Mesangial cell gelatinase A synthesis is attenuated by oscillating hyperbaric pressure

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Glomerular hypertension has been established as a major factor contributing to glomerular scarring. Underlying cellular mechanisms leading to matrix accumulation are largely unknown. The isolated effect of oscillating hyperbaric pressure [OP; $P_{\max}$ 50 mmHg (1 mmHg = 0.133 kPa), P_{mean} 24 mmHg, with a fixed oscillation of 60/min] on matrix-degrading protease secretion by rat mesangial cells (MCs) was analysed using a pressure chamber model described previously [Mertens, Espenkott, Venjakob, Heintz, Handt and Sieberth (1998) *Hypertension* **32**, 945–952]. MCs were grown under atmospheric pressure (AP) or a controlled OP, and protease synthesis and gene transcription were analysed. A distinct biphasic cellular response to OP with stimulated gelatinase A protein expression and enzyme activity during the initial 24 h, and subsequent inhibition, was apparent, as shown by gelatin zymography. Gelatinase B activity remained unchanged. The abundance of gelatinase A transcripts, determined by reverse transcriptase-PCR, indicated a concordant

regulation of gene transcription. To elucidate underlying regulatory events, reporter constructs were transfected. In these experiments, a recently identified response element, RE-1, conferred a significant stimulatory effect within the initial 4 h of OP. Nuclear protein/RE-1 binding studies revealed additional complexes from 5 min up to 3 h after OP exposure, with intensities dependent on $P_{\max}$. STAT3 was identified as a component of these novel complexes. Down-regulation of *cis*-activity after 48 h of OP exposure was not transferred via the proximal 1686 bp of the gelatinase A regulatory sequence. In conclusion, hyperbaric OP elicits time-dependent changes in rat MC gelatinase A gene transcription.

Key words: enhancer element, extracellular matrix, gene transcription, pressure-responsive regulatory sequence, signal transduction and activation of transcription factors.

INTRODUCTION

Intraglomerular hypertension is a central determinant of glomerulosclerosis progression, characterized by extended extracellular matrix deposition. Therapeutic interventions that reduce the transcapillary pressure gradient, and thereby intracapillary pressure, e.g. with angiotensin-converting enzyme inhibitors and low-protein diets, can attenuate progression of glomerulosclerosis [1–4]. Laboratory studies have shown that a loss of afferent arteriole pressure autoregulation [5] is accompanied by increased intracapillary pressure. Nevertheless, little information is available on direct pathophysiological effects of glomerular intracapillary pressure on intrinsic glomerular cells and matrix turnover. The normal mesangial matrix mainly consists of type IV collagen, laminin, fibronectin and proteoglycans. In mesangio-proliferative disease, additional deposition of interstitial type I and III collagens occurs. Gelatinase A [or matrix metalloproteinase-2 (MMP-2); also known as 72 kDa type IV collagenase] is a key component involved in the turnover of glomerular extracellular matrix under (patho-)physiological conditions [6]. This enzyme belongs to a family of metalloproteinases that are characterized by activity at neutral pH, dependence upon zinc and bivalent cations for catalytic activity, secretion as latent proenzymes and inhibition by tissue inhibitors of metalloproteinases ('TIMPs'). Gelatinase A expression in mesangial cells (MCs) is highly regulated *in vivo* and *in vitro* [6,7]. Harendza et al. [7] observed induced gelatinase A expression in a rat model of mesangioproliferative glomerulonephritis. At the same time,

inhibition of protease activity ameliorated changes in the same model [8]. Controversial opinions exist concerning whether the effects of gelatinase A are beneficial or deleterious during the course of glomerular disease: on the one hand, its action as a protease might contribute to matrix degradation with prevention of matrix accumulation; on the other hand, gelatinase A has been shown to directly activate MCs to the pro-sclerotic phenotype [9]. In this respect, increased expression of gelatinase A in the anti-Thy1.1 model might contribute to restoration of the mesangial architecture, whereas decreased expression might contribute to glomerular scarring. Another member of the MMP gene family, gelatinase B, is also synthesized by MCs at low levels, although its involvement in mesangioproliferative diseases has not been thoroughly established.

Numerous studies have investigated the mesangial matrix metabolism and, amongst others, identified shear stress forces as regulatory events [10–13]. A cyclical stretch–relaxation model (e.g. of flexible culture-plate undersurfaces) was derived from observations with isolated glomeruli, in which enhanced perfusion pressure led to progressive glomerular volume expansion [4,10–14]. In such studies, shear forces profoundly affected mesangial cell growth, matrix synthesis and intermediary filament distribution [4,15–19]. Besides these shear forces, profound increases of intraglomerular pressure have been postulated, since intraglomerular capillary pressure alterations might be conferred on the mesangial compartment, because an interfering basement membrane is absent and a close spatial relationship between the mesangium and the surrounding intracapillary space exists

Abbreviations used: AP, atmospheric pressure; AP-2, activating protein-2; CE, cytoplasmic extract; MC, mesangial cell; MMP, matrix metalloproteinase; NE, nuclear extract; OP, oscillating hyperbaric pressure; RE-1, response element-1; RT, reverse transcription; SIE, serum-inducible element; STAT, signal transduction and activation of transcription factors; SV40, simian virus 40; YB-1, Y-box protein-1.

