT1 mRNA levels. To assess the effects of HG conditions on AT1 mRNA levels, real-time PCR was performed. Results shown in Fig. 5 indicate that HG concentration significantly increased AT1 mRNA expression approximately eightfold compared with NG concentration (\( P < 0.05 \)).

Effects of vitamin E on HG concentration-induced changes in CF function and AT1 mRNA levels. To examine whether vitamin E reverses HG concentration-induced increases in CF protein and collagen synthesis, 45 \( \mu \)M vitamin E was added to CF culture media. As illustrated in Fig. 6, the addition of vitamin E to cultures of CFs significantly reversed HG concentration-induced effects on total protein synthesis (\( P = 0.29 \))
vs. NG; \( P = 0.033 \) vs. HG). However, the HG concentration-induced increase in collagen synthesis remained unaffected by vitamin E treatment \( (P = 0.03 \) vs. NG; \( P = 0.76 \) vs. HG). As shown in Fig. 7, vitamin E treatment stimulated MMP activity above control levels in NG concentration-treated CFs and reversed HG concentration-induced decreases on MMP activity \( (P < 0.05) \). Vitamin E treatment did not modify HG concentration-induced increases in AT1 mRNA levels (data not shown).

**DISCUSSION**

DM and heart-failure development may be causally related by at least three mechanisms that include 1) the presence of associated comorbidities, 2) the development of coronary atherosclerosis, and 3) via a specific diabetic cardiomyopathy \( (2, 6) \). In diabetic patients without known cardiac disease, abnormalities of left ventricular mechanical function have been demonstrated in 40–50\% of subjects, and it is primarily a diastolic phenomenon \( (3, 6, 11) \). Advanced glycosylation end products have been related to the increased stiffness seen in DM, because they are able to cross-link glycated collagen and make it resistant to enzymatic proteolysis and subsequent degradation \( (3) \). However, DM diastolic dysfunction is also related to increases in interstitial collagen deposition \( (i.e., \) fibrosis; Refs. 11, 40). Few studies are presently available that examine the effects of HG conditions on CF functions. However, in proximal tubular cells from human kidney, HG treatment decreased cell protein content \( 40\% \), whereas in renal cortical fibroblasts, no differences were observed \( (24) \). In
fibroblasts from human skin explants, HG concentration increased protein synthesis only with high cell passage (7). Our results indicate that HG conditions increased protein synthesis by $\frac{1}{3}$5% in CFs, as reflected by [3H]leucine-incorporation measurements. We also observed that HG concentration stimulates CF collagen synthesis by $\frac{1}{3}$40% as reflected by [3H]proline-incorporation measurements. This is consistent with findings in other cell types such as mouse renal cortical fibroblasts (21) or human proximal tubule cells and cortical fibroblasts (24) where HG concentration increased [3H]proline incorporation by $\frac{1}{3}$37%. In human skin fibroblasts, HG concentration also elevated levels of pro-\(\alpha\)-collagen III and fibronectin mRNAs (7).

When evaluating potential additive and/or synergistic effects of ANG II and HG conditions in CFs, we observed ANG II-induced increases in leucine and proline incorporation in the NG- but not HG-treated CFs. In rat mesangial cells, Singh et al. (42) showed that HG concentration increases transforming growth factor-\(\beta\) activity in culture media but did not demonstrate additive effects of ANG II with HG concentration. Amiri et al. (1) showed additive effects of ANG II and HG concentration on collagen IV synthesis in rat glomerular mesangial cells. They also provided evidence that the activation of the JAK/STAT pathway by HG concentration and/or ANG II may be important for increases in collagen synthesis. We found no additive or synergistic effects on leucine and proline incorporation with ANG II and HG cotreatment of CFs. One possible explanation for these observations is that HG concentration effects may operate at least in part via the stimulation of AT1-related signaling systems. Indeed, in our experiments, the AT1 blocker losartan was able to prevent HG concentration-induced increases in CF leucine and proline incorporation.

MMPs are critically involved in ECM turnover by degrading collagens (52). Results indicate that CFs cultured either in HG conditions or with ANG II (regardless of the glucose concentration) decreased global MMP activity in conditioned media.

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**Fig. 6.** Effects of NG and HG levels in the absence or presence of 45 \(\mu\)M vitamin E (Vit E) on protein (A) and collagen (B) synthesis in rat CFs as measured by [3H]leucine and [3H]proline incorporation, respectively. Data are normalized to control group values (NG). *\(P < 0.05\); \(n = 9\) cultures.

**Fig. 7.** Effects of NG and HG levels in the absence or presence of 45 \(\mu\)M vitamin E on MMP activity in CF culture media. MMP activity was measured as the maximum slope of the kinetic curve in RFU per minute (normalized to total protein contents) of the cleavage reaction for an MMP-specific fluorescent peptide. Data are normalized to control group values (NG). *\(P < 0.05\); \(n = 6\) cultures.

