Background. Platelet-derived growth factors (PDGF)-AA and -CC mediate renal fibroblast proliferation and/or renal fibrosis. Whereas PDGF-CC binds to both the PDGF receptors (PDGFRs)-αα- and -αβ, PDGF-AA binds more selectively to the αα-receptor, suggesting potential differences in the biological activities.

Methods. We compared signal transduction, gene expression as well as changes in the proteome induced by PDGF-AA and -CC in rat renal fibroblasts, which express both PDGFR subunits. The growth factor concentrations used were chosen based on their equipotency in inducing rat renal fibroblast proliferation.

Results. Both PDGF-AA and PDGF-CC induced phosphorylation and activation of extracellular signal-regulated kinase 1 (ERK1) and ERK2. Renal fibroblast proliferation induced by either PDGF-AA or -CC could be blocked by signal
transduction inhibitors of the mitogen-activated protein kinase (MAPK)-, Janus-kinase (JAK)/signal transducers and activators of transcription (STAT) and phosphatidylinositol-3-kinase (PI3K) pathway, pointing to the involvement of all the three pathways. However, quantitative differences between both the stimulations were minor. Additive or synergistic effects by stimulating simultaneously with PDGF-AA and -CC were not observed. Using a proteomic approach we found eleven differentially expressed proteins, which were quantitatively altered after treatment with either PDGF-AA or PDGF-CC. The regulation of calreticulin and inorganic pyrophosphatase 1 could be verified by western blotting.

Conclusions. PDGF-AA and -CC exhibit almost identical biological effects on signal transduction and proteome in cultured renal fibroblasts, suggesting that the ligands exert their activity essentially through the commonly bound PDGFR-αα. Nonetheless, two differentially expressed proteins were identified which might be involved in the development of renal failure.

INTRODUCTION

The platelet-derived growth factor (PDGF) system comprises four isoforms (PDGF-A, -B, -C and -D) and two receptor chains [PDGF receptor (PDGFR)-α and -β chains] plays an important role in the development of fibrosis, wound healing, angiogenesis and malignancy [1–3]. PDGF-A and -B are secreted as homo- or heterodimers and bind to and induce PDGFR homo- and/or heterodimers. PDGF-C and -D are only released as homodimers. They contain an additional complement C1r/C1s-UEGF-Bmp1 (CUB) domain, which has to be proteolytically cleaved until the ligands can bind to their receptors [4–6]. Whereas PDGF-AA is a specific ligand for the αα-receptor, PDGF-CC binds to both the αα- and the αβ-receptors with high affinity. PDGF-DD is a specific ligand for the ββ-receptor and PDGF-BB is known to bind to all receptor types [2, 3]. Each PDGFR possesses tyrosine kinase activity and upon ligand binding, PDGFR dimerization and autophosphorylation provide a docking site for cytoplasmic downstream signalling molecules, predominantly those containing Src homology 2 (SH2) domains and phosphotyrosine binding domains [1–3, 7]. Further signalling includes molecules with SH3 domains, which recognize proline-rich regions, molecules with pleckstrin homology domains, which recognize membrane phospholipids and others [8]. Thus, PDGFRs engage in several well-characterized signalling pathways. For example, the rat sarcoma-mitogen-activated protein kinase (Ras-MAPK), phosphatidylinositol-3-kinase (PI3K), phospholipase C-γ (PLC-γ) and Janus-kinase/signal transducer and activator of transcription (JAK/STAT) pathways are activated [2]. PDGF-AB, PDGF-BB and PDGF-CC, but not PDGF-AA, are able to bind and activate the PDGFR-αβ heterodimer [9]. Since PDGFR-αβ shows autophosphorylation on tyrosine residues other than PDGFR-αα and -ββ [2], the biological effects of PDGF-AA and PDGF-CC might be similar but not identical. Preliminary studies demonstrated a potent mitogenic effect of both PDGF-AA and -CC on renal rat fibroblasts but with a 20-fold stronger effect of PDGF-CC [10].

