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PROFIBROTIC INFLUENCE OF HIGH GLUCOSE ON CARDIAC FIBROBLAST FUNCTIONS: EFFECTS OF LOSARTAN AND VITAMIN E.

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ABSTRACT

Long-standing diabetes can result in the development of a cardiomyopathy, which can be accompanied by myocardial fibrosis. Whereas exposure of cultured kidney and skin fibroblasts to high glucose (HG) is known to increase collagen synthesis, little is known about cardiac fibroblasts (CF). Therefore, we determined the influence of HG on CF functions, and the effects of losartan and vitamin E in these responses. We cultured rat CF in either normal (NG, 5.5 mM) or HG media (25 mM) and assessed changes in protein and collagen synthesis, matrix metalloproteinase (MMP) activity, and levels of mRNA for the angiotensin II type 1 (AT_1) receptor. Results indicate that HG CF synthesized more protein and collagen and that the effects were not due to changes in osmotic pressure. The addition of angiotensin II (Ang II) stimulated protein and collagen synthesis in NG but not HG CF. Interestingly, losartan pre-treatment 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. AT_1 mRNA levels were upregulated with HG. Vitamin E pre-treatment blocked HG effects on total protein synthesis and stimulated MMP activity. Results suggest that HG may promote fibrosis by increasing CF protein and collagen synthesis and decreasing MMP activity. HG may cause these effects via the upregulation of AT_1 receptors, which can be blocked by losartan. However, vitamin E can alter HG-induced changes in CF functions independently of AT_1 mRNA levels.

Keywords: Diabetes, myocardial fibrosis, collagen, matrix metalloproteinases

INTRODUCTION

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 are unknown. Several possibilities have been proposed and include: 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, mostly collagens (12, 15, 17). When isolated, cultured fibroblasts from human or animals species are treated with HG concentrations (simulating DM conditions), increases in ECM protein synthesis can occur. Pro-fibrotic effects have been observed in cultures of human (24, 25) and rat kidney cells (42), 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.

Angiotensin II (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 (AT_1) receptors (9, 27). In cultured CF, Ang II is known to favor the development of fibrosis through the stimulation of ECM synthesis and the inhibition of its degradation (i.e., inhibition of matrix metalloproteinase activity) (19, 23, 33). Recent studies indicate that co-treatment of mesangial cells with Ang II and HG had

an additive effect on collagen production (1) or matrix degradation (42) compared to treatment with either factor alone. Recent studies also indicate that treatment of vascular smooth muscle cells with HG upregulates AT_1 mRNA levels (45). The ability of Ang II and HG co-treatment to alter CF cell functions such as protein and collagen synthesis and matrix metalloproteinase (MMP) activity is unknown, as is the ability of HG to stimulate AT_1 mRNA levels.

The development of tissue fibrosis as stimulated by HG may also follow the development of a pro-oxidative environment (16). An emerging hypothesis is that antioxidative agents may serve to counter pro-fibrotic effects. Indeed, various *in vitro* and *in vivo* studies indicate that the use of antioxidants may diminish the development of tissue fibrosis and/or improve cell survival (41, 47, 53). Treatment with vitamin E (a known antioxidant) has been shown to be beneficial in animal models where oxidative stress plays an important role (18, 35) and also in the prevention/treatment of diabetic complications (10, 30, 38).

The purpose of this study was to assess the effects of HG on CF protein and collagen synthesis and global MMP activity. Furthermore, we wished to determine potential additive and/or synergistic effects of HG with Ang II, the involvement of AT_1 receptors, and to 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 NIH guidelines for animal research. [^{3}H]proline and [^{3}H]leucine were purchased from PerkinElmer (Boston, MA). Collagenase was from Worthington Biomedical Corporation (Lakewood, NJ), and pancreatin, as well as vitamin E (dl-alpha tocopherol) was acquired from ICN Biomedicals (Aurora, OH). Cell culture media, trypsin, and antibiotics were from Gibco BRL (Invitrogen, Carlsbad, CA) and fetal bovine serum (FBS) from Omega Scientific (Tarzana, CA). Ang II, D- and L-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. Gurantz (19).

Cell culture. Primary adult rat CF cultures were generated from ventricular tissues of 6-8 week old male Sprague-Dawley rats (250-275g) as previously described (51). Briefly, rats were sacrificed by CO_2 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 CF were pooled together, centrifuged and resuspended in growth media containing DMEM (pH 7.4), 5.5 mM D-glucose (normal glucose, NG), 10% FBS and 1% PSF (penicillin, streptomycin, fungizone). The CF suspension was plated onto tissue culture dishes for 30 minutes; this allowed preferential attachment of CF to culture dishes. After this incubation time, non-adherent cells were washed away and the media replaced. CF were

maintained in growth media and incubated in a humidified atmosphere of 7% CO_2 at 37^o C until fully confluent. Cells were passaged with 0.05% trypsin-EDTA. All studies were performed with cells at passage 2-3.

