

Parathyroid hormone-related protein induces hypertrophy in podocytes via TGF- β_1 and p27^{Kip1}: implications for diabetic nephropathy

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Abstract

Background. Hypertrophy of podocytes is characteristic in diabetic nephropathy (DN). Previously, we observed the upregulation of parathyroid hormone-related protein (PTHrP) and its receptor PTH1R, in experimental DN, associated with renal hypertrophy. Herein, we test the hypothesis that PTHrP participates in the mechanism of high glucose (HG)-induced podocyte hypertrophy.

Methods. On mouse podocytes, hypertrophy was assessed by protein content/cell and [H^3]leucine incorporation. Podocytes were stimulated with HG (25 mM), PTHrP(1–36) (100 nM), angiotensin II (AngII) (100 nM) or TGF- β_1 (5 ng/mL) in the presence or absence of PTHrP-neutralizing antibodies (α -PTHrP), the PTH1R antagonist JB4250 (10 μ M), PTHrP silencer RNA (siRNA) or TGF- β_1 siRNA. Protein expression was analysed by western blot and immunohistochemistry.

Results. HG-induced hypertrophy was abolished in the presence of either α -PTHrP or PTHrP siRNA. This effect was associated with an inhibition of the upregulation of TGF- β_1 and p27^{Kip1}. JB4250 also inhibited HG-induced p27^{Kip1} upregulation. Interestingly, whilst HG and AngII were unable to stimulate the expression of p27^{Kip1} on PTHrP siRNA-transfected podocytes, TGF- β_1 was still able to upregulate p27^{Kip1} in these cells. Moreover, HG and PTHrP-induced hypertrophy as well as p27^{Kip1} upregulation were abolished on TGF- β_1 siRNA-transfected podocytes. Furthermore, the glomeruli of transgenic PTHrP-overexpressing mice showed a constitutive overexpression of TGF- β_1 and p27^{Kip1} to a degree similar to that of diabetic animals.

Conclusions. PTHrP seems to participate in the hypertrophic signalling triggered by HG. In this condition, AngII induces the upregulation of PTHrP, which might induce the expression of TGF- β_1 and p27^{Kip1}. These findings provide new insights into the protective effects of AngII antagonists in DN, opening new paths for intervention.

Keywords: diabetic nephropathy; hypertrophy; parathyroid hormone-related protein; podocyte; PTH1R receptor

Introduction

Diabetic nephropathy (DN), the most common cause of end-stage renal disease in developed countries, has been recognized as a worldwide medical catastrophe [1]. DN is characterized by the development of proteinuria and subsequent glomerulosclerosis, conditions which are always preceded by the development of an early hypertrophic process in the glomerular compartment [2]. Hypertrophy of podocytes, associated with a decrease in their number per glomerulus, is known to occur in diabetes [3–5]. Over time, podocyte hypertrophy might become a maladaptive response leading to glomerulosclerosis.

Although the mechanisms by which high glucose (HG) leads to renal cell hypertrophy are still incompletely understood, it appears to involve cell entry into the cell cycle and subsequent arrest at the G1/S interphase followed by cell hypertrophy [6].

Recent studies have shown that HG-induced podocyte hypertrophy involves an early activation of angiotensin II (AngII), followed by an induction of TGF- β_1 , which in turn activates a cell cycle regulatory protein, the cyclin-dependent kinase inhibitor p27^{Kip1} [7–10].

Parathyroid hormone-related protein (PTHrP) is a widespread factor in normal tissues where it has autocrine/paracrine/intracrine actions [11]. Renal PTHrP and AngII are upregulated in several experimental nephropathies [12,13]. We previously observed an early activation of PTHrP and its receptor (PTH1R) associated with renal hypertrophy. Moreover, HG condition was found to increase PTHrP expression in cultured podocytes by a mechanism which involves AngII [14]. Since recent data suggest that PTHrP might exert a reciprocal control on or recapitulate some AngII effects in the

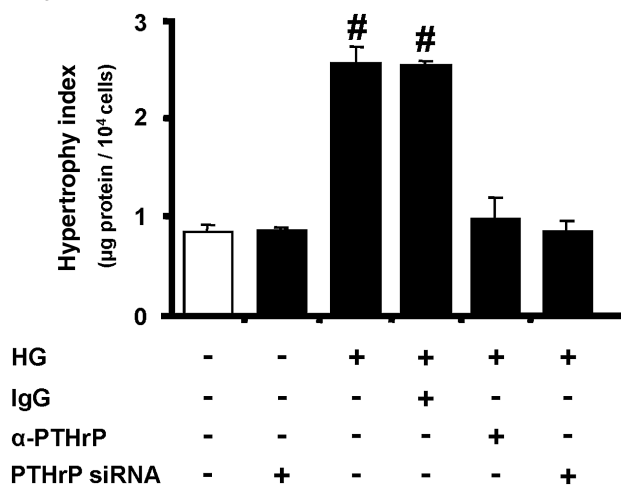


Fig. 1. PTHrP(1–36) is involved in HG-induced podocytes hypertrophy. Podocytes were cultured in control and HG concentrations for 72 h. In some experiments, podocytes were cultured in the presence of PTHrP-neutralizing antibodies (α -PTHrP), non-immune-specific rabbit IgG (as an internal control) or those transfected with PTHrP siRNA before the stimulation with HG. The hypertrophy index was determined as described in the Materials and methods section. Values are the mean \pm SEM from three independent experiments, [#] $P < 0.01$ vs all other values.

damaged kidney [15,16], herein, we test the hypothesis that PTHrP is involved in the mechanism of HG-induced podocyte hypertrophy.