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[20,21]. Recent models have focused on pressure-related effects on cell phenotypes, signalling events and changes in matrix synthesis and composition [22–25]. In this and other models with pressure-load, a significant matrix accumulation was detected, whereas, at the same time, cell proliferation was inhibited [23,25]. In the present study, we set out to elucidate the influence of oscillating hyperbaric pressure (OP) on metalloprotease secretion, and address the question of whether a direct effect of OP on gene transcription occurs.

MATERIALS AND METHODS

Cell culture

Rat MCs were established as described previously [9], and grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 µg of streptomycin and 100 units/ml penicillin (all products were purchased from Invitrogen, Groningen, The Netherlands) at 37 °C in a mixture of CO₂/air (1:19).

Pressure chamber

The pressure chamber was constructed as described in detail previously [23]. Pressure values were adjusted by direct measurements of intrachamber pressures via an in-lying line and set at a $P_{\max}$ of 30 and 50 mmHg (1 mmHg = 0.133 kPa), respectively, with a fixed oscillation (f) of 60/min. Oscillations were rhomboid, and $P_{\min}$ was equivalent to atmospheric pressure (AP). As described previously, no significant differences for pH, P_{O_2} or P_{CO_2} were found between AP and OP [23].

Semi-quantitative zymography

Zymography was performed as described previously [9]. Rat MCs were fed with serum-free RPMI 1640 medium containing 0.2% (v/v) serum albumin 24 h after outgrowth in six-well culture plates, and conditioning of supernatants was performed for 12, 24 and 48 h under AP or OP with a $P_{\max}$ of 50 mmHg, $P_{\min}$ of 24 mmHg and f of 60/min. In addition, serum-free medium was exchanged after 24 h, and further conditioning for a period of 24 h was performed (indicated by label '48/24' on the relevant Figures) to determine differences of gelatinase A synthesis in the last 24 h during a total OP period of 48 h. Supernatants were briefly centrifuged to remove cellular debris. The amount of supernatant loaded for gelatin zymography was normalized for total cellular protein content, since no change in protein content per cell was determined with OP compared with AP. Conditioned media were added to non-reducing sample buffer before loading, electrophoresed on an SDS/7.5% polyacrylamide gel containing 2 mg/ml gelatin, washed twice in renaturation buffer [50 mM Tris/HCl (pH 8.0)/2.5% Triton X-100] for 30 min each wash, and incubated overnight at 37 °C in 50 mM Tris/HCl, pH 8.0/1 µM ZnSO₄/5 mM CaCl₂. Fixed and Coomassie Blue R250-stained gels were analysed by scanning, and relative band intensities were assessed using computer densitometry software (Biostep, Jahnshof, Germany). All experiments were performed as duplicates and repeated at least three times.

Reverse transcription (RT)-PCR

After MCs were exposed to AP or OP for different periods, mRNA was isolated using Oligotex (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription for cDNA synthesis was performed with 5 µg of total RNA

transcribed in reaction buffer [50 mM Tris/HCl (pH 8.3)/50 mM KCl/10 mM MgCl₂/10 mM dithiothreitol/0.5 mM spermidine] containing 4 units of RNase inhibitor (Boehringer Mannheim, Mannheim, Germany), 0.5 µg of oligo d(T)_{12–18} (Invitrogen), 0.5 nM dNTPs Mix (Amersham Biosciences, Little Chalfont, Bucks., U.K.) and 8 units of avian myeloblastosis virus reverse transcriptase (Promega, Mannheim, Germany) for 2 h at 42 °C, followed by termination at 95 °C for 5 min. Subsequently, cDNAs were precipitated and PCR was performed by addition of 2 units of *Taq* polymerase (Invitrogen) in reaction buffer [200 mM Tris/HCl (pH 8.3)/0.5 M KCl/1.5 mM MgCl₂] containing 0.25 pmol of dNTPs and 25 pmol of respective primer oligonucleotides. Primers for rat gelatinase A were: sense, 5'-CAATACCTGAACACCTT-3'; antisense, 5'-CTGTATGTGATCTGGTT-3' [26], yielding a 227 bp amplification product. Primers for cyclophilin were: sense, 5'-CAACTCTAATTTCTTGTGACTTGCGGG-3'; antisense, 5'-AGAGATTACAGGGTATTGCGAG-3' [27], yielding a 100 bp amplification product. Thermal cycling conditions were equal for both reactions: after heating at 94 °C for 4 min, a total of 35 cycles were performed with 20 s at 94 °C, 25 s at 53 °C and 25 s at 72 °C. The final reaction was kept at 72 °C for 7 min, and amplified fragments were electrophoresed on an ethidium-bromide-containing 1.5% agarose gel.

Nuclear extracts

MCs were grown to approx. 80% confluence in tissue-culture flasks, exposed to AP or OP (30 or 50 mmHg respectively) for 5, 30, 60 and 180 min, washed twice with ice-cold PBS without calcium and magnesium, and scraped in 10 ml of cold PBS. Nuclear cell-extract preparations were performed exactly as described previously [28–30].