Treatment. CF were grown to full confluency in growth media. CF were rendered quiescent by serum starving for 24 hours in DMEM supplemented with 0.1% heat-inactivated FBS (HI-FBS) and 1% PSF. CF were then treated for 48 hours with DMEM 0.5% FBS, 1% PSF at two different glucose concentrations (5.5 mM, NG, and 25 mM D-glucose, HG) or with an osmotic control media (5.5 mM of D-glucose plus 19.5 mM of L-glucose, OC). For the subsequent 24 hours CF were treated using the same glucose concentrations in serum-deprived (0.1% HI-FBS) medium. Drugs (or their corresponding vehicles) were added to each well during this time period. Ang II was used at 1 μM, losartan at 10 μM, and vitamin E at 45 μM. Long term treatment (72 h total culture time) was modeled as per previous publications on HG-induced effects on cells functions (22).

[^{3}H]Leucine and [^{3}H]proline incorporation assays. [^{3}H]leucine and [^{3}H]proline incorporation assays were used as indirect indicators of total protein and collagen synthesis, respectively (13, 14). CF were plated in 12-well tissue culture dishes and allowed to reach 100% confluence. During the last 16 hours of treatment, CF were pulsed with [^{3}H]leucine or [^{3}H]proline (1 μCi/mL). To stop the experiments, each well was washed twice with cold phosphate-buffered saline solution (PBS). Cold 10% trichloroacetic acid (TCA) was added for 30 minutes to lyse the cells and precipitate cellular proteins (500 μl/well). After washing three times with TCA, 250 μL of 1N NaOH was added to each well to hydrolyze the proteins. Samples were neutralized with 250 μl of 1N HCl for 30 minutes and radioactivity was counted after the addition of Ecolite

scintillation fluid (ICN Biomedicals, Aurora OH).

Global MMP activity. Global MMP activity was assessed by reacting culture media or total cell lysate samples with 10 μmol/L OmniMMP 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 which represent the sum of all MMP proteolytic activities and inhibitors present in the culture (e.g., tissue inhibitors of MMP or TIMPs). Cells were washed with cold phosphate buffer saline (PBS) and collected in fluorescence assay buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 0.2 mM NaN_3, pH 7.6). Samples were centrifuged and the supernatant stored at -80^{o}C until used. Total protein was determined by the Bradford assay (BioRad, Hercules, CA). Prior to assaying, fluorescence assay buffer and 1 μl of fluorescent MMP-specific substrate were added to each sample. Kinetic measurements were performed at 3-minute intervals with a FLX800 Microplate Fluorescence Reader (Bio-Tek Instruments Inc.) using wavelengths of 340 nm (excitation) and 405 nm (emission). Results are expressed as relative fluorescent units per minute (RFU/min) 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% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 and exposed to a mouse anti-type I collagen primary antibody (Calbiochem, San Diego, CA)

used at a dilution of 1:500 in 5% non-fat milk in TBST. A monoclonal antibody against β-actin was used at 1:5000 dilution (Sigma, St Louis MO) for normalization purposes. Incubation for 1 h at room temperature with their respective secondary horseradish peroxidase-labeled antibody was then performed. Membranes were developed by ECL plus detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).

Isolation of Total mRNA, reverse transcription and real-time Polymerase Chain Reaction (Real-Time PCR). Total mRNA from treated CF was extracted using RNeasy mini kit (Qiagen, Valencia, CA). Reverse transcription was performed using Invitrogen's Thermoscript RT-PCR kit (Carlsbad, CA). From the cDNA obtained, 50 ng were used in real-time PCR using QuantiTect SYBR Green PCR kit, following manufacturers instructions (Qiagen, Valencia, CA). Samples were run in duplicate, and primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the AT_1 receptor were loaded in different wells. GAPDH forward primer sequence used was 5'-CTCATGACCACACTCCATGC-3' and GAPDH reverse primer was 5'-TTCAGCTCTGCGATGACCTT-3'. The AT_1 PCR forward and reverse primers used have been previously described (19) and the sequences for them were 5'-CACCTATGTAAGATCGCTTC-3' and 5'-GCACAATCGCCATAATTATCC-3', respectively. PCR conditions were 15 min at 95^{o}C to activate the HotStarTaq DNA polymerase, and then 42 cycles of 94^{o}C for 30 s, 46^{o}C for 45 s, and 72^{o}C for 60 s. Real-time fluorescence detection was performed in a DNA Engine Opticon-2 instrument (MJ Research Inc, 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 (at