To this end, we performed *in vitro* and *in vivo* studies. We found that PTHrP participates in the hypertrophic signalling processes triggered by HG on podocytes by a mechanism which involves both TGF- β_1 and p27^{Kip1}.

Materials and methods

Cell culture and siRNA transfection

Conditionally immortalized mouse podocytes were cultured as reported [14,17,18]. Podocytes were maintained in RPMI 1640 medium (Gibco-BRL, Eggenstein, Germany) supplemented with 10% FBS and antibiotics. To propagate podocytes, cells were cultured on type I collagen (Sigma, St. Louis, MO) at 33°C and the culture medium was supplemented with 10 U/ml recombinant interferon- γ to enhance T antigen expression. To induce differentiation, podocytes were maintained in DMEM (Gibco-BRL) supplemented with 5% FBS on Type I collagen at 37°C without interferon- γ . Differentiation of podocytes grown for 14 days at 37°C was confirmed by the identification of synaptopodin, a podocyte differentiation marker, by immunocytochemistry. Podocytes were grown to near confluency and serum-deprived for 24 h before experiments.

Podocytes were cultured in control (5 mM) or in HG (25 mM) concentrations. In some experiments, cells were grown in the presence or absence of PTHrP(1–36) (at 100 nM unless otherwise specified) as previously described [19,20], 100 nM Ang II (Sigma), 5 ng/mL TGF- β_1 (Sigma) and the specific N-terminal C₁₃ PTHrP-neutralizing antibody (α -PTHrP) (at 1:100 dilution) [12,19]. Moreover, predesigned mice PTHrP or TGF- β_1 RNA silencers (siRNA) (Ambion, Austin, TX) were transfected into podocytes using the Lipofectamine 2000 Reagent (Invitrogen; Groningen, The Netherlands). Briefly, 80 μ M of each siRNA was mixed with Lipofectamine in Opti-MEM I (Gibco-BRL) medium. Each siRNA-Lipofectamine mixture was added to the cells and incubated overnight at 37°C and 5% CO₂. After washing, podocytes were incubated with normal culture medium. The cells were harvested for protein extraction 72 h after transfection.

The pharmacological blockade of PTH1R was performed using the PTH1R antagonist JB4250, a generous gift from Robert Hull, Head of Pharmacology, James Black Foundation, London, UK.

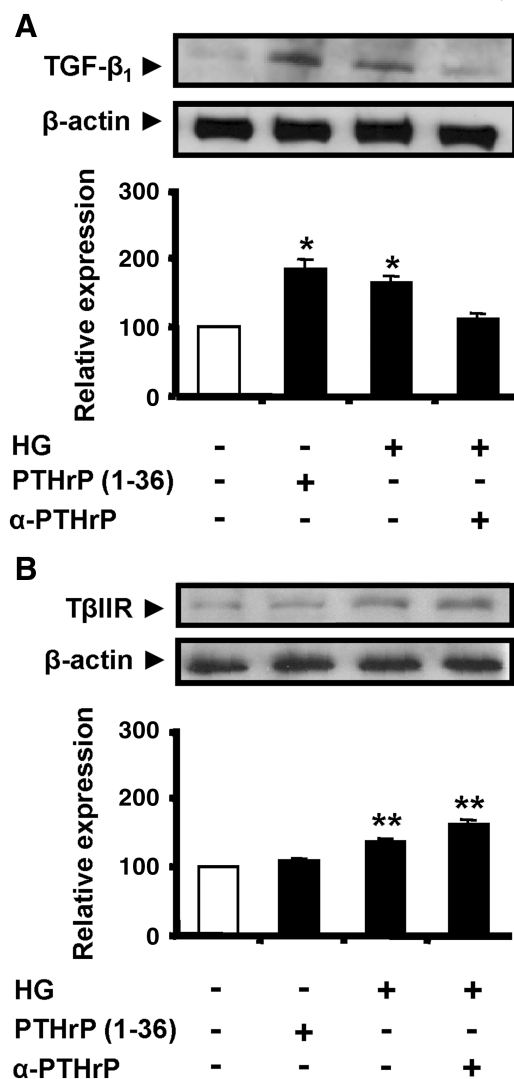


Fig. 2. PTHrP(1–36) mediates HG-induced expression of TGF- β_1 in mice podocytes. Podocytes were treated with 100 nM PTHrP(1–36) in the presence of control glucose concentrations, HG or HG in the presence of PTHrP-neutralizing antibodies (α -PTHrP) for 24 h. Cell extracts were prepared and equal volumes were analysed by western blot, using anti-TGF β_1 antibodies (A) or anti-T β IIIR antibodies (B). Densitometric analysis of TGF β_1 and its receptor T β IIIR were performed and normalized against β -actin protein expression as quantity loading control. Values are the mean \pm SEM from at least three independent experiments. * $P < 0.01$ vs all other values; ** $P < 0.05$ vs all other values.

