Pressure Oscillation Regulates Human Mesangial Cell Growth and Collagen Synthesis

Peter Rene Mertens, Volker Esenkott, Birgit Venjakob, Bernhard Heintz, Stefan Handt, Heinz-Günther Sieberth

Abstract—Experimental renal disease models establish glomerular hypertension as a crucial determinant in glomerulosclerosis progression and demonstrate that glomerular capillary pressure reduction delays sclerosis development. An oscillating pressure (OP) chamber was constructed as an in vitro model to study human mesangial cells. Cell cultures were grown under atmospheric pressure (AP) and a controlled OP corresponding to intraglomerular capillary pressure. We show that OP significantly decreases mesangial cell proliferation within 24 hours and attenuates DNA synthesis throughout a 7-day period. To explore the effects of OP on cell metabolism, cell-associated and medium-secreted extracellular (CA and EC, respectively) collagen synthesis were measured by [3 H]proline incorporation. In subconfluent cultures, total CA and EC collagen synthesis was unaffected by OP, while in confluent cultures total EC collagen [3 H]proline incorporation was increased. To determine whether OP influenced mesangial cell growth induction, the effects of increasing glucose in the cell culture media were investigated. Our data show that the high glucose growth stimulatory effect on cell number and DNA synthesis was suppressed by OP. Under high glucose conditions, total CA collagen synthesis was increased in confluent cultures, whereas the EC collagen fraction remained unchanged. In these cultures, OP caused an additional increase in CA collagen synthesis. This study shows that mesangial cell growth and collagen synthesis are influenced by hyperbaric OP, supporting the hypothesis that glomerular capillary pressure plays a role in progressive glomerulosclerosis development. (Hypertension. 1998;32:945-952.)

Key Words: glomerular mesangium ■ pressure, oscillating ■ hyperglycemia ■ collagen

Laboratory and clinical studies have established glomerular hypertension as a contributing factor in glomerulosclerotic disease progression.1–4 Experimental models have shown that changes in intracapillary pressure are accompanied by a loss of afferent arteriole glomerular capillary pressure autoregulation.5 Furthermore, therapeutic interventions with angiotensin-converting enzyme inhibitors and low protein diets that reduce glomerular intracapillary pressure can attenuate progression of glomerulosclerosis.6,7 Nevertheless, compelling evidence has not yet been collected about the direct pathophysiologic effects of glomerular intracapillary pressure on intrinsic glomerular cells.

A stretch/relaxation model (eg, by cyclic stretch/relaxation of culture plate undersurfaces) has been developed from observations of isolated glomeruli, in which glomerular volume progressively increases with enhanced perfusion pressure.8–10 In such studies, shear forces profoundly affect mesangial cell (MC) growth, matrix synthesis, and intermedia filament distribution.8,9,11–13 However, fixed mesangial compartment expansion and glomerular hypertrophy are commonly found only in advanced stages of glomerulosclerosis, while intraglomerular pressure elevation generally precedes these changes.

Mesangial structures with inherent centripetal forces are maintained by specific MC properties, including a smooth muscle cell phenotype, contractility, and contact points with the capillary basal membrane.14 Furthermore, the unique central localization of MCs in the glomerular tuft, direct connection to the blood compartment, and absence of an intervening basement membrane support the notion that in addition to shear forces, intraglomerular pressure changes may mediate MC injury.9 In the present study, a pressure chamber was constructed to study the influence of oscillating hyperbaric pressure on MC growth and metabolism directly. Cell proliferation rates and matrix synthesis were determined and compared with parallel cultures grown under atmospheric pressure.

Methods

Establishment of Human MC Cultures

Human MC isolation and primary cell culture maintenance are described in detail elsewhere.5,16 Briefly, human MCs were harvested from intact renal tissue obtained from a 7-year-old nephrectomized patient with Wilms tumor. After macroscopic separation of unaffected renal medullary tissue, glomeruli were collected on 75-μm sieves and digested with 1% type VII collagenase (Sigma Chemical Co) for 30 minutes at 37°C. MC cultures were developed