By investigating signal transduction pathways and using a proteomic approach with equipotent proliferative concentrations of growth factors, we aimed to explore potential differences in signalling and protein expression concerning the response of renal fibroblasts upon stimulation with PDGF-AA and -CC.

MATERIALS AND METHODS

Reagents and cells

Active recombinant human PDGF-CC lacking the CUB domain was produced as described [6]. Human PDGF-AA and PDGF-BB were purchased from Sigma Aldrich (Deisenhofen, Germany). A cell line of rat renal fibroblasts (NRK-49F) was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The responsiveness of rat PDGFRs to human PDGF was demonstrated previously [10–12].

Cell culture

NRK-49F rat renal fibroblasts were cultured as previously described [10]. Incubation of 72-h serum-starved cells with PDGF-CC resulted in an ~20-fold higher proliferation compared with PDGF-AA [10]. In all of the following experiments, we therefore used 100 ng/mL PDGF-AA and 5 ng/mL PDGF-CC and regularly verified their equipotent proliferative effect. Controls included non-stimulated serum-starved cells and proliferating cells (DMEM containing 5% fetal calf serum, FCS; Invitrogen, Paisley, Scotland).

To study PDGF signalling pathways, pharmacological inhibitors were added 10 min prior to stimulation of NRK-49F with PDGF-AA or -CC for 24 h: U0126 [inhibits the ERK-activating kinases [MAPK/ERK kinase 1 (MEK1 and MEK2); final concentration 5 and 25 µM, Sigma Aldrich]], AG490 [inhibits the Janus-activated kinase 2 (JAK-2); final concentration 50 and 100 µM, Sigma Aldrich] and LY294002 [inhibits the phosphoinositide-3-kinase (PI3K); final concentration 5 µM, Sigma Aldrich] [13]. By trypan blue exclusion, we confirmed that the viability of NRK-49F cells was not affected by the inhibitor concentrations used. All experiments were performed in triplicate.

Extracellular signal-regulated kinase (ERK) 1/2 activity assay

The ERK 1/2 activity assay measuring phosphorylation of the ERK substrate GST-Elk1 (Santa Cruz Biotechnology, Santa Cruz) was performed as previously described [14].

Electrophoretic mobility shift assay (EMSA)

Following stimulation of NRK-49F with PDGF-AA and -CC, the cells were washed twice in ice-cold phosphate-buffered saline and harvested with a cell lifter. Nuclear extracts were isolated and probed with a double-stranded mutated sis-inducing element (SIE) oligonucleotide from the c-fos promoter (m675IE: 5′-GAT CCG GGA GGG ATT TAC GGG AAA TGC TG-3′) as described [15]. Nuclear extracts of interleukin-6 (IL-6)-stimulated NRK-49F and oncostatin M
(OSM)-stimulated melanoma cells A375 served as positive controls.

Fluorescence-activated cell sorting (FACS)

Subconfluent NRK-49F cells were harvested in a cell dissociation buffer (BD biosciences, Heidelberg, Germany) and washed in a fluorescence-activated cell sorting (FACS) buffer (0.5% BSA, 2 mM EDTA, 0.02% NaN3). Cell staining was performed with phycoerythrin (PE)-labelled PDGFR-α (CD140a–PE, 1:50, Miltenyi Biotech, #130–096–276) and PDGFR-β (CD140b–PE, 1:50 Miltenyi Biotech, #130–096–266) antibodies for 20 min at 4°C. For flow cytometry analysis (FACSCalibur; BD biosciences), the cell suspension was centrifuged (12,000 r.p.m., 5 min, 4°C) and the pellet resuspended in a 500 µL FACS buffer to a concentration of 5 × 10⁶ cells/mL. Data were analysed by the CellQuestPro analysis software.