[³H]leucine incorporation

Podocytes were pulsed with 2 μ Ci/ml [³H]leucine, washed with phosphate-buffered saline (PBS), solubilized with 0.1% sodium dodecyl sulphate (SDS) and transferred into a tube containing 10% bovine serum albumin (BSA). Precipitated proteins (20% TCA) were centrifuged at 2000 g for 30 min at 4°C, the supernatant was discarded and the pellet was resuspended in 0.5 N NaOH. Duplicate aliquots were removed and counted in a scintillation counter [21].

Cell hypertrophy index

Podocytes were trypsinized, washed with PBS and counted using a Neubauer haemocytometer. Equal numbers of cells were lysed in RIPA buffer (0.1% SDS, 0.5% sodium deoxycholate and 1.0% Nonidet P-40 in PBS) and the total protein content was determined by the Bradford's method

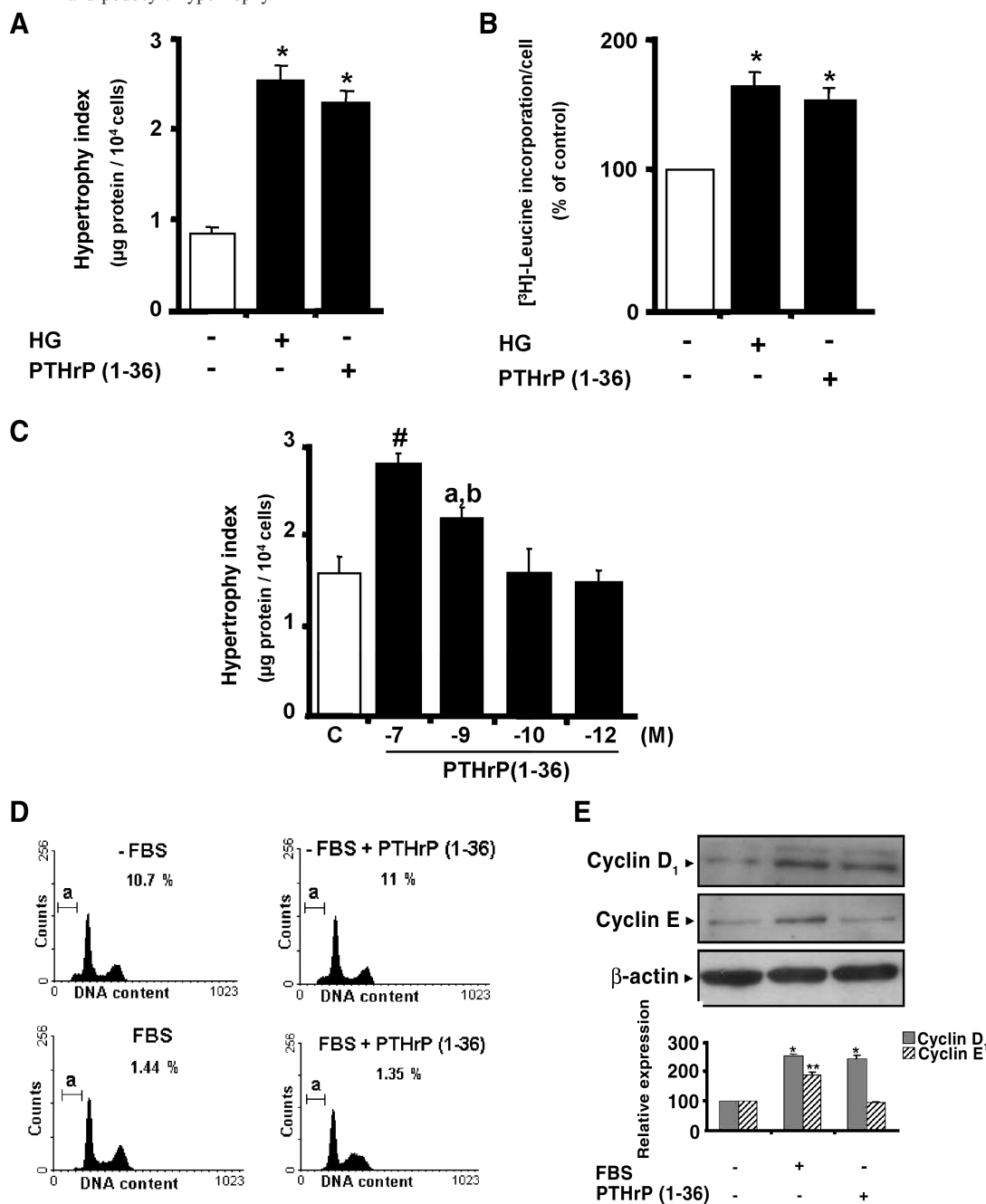


Fig. 3. PTHrP(1–36) has a direct effect on podocyte hypertrophy. (A and B) Podocytes were treated with PTHrP(1–36) at 100 nM in the presence of control glucose concentrations or HG for 72 h. (C) Podocytes were treated with several doses of PTHrP(1–36) (from 100 nM to 1 pM) for 72 h. (D) Podocytes were treated with PTHrP(1–36) at 100 nM in control glucose concentration in the presence or absence of 10% FBS. Cell viability was assessed by flow cytometry. The percentage of apoptosis (a) is indicated at the top. (E) Protein