which the fluorescence reaches a given value in the log-linear range of amplification) between the housekeeping gene (GAPDH) and the target gene (AT_1): (ΔC_T). Comparative C_T calculations for AT_1 gene expression were all relative to NG-treated (calibrator) cultures. $\Delta\Delta C_T$ values were achieved by subtracting the average calibrator ΔC_T value from the ΔC_T value in each sample. AT_1 gene expression relative to the calibrator with different treatments was evaluated using the expression $2^{-\Delta\Delta CT}$. Specificity of the amplified products was determined using melting curve analysis.

Statistical Analysis. All groups were tested for normality and equal variance. Analysis of variance (ANOVA) was used for statistical comparisons. Post-hoc analysis was performed using Bonferroni's test if statistical significances were observed between groups ($p < 0.05$). Results shown are mean $\pm$ standard error of the mean (SEM).

RESULTS

Effects of HG, Ang II and losartan on leucine and proline incorporation. As shown in figure 1, when confluent CF are treated for 72 hours with HG, significant increases in [^{3}H]leucine and [^{3}H]proline incorporation occur. On average, [^{3}H]leucine increased 35%, (p=0.038) whereas [^{3}H]proline increased by 40% (p<0.01). These effects were not observed in high osmolarity-treated CF, ruling 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. Figure 2 shows the results of these experiments. Addition of Ang II to NG yielded a statistically significant enhanced incorporation of leucine (28.8%, p=0.001) and proline (39.5%, p=0.03) when compared to controls. However, addition of micromolar Ang II to HG-treated CF did not yield further increases in leucine and proline incorporation vs. those noted for NG conditions. We also explored the capacity of the AT_1 blocker losartan to reverse HG effects on leucine and proline incorporation. Results are illustrated in figure 3 and indicate that losartan is capable of reversing the effects of HG on [^{3}H]leucine and [^{3}H]proline incorporation.

Effects of HG, Ang II and losartan on global MMP activity. We evaluated the effects of HG on global MMP activity through fluorescent peptide probe assays. The results shown in figure 4 indicate that total MMP activity present in cell culture media was decreased in HG-treated cells vs. NG CF (p<0.001), as well as in the Ang II-treated groups, regardless of the glucose concentration (p=0.001 for both compared to NG). Thus, the addition of Ang II to HG-treated CF did not further inhibit MMP activity. The addition of losartan by

itself had a non-significant (p=0.065) decrease on total MMP activity under NG conditions, but further blocked the effects of HG on total MMP activity (p=0.057 versus NG). In total cell lysate assays, no differences were observed in global MMP activity between treatments (data not shown).

Effects of HG on AT_1 mRNA levels. To assess the effects of HG on AT_1 mRNA levels real-time PCR was performed. Results shown on figure 5 indicate that HG significantly increased AT_1 mRNA expression by ~8 fold compared to NG (p<0.05).

Effects of vitamin E on HG-induced changes in CF function and AT_1 mRNA levels. In order to examine whether vitamin E reverses HG-induced increases in CF protein and collagen synthesis, vitamin E (dl-α-tocopherol, 45 μM) was added to CF culture media. As illustrated in figure 6, the addition of vitamin E to cultures of CF significantly reversed HG-induced effects on total protein synthesis (p=0.29 vs. NG, p=0.033 vs. HG). However, the HG-induced increase in collagen synthesis remained unaffected by vitamin E treatment (p=0.03 vs. NG, p=0.76 vs HG). As shown in figure 7, vitamin E treatment stimulated MMP activity above control levels in NG-treated CF, and reversed HG-induced decreases on MMP activity (p<0.05). Vitamin E treatment did not modify HG-induced increases in AT_1 mRNA levels (data not shown).

Effects of HG on type I collagen immunoblotting. Data derived from immunoblotting are shown in figure 8. Results indicate that HG treatment stimulates the deposition of type I collagen in CF. HG-induced increases were blocked by losartan pre-treatment but not by vitamin E.