Two-dimensional difference in gel electrophoresis (2D-DIGE) and mass spectrometry

Cell lysate. Serum-starved NRK-49F fibroblasts stimulated with PDGF-AA and PDGF-CC were washed two times in a 250 mM sterile sucrose solution (250 mM sucrose, 10 mM Tris, pH 8.5). Cells (10 × 75 cm² flasks) were harvested in a 350 µL lysis buffer [8 M urea, 4% CHAPS, 5 mM Tris, pH 8.5 and proteinase inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany)]. After incubation for 30 min at 4°C, ultrasound treatment (Bandelin Sonopuls HD 200, Bandelin electronic, Berlin, Germany) (3 × 10⁵) and centrifugation at 4°C for 20 min at 13,000 r.p.m. were performed. The 2D-cleanup kit (GE Healthcare Bio-Sciences, Buckinghamshire, UK) was used to decontaminate the cell lysate. The pellet was resuspended in 150 µL of DIGE lysis buffer (8 M urea, 5 mM Tris, 4% CHAPS, pH 8.5). Protein concentrations were determined using the 2-D Quant kit (GE Healthcare Bio-Sciences).

Protein labelling. Fifty micrograms of lysates of PDGF-AA-stimulated cells were labelled with Cy3 (200 pmol each), proteins of PDGF-CC-stimulated cells with Cy5 and a mixture of both samples as internal standard with Cy2 as described [16, 17] and processed according to the manufacturer’s instructions (GE Healthcare Bio-Sciences, Freiburg, Germany).

Gel electrophoresis. Isoelectric focussing was performed with the IPGphor™ system (GE Healthcare Bio-Sciences) [18] utilizing immobilized pH gradient (IPG) strips (Immobiline™ DryStrip, pH 3–10, 18 cm, GE Healthcare Bio-Sciences), as described [16]. The second dimension was carried out with sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) with a gradient of 9–18%. Gels were scanned using the Typhoon™ 9410 Imager (GE Healthcare Bio-Sciences) at the appropriate wavelength for Cy5 (649 nm), Cy3 (550 nm) and Cy2 (489 nm).

Analysis. Images were analysed using the Delta2D 4.0 software (Decodon, Greifswald, Germany). A setting of 1.5-fold protein expression change was chosen as differential threshold and a P < 0.05 was the criterion for proteins picked for further identification. In the case of normal distributions of spot quantities within replicated gels, Student’s t-test was applied. In the case of non-normally distributed spot quantities, the Wilcoxon–Mann–Whitney test was applied.

Protein digestion and mass spectrometry. Differentially expressed proteins were excised using a biopsy punch from a preparative (500 µg protein/sample) Sypro® Ruby-stained gel (Invitrogen, Carlsbad). The spots were in-gel digested with trypsin (Promega, Mannheim, Germany) and extracted from the gel as described [19]. For MALDI sample preparation, we applied the AnchorChip™ technology (Bruker Daltonics, Bremen, Germany) according to the manufacturer’s instructions using α-cyano-4-hydroxycynamic acid as MALDI matrix. For analysis of tryptic peptides, MALDI-TOF-MS was performed using the UltraFlex™ II (Bruker Daltonics) according to the manufacturer’s instructions. The spectra were acquired and calibrated as described [16]. The peptide mass fingerprint (PMF) spectra were processed using the FlexAnalysis™ software (Bruker Daltonik) and generated mass lists were subsequently sent to Mascot (http://www.matrixscince.com/search). The mass tolerance was set to 50 p.p.m. for internal calibration and 100 p.p.m. for external calibrated spectra. The following search parameters were used: variable modification due to methionine oxidation, one maximal missed cleavage site in the case of incomplete trypsin hydrolysis and no details about 2-DE-derived protein mass and pl. The searches were run against the rat protein sub-database of National Center for Biotechnology Information (NCBI). All proteins reaching a Mascot score of >65 were considered as identified.