Electrophoretic mobility-shift analysis

Synthetic oligonucleotides encompassing the response element-1 sequence (5'-AGCCTGCTGGGCAAGTCTGAACCTTGTCAGAAACCCACTAG-3'; the sense strand) [31] was radiolabelled by means of T4 polynucleotide kinase using [γ -³²P]ATP. Single- and double-stranded probes were purified on 14% polyacrylamide gels, eluted, and 2 × 10³ c.p.m. of labelled probe was included per binding reaction. Binding reactions were performed in binding buffer A [20 mM Hepes (pH 7.9)/20% (v/v) glycerol/0.1 M NaCl/0.2 mM EDTA] containing 0.2 mM PMSF, 0.5 mM dithiothreitol, 300 µg/ml acetylated BSA and 2 µg of poly(dI-dC) in a total vol. of 25 µl upon addition of nuclear and cytoplasmic extracts (total protein amount of 10 µg per binding reaction) at 22 °C for 30 min. Samples were electrophoresed on non-denaturing 4% polyacrylamide/5% glycerol gels in a buffer containing 1 × TBE (45 mM Tris/borate/1 mM EDTA), followed by autoradiography at –80 °C. For supershift studies, monospecific polyclonal antibodies raised against two members of the signal transduction and activation of transcription factors (STAT) family, STAT1 and STAT3 (anti-GNN31, kindly provided by W. Mueller-Esterl; [32]), were added to the binding reaction 2 h before the addition of radiolabelled probe. A double-stranded nucleotide containing a STAT3-consensus binding site derived from the *c-fos* gene (*sis*-inducible element, or 'SIEM'; 5'-CATT-TCCCGTAAATCTTGTCG-3') was used as competitor DNA.

Transient transfection studies

Transient transfections of MCs were performed using the liposomal Tfx-50 preparation (Promega) in six-well cell culture plates, as described in detail previously [28].

Figure 1 OP elicits a biphasic response of gelatinase A activity

(A) Gelatin zymography was performed with conditioned media from MCs. Cells were grown for 24 h under AP to allow for plastic surface attachment, washed and the medium was then exchanged with serum-free, albumin-supplemented medium and kept under AP or OP ($t = 60/\text{min}$) for the periods indicated. To detect changes within the last 24 h of a 48 h period, medium was exchanged again after 24 h and cells were kept under AP or OP for an additional 24 h (indicated as '48/24'). Total cellular protein was determined and conditioned media normalized for protein amounts. A major lucent band corresponding to 66 kDa could be detected. A minor lucent band of 92 kDa, which remained unchanged by OP, is not visible by means of gel scanning. Shown is a representative result of three experiments performed with duplicate determinations. (B) Densitometric analysis of zymography was performed using Phoretix software. Values obtained in the AP group were arbitrarily set as 1. Bars indicate S.D. for three experiments performed in duplicate; * $P < 0.05$ between AP and OP groups. (C) Nucleoprotein complex formation with the double-stranded (DS) RE-1 probe is dependent on MC growth characteristics. Compared with nuclear extracts (NE) prepared in exponential-growth phase (lane 2), diminished complex formation was detected with extracts prepared from confluent cells (lane 3).

Plasmids used included a luciferase reporter construct harbouring the rat gelatinase A regulatory sequences up to -1686 bp relative to the translation start site subcloned into pGL2-Basic (pT4 Luc1686), and the isolated response element-1 (RE-1) subcloned into reporter construct pGL2-Promoter (pGL2-P-RE-1). Cells were transfected, grown under AP and subsequently kept at either AP or OP for 2, 4, 24 or 48 h. The total transfection period was 48 h for all determinations. Luciferase activities were normalized by means of a co-transfected simian virus 40 (SV40)- β -galactosidase plasmid (Stratagene). All transfections were performed in triplicate and repeated at least three times.

RESULTS

Time-dependent effect of OP on MC gelatinase A synthesis

To investigate the effects of OP on MC gelatinase A activity, conditioned media of cells grown under AP or OP were analysed by zymography. As can be seen in Figures 1(A) and 1(B), there was a single prominent lucent band with a molecular mass of 66 kDa, corresponding to the size of activated gelatinase A, which was increased within 12 h of OP compared with AP. A minor gelatinolytic band with molecular mass of 92 kDa, corresponding to gelatinase B (MMP-9), was also detected; however, this was too faint for visualization by gel scanning. After 24 h, gelatinolytic activity was still increased in the OP group. At the time point of 48 h, a decreased gelatinolytic activity was detected in the OP group compared with the AP group. A second protocol was set up to observe changes in gelatinase A synthesis within the last 24 h of a period of 48 h of OP by performing an additional change of media. OP was continued for 24 h, and supernatants were processed as described above (indicated in Figures 1A and 1B by '48/24'). As can be seen in lanes 13–16 in Figure 1(A), gelatinolytic activity was markedly decreased in the OP group compared with the AP group. To exclude differences in cell viability between both groups, Trypan Blue exclusion staining was performed as reported previously [23]. Here, no significant differences were detected between the groups ($90.0 \pm 5.2\%$ with AP as compared with $88.0 \pm 8.2\%$ with OP on day 4).

Previous studies performed with the OP model demonstrated a down-regulation of gelatinase A synthesis within 24 h of OP when OP was applied to cells that were grown to confluence [23]. The differences in gelatinase A synthesis under OP with confluent and non-confluent cells indicated growth-dependent differences of the pressure-related effect that might reside in growth-dependent transcriptional regulation. Within the rat gelatinase A regulatory sequence, a potent enhancer element designated RE-1 at $-1282/-1322$ bp relative to the translational start site has been identified previously by Harendza et al. [31], which confers approx. 80% of constitutive gene activity of the proximal 1686 bp. To elucidate whether binding activities to this element differ in actively dividing cells compared with confluent cells, MC nuclear extracts were prepared from cells exhibiting exponential growth (approx. 40% confluence on day 2) and cells grown to confluence for 6 days before OP was applied. In DNA-binding assays, nucleocore complex formation with the double-stranded RE-1 probe was drastically reduced with nuclear proteins from confluent cells compared with proteins obtained from exponentially growing cells (Figure 1C), suggesting that RE-1 trans-activating factors are growth-dependent and less abundant with confluence.