DISCUSSION

DM and heart failure development may be causally related by at least three mechanisms: 1) associated co-morbidities, 2) development of coronary atherosclerosis, and 3) through a specific diabetic cardiomyopathy (2, 6). In diabetics 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 (AGEs) have been related to the increased stiffness seen in DM, since they are able to crosslink 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) (11, 40). Few studies are currently available that examine the effects of HG on CF functions. However, in proximal tubular cells from human kidney, HG treatment decreased cell protein content ~40%, while in renal cortical fibroblasts, no differences were observed (24). In fibroblasts from human skin explants, HG increased protein synthesis only with high cell passage (7). Our results indicate that HG increased protein synthesis by ~35% in CF as reflected by [^{3}H]leucine incorporation measurements. We also observed that HG stimulates CF collagen synthesis by ~40% as reflected by [^{3}H]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 increased [^{3}H]proline incorporation by ~37%. In human skin fibroblasts, HG also elevated levels of pro-alpha collagen III and fibronectin mRNAs (7).

When evaluating potential additive/synergistic effects of Ang II and HG in CF, we observed Ang II-induced increases in leucine and proline incorporation in the NG- but not in HG-treated CF. In rat mesangial cells, Singh et al. showed that HG increases transforming growth factor-β_1 (TGF-β_1) activity in culture media but did not demonstrate additive effects of Ang II with HG (42). Amiri et al. (1) showed additive effects of Ang II and HG on collagen IV synthesis in rat glomerular mesangial cells. They also provided evidence that the activation of the JAK/STAT pathway by HG and/or Ang II may be important for increases in collagen synthesis. We found no additive and/or synergistic effects on leucine and proline incorporation with Ang II and HG co-treatment of CF. One possible explanation for these observations is that HG effects may operate at least in part, via the stimulation of AT_1-related signaling systems. Indeed, in our experiments, the AT_1 blocker losartan was able to prevent HG-induced increases in CF leucine and proline incorporation.

MMPs are critically involved in ECM turnover by degrading collagens (52). Results indicate that CF cultured either in HG conditions or Ang II (regardless of the glucose concentration) decreased global MMP activity in conditioned media. Decreases in global MMP activity may translate into less collagen degradation, ultimately favoring cardiac fibrosis. Indeed, immunoblotting results support the observation that HG treatment of CF induces increases in type I collagen deposition. Losartan blocked these effects, indicating the involvement of AT_1 receptors in these responses. The decrease we observe in global MMP activity reflects reduced levels of MMP production/activation and/or increased production of tissue inhibitor of MMPs (TIMPs). It has been demonstrated that the treatment of cultured CF with Ang II shifts the production of

MMP/TIMP levels to favor the development of fibrosis (33). The assessment of specific types of MMPs/TIMPs that are stimulated by HG glucose treatment warrants further investigation.

The ability of losartan to block HG-induced stimulation of CF functions prompted us to determine whether HG stimulated AT_1 receptor mRNA levels. HG-induced increases in AT_1 receptor levels/activation may explain changes on CF functions. HG-stimulated increases in AT_1 mRNA levels have been demonstrated in vascular smooth muscle cells (45). However, other studies have shown that HG can downregulate AT_1 receptors as seen in rabbit renal proximal tubule cells (32). Our results indicate that HG stimulates AT_1 mRNA levels. Thus, HG stimulation of CF leucine and proline incorporation, as well as the inhibition of global MMP activity, may be secondary to increased AT_1 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 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 treatment 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 a oxygen radical scavenger (16, 39). However, non-antioxidant mechanisms have

also been proposed for vitamin E actions, including inhibition of 5-lipoxygenase and decreased protein kinase C activation (PKC) (4). The latter effect occurs via at least two mechanisms: dephosphorylation of PKC by a phosphatase (36), or decreasing intracellular diacylglycerol (DAG) levels by activating the DAG kinase (26).

We tested the ability of vitamin E to counter the effects of HG on CF leucine, 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 affecting degradation rather than synthesis of collagen (18).

In our cultures of CF, vitamin E treatment reversed HG-induced decreases in total MMP activity yielding 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/activity of MMP-9 are increased in response to oxidative stress (50). Indeed, Siwik et al. found that rat CF treated with either H_2O_2 or xanthine-xanthine oxidase increased MMP activity (44). Given the ability of vitamin E to act as a ROS 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 messengers 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 AT_1 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, approximately 15 to 25% of patients with heart failure are diabetics. Its 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 AT_1-blockers slow the progression of diabetic nephropathy in patients with type 2 diabetes and that this beneficial effect extends beyond their ability 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 to patients treated with placebo (54). Understanding the relationship between HG and the Ang II receptor system may significantly impact 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 the treatment of DM cardiomyopathy as supported by results observed in other organs (10, 20, 49). Further studies are needed to fully elucidate the effects of HG in the Ang II signaling pathway, the effects of vitamin E, as well as uncover possible targets for developing or improving our current therapeutics for DM.