Western blot analysis

NRK-49F were growth arrested and stimulated for 24 h with PDGF-AA (100 ng/mL) and PDGF-CC (5 ng/mL) as described above. Preparation of cell lysates and subsequent western blot analyses were performed as previously described [14]. The membranes were probed with cell rabbit polyclonal antibodies to phosphorylated MAP kinases ERK1/ERK2 (Millipore, Schwalbach, Germany), SM22 (transgelin) (Abcam, Cambridge, UK), 14-3-3 ε (Abcam), calregulin (Santa Cruz), pyrophosphatase 1 (Abcam), PDLIM 1 (Life-scan biosciences, Seattle), glucosidase II beta (affinity-purified, Acris, Herford, Germany) and with a goat polyclonal antibody to glutathione-S-transferase-α (Abcam). For normalization, we co-incubated the membranes with an anti-p44/42 MAPK (ERK1/2) rabbit antibody (Cell Signaling, Boston) or stripped the membranes (2 × 30 min incubation in 200 mM glycine, 0.1% SDS, 1% Tween 20, pH 2.2) prior to incubation with the normalization antibody. For the detection of PDGFR-α and -β, lysates from growth-arrested NRK-49F underwent western blot analysis with a polyclonal PDGFR-α antibody (R&D) or a polyclonal PDGFR-β antibody (Santa Cruz) as described above.
<table>
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Tgl2, transgelin 2 (SM22); YWhae/14-3-3 ε, tyrosine 3-monooxygenase/tryptophan 5–monooxygenase-activation protein epsilon (14-3-3 ε); Ppa 1, inorganic pyrophosphatase 1; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
RESULTS

Rat renal fibroblasts NRK-49F express both PDGFR chains

Expression of PDGFR-α and -β chains by non-stimulated NRK-49F cells was confirmed by both western blotting and FACS analysis (Figure 1A and B). The cells exhibited higher expression of the PDGFR-β-chain. PDGFR expression was also confirmed at the mRNA level (Figure 2D).

Effects of PDGF-AA and/or -CC on the expression of PDGF ligands and receptors in renal fibroblasts

Transcripts of PDGF-A, -B and -C but almost none of -D were detectable in unstimulated NRK-49F (Figure 2A–C). Following stimulation with PDGF-AA and/or -CC, we observed an increase of PDGF-A transcript expression peaking at 2 h, followed by a slight decrease after 24 h (Figure 2A). Effects of PDGF-AA, -CC and -AA/CC did not differ significantly (Figure 2A). A similar expression pattern was observed for PDGF-B transcripts (Figure 2B). PDGF-C transcript expression was not affected by PDGF-AA, PDGF-CC or their combination (Figure 2C). PDGF-D expression in stimulated cells remained too low for quantitative analysis (data not shown). Both PDGFR-α and -β chains were slightly, but non-significantly, downregulated following 24-h-stimulation with PDGF-AA, -CC or their combination (Figure 2D).

PDGF-AA and -CC induce similar signalling pathways

Western blot analysis for phosphorylated (i.e. activated) ERK1/2 and a functional ERK1/2 kinase assay demonstrated that PDGF-CC, like PDGF-AA, activates ERK1/2 within a similar time course (Figure 3A). PDGF-AA induced a somewhat stronger ERK1/2 activation after 2 min compared with PDGF-CC. Vice versa, kinase activity after 30 min of stimulation with PDGF-CC was higher. Upon combination of PDGF-AA and -CC no additive effects could be detected.

By EMSA we could not detect activated STAT1 and STAT3 in response to stimulation with either PDGF-CC or -AA or both, whereas both transcription factors increased in NRK-49F following stimulation with IL-6 (20 ng/mL) (Figure 3B).

To assess the induction of the MAPK, JAK/STAT and PI3K signalling pathways by PDGF-AA and -CC, NRK-49F cells were pre-incubated with U0126 (blocks MAPK-signalling), AG490 (blocks the JAK/STAT-pathway) and LY294002 (blocks PI3K-signalling pathways). Blockade of any of the three pathways blunted the PDGF-AA-, -CC- as well as -AA- plus -CC-induced proliferation, indicating that both PDGF isoforms use the same pathways to induce cell proliferation (Figure 4).