Influence of OP on gelatinase A mRNA expression

To determine whether the changes of gelatinolytic activity were due to transcriptional or post-transcriptional regulatory

Figure 2 OP attenuates gelatinase A gene expression

RT-PCR was performed with mRNA prepared at the indicated time points from cells grown under AP or OP using pairs of primers that are specific for gelatinase A and cyclophilin. The total amount of gelatinase A transcripts changed over time under OP, with a transient up-regulation at time point 12 h, and subsequent down-regulation at time point 48 h. Shown is a representative result of two independent experiments performed as duplicates.

mechanisms, semi-quantitative RT-PCR of gelatinase A transcripts was performed with mRNA isolated from MCs grown under AP and OP for 12, 24 and 48 h (Figure 2). The 'house-keeping' gene cyclophilin was amplified from the same mRNA samples, and served as an internal control. Comparison of gelatinase A mRNA abundance under conditions of OP and AP demonstrated a transient up-regulation of MMP-2 transcripts after 12 h of OP (lanes 1 and 2), whereas mRNA levels were equal in both groups after 24 h (lanes 3 and 4). After 48 h of OP, gelatinase A transcripts were reduced compared with the AP group (Figure 2, lanes 5 and 6). These results indicate that differences in gelatinolytic activities observed by gelatin zymography are due to concordant changes in gelatinase A gene transcription.

OP stimulates gelatinase A gene expression via the enhancer element RE-1

Transient transfection studies using reporter constructs harbouring the isolated regulatory element RE-1 were conducted to evaluate whether this element participates in the transient up-regulation of gelatinase A expression. As can be seen in Figure 3, reporter construct activity was significantly stimulated (2-fold) under OP within 2 h, whereas no *trans*-activation was apparent after prolonged OP of 24 and 48 h. This result suggested that most, if not all, of the initial pressure-related *trans*-activation of the gelatinase A gene was transferred by RE-1. When construct pT4 Luc1686 was tested with prolonged OP, no difference in reporter activity was detected (results not shown). Thus transcriptional repression is conferred through regulatory elements that reside outside of the analysed sequence.

Additional RE-1-binding activities appear with OP

To characterize further the initial stimulatory events taking place under OP within the context of RE-1, DNA-binding studies were performed using the double-stranded and single-stranded (sense strand) RE-1 as probe, as the latter has been shown to be specifically bound by several relevant transcription factors [28]. Nuclear extracts were prepared at time points 0, 5, 30, 60 and 180 min of OP. $P_{\max}$ was set at 50 mmHg, as was the case during the gelatin zymography experiments. There was a rapid change of binding pattern within 5 min to both the double-stranded RE-1 (Figure 4A) and the sense-strand probe (Figure 4B) with the appearance of additional bands (indicated by the arrowheads labelled '2', '3' and '6' in Figure 4A, and '1' in Figure 4B). The

Figure 3 Transcriptional *trans*-activation is conferred through RE-1

Transient transfection experiments were performed with MC using hybrid reporter constructs harbouring RE-1 in conjunction with a heterologous SV40 promoter (pGL2-P-RE-1). Upon transfection, cells were grown under AP and subsequently exposed to OP for time periods indicated. Transfections lasted in all groups for 48 h. Luciferase (LUC) measurements were corrected for transfection efficiencies by co-introducing a β -galactosidase vector and measuring galactosidase activities. Data are given from triplicate determinations, which were confirmed in three independent experiments and are expressed as means $\pm$ S.D.

differences in banding pattern lasted for at least 180 min. Notably, changes were obvious for cytoplasmic proteins (lanes 6–10 in Figure 4B); that is, the intensities of major nucleocomplexes 1 and 2 were down-regulated and returned to basal levels after 180 min, implying a rapid nuclear translocation of pre-formed cytoplasmic activities. The formation of specific nucleoprotein complexes was ascertained by competition experiments using specific and non-specific competitor DNA (Figure 4A, lanes 7 and 8) in 100-fold molar excess.

To elucidate whether this change of RE-1 binding could be attributed to pressure-related cellular changes, additional binding assays were performed using nuclear extracts that were prepared at a lower pressure level with a $P_{\max}$ of 30 mmHg ($f = 60$ /min). As can be seen in Figure 4(C), there was a similar rapid change of binding activity to the sense strand; however, differences in binding were only present for a short time period of less than 30 min. Again, a concordant change of cytoplasmic binding activities was apparent. A similar transient change of nucleocomplex formation was also present with the double-stranded probe (results not shown). Taken together, these results support the notion that the pressure-related transcriptional up-regulation of gelatinase A gene expression was conferred by the previously described RE-1. The extent of binding-activity changes correlated with the maximum pressure level applied, supporting the notion of pressure-related transcriptional regulation.