expression of cyclin D₁ and E was analysed by western blot, using specific antibodies. All densitometric analyses were performed and normalized against β-actin protein expression as quantity loading control. Values are the mean ± SEM from at least three independent experiments in triplicate, *P < 0.01 and **P < 0.05 vs control; ^aP < 0.05 vs PTHrP(1–36) at 100 nM values; ^bP < 0.05 vs control and PTHrP(1–36) at 100 or 1 pM values; #P < 0.01 vs control and PTHrP(1–36) at 100 or 1 pM values.

(Pierce, Rockford, IL), using BSA as standard [22]. Total protein was expressed as micrograms of protein per 10⁴ cells.

Cell viability by fluorescence-activated cell sorter

Pooled adherent and non-adherent cells were incubated in the dark for 1 h at 4°C in 60 µg/ml RNase A, 50 µg/mL propidium iodide and 0.05% Nonidet P-40 in PBS. FACScan analysis was then performed using the LYSIS II software. The percentage of hypodiploid cells,

corresponding to apoptotic cells, was calculated on the basis of the evaluation of 10 000 cells per experimental condition [23].

Immunostaining

Podocytes were fixed with 64% isopropanol/15% polyoxyethylene (Cell-fixx; Shandon, Pittsburgh, PA) and permeabilized with 0.1% Triton X-100. After blocking with 1.5% normal goat serum in PBS, the anti-p27^{Kip1} antibody (sc-528, Santa Cruz Biotechnology, Santa Cruz, CA)