Identification of differentially regulated proteins by PDGF-AA and PDGF-CC in renal fibroblasts by 2D-DIGE

We next analysed the changes in the proteome of PDGF-AA- or PDGF-CC-stimulated renal fibroblasts using 2D-DIGE and subsequent mass spectrometry (Figure 5A–C and Table 2). Image analysis revealed 11 differentially expressed protein spots (P < 0.05, expression change >1.5-fold) (Figure 5B and C and Table 2).

Proteins upregulated by PDGF-CC versus PDGF-AA. A significantly higher expression by PDGF-CC-stimulated fibroblasts could be detected for transgelin 2, glutathione-S-transferase, the PDZ and LIM domain protein 1 (Elfin) and adenylyl kinase 1 (Table 2). However, no regulation of the first three proteins could be verified by western blot analyses (data not shown).

Proteins downregulated by PDGF-CC versus PDGF-AA. A significantly lower expression of PDGF-CC-stimulated fibroblasts was detected for seven proteins: myosin light chain 6, inorganic pyrophosphatase 1 (Ppa1), calumenin isoform A, tyrosine-3-monoxygenase/tryptophan-5-monoxygenase activation protein epsilon isoform (14-3-3 ε) and the theta isoform (14-3-3 θ), calreticulin and protein kinase C substrate 80K-H (glucosidase II beta) (Figure 4). Western blot analyses were performed for Ppa 1, 14-3-3 ε, calreticulin and protein kinase C substrate 80K-H (glucosidase II beta) to confirm the results. While we were able to reproduce the 2-D results for calreticulin and Ppa 1 (Figure 6), western blot analyses failed to confirm the differential regulation of 14-3-3 ε and protein kinase C substrate 80K-H (glucosidase II beta) (data not shown).

Transcript expression of calreticulin, transgelin 2, 14-3-3 ε and pyrophosphatase in renal fibroblasts in response to PDGF-AA and PDGF-CC

Given their pathophysiologic relevance for cell signalling processes, quantitative RT-PCR was performed for transgelin 2, calreticulin, 14-3-3 ε and pyrophosphatase (Ppa 1) (Figure 7A–D). There were no significant differences in mRNA expression for any of these transcripts upon stimulation of renal fibroblasts with PDGF-AA or PDGF-CC compared with non-stimulated cells (control). In contrast, 14-3-3 ε transcript expression in NRK-49F cells decreased significantly after stimulation of cells with either PDGF-AA or -CC.
However, the differences between both stimulations were not detectable.

**DISCUSSION**

PDGF-AA and PDGF-CC are potent mitogens for fibroblasts and high-affinity ligands of the PDGFR-αα homodimer, binding to this receptor with almost identical affinity [6]. PDGF-CC is additionally a ligand of the heterodimeric PDGFR-αβ [21, 22]. Distinct biological responses following PDGFR-αα and -αβ signalling have been reported, which may partly be explained by different signalling pathways of both the receptors [21, 23, 24]. We expected similar differences following stimulation of renal fibroblasts with PDGF-
FIGURE 3: ERK1/2 and STAT1/3 signalling in NRK-49F following PDGF-AA and/or -CC stimulation. (A) Activation of MAP kinases ERK1/2 was measured after stimulation of NRK-49F with PDGF-AA and -CC (n = 3) in a western blot specific for phosphorylated MAP kinases (upper panel) and in a MAP kinase assay (lower panel, n = 3) with the Ets-like transcription factor (Elk)-1 protein as a substrate. At equipotent proliferative concentrations, both PDGF-AA (100 ng/mL) and -CC (5 ng/mL) or their combinations (A/C) phosphorylate and activate the MAP kinases with a similar efficiency and kinetics. (B) STAT 1/3 activation was measured by electrophoretic mobility shift assay (EMSA) with nuclear extracts of NRK-49F after stimulation with PDGF-AA (100 ng/mL) and/or -CC (5 ng/mL) at the indicated time points (n = 3). Both the factors, either alone or in combination, did not activate STAT1/3. In general, NRK-49F are competent to signal via this pathway which is demonstrated by the EMSA for STAT1/3 following stimulation with IL-6 (20 ng/mL) and subsequent binding of STAT1/1 homodimers, STAT1/3 heterodimers and STAT3/3 homodimers. Another positive control was oncostatin M (OSM)-stimulated human melanoma cells A375 (0.5–5 µg/mL of nuclear extracts loaded).