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LEGENDS

Figure 1. Effects of 5.5 mM D-glucose (normal glucose; NG), 25 mM D-glucose (high glucose; HG), and 5.5 mM D-glucose supplemented with 19.5 mM L-glucose (osmotic control; OC) on rat CF protein (Panel A) and collagen (Panel B) synthesis as measured by [^{3}H]leucine and [^{3}H]proline incorporation, respectively. Data are normalized to control group values (NG=100%). n=9. *p<0.05

Figure 2. Effects of 5.5 mM D-glucose (NG) and 25 mM D-glucose (HG) in the absence or presence of angiotensin II (Ang II, 1 μM) on rat CF protein (Panel A) and collagen (Panel B) synthesis as measured by [^{3}H]leucine and [^{3}H]proline incorporation, respectively. Data are normalized to control group values (NG). n=9. *p<0.05

Figure 3. Effects of 5.5 mM D-glucose (NG) and 25 mM D-glucose (HG) in the absence or presence of losartan (Los, 10 μM) on rat CF protein (Panel A) and collagen (Panel B) synthesis measured by [^{3}H]leucine and [^{3}H]proline incorporation, respectively. Data are normalized to control group values (NG). n=6. *p<0.05

Figure 4. Effects of 5.5 mM D-glucose (NG) and 25 mM D-glucose (HG) in absence or presence of angiotensin II (Ang II, 1μM) or losartan (Los, 10 μM) on MMP activity in CF culture media. MMP activity was measured as the maximum slope of the kinetic curve in relative fluorescence units (RFU) per minute (normalized to total protein contents) of the cleavage reaction for a MMP-specific fluorescent peptide. Data are

expressed as a percentage of change versus control group (NG). n=8. *p<0.05 ns=not significant.

Figure 5. Effects of normal (NG) and high glucose (HG) levels in the mRNA expression of the AT_1 receptor. Units represent fold-increase in mRNA abundance. GAPDH expression was used to normalize between reactions. n=2 *p<0.05

Figure 6. Effects of 5.5 mM D-glucose (NG) and 25 mM D-glucose (HG) in the absence or presence of vitamin E (Vit E, 45 μM) on protein (Panel A) and collagen (Panel B) synthesis on rat CF as measured by [^{3}H]leucine and [^{3}H]proline incorporation, respectively. Data are normalized to control group values (NG). n=9. *p<0.05

Figure 7. Effects of 5.5 mM D-glucose (NG) and 25 mM D-glucose (HG) in absence or presence of vitamin E (Vit E, 45 μM) on MMP activity in CF culture media. MMP activity was measured as the maximum slope of the kinetic curve in relative fluorescence units (RFU) per minute (normalized to total protein contents) of the cleavage reaction for a MMP-specific fluorescent peptide. Data are normalized to control group values (NG). n=6. *p<0.05

Figure 8. Effects of 5.5 mM D-glucose (NG) and 25 mM D-glucose (HG) in the absence or presence of vitamin E (Vit E, 45 μM) or losartan (Los, 10 μM) on type I collagen deposition as assessed by immunoblotting. Data show densitometry analysis of 115 kDa

bands normalized by β-actin expression, as percentage of the control group values (NG). n=3. *p<0.05.

Figure 1.