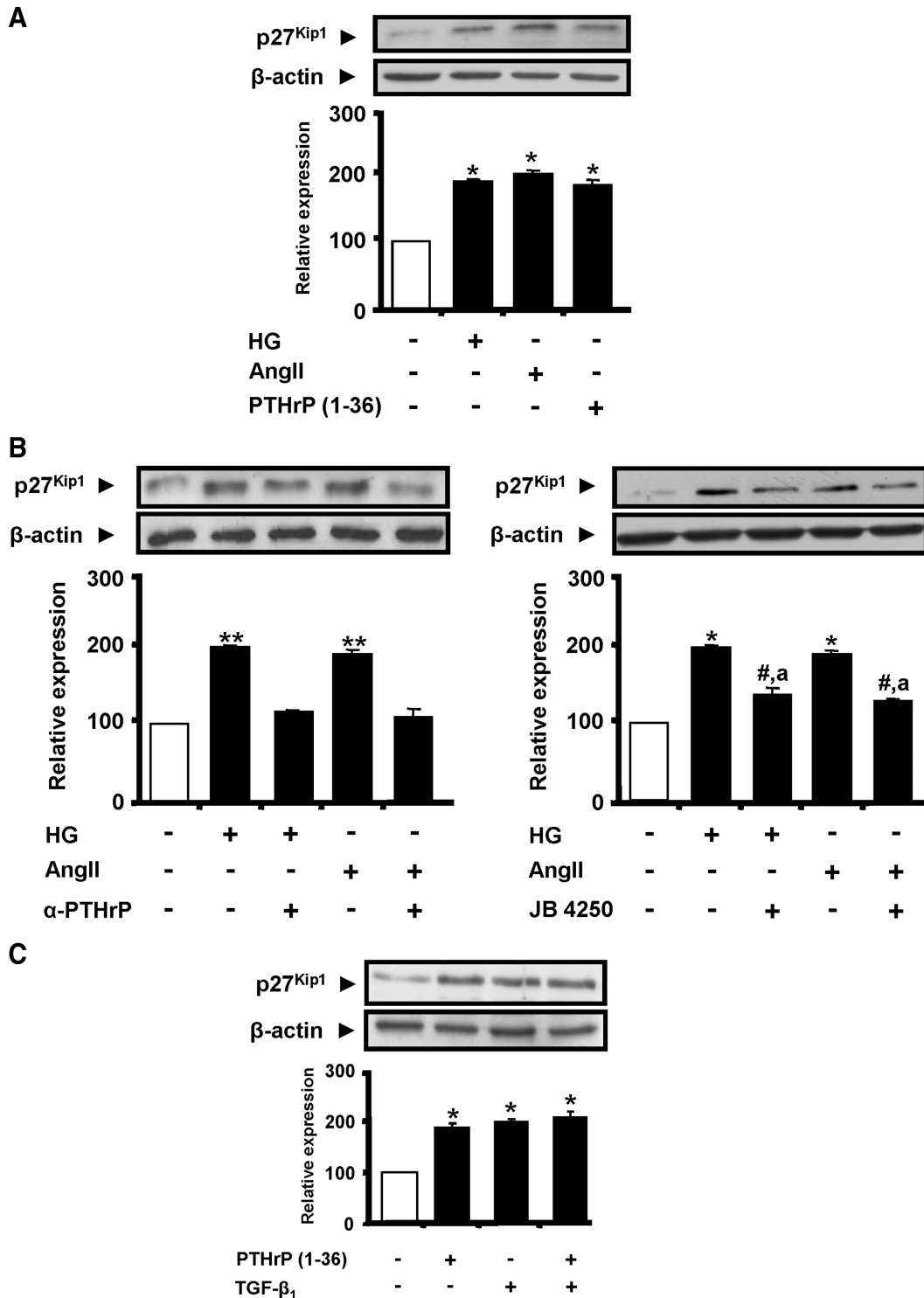


Fig. 4. Effect of several treatments on p27^{Kip1} protein expression. (A) 25 mM glucose (HG), 100 nM AngII or 100 nM PTHrP(1–36) were added on mouse podocytes for 24 h. (B) PTHrP-neutralizing antibodies (α -PTHrP) (left) or 100 nM PTH1R antagonist (JB4250) (right) were added on mouse podocytes 1 h before the stimulation with either HG or AngII for 24 h. (C) TGF- β 1 (5 ng/mL) and/or 100 nM PTHrP(1–36) were added to mouse podocytes during a period of 24 h. In all cases, protein loading was similar in each well, as assessed by β -actin as an internal control. * $P < 0.01$ or # $P < 0.05$ vs control values; ** $P < 0.01$ vs all other values; ^a $P < 0.05$ vs corresponding control values.

was added at 1:200 dilution. Then, fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (Sigma), at 1:200 dilution, was added. After washing, samples were counterstained with propidium iodide and mounted in Mowiol (Calbiochem). Immunofluorescence analysis was performed with a Leica DM-IRB confocal microscope.

Fixed renal tissue sections were dehydrated and embedded in paraffin. Immunohistochemistry was performed using rabbit polyclonal antibodies for p27^{Kip1} (previously mentioned) (1:500), TGF- β 1 (sc-146, Santa Cruz) (1:250), TGF- β RII (sc-220, Santa Cruz) (1:200) and WT-1 (NeoMarkers, Fremont, CA) (1:100) as a podocyte marker. To identify p27^{Kip1} expres-