FIGURE 4: PDGF-AA and -CC use the same intracellular pathways for mediating fibroblast proliferation. At 24 h following PDGF-AA and/or -CC stimulation, proliferation of NRK-49F was measured via BrdU incorporation after selective inhibition of the JAK/STAT, PI3K and the MAPK signal transduction pathways. ERK1/2 activation was inhibited by 5 µM and 25 µM U0126, the JAK/STAT pathway was blocked by 50 µM and 100 µM AG490 and signalling through PI3K was blocked by 5 µM LY294002. PDGF-AA- and -CC-induced proliferation of NRK-49F was inhibited by blocking each of the three signalling pathways. Data are means ± SD of four independent experiments. * indicates a P value of <0.05 of the PDGF-AA and inhibitor-treated group against the PDGF-AA alone treatment group. # indicates a P value of <0.05 of the PDGF-CC- and inhibitor-treated group against the PDGF-CC alone treatment group. FCS, fetal calf serum.
AA and/or PDGF-CC. Thus, the receptor-binding profile of PDGF-CC is expected to be at least partially similar to that of the PDGF-AB heterodimer. For instance, in mesangial cells it was demonstrated that PDGF-AB can potently induce an increase in DNA synthesis, activation of PLC and autoinduction of PDGF-A- and -B-chain mRNAs, whereas PDGF-AA lacks these effects [25]. Furthermore, PDGF-AB stimulated the chemotactic activity on vascular smooth muscle cells, whereas PDGF-AA had no stimulatory effect [26].

**FIGURE 5:** 2D-DIGE proteome pattern of NRK-49F lysates after stimulation with either PDGF-AA or PDGF-CC and identification of differentially expressed proteins. (A) Cy2-, Cy3- and Cy5-labelled lysates on a 2D-DIGE. Samples were first labelled (Cy3 = PDGF-AA treated, Cy5 = PDGF-CC treated and Cy 2 = internal standard, data of four independent experiments) and then separated in the first dimension by isoelectric focusing and subsequently in the second dimension by SDS-DIGE. Gel images were scanned using the Typhoon 9410 imager. Figure 6A shows an overlay of Cy3- and Cy5-fluorescence. The X-axis shows the pH value, and the Y-axis shows the molecular weight (MW). This allocation was done following identification of several known protein spots. (B) Depiction of the labelled lysates with localization of identified, differentially regulated proteins (arrows). (C) 2D-DIGE image, showing exemplarily the 14-3-3 ε protein spot from four independent experiments. All spots were analysed using the Delta2D 4.0 software (Decodon, Greifswald, Germany). Quantification in the case of 14-3-3 ε resulted in a 1.5-fold reduction of protein expression after PDGF-CC stimulation compared with PDGF-AA stimulation (see also Table 2, n = 4).
Here, we first demonstrated the expression of both PDGFR chains in NRK-49F with PDGFR-β being the dominant receptor chain, suggesting that renal fibroblasts are able to express all three dimeric PDGFR subtypes. This would be compatible with our prior observation that PDGF-CC via binding to both PDGFR-αα and -αβ is a more potent mitogenic stimulus for these cells than PDGF-AA with its higher receptor selectivity [10]. It is also noteworthy that in renal fibroblasts, PDGF-AA and -CC failed to exert a negative feedback on PDGF-α- or -β-chain mRNA expression. This contrasts with the effects of PDGF-BB and -DD in mesangial cells [13] and indicates that PDGF-AA and -CC might exert more prolonged effects in vivo.