STAT3 is part of the novel binding activities at RE-1

Closer inspection of the RE-1 sequence revealed the presence of a nearly perfect STAT3-binding motif within the 3' portion of RE-1 (Figure 5A) [33,34], which is highly homologous with STAT3 binding sites in the *c-myc* and α 1-antichymotrypsin genes. This motif is not contiguous with the previously localized activating protein-2 (AP-2) binding site; however, it overlaps with a region that is protected by Y-box protein-1 (YB-1) in dimethylsulphate methylation footprinting assays (Figure 5B) [28,29]. As the mobilities of the novel binding activity clearly differed from the ones obtained under AP, and rapid signalling was evident

Figure 4 DNA-binding activities at RE-1 under OP

(A) DNA-binding studies were performed using the double-stranded (DS) RE-1 as probe and nuclear extracts prepared at the time points indicated after cell exposure to OP (P_{max} 50 mmHg; $f = 60/\text{min}$). For cells grown under AP, three major bands can be detected (designated 1, 4 and 5), whereas OP led to the formation of additional complexes within 5 minutes, designated 2, 3 and 6. These additional nucleoprotein complexes were present for at least 180 min after OP exposure. Binding specificity was determined by inclusion of specific (s) and non-specific (ns) competitor DNA in 100-fold molar excess to the binding reaction (lanes 7 and 8). (B) When DNA-binding studies were repeated with the sense strand (SS1) of RE-1 as probe and nuclear extract (NE) prepared under OP of 50 mmHg ($f = 60/\text{min}$), a similar change of binding activities could be detected; that is, enhanced binding of complex 2 and presence of a novel low-mobility complex, designated 1. Again, the appearance of these complexes is rapid, whereas at the same time cytoplasmic binding complexes (CE) are diminished (lanes 6–10). (C) To evaluate whether changes of nucleoprotein-complex formation were pressure-dependent, binding studies were repeated with nuclear extracts obtained after exposure to a lower OP of 30 mmHg ($f = 60/\text{min}$) and the sense RE-1 strand as probe. Again, a dramatic change of nuclear binding activities (NE) and appearance of a low-mobility band was apparent within 5 min. At the same time, complexes obtained with cytoplasmic extracts (CE) were diminished. Binding pattern changes with lower pressure levels were of short duration, as they returned to the 'baseline' pattern within 30 min.

Figure 5 STAT3 specifically binds to RE-1

(A) Sequence comparison with known transcription factor binding motifs revealed the presence of a STAT3 binding site within RE-1. Matching bases for the centres of core-half sites that have been found to be bound by STAT3 [34] are shown in **bold**. (B) Summary of known transcription factor binding sites within RE-1, as determined by electrophoretic mobility-shift analysis or DNA footprinting analysis. Notably, the STAT-binding motif is contiguous with YB-1, NM23-β and one half-site of the p53 binding motifs. (C) DNA-binding studies were performed using nuclear extracts obtained after 3 h of pressure exposure (P_{max} 50 mmHg) and the double-stranded RE-1 probe. As can be seen in lane 3, inclusion of a polyclonal anti-STAT3 antibody led to a supershift (indicated by *), whereas bands 1–4 were diminished. Inclusion of unrelated IgG did not alter the banding pattern. Competition experiments were performed using non-labelled SIE and scrambled oligonucleotides. Here, specific competition for binding could be detected with complexes 1–4, whereas complexes 5 and 6 were slightly diminished.

as has been described for STAT factors, supershift studies were performed using polyclonal anti-STAT1 and -STAT3 antibodies, double-stranded RE-1 probe and nuclear extracts obtained from MCs grown under OP for 3 h. As can be seen in Figure 5(C), inclusion of anti-STAT3 antibody in the binding reaction led to

diminished bands (indicated by arrowheads 1–4 in lane 3), while at the same time a low-mobility band appeared (supershift indicated by *). Similar experiments using an anti-STAT1 antibody failed to produce a supershift or diminished bands (results not shown). Addition of unrelated IgG did not change the binding pattern (lane 4). To confirm the involvement of STAT factor(s) in nucleocomplex formation with RE-1, DNA-competition experiments were performed using the serum-inducible element (SIE) oligonucleotide (at 500-fold molar excess), which is derived from the *c-fos* promoter sequence and constitutes a known binding site for STAT1 and STAT3. As can be seen in lane 5 of Figure 5(C), major complex formation of bands 1–4 was abrogated with the addition of SIE competitor DNA, whereas complexes 5 and 6 were slightly reduced. The reduced formation of complexes 5 and 6 might be due to competition for the second YB-1 binding site in the 3' portion of the probe. Specificity of competition was determined by addition of unrelated DNA, shown in lane 6, which had no effect on nucleocomplex formation.

DISCUSSION

In the present study, advantage was taken of a model system that allows for the generation of a defined OP without affecting the remainder of the culture conditions [23]. In this way, isolated effects of OP on matrix metabolism from MCs can be detected *in vitro*. Previous data have showed increased collagenase-sensitive matrix generation under pressure-load [23]. In the present study, two members of the MMP superfamily were detected by zymography from MC culture supernatants, gelatinases A and B. Whereas the amount of gelatinase B secretion and activation was unaffected by OP, significant changes in gelatinase A secretion were apparent with a biphasic response, consisting of a transient up- and subsequent down-regulation. A similar increase in gelatinase A activity by pressure-load for 24 h was detected by Singhal et al. [35]. In their study, pressure levels up to 105 mmHg were tested, which are far outside the range achieved with our model system. Most interestingly, a similar increase of gelatinase A activity was observed with the shear-stress model [19,35]. To extend the findings by Singhal et al. [35], which were confined to a 24 h observational period, our results demonstrate a biphasic response with subsequent down-regulation of gelatinase A activity. Using different methodologies, the present study shows that these changes can be attributed to differences in transcriptional regulation, and point towards RE-1 as the major *cis*-element for the initial transcriptional up-regulation. This element has been shown to be specifically bound by several transcription factors in its 5' portion, i.e. AP-2, YB-1 and p53, the binding sites for which are contiguous [28–30,36]. DNA-binding studies reveal that, upon pressure exposure, additional binding activities to this element are rapidly translocated from the cytoplasm into the nucleus. Furthermore, the duration of novel binding activities present was dependent on the maximum pressure level applied. Sequence comparison of RE-1 with known STAT binding sites [34] revealed a nearly perfect match in the 3' portion of RE-1. In supershift studies, a member of the STAT family of transcription factors, STAT3, was identified as a component of the novel binding activities. DNA competition experiments support the notion of specific binding of STAT3 to the response element. NM23-β has been identified as yet another transcription factor binding to the RE-1 (S. Cheng, M. A. Alfonso-Jaume, P. R. Mertens and D. H. Lovett, unpublished work), rendering an even more complex picture of binding activities (summarized in Figure 5B). Binding motifs for NM23-β, p53 and YB-1 overlap with the predicted STAT3 binding site; therefore mapping of STAT3 contacts to the DNA by mutagenesis of