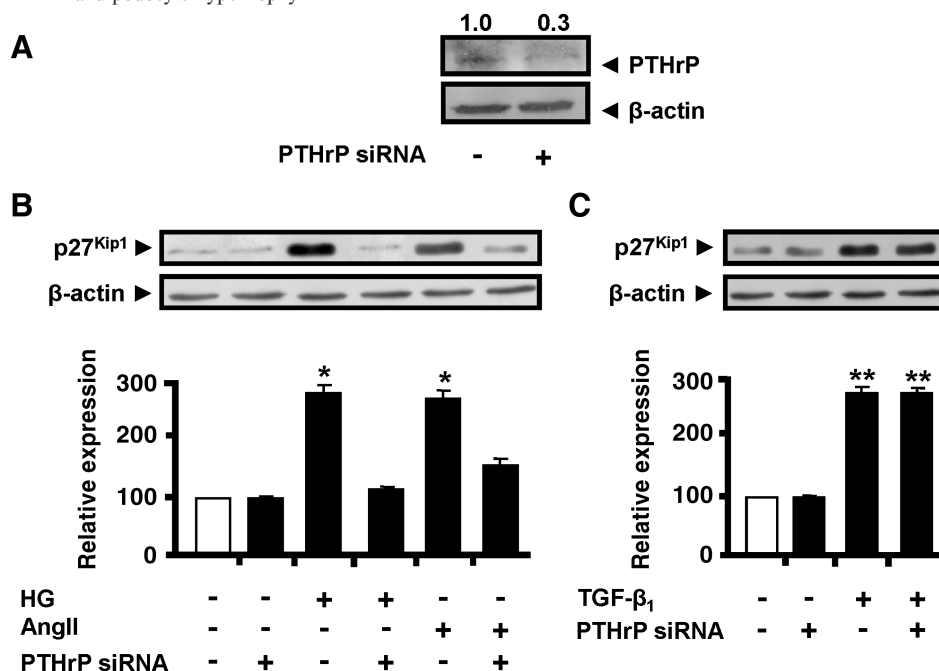


Fig. 5. The effect of PTHrP siRNA on the stimulation of p27^{Kip1} expression. p27^{Kip1} induction by 25 mM glucose (HG), 100 nM AngII and TGFβ₁ (5 ng/mL) on cultured mouse podocytes was assessed by western blot. (A) PTHrP protein expression on mouse podocytes transfected with PTHrP siRNA analysed by western blot. Relative intensities of PTHrP signal are indicated at the top. Mouse podocytes with and without PTHrP siRNA transfected were cultured in the presence of HG, AngII (B) or TGFβ₁ (C). The relative expression of p27^{Kip1} was normalized against β-actin protein expression. Data were obtained from at least three different experiments and are shown as the mean ± SEM. *P < 0.01 or **P < 0.05 vs all other values.

sion in glomerular podocytes, immunostaining in consecutive tissue sections for this protein, as well as for WT-1, was performed. As secondary antibodies, we used a biotinylated anti-rabbit antibody (p27^{Kip1}, TGF-β₁ and TGF-β RII) and FITC-conjugated anti-rabbit IgG antibody (WT-1). Some tissue samples were incubated without the primary antibody and served as negative controls.

The immunostaining was scored as follows: for p27^{Kip1}, the number of stained glomerular cells was counted per glomerulus in a total of 20 glomeruli per animal. TGF-β₁ and TGF-β RII were graded as 0, negative staining; 1, mild staining; 2, moderate staining; and 3, intense staining. The final score was always the mean of the two independent observers in a blinded fashion.

Western blot analysis

Kidney tissue or cell samples were homogenized in RIPA buffer [14,19,23]. After protein electrophoresis and transfer, membranes were blocked and incubated with the following rabbit polyclonal antibodies: anti-p27^{Kip1} (sc-528, Santa Cruz) (1:300); anti-p21^{Waf1/Cip1} (eBioscience, San Diego, CA) (1:500); anti-TGF-β₁ (sc-146, Santa Cruz) (1:500); anti-TβRII (sc-220, Santa Cruz) (1:500); anti-PTHrP antiserum (sc-9680, Santa Cruz) (1:500); anti-cyclin D₁ antibody (Neomarkers) (1:500); and anti-cyclin E antibody (Neomarkers) (1:500). β-Actin was used as constitutive control. Then, membranes were washed and incubated with peroxidase-conjugated goat anti-rabbit or donkey anti-goat (PTHrP) and developed by ECL (Amersham, Buckinghamshire). The corresponding fluorogram bands were quantified by densitometric scanning (ImageQuant, Molecular Dynamics; Sunnyvale, CA).

Experimental model of diabetes

CD1 mice and transgenic PTHrP-overexpressing mice were used. Targeted overexpression of PTHrP to the mouse renal proximal tubule has been previously reported [14,24]. Experimental protocols were approved by the Institutional Animal Care and Use Committees at the University of Alcalá.

Diabetes was induced by three consecutive daily intraperitoneal injections of streptozotocin (STZ) (Sigma), 65 mg/kg body weight in citrate buffer, pH 4.5 (vehicle) [14]. Mice with blood glucose >300 mg/dL were included in the study. Animals were sacrificed under ether anaesthesia 6 weeks following the development of diabetes (n = 6–7, at each time

period). Another group (n = 4–6) of weight-matched PTHrP-overexpressing mice or their corresponding normal littermates received the same volume of vehicle and were used as non-diabetic controls. One kidney of each animal was removed for subsequent total protein extraction. The remaining kidney of each animal was fixed in 4% buffered p-formaldehyde for immunohistochemical study.

Statistical analysis

All results are expressed as the mean ± SEM. Statistical significance (P < 0.05) was assessed by Kruskal–Wallis test or Mann–Whitney test, when appropriate.

Results

PTHrP as a mediator of HG-induced hypertrophy on podocytes: role of PTHrP on the regulation of the TGF-β system protein expression

We first studied the hypertrophy response to HG on podocytes treated with α-PTHrP [12], and then the same was done on podocytes transfected with a silencer RNA designed to inhibit PTHrP expression (PTHrP siRNA). We found that both techniques were able to inhibit the hypertrophy response to HG in cultured podocytes (Figure 1).

Then, we analysed whether PTHrP(1–36) could modulate the expression of the TGF-β₁ system in these cells. We observed that, whilst pre-incubation with α-PTHrP completely abolished HG-induced TGF-β₁ expression on podocytes (Figure 2A), no significant changes on the expression of the receptor TβIIIR occurred (Figure 2B).