We next investigated potential differences or additive effects in the signalling pathways of both growth factors in renal fibroblasts. Upon ligand binding, PDGFR-αα, -αβ or -ββ engage the MAPK, JAK/STAT, phosphoinositide-3-kinase (PI3K) and PLC pathways as the most prominent signal cascades [2, 13, 14, 27, 28]. In two approaches, we found a transient induction of MAPK activity in NRK-49F with no differences between PDGF-AA and -CC or any additive effects. At the growth factor concentrations used, we were unable to detect any STAT1/3 activation. However, proliferation assays using specific inhibitors of the MAPK-, JAK/STAT- and PI3K-pathway clearly demonstrated the involvement of all three pathways in driving PDGF-AA- and -CC-triggered renal fibroblast proliferation. In contrast to renal fibroblasts, PDGF-AA and -CC have been demonstrated to exert different mitogenic effects in glomerular mesangial cells. Thus, in mesangial cells, PDGF-AA activates the PDGFR-αα with a subsequent increase in PLC- and PI3K activity; however, these signals were not sufficient to induce mesangial cell proliferation or migration [27]. Indeed, whereas PDGF-AA is an effective mitogen for fibroblasts [29], it is at best a weak mitogen for mesangial cells [30]. In contrast, PDGF-CC was identified as a potent mitogen for mesangial cells in vitro [12]. Potential explanations for these differing observations in fibroblasts and mesangial cells may include a different expression of the PDGFR-α and β chains on the two cell types or differences in their autocrine amplification loops, e.g. PDGF-AA- and/or -CC-induced production of PDGF isomers.

In a non-hypothesis-driven proteomic approach, we finally searched for differentially expressed proteins following stimulation of renal fibroblasts with PDGF-AA and -CC and identified 11 proteins using DIGE and mass spectrometry. The differential expression of all these proteins was rather weak, mostly 1.5 to 1.8-fold, in PDGF-CC- versus PDGF-AA-stimulated fibroblasts (Table 2). We also investigated potential regulations on the transcript level, but did not detect any significant differences between PDGF-AA- and -CC-stimulated cells. Thus, not surprisingly, out of the above 11 proteins, only two could be verified independently by western blot analysis: inorganic pyrophosphatase 1 (Ppa 1) and calreticulin.
Ppa1 catalyses the hydrolysis of inorganic pyrophosphate PPi to inorganic orthophosphate Pi. It induces collagen I expression in osteoblasts and stimulates calcification [31]. Importantly, plasma pyrophosphate is negatively associated with vascular calcification in end-stage-renal disease [32], suggesting that a deregulated Ppa1 may have implications for renal function.

Calreticulin is an endoplasmic reticulum Ca2+-binding chaperone with functions in intracellular Ca2+ homeostasis, ...

**FIGURE 6:** Calreticulin and pyrophosphatase western blot analyses. To verify the differential regulation of proteins identified in the DIGE approach, western blots were performed. NRK-49F cells were again stimulated with either PDGF-AA or -CC in equiproliferative dosages and their lysates were applied to western blot analyses. The differential regulation of calreticulin and inorganic pyrophosphatase 1 (Ppa1) following either PDGF-AA or CC stimulation could be reproduced. The bands were quantified and the intensity normalized to ERK expression. Data are means ± SD of three independent experiments. * indicates a P value of <0.05 versus PDGF AA treated samples.

**FIGURE 7:** Transcript expression of transgelin 2, 14-3-3 ε, calreticulin and pyrophosphatase. Real-time RT-PCR-based expression data were normalized to GAPDH mRNA. Transcript expression of (A) transgelin 2 (Tagl2), (B) calreticulin, (C) 14-3-3 ε (YWhae) and (D) inorganic pyrophosphatase 1 (Ppa1) of unstimulated and PDGF-AA- or -CC-stimulated NRK-49F is shown. Data are means ± SD of four independent experiments. * indicates a P-value of <0.05 versus unstimulated control.
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None declared.

CONFLICT OF INTEREST STATEMENT

None declared.

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