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From the Medical Clinic II (P.R.M., V.E., B.V., B.H., H.-G.S.) and the Institute of Pathology (S.H.), University of Aachen, Aachen, Germany.
Correspondence to Peter Rene Mertens, Medizinische Klinik II, RWTH Aachen, Pauwelsstraße 30,52057 Aachen, Germany.
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in RPMI 1640 medium (Gibco) supplemented with 1% nonessential amino acids, 8 mmol/L L-glutamine, 50 μg/mL streptomycin sulfate, 50 U/mL penicillin, and 10% fetal bovine serum (Gibco) in a 95% air/5% CO₂ environment. Fibroblast growth was eliminated by substitution of L-valin by D-valin in the first 3 passages. After 3 weeks in primary culture, cells were trypsinized and subcultured. Propagation on sterile plastic flasks was repeated at 7- to 10-day intervals. Glomerular epithelial and endothelial cells were excluded as described.¹⁵,¹⁶ Final cultures were negative for cytokeratin and factor VIII and positive for actin, vimentin, and myosin, confirming uniform MC gene expression. In addition, prolonged growth of these cells resulted in the appearance of “cell hillocks” containing abundant amounts of extracellular matrix (ECM) material.¹⁷ Uniform populations were stored in liquid nitrogen at the fourth through sixth passages.

In addition, primary Sprague-Dawley rat MC cultures were established and characterized as described previously.¹²,¹⁸ Cell cultures were used from passages 6 through 10 for proliferation experiments.

Pressure Chamber

A pressure chamber was constructed using 5-mm Plexiglas plates, depicted in Figure 1. A rubber bellows connected to the chamber allowed generation of oscillating hyperbaric intrachamber pressures at a frequency of 60 per minute (F) by means of motorized compression and expansion. Two valves are inserted into the pressure chamber top. Valve A functions as a pressure relief valve, with a cutout at 40 mm Hg, while valve B is constantly closed at positive pressures, opening with negative intrachamber pressure when the bellows expands. The pressure chamber was placed into a humidified 37°C cell incubator with 5% CO₂. The interplay between valves A and B permitted a continuous exchange of intrachamber (E) and incubator atmosphere. A chamber water reservoir (D) maintained saturated humidity. The intrachamber pressure was continuously monitored via an airtight access (C). Further parameters including intrachamber temperature, P O₂, and P CO₂ of the culture medium were determined in 6- to 24-hour intervals.

TABLE 1. Effect of OP on MC Viability

<table>
<thead>
<tr>
<th>Days of Growth</th>
<th>AP</th>
<th>OP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>89.2±8.2</td>
<td>87.8±9.1</td>
</tr>
<tr>
<td>2</td>
<td>90.1±7.6</td>
<td>89.3±11.0</td>
</tr>
<tr>
<td>4</td>
<td>90.0±5.2</td>
<td>88.4±9.5</td>
</tr>
<tr>
<td>7</td>
<td>86.9±10.1</td>
<td>84.3±8.9</td>
</tr>
</tbody>
</table>

Equal numbers of MCs were plated in 12-well plates and grown under AP or OP for the indicated time period. After trypsinization, cell viability was assessed by trypan blue exclusion technique. Results are expressed as mean±SEM from 3 series of experiments.

Determination of Cell Viability and Cellular Proliferation

Frozen MCs were thawed, cultured for 1 passage until normal growth characteristics resumed, and plated at 2×10⁴ cells/mL on
TABLE 3. Rat MC Proliferation Under AP and OP

<table>
<thead>
<tr>
<th>Days</th>
<th>AP, $10^5$ cells/mL</th>
<th>OP, $10^5$ cells/mL</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.5±2.1</td>
<td>19.0±1.9</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>35.0±2.7</td>
<td>28.1±3.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>4</td>
<td>94.0±7.1</td>
<td>63.8±9.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>7</td>
<td>321.1±22.8</td>
<td>231.4±28.4</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Primary rat MCs were seeded at 20×10^5 cells/mL, grown under AP for 24 hours, and subsequently divided into 2 groups. For cells grown under OP, (P_{max}, 40 mm Hg), proliferation rates were significantly lower from days 2 to 7 compared with cells that were grown under AP.

12-well sterile culture plates in medium containing 1000 mg/L or 4500 mg/L glucose. Cell cultures were divided after 24 hours: 1 group was grown under atmospheric pressure (AP) and the other was grown in the pressure chamber with applied oscillating pressure (OP) adjusted to a maximum pressure (P_{max}) of 40 mm Hg. Cellular proliferation rates and viability were determined at daily intervals by trypan blue staining. Each experiment was performed in triplicate and repeated at least 3 times.