Moreover, the addition of PTHrP(1–36) on cultured podocytes significantly increased the protein expres-

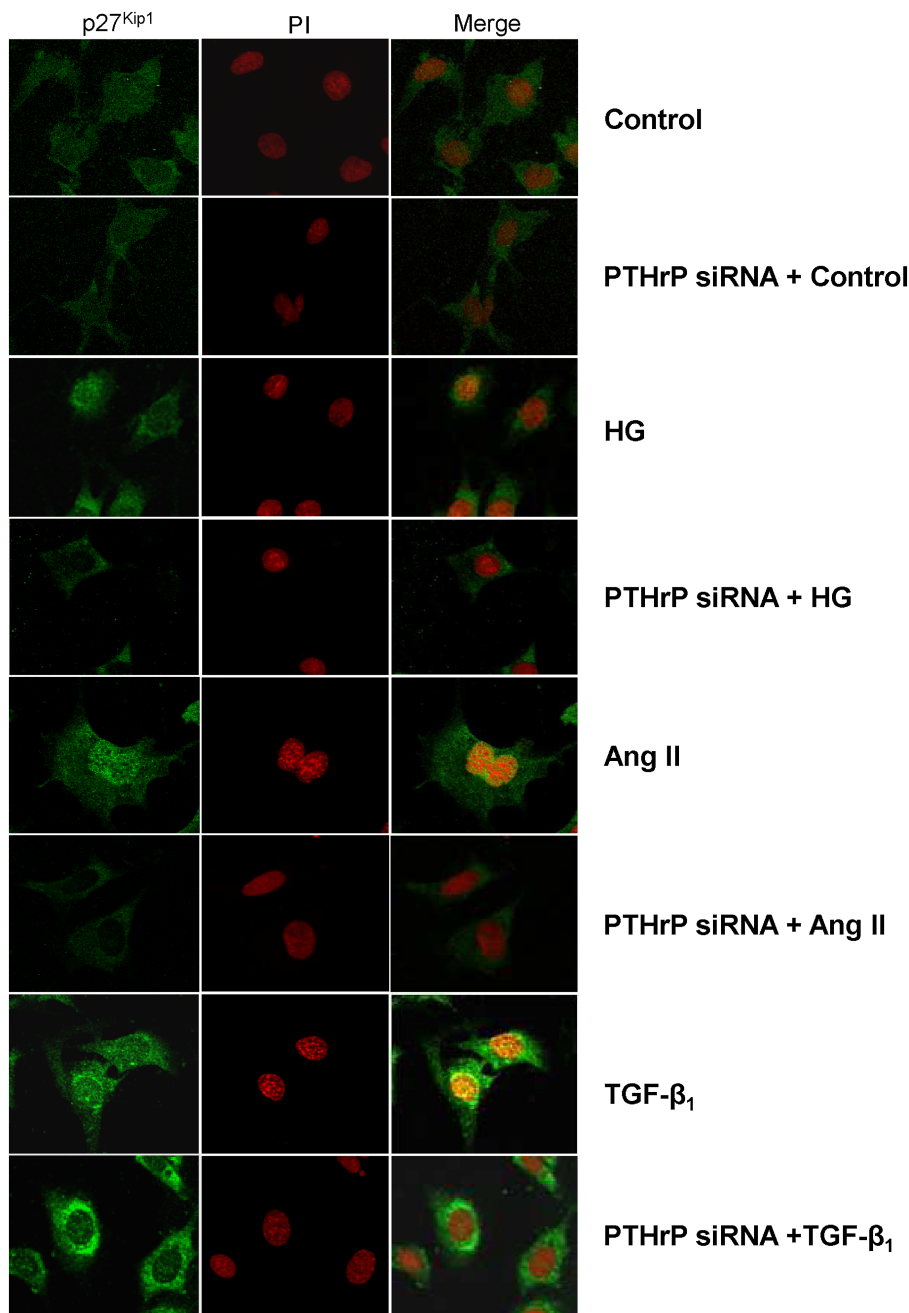


Fig. 6. Immunostaining with a fluorescently labelled IgG (green) for p27^{Kip1} detection. PTHrP siRNA-transfected and non-transfected mouse podocytes were grown on multi-well chambers and treated with 25 mM glucose (HG), 100 nM AngII or TGFβ₁ (5 ng/ml) for 24 h. Double immunofluorescence staining was assessed for the nucleus using propidium iodide (PI) (in red) and using a FITC-labelled IgG (in green) for p27^{Kip1} detection. The overlaid images in red and green (merge) showed an increase of p27^{Kip1} expression in the cytosol as well as in the nucleus (orange staining) of cells treated with HG, Ang II, TGF-β₁ and PTHrP siRNA + TGF-β₁. These effects were not observed in cells treated with PTHrP siRNA + HG or PTHrP siRNA + Ang II. This represents the results of three independent experiments.

sion of TGF-β₁ (Figure 2A), but not that of TβIIIR (Figure 2B), suggesting a possible direct effect of PTHrP on podocyte hypertrophy.