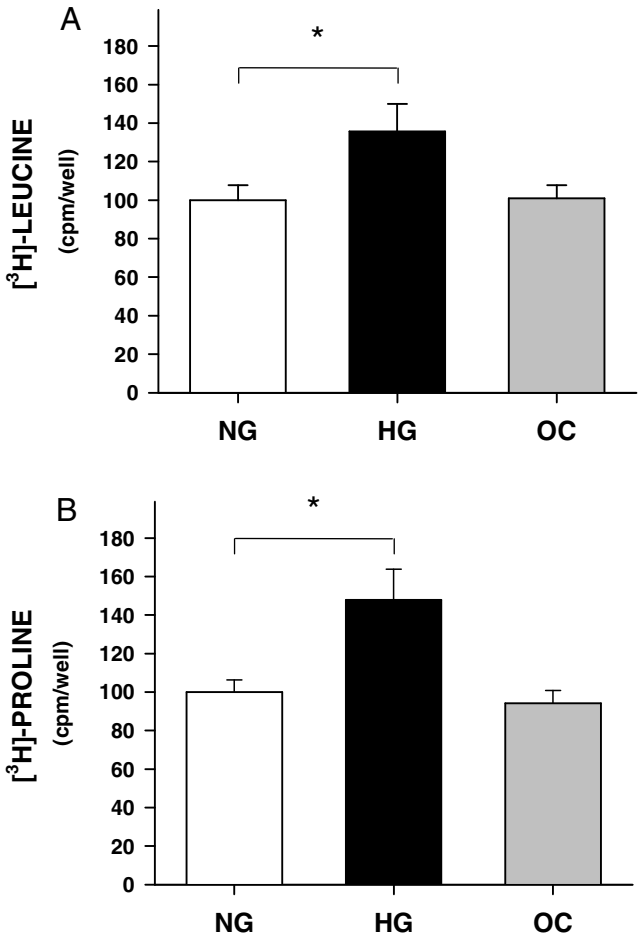

A
*
180
160
140
120
100
80
60
40
20
0
[3H]-LEUCINE
(cpm/well)
NG
HG
OC
B
*
180
160
140
120
100
80
60
40
20
0
[3H]-PROLINE
(cpm/well)
NG
HG
OC

Figure 2.

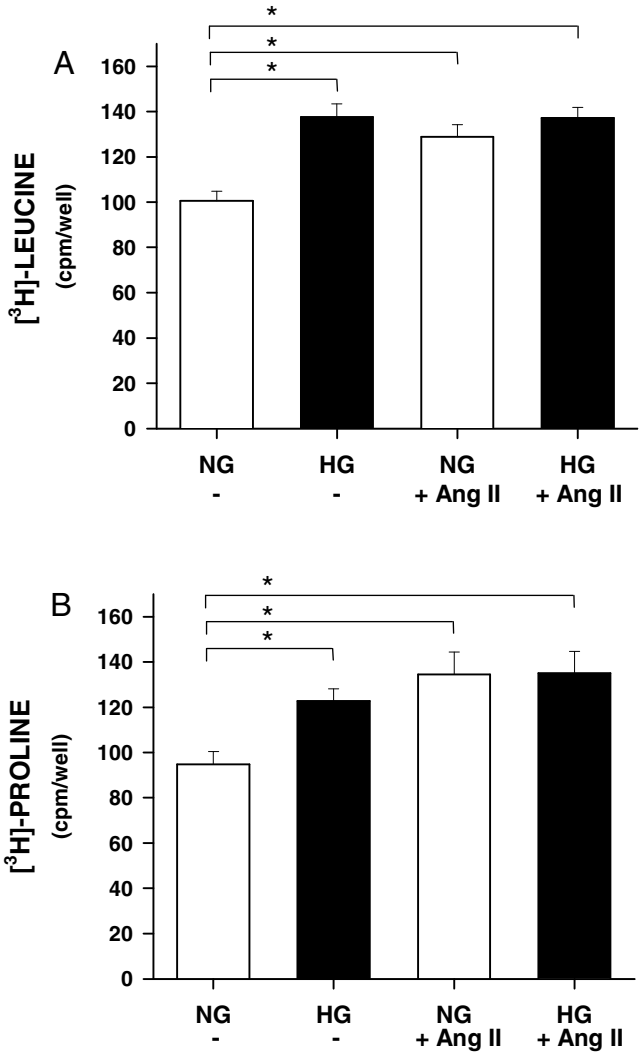

A
[3H]-LEUCINE
(cpm/well)
160
140
120
100
80
60
40
20
0
*
*
*
NG
-
HG
-
NG
+ Ang II
HG
+ Ang II
B
[3H]-PROLINE
(cpm/well)
160
140
120
100
80
60
40
20
0
*
*
*
NG
-
HG
-
NG
+ Ang II
HG
+ Ang II

Figure 3.