For DNA synthesis measurement, cells were seeded at 2×10^4 cells/mL in 24-well plates and grown under AP and OP. Cell [^3]H]thymidine incorporation was determined at days 1, 2, 4, and 7 by addition of 1.0 μCi/mL [^3]H]thymidine (30 Ci/mmol). After 24 hours, the supernatant was removed, cells were washed 3 times with ice-cold PBS, and DNA was precipitated with 5% trichloroacetic acid (TCA) overnight. Precipitates were washed 3 times with 5% TCA solubilized (1 hour at 37°C) in 750 μL of 0.25 mol/L NaOH, 0.1% SDS. The solution was neutralized with 50 μL of 4 mol/L HCl, and a 500-μL aliquot was counted in a scintillation counter.

Quantitative Measurement of Cell-Associated and Secreted Matrix Proteins
[^3]H]proline incorporation was performed essentially as described by Singhal et al., with minor modifications. MCs were plated at 2×10^5 cells/well in 12-well plates in RPMI medium containing 1000 mg/L or 4500 mg/L glucose and grown under AP and OP (P_{max}, 40 mm Hg; P_{max}, 18 mm Hg). Matrix component labeling was performed during 2 hours, and treated separately as described for CA collagens. For each CA and OG collagen, 6 sets of experiments were carried out (with triplicate determinations).

Immunocytochemistry
Cells were seeded on chamber slides and grown for 4 days under control conditions and OP. After fixation in acetone, cells were permeabilized with 0.5% Tween 20 and PBS and blocked with 0.5% BSA in PBS. For collagen detection, cells were incubated with primary monoclonal antibodies against human type I (0.5 μg IgG/mL, Bio Trend), type IV collagen (0.8 μg IgG/mL, Dako), and polyclonal goat anti-human type III and type V collagen antibodies (both 1.7 μg/mL, Southern Biotechnology Associates). For all antibodies, <10% cross-reactivity to collagen isotypes has been described. As secondary antibodies, biotinylated F(ab')2 rabbit anti-mouse IgG (40 μg/mL, Dako) and rabbit anti-goat antibodies (8 μg/mL, Immuno Research) were used, followed by incubation with FITC-conjugated streptavidin (4 μL/mL, Dako). Mouse monoclonal anti-smooth muscle α-actin (5 μg/mL, Dako) and vimentin antibody (2 μg/mL, Dako) were followed with biotinylated F(ab')2 rabbit anti-mouse IgG (Dako) and FITC-streptavidin.

Gelatin Zymography
Gelatin zymography was performed essentially as described previously. In brief, cells were grown to near confluence for 6 days and washed twice with PBS, and medium containing 0.2% BSA instead of fetal bovine serum was added. After exposure to control conditions or OP for 24 hours, cell supernatants were subjected to electrophoresis on 7.5% SDS–polyacrylamide gels containing 2 mg/mL gelatin, and zymography proceeded as reported.
Results

Effect of OP on MC Viability
To establish the effects of pressure change on MC viability, we maintained MC cultures under OP (P \text{max}, 40 mm Hg; P \text{mean}, 18 mm Hg; 60 cycles per minute) and AP in parallel. Cell viability was assessed at days 1, 2, 4, and 7 by trypan blue exclusion. As shown in Table 1, there was no significant difference in the percentage of viable MCs between the groups, indicating cell death was not measurably affected by the pressure chamber growth conditions within 7 days of culture. In addition, measurements of PCO2 and PO2 content and culture medium pH outlined in Table 2 revealed no significant differences between the intrachamber and AP groups after 48 hours, indicating the pressure chamber valves allowed for a continuous 5% CO2/air atmosphere exchange.

MC Proliferation at Increased Pressure
The effects of OP on MC proliferation were next evaluated by determination of cell number and [3H]thymidine incorporation as a marker for cell DNA synthesis. Four different treatment groups were established: group A, cells grown at AP and 1000 mg/L glucose; group B, cells grown at AP and 1000 mg/L glucose; group C, cells grown at AP and 4500 mg/L glucose; and group D, cells grown at OP and 4500 mg/L glucose.