PTHrP induces hypertrophy on podocytes in culture

We studied the direct effect of PTHrP(1–36) on podocyte hypertrophy *in vitro*. To this end, we determined the hypertrophy index after the addition of PTHrP(1–

36) at 72 h. As shown in Figure 3A, PTHrP(1–36) at 100 nM induced hypertrophy of podocytes in a fashion similar to HG. To further confirm these results, podocyte hypertrophy was measured by the incorporation of [³H]leucine, a measure of protein synthesis. Exposure of podocytes to PTHrP(1–36) stimulated [³H]leucine incorporation (Figure 3B). Moreover, this peptide was able to induce podocyte hypertrophy in a dose-dependent manner (Figure 3C).

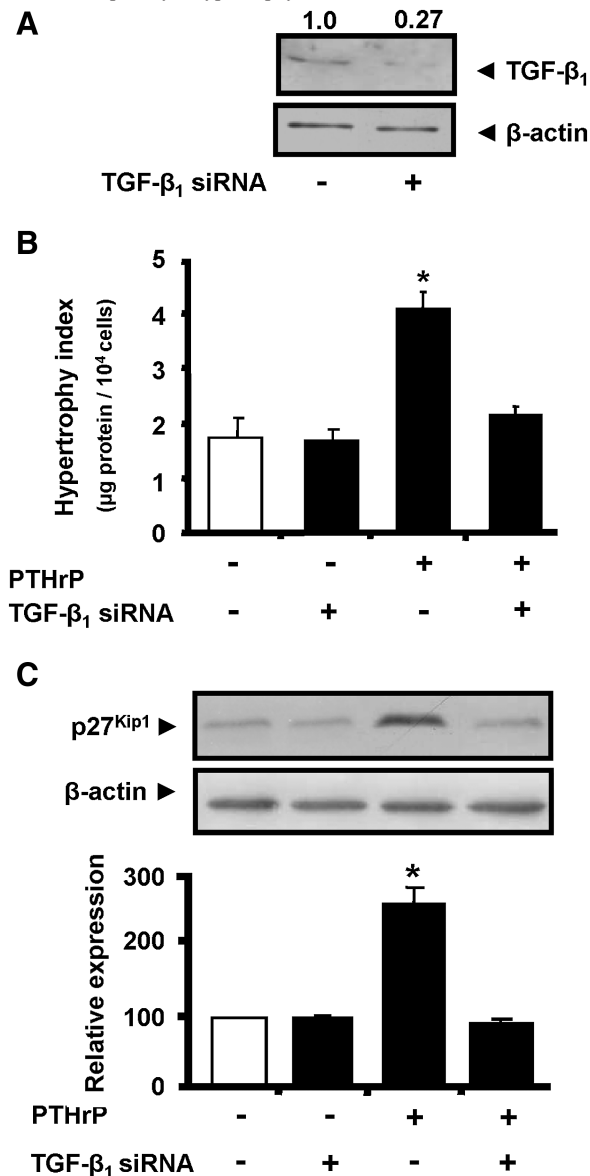


Fig. 7. TGF-β₁ mediates the upregulation of p27^{Kip1} induced by PTHrP (1–36). Cell hypertrophy and p27^{Kip1} induction by 100 nM PTHrP(1–36) on cultured mouse podocytes were assessed by hypertrophy index and western blot, respectively (**B** and **C**). (**A**) TGF-β₁ protein expression on mouse podocytes transfected with siRNA was analysed by western blot. Relative intensities of the TGF-β₁ signal are indicated at the top. Mouse podocytes with and without TGF-β₁ siRNA transfected were cultured in the presence of PTHrP(1–36). The relative expression of p27^{Kip1} was normalized against β-actin protein expression. Data were obtained from at least three different experiments and are shown as the mean ± SEM. *P < 0.01 vs all other values.

Due to the fact that DN is commonly associated with a decrease in podocyte number [3–5], we next studied whether PTHrP(1–36) could also affect podocyte viability. We observed that PTHrP(1–36) did not affect apoptosis on these cells (Figure 3D). We then studied the expression of several cell cycle regulatory proteins. We found that, whilst PTHrP(1–36) was able to stimulate cyclin D₁, promoting podocytes to enter into G₁, it also downregulates cyclin E, blocking the cell cycle later in G₁ (Figure 3E).

PTHrP as a modulator of the expression of the cyclin-dependent kinase inhibitor p27^{Kip1}: evidence of the pharmacological blockade of the PTH1R

We then investigated whether PTHrP was able to modulate the expression of two well-characterized CDKIs (p27^{Kip1} and p21^{Waf1/Cip1}) which mediate the hypertrophy response on several renal cell types [25,26].

We observed that PTHrP(1–36) stimulated the expression of the cell cycle-negative regulator p27^{Kip1} (Figure 4A), but it was unable to induce p21^{Waf1/Cip1} expression (data not shown). Moreover, the addition of α-PTHrP 1 h before the stimulation with HG or AngII prevented the upregulation of p27^{Kip1} protein expression (Figure 4B, left panel).

We also found that the PTH1R antagonist JB4250, at 100 nM, was able to abrogate the capabilities of both HG and