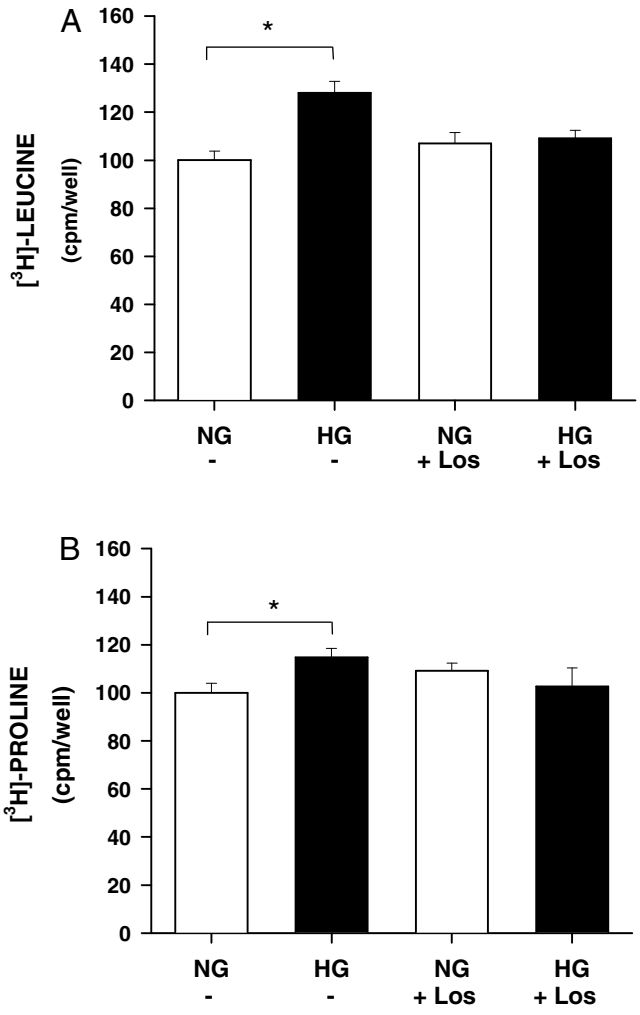

A
160
140
120
100
80
60
40
20
0
*
[3H]-LEUCINE
(cpm/well)
NG
-
HG
-
NG
+ Los
HG
+ Los
B
160
140
120
100
80
60
40
20
0
*
[3H]-PROLINE
(cpm/well)
NG
-
HG
-
NG
+ Los
HG
+ Los

Figure 4.

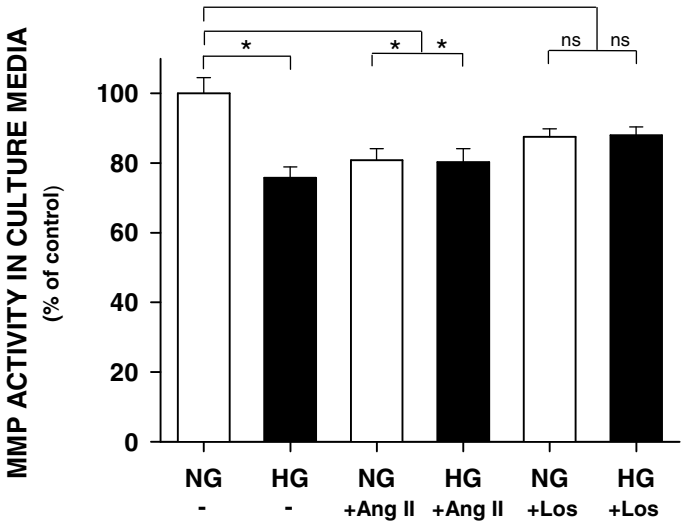
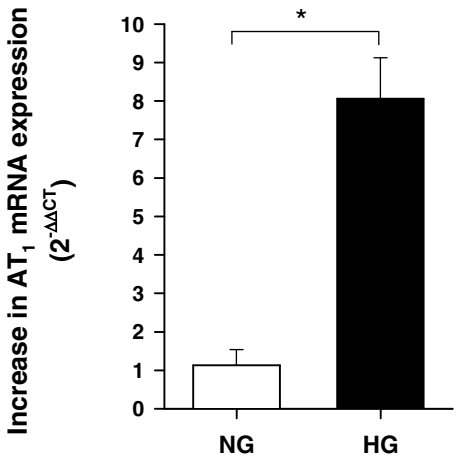

MMP ACTIVITY IN CULTURE MEDIA
(% of control)
100
80
60
40
20
0
*
*
*
ns
ns
NG
-
HG
-
NG
+Ang II
HG
+Ang II
NG
+Los
HG
+Los

Figure 5.