this sequence will most likely also affect the binding of other proteins.

The cellular sensing and signalling events that take place with hyperbaric pressure are poorly understood. For pressure loading with a $P_{\max}$ of 90 mmHg, a rapid activation of mitogen-activated protein kinase in MCs has been described recently [37]. This activation was transient, and lasted about 15 min. Subsequently, nuclear c-Fos levels were increased more than 10-fold for a prolonged period of at least 48 h. Thus a prolonged activation of transcription factors by pressure load may be observed, which could profoundly influence corresponding target genes. Both STAT1 and STAT3 are activated by osmotic shock via p38 [38,39]. In this model, a prolonged activation of STAT factors for more than 180 min was detected after exposure to 600 mM sorbitol, a period similar to the one observed with pressure load. It is conceivable that changes in the cytoskeleton architecture occur in both models, which activate similar proximate signalling events.

Our results indicate that RE-1 provides a sequence motif for early pressure-related transcriptional changes, since it serves as a template for STAT3 binding. Notably, shear-stress-responsive elements ('SSREs'; sequence GAGACC) are not present within this element [40,41]. The remaining pressure-related RE-1 binding activities could not be related to any of the previously identified binding factors (AP-2, YB-1 and p53), since attempts to 'supershift' these failed (results not shown). For shear-stress-related transcriptional regulation, a multitude of proximal signalling events have been detected [20,42–44], which, among others, activate the transcription factors nuclear factor- κ B [45], c-Fos, c-Jun, Egr-1 and c-Myc [46,47]. However, binding sites for these transcription factors are not present in RE-1.

In previous experiments, cells were grown to confluence before pressure was applied, and we observed a down-regulated gelatinase A activity after 24 h of OP (40 mmHg) compared with AP. This discrepancy in the response compared with our present findings with cells in the exponential-growth phase prompted further investigation of RE-1 binding activities in exponentially growing cf. confluent cell cultures. In these experiments, we observed a major decrease in binding activities at RE-1 with cell confluence, suggesting that the stimulatory response to OP is abrogated under these culture conditions.

In the present study, we were not able to identify sequence elements within the proximal 1686 bp of the regulatory sequence responsible for down-regulated gelatinase A expression. Studies are in progress to elucidate whether the recently described silencing element [48] located between –1903 and 1847 bp is operative in gene silencing with OP and prolonged pressure exposure. Given the importance of pressure-related end organ damage, which is not confined to the kidney, and its relevance for changes in the vasculature, it will be of great interest to understand the underlying transcriptional mechanisms and identify pressure-regulated target genes. Future work will also be aimed at the elucidation of proximal signalling events with pressure load, and the involvement of STAT factors.