**Fig. 8.** Effects of NG and HG levels in the absence or presence of 45 \(\mu\)M vitamin E or 10 \(\mu\)M losartan on type I collagen deposition as assessed by immunoblotting. Data show densitometry analysis of 115-kDa bands normalized by \(\beta\)-actin expression, as percentage of the control group values (NG). *\(P \leq 0.05\); \(n = 3\) cultures.
Decreases in global MMP activity may translate into less collagen degradation and ultimately favor cardiac fibrosis. Indeed, immunoblotting results support the observation that HG treatment of CFs induces increases in type I collagen deposition. Losartan blocked these effects, which indicates the involvement of AT1 receptors in these responses. The decrease we observed in global MMP activity reflects reduced levels of MMP production or activation and/or increased production of TIMPs. It has been demonstrated that treatment of cultured CFs with ANG II shifts the production of MMP/TIMP levels to favor the development of fibrosis (33). The assessment of specific types of MMPs and TIMPs that are stimulated by HG treatment warrants further investigation.

The ability of losartan to block HG concentration-induced stimulation of CF functions prompted us to determine whether HG stimulated AT1 receptor mRNA levels. HG concentration-induced increases in AT1 receptor levels or activation may explain changes in CF functions. HG-stimulated increases in AT1 mRNA levels have been demonstrated in vascular smooth muscle cells (45). However, other studies have shown that HG concentration can downregulate AT1 receptors as seen in rabbit renal proximal tubule cells (32). Our results indicate that HG concentration stimulates AT1 mRNA levels. Thus the HG stimulation of CF leucine and proline incorporation as well as the inhibition of global MMP activity may be secondary to increased AT1 receptor levels and the enhanced activation of associated signaling pathways. This possibility is supported by the fact that the addition of ANG II to HG conditions could not further modulate CF functions. An attempt was made to determine ANG II levels in conditioned media using a commercially available immunoassay kit. No detectable levels of ANG II were present in cell media. The lack of detectable ANG II levels does not exclude its participation in the responses but may reflect the well-known highly unstable nature of the oligopeptide in cell culture media recovered after long-term treatments (72 h).

Antioxidants such as vitamin E have been proposed as suitable treatments to prevent oxidative stress-related complications associated with HG conditions (30). Indeed, vitamin E amelioration of DM-associated damage has been related to its ability to work as an oxygen radical scavenger (16, 39). However, non-antioxidant mechanisms have also been proposed for vitamin E actions including inhibition of 5-lipoxygenase and decreased PKC activation (4). The latter effect occurs via at least two mechanisms: dephosphorylation of PKC by a phosphatase (36) or decreasing intracellular diacylglycerol levels by activation of the diacylglycerol kinase (26).

We tested the ability of vitamin E to counter the effects of HG concentration on CF leucine and proline incorporation and collagen deposition. HG-stimulated CF leucine incorporation was blocked by vitamin E treatment. However, no effects were observed in CF proline incorporation or type I collagen deposition. The ability of vitamin E to specifically block the activity of an isoform of PKC (5) may explain its effects on leucine incorporation. Its failure to block HG-stimulated increases in proline incorporation or collagen deposition suggests again a specific mechanism that affects degradation rather than synthesis of collagen (18).

In our CF cultures, vitamin E treatment reversed HG-induced decreases in total MMP activity and yielded a stimulatory effect. These results are compatible with observations derived from cell culture and animal studies of diabetic nephropathy, where it has been shown that HG conditions can decrease the expression of MMPs and increase the expression of TIMPs (31, 43). However, it is worth noting that in animal models of DM, studies have also shown that vascular tissue and plasma expression and/or activity of MMP-9 are increased in response to oxidative stress (50). Indeed, Siwik et al. (44) found that rat CFs treated with either H2O2 or xanthine-xanthine oxidase increased MMP activity. Given the ability of vitamin E to act as a reactive oxygen species scavenger, it can be expected that this treatment may inhibit MMP activity. Thus the mechanism responsible for the effects observed in our CF cultures cannot be attributed to its antioxidant actions but may be related to its ability to modify second-messenger systems such as PKC that may affect the production of MMPs and/or TIMPs (34). Ultimately, vitamin E treatment may prevent greater levels of collagen deposition in the myocardium by enhancing its degradation via a mechanism distinct from that observed by the use of losartan. Indeed, vitamin E treatment did not significantly suppress HG-induced increases in AT1 mRNA levels.

DM is a well-known and important risk factor for cardiac disease. Even though coronary artery disease is the most common cardiac manifestation seen in diabetic patients, there are studies linking DM with heart failure. Moreover, ~15 to 25% of patients with heart failure are diabetic. It has been suggested that DM may play an important role in the pathogenesis, prognosis, and response to treatment of heart failure (6). Clinical trials have proven that AT1 blockers slow the progression of diabetic nephropathy in patients with type 2 diabetes, and that this beneficial effect extends beyond the ability of the blockers to lower systemic blood pressure (48). Some studies even found that patients at high risk of major cardiovascular events treated with angiotensin-converting enzyme inhibitors had fewer new cases of DM compared with patients treated with placebo (54). Understanding the relationship between HG concentration and the ANG II receptor system may significantly affect the way that these compounds are used in patients (28). Our results also support the notion that vitamin E treatment may be a worthwhile consideration for treatment of DM cardiomyopathy as supported by results observed in other organs (10, 20, 49). Additional studies are needed to fully elucidate the effects of HG concentration in the ANG II signaling pathway and the effects of vitamin E as well as uncover possible targets for developing or improving our present therapeutics for DM.

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