In Figure 2A, AP and OP effects on MC growth rates are compared under normal glucose concentration. As shown, MC numbers in group B were significantly lower than in group A after 24 hours (group A, 23 000 ± 2 500 cells/mL; group B, 19 000 ± 900 cells/mL; P < 0.05) and continued to be lower over 7 days (group A, 208 100 ± 11 200 cells/mL; group B, 119 800 ± 20 500 cells/mL; P < 0.01). As shown in Figure 2B, cell numbers were higher under elevated glucose concentrations (on day 7: group A, 208 100 ± 11 200 cells/mL; group B, 429 000 ± 58 000 cells/mL; P < 0.01). OP application, however, suppressed cell proliferation markedly (day 7: group D, 143 000 ± 69 000 cells/mL). A similar growth inhibitory effect could be observed with primary rat MCs, as shown in Table 3.

As can be seen in Figure 2B, the proliferation-suppressive effect of OP is enhanced at elevated glucose concentrations. To rule out the possibility that elevated glucose affected cell growth via an osmolality change, experiments were performed using mannitol, which maintained the high osmolality. Under these conditions, growth-stimulatory effects were not observed (data not shown).

We next determined the effects of OP on MC DNA synthesis by measuring the incorporation of [3H]thymidine. MCs grown under OP (P \text{max}, 40 mm Hg; P \text{mean}, 18 mm Hg; groups B and D) showed a significantly lower [3H]thymidine incorporation throughout days 2 to 7 (on day 7: group B, 30 557 ± 2353 cpm; group D, 58 724 ± 5461 cpm) compared with cells grown under AP (group A, 68 012 ± 4148 cpm; group C, 102 659 ± 5440 cpm; for both comparisons, P < 0.01), as shown in Figure 3A. In agreement with cell counting data (Figure 2A and 2B), elevating culture medium glucose at AP stimulated [3H]thymidine incorporation from days 2 to 7 (day 2: group A, 49 751 ± 2388 cpm; group C, 61285 ± 3677 cpm; P < 0.05), shown in Figure 3A and 3B.

OP Regulates MC Collagen Synthesis
To determine the effects of OP on MC metabolism, we measured the levels of proline incorporation into CA and medium-released (EC) collagen. For these studies, [1H]proline labeling was performed over two 72-hour incubation periods; days 2 to 4 and days 4 to 6. Absolute value comparison ([1H]proline incorporation per well), expressed in Table 4, shows that the CA fractions do not differ significantly under AP and OP, whereas the absolute EC collagen fraction increased markedly under OP for confluent cultures. As shown in Figure 4, OP significantly enhanced relative CA and EC [1H]proline incorporation (cpm/10^5 cells, mean ± SD) into collagens of cells grown in low-glucose culture medium during the incubation period of days 2 to 4 (group A: CA, 696 ± 12 cpm/10^5 cells; EC, 848 ± 124 cpm/10^5 cells; group B:

<table>
<thead>
<tr>
<th>Glucose concentration 1000 mg/L</th>
<th>Days</th>
<th>Total [3H]Proline Uptake, cpm/well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AP (Group A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA</td>
</tr>
<tr>
<td>2–4</td>
<td>54 993 ± 949</td>
<td>67 020 ± 9855</td>
</tr>
<tr>
<td>4–6</td>
<td>108 237 ± 6985</td>
<td>217 134 ± 11 474</td>
</tr>
<tr>
<td>Glucose concentration 4500 mg/L</td>
<td>2–4</td>
<td>67 155 ± 9390</td>
</tr>
<tr>
<td>4–6</td>
<td>135 958 ± 989</td>
<td>227 032 ± 22 234</td>
</tr>
</tbody>
</table>

MC matrix synthesis was determined by [1H]proline incorporation over 72 hours during culture time periods days 2 to 4 and days 4 to 6. CA and medium-secreted (EC) radiolabeled collagens were determined separately as described in Methods and are expressed as cpm/well. Values are presented as mean ± SD from triplicate determinations and were confirmed in 6 sets of experiments.