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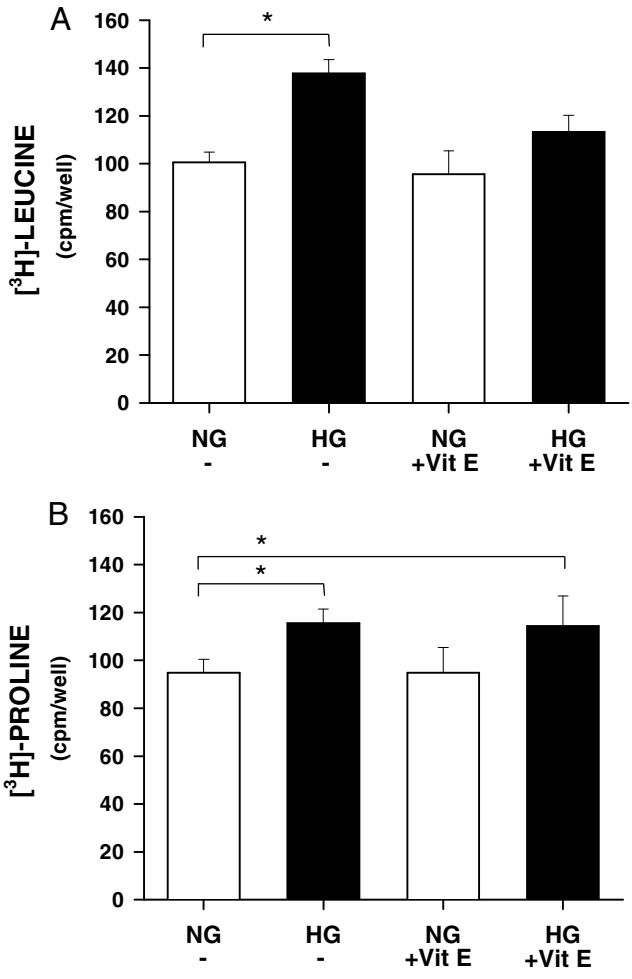

A
[3H]-LEUCINE
(cpm/well)
160
140
120
100
80
60
40
20
0
*
NG
-
HG
-
NG
+Vit E
HG
+Vit E
B
[3H]-PROLINE
(cpm/well)
160
140
120
100
80
60
40
20
0
*
*
NG
-
HG
-
NG
+Vit E
HG
+Vit E

Figure 7.

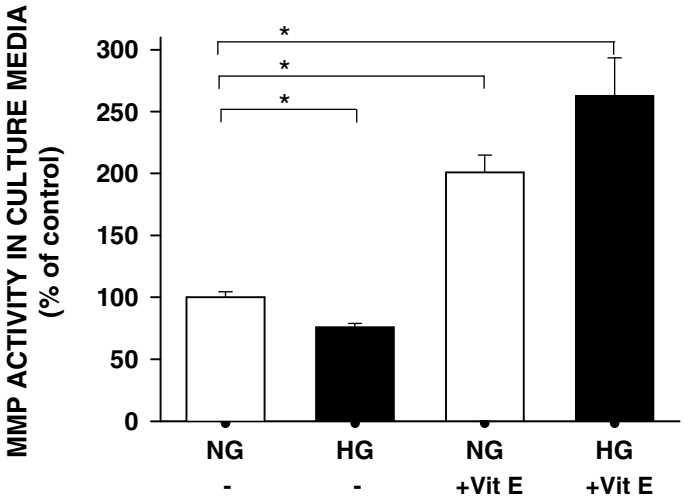
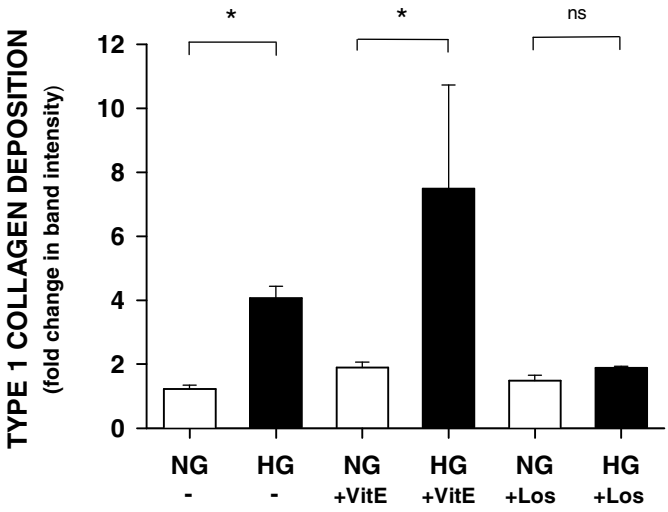

MMP ACTIVITY IN CULTURE MEDIA
(% of control)
300
250
200
150
100
50
0
*
*
*
NG
-
HG
-
NG
+Vit E
HG
+Vit E

Figure 8


TYPE 1 COLLAGEN DEPOSITION
(fold change in band intensity)
*
*
ns
12
10
8
6
4
2
0
NG
-
HG
-
NG
+VitE
HG
+VitE
NG
+Los
HG
+Los