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REFERENCES

- Brenner, B. M., Meyer, T. W. and Hostetter, T. H. (1982) Dietary protein intake and the progressive nature of kidney disease: the role of hemodynamically mediated glomerular injury in the pathogenesis of progressive glomerular sclerosis in aging, renal ablation, and intrinsic renal disease. *N. Engl. J. Med.* **307**, 652–659
- Hostetter, T. H., Rennke, H. G. and Brenner, B. M. (1982) Compensatory renal hemodynamic injury: a final common pathway of residual nephron destruction. *Am. J. Kidney Dis.* **1**, 310–314
- Klahr, S., Schreiner, G. and Ichikawa, I. (1988) The progression of renal disease. *N. Engl. J. Med.* **318**, 1657–1666
- Riser, B. L., Cortes, P., Zhao, X., Bernstein, J., Dumler, F. and Narins, R. G. (1992) Intraglomerular pressure and mesangial stretching stimulate extracellular matrix formation in the rat. *J. Clin. Invest.* **90**, 1932–1943
- Pelayo, J. C. and Westcott, J. Y. (1991) Impaired autoregulation of glomerular capillary hydrostatic pressure in the rat remnant nephron. *J. Clin. Invest.* **88**, 101–105
- Lovett, D. H., Johnson, R. J., Marti, H. P., Martin, J., Davies, M. and Couser, W. G. (1992) Structural characterization of the mesangial cell type IV collagenase and enhanced expression in a model of immune complex-mediated glomerulonephritis. *Am. J. Pathol.* **141**, 85–98
- Harendza, S., Behrens, U., Zahner, G., Schneider, A. and Stahl, R. A. (1997) In vitro characterization of the mesangial phenotype in a proliferative glomerulonephritis of the rat. *Nephrol. Dial. Transplant.* **12**, 2537–2541
- Steinmann-Niggli, K., Ziswiler, R., Kung, M. and Marti, H. P. (1998) Inhibition of matrix metalloproteinases attenuates anti-Thy1.1 nephritis. *J. Am. Soc. Nephrol.* **9**, 397–407
- Turck, J., Pollock, A. S., Lee, L. K., Marti, H. P. and Lovett, D. H. (1996) Matrix metalloproteinase 2 (gelatinase A) regulates glomerular mesangial cell proliferation and differentiation. *J. Biol. Chem.* **271**, 15074–15083
- Border, W. A., Okuda, S. and Nakamura, T. (1989) Extracellular matrix and glomerular disease. *Semin. Nephrol.* **9**, 307–317
- Floege, J., Burns, M. W., Alpers, C. E., Yoshimura, A., Pritzl, P., Gordon, K., Seifert, R. A., Bowen-Pope, D. F., Couser, W. G. and Johnson, R. J. (1992) Glomerular cell proliferation and PDGF expression precede glomerulosclerosis in the remnant kidney model. *Kidney Int.* **41**, 297–309
- Floege, J., Eng, E., Lindner, V., Alpers, C. E., Young, B. A., Reidy, M. A. and Johnson, R. J. (1992) Rat glomerular mesangial cells synthesize basic fibroblast growth factor. Release, upregulated synthesis, and mitogenicity in mesangial proliferative glomerulonephritis. *J. Clin. Invest.* **90**, 2362–2369
- Riser, B. L., Cortes, P., Yee, J., Sharba, A. K., Asano, K., Rodriguez-Barbero, A. and Narins, R. G. (1998) Mechanical strain- and high glucose-induced alterations in mesangial cell collagen metabolism: role of TGF- β . *J. Am. Soc. Nephrol.* **9**, 827–836
- Cortes, P., Riser, B. L., Yee, J. and Narins, R. G. (1999) Mechanical strain of glomerular mesangial cells in the pathogenesis of glomerulosclerosis: clinical implications. *Nephrol. Dial. Transplant.* **14**, 1351–1354
- Cortes, P., Riser, B. L., Zhao, X. and Narins, R. G. (1994) Glomerular volume expansion and mesangial cell mechanical strain: mediators of glomerular pressure injury. *Kidney Int. Suppl.* **45**, S11–S16
- Homma, T., Akai, Y., Burns, K. D. and Harris, R. C. (1992) Activation of S6 kinase by repeated cycles of stretching and relaxation in rat glomerular mesangial cells. Evidence for involvement of protein kinase C. *J. Biol. Chem.* **267**, 23129–23135
- Raghow, R. and Thompson, J. P. (1989) Molecular mechanisms of collagen gene expression. *Mol. Cell. Biochem.* **86**, 5–18
- Riser, B. L., Cortes, P., Heilig, C., Grondin, J., Ladson-Wofford, S., Patterson, D. and Narins, R. G. (1996) Cyclic stretching force selectively up-regulates transforming growth factor- β isoforms in cultured rat mesangial cells. *Am. J. Pathol.* **148**, 1915–1923
- Yasuda, T., Kondo, S., Homma, T. and Harris, R. C. (1996) Regulation of extracellular matrix by mechanical stress in rat glomerular mesangial cells. *J. Clin. Invest.* **98**, 1991–2000
- Ingram, A. J. and Scholey, J. W. (2000) Stress-responsive signal transduction mechanisms in glomerular cells. *Curr. Opin. Nephrol. Hypertens.* **9**, 49–55
- Riser, B. L., Cortes, P. and Yee, J. (2000) Modelling the effects of vascular stress in mesangial cells. *Curr. Opin. Nephrol. Hypertens.* **9**, 43–47
- Kawata, Y., Mizukami, Y., Fujii, Z., Sakumura, T., Yoshida, K. and Matsuzaki, M. (1998) Applied pressure enhances cell proliferation through mitogen-activated protein kinase activation in mesangial cells. *J. Biol. Chem.* **273**, 16905–16912
- Mertens, P. R., Espenkott, V., Venjakob, B., Heintz, B., Handt, S. and Sieberth, H. G. (1998) Pressure oscillation regulates human mesangial cell growth and collagen synthesis. *Hypertension* **32**, 945–952
- Singal, D. P. and Qiu, X. (1996) Polymorphism in both X and Y box motifs controls level of expression of HLA-DRB1 genes. *Immunogenetics* **43**, 50–56
- Singhal, P. C., Gibbons, N., Franki, N., Reddy, K., Sharma, P., Mattana, J., Wagner, J. D. and Bansal, V. (1998) Simulated glomerular hypertension promotes mesangial cell apoptosis and expression of cathepsin-B and SGP-2. *J. Invest. Med.* **46**, 42–50
- Ailenberg, M. and Silverman, M. (1996) Cellular activation of mesangial gelatinase A by cytochalasin D is accompanied by enhanced mRNA expression of both gelatinase A and its membrane-associated gelatinase A activator (MT-MMP). *Biochem. J.* **313**, 879–884