Intergroup comparisons were performed by ANOVA, q values determined by Keuls multiple range test. *P = NS compared with CA group A, days 4–6; †P < 0.01 compared with CA group A, days 4–6; ‡P < 0.01 compared with CA group C, days 4–6; §P < 0.05 compared with EC group A, days 2–4; ¶P < 0.01 compared with EC group A, days 2–4; |P < 0.01 compared with EC group C, days 4–6.
fraction not significantly elevated, suggesting that the CA collagen fraction is preferentially augmented (Table 4). Remarkably, when elevated glucose and OP treatment were combined (group D), the relative and absolute values for CA and EC collagen fractions showed a significant increase. In comparison to cells grown with 1000 mg/L glucose and 40 mm Hg OP, absolute labeling of the CA collagen was significantly increased \((P<0.01)\) with 4500 mg/L glucose and OP; however, the EC fraction remained unchanged. These findings support the notion that elevated glucose and OP act synergistically on CA collagen synthesis.

**Immunocytochemistry**

Immunocytochemical studies were performed for smooth muscle \(\alpha\)-actin, vimentin, and ECM proteins. As assessed by semiquantitative comparison, a fibrillary intense staining pattern could be seen for smooth muscle \(\alpha\)-actin and vimentin, with no apparent differences for cells grown under AP and OP conditions. In contrast, differences could be observed for the fine granular staining pattern observed with ECM proteins type I and, even more pronounced, type III collagen (Figure 5). With OP, a more patchy staining pattern was present, especially within cell clusters. No apparent differences could be found for type IV and type V collagen staining, which was under both conditions intense and granular for type IV and rather sparse for type V collagen (not shown).

**OP Reduces Gelatinase A Activity**

Gelatin zymography studies were performed to evaluate the effect of OP on matrix-degrading enzymes. In MC culture supernatants, a single gelatinolytic band of 72 kDa could be detected, corresponding to gelatinase A activity. As shown in Figure 6, the gelatinolytic activity was significantly reduced after exposure to OP compared with control conditions (AP).

**Discussion**

Our studies have explored the hypothesis that intracapillary pressure changes are transmitted to the mesangial compartment and can be translated by MCs to specific metabolic and growth responses. Although glomeruli are highly distensible over the normal and abnormal ranges of pressure, data with isolated perfused glomeruli of subtotally nephrectomized rats demonstrate glomerular compliance increases by 59% in later stages of glomerulosclerotic disease. One half of the overall glomerular volume expansion is attributable to capillary dilation, while the other half is a consequence of stretching/dilation of other glomerular structures. Shear forces are likely to accompany glomerular expansion, which have been studied by different groups in an in vitro model consisting of flexible culture plate undersurfaces. In addition to distension, mechanical deformation, and transcellular pressure gradients, there is also an absolute increase in pressure occurring in a pulsatile pattern within the glomerular MC environment. These studies are focused on these alterations of absolute pressure. Micropuncture studies have documented intracapillary glomerular pressure changes in the range of 35 to 40 mm Hg and a transglomerular pressure difference of 3.
These studies, however, have not provided data concerning actual pressure changes residing in the glomerular mesangial compartment. Because a physiological pressure range remains undefined, we chose to examine effects through a $P_{\text{max}}$ of 40 mm Hg and $P_{\text{mean}}$ of 18 mm Hg, which correspond to afferent arteriole micropuncture data. In these studies, we have shown that MC proliferation and DNA synthesis were significantly inhibited by OP within 24 hours. The observed effects are in agreement with the only published study in which positive pressure has been applied directly to MC cultures. In this study, MC growth was inhibited at high constant positive pressure of 40 to 50 mm Hg. A drawback of this model is that significant differences for pH, $P_{\text{CO}_2}$, and $P_{\text{O}_2}$ values were reported for treatment and control groups, and high intrachamber pressure was not varied. In our model, the latter parameters were controlled. These results stand in contrast to the growth-stimulatory effects observed with the stretch/relaxation model.

To rule out clonal expansion of a subset of human MCs in culture with distinct growth characteristics, separate cell cultures were established from primary kidney tissue and

Figure 5. Expression of ECM proteins. Immunocytochemical studies demonstrate a more intense, granular type III collagen staining pattern for cells grown under OP (bottom) compared with cells grown under AP (top).
were evaluated for growth and collagen synthesis under OP. In these experiments, the growth-inhibitory pressure effect could be observed with all MC cultures. Further studies were conducted to test for interspecies differences using primary rat MC cultures. A similar pressure-related growth inhibitory effect could be demonstrated with all cells examined.

To study the effect that OP has on growth-induced MCs, cells were grown in elevated glucose. Consistent with previous data, those data show that elevated glucose significantly stimulated MC cell division. Under OP, this growth-stimulatory effect of glucose was markedly suppressed, as determined for cell numbers and \([^{3}H]\)thymidine incorporation, reaching a level that was remarkably close to the value observed under normal glucose growth condition.

In cell culture, MCs acquire the activated prosclerotic phenotype, characterized by increased proliferation, synthesis of interstitial collagens, and expression of activation marker smooth muscle \(\alpha\)-actin. Because data describing actual intramesangial pressure changes in vivo are lacking, the present results may be interpreted in 2 ways. On one hand, our model with OP may closely parallel physiological pressure changes, which may induce MCs to acquire a lowered growth rate characteristic of MCs in vivo. On the other hand, the decreased proliferation rate may correspond to the sclerotic glomerulus in late-stage disease, which is generally hypocellular. The latter interpretation might be supported by our findings of increased collagen synthesis accompanying growth suppression under OP.

In addition, we performed immunocytochemical studies using specific antibodies raised against types I, III, IV, and V collagen, vimentin, and smooth muscle \(\alpha\)-actin. In these studies, cells grown under either experimental conditions, AP or OP, stained intensively for vimentin and smooth muscle \(\alpha\)-actin, indicating their activated phenotype. Staining pattern differences were not apparent. Furthermore, a granular cellular staining pattern for interstitial collagen isotypes I and III, as well as isotypes IV and V, could be detected under AP and OP. Semiquantitative assessment of staining intensities suggested that under OP, collagen depositions of types I and III were more prominent. However, quantitative determinations have not been performed in this study.

MCs play a crucial role in matrix synthesis of collagen types I, III, IV, and V, fibronectin and laminin, among others, and influence matrix turnover by matrix metalloproteinase and cystein protease secretion (reviewed in Reference 24). In this work we have measured CA and medium-secreted (EC) matrix synthesis by \([^{3}H]\)proline labeling and isolation of collagenase-susceptible proteins. Our data demonstrate that OP preferentially increases CA matrix synthesis in subconfluent and confluent cultures, whereas total CA matrix synthesis was only slightly increased in confluent cultures. A similar effect has been reported by Mattana and Singhal under continuous positive pressure of 50 mm Hg.

Changes in matrix net synthesis rates may be attributable to differences in synthesis and/or degradation. To evaluate whether matrix degradation is influenced by OP, gelatin zymography studies were performed with cell culture supernatant. In these studies, a major band corresponding to the 72-kDa type IV collagenase, MMP-2, could be detected, which was significantly reduced after cells were exposed to OP for 24 hours. This finding supports the notion that decreased matrix degradation may contribute to EC matrix accumulation under OP.

Furthermore, we studied the effect of combined treatment with elevated glucose and applied OP. Elevated glucose under AP stimulated CA matrix synthesis as previously reported, whereas the EC fraction was unchanged. Interestingly, the total and relative (cpm/cells) CA collagen synthesis increased synergistically under applied OP.

In summary, these results indicate that with subconfluent cell cultures grown in 1000 mg/L glucose (labeling period, days 2 to 4), OP increases the total EC matrix pool. However, this growth medium does not significantly affect CA collagen synthesis. With confluent cells and a 4- to 6-day labeling period, a major increase in EC matrix synthesis occurs. Elevated glucose stimulates mainly the absolute CA collagen fraction synthesis, which further increases under OP. Thus, OP itself, in the absence of shear force, deformation, transcellular pressure gradients, or fluid flows, affects collagen net synthesis rates.

The present study is limited in that it does not address the question of reversibility of the pressure effect, and different pressure levels have not been tested because of structural limitations of the pressure chamber, which did not allow generation of larger pressure levels.

The pressure chamber model may be useful to study the effect of pressure in isolation as a modulator of glomerular cell properties, which contribute to glomerulosclerotic disease progression. For the stretch/relaxation model, gene expression is regulated specifically via the inositol phosphate and protein kinase C pathway. Further study is required to delineate the mechanism and proximate steps in the OP pathways.

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References
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