- 27 Zhong, H. and Simons, J. W. (1999) Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. *Biochem. Biophys. Res. Commun.* **259**, 523–526
- 28 Mertens, P. R., Harendza, S., Pollock, A. S. and Lovett, D. H. (1997) Glomerular mesangial cell-specific transactivation of matrix metalloproteinase 2 transcription is mediated by YB-1. *J. Biol. Chem.* **272**, 22905–22912
- 29 Mertens, P. R., Alfonso-Jaume, M. A., Steinmann, K. and Lovett, D. H. (1998) A synergistic interaction of transcription factors AP2 and YB-1 regulates gelatinase A enhancer-dependent transcription. *J. Biol. Chem.* **273**, 32957–32965
- 30 Mertens, P. R., Alfonso-Jaume, M. A., Steinmann, K. and Lovett, D. H. (1999) YB-1 regulation of the human and rat gelatinase A genes via similar enhancer elements. *J. Am. Soc. Nephrol.* **10**, 2480–2487
- 31 Harendza, S., Pollock, A. S., Mertens, P. R. and Lovett, D. H. (1995) Tissue-specific enhancer-promoter interactions regulate high level constitutive expression of matrix metalloproteinase 2 by glomerular mesangial cells. *J. Biol. Chem.* **270**, 18786–18796
- 32 Haan, S., Kortylewski, M., Behrmann, I., Muller-Esterl, W., Heinrich, P. C. and Schaper, F. (2000) Cytoplasmic STAT proteins associate prior to activation. *Biochem. J.* **345**, 417–421
- 33 Wegenka, U. M., Luttkien, C., Buschmann, J., Yuan, J., Lottspeich, F., Muller-Esterl, W., Schindler, C., Roeb, E., Heinrich, P. C. and Horn, F. (1994) The interleukin-6-activated acute-phase response factor is antigenically and functionally related to members of the signal transducer and activator of transcription (STAT) family. *Mol. Cell. Biol.* **14**, 3186–3196
- 34 Ehret, G. B., Reichenbach, P., Schindler, U., Horvath, C. M., Fritz, S., Nabholz, M. and Bucher, P. (2001) DNA binding specificity of different STAT proteins: comparison of in vitro specificity with natural target sites. *J. Biol. Chem.* **276**, 6675–6688
- 35 Singhal, P. C., Sagar, S. and Garg, P. (1996) Simulated glomerular pressure modulates mesangial cell 72 kDa metalloproteinase activity. *Connect. Tissue Res.* **33**, 257–263
- 36 Bian, J. and Sun, Y. (1997) Transcriptional activation by p53 of the human type IV collagenase (gelatinase A or matrix metalloproteinase 2) promoter. *Mol. Cell. Biol.* **17**, 6330–6338
- 37 Kawata, Y., Fujii, Z., Sakumura, T., Kitano, M., Suzuki, N. and Matsuzaki, M. (1998) High pressure conditions promote the proliferation of rat cultured mesangial cells in vitro. *Biochim. Biophys. Acta* **1401**, 195–202
- 38 Gatsios, P., Terstegen, L., Schliess, F., Haussinger, D., Kerr, I. M., Heinrich, P. C. and Graeve, L. (1998) Activation of the Janus kinase/signal transducer and activator of transcription pathway by osmotic shock. *J. Biol. Chem.* **273**, 22962–22968
- 39 Bode, J. G., Gatsios, P., Ludwig, S., Rapp, U. R., Haussinger, D., Heinrich, P. C. and Graeve, L. (1999) The mitogen-activated protein (MAP) kinase p38 and its upstream activator MAP kinase kinase 6 are involved in the activation of signal transducer and activator of transcription by hyperosmolarity. *J. Biol. Chem.* **274**, 30222–30227
- 40 Shinoki, N., Kawasaki, T., Minamino, N., Okahara, K., Ogawa, A., Ariyoshi, H., Sakon, M., Kambayashi, J., Kangawa, K. and Monden, M. (1998) Shear stress down-regulates gene transcription and production of adrenomedullin in human aortic endothelial cells. *J. Cell. Biochem.* **71**, 109–115
- 41 Resnick, N., Collins, T., Atkinson, W., Bonthron, D. T., Dewey, C. F. and Gimbrone, M. A. (1993) Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4591–4595
- 42 Saarialho-Kere, U.K., Welgus, H. G. and Parks, W. C. (1993) Distinct mechanisms regulate interstitial collagenase and 92-kDa gelatinase expression in human monocytic-like cells exposed to bacterial endotoxin. *J. Biol. Chem.* **268**, 17354–17361
- 43 Sadoshima, J. and Izumo, S. (1997) The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu. Rev. Physiol.* **59**, 551–571
- 44 Ingram, A. J., Ly, H., Thai, K., Kang, M. and Scholey, J. W. (1999) Activation of mesangial cell signaling cascades in response to mechanical strain. *Kidney Int.* **55**, 476–485
- 45 Khachigian, L. M., Resnick, N., Gimbrone, M. A. and Collins, T. (1995) Nuclear factor-kappa B interacts functionally with the platelet-derived growth factor B-chain shear-stress response element in vascular endothelial cells exposed to fluid shear stress. *J. Clin. Invest.* **96**, 1169–1175
- 46 Komuro, I., Kaida, T., Shibasaki, Y., Kurabayashi, M., Katoh, Y., Hoh, E., Takaku, F. and Yazaki, Y. (1990) Stretching cardiac myocytes stimulates protooncogene expression. *J. Biol. Chem.* **265**, 3595–3598
- 47 Sadoshima, J., Jahn, L., Takahashi, T., Kulik, T. J. and Izumo, S. (1992) Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. An in vitro model of load-induced cardiac hypertrophy. *J. Biol. Chem.* **267**, 10551–10560
- 48 Harendza, S., Lovett, D. H. and Stahl, R. A. (2000) The hematopoietic transcription factor PU.1 represses gelatinase A transcription in glomerular mesangial cells. *J. Biol. Chem.* **275**, 19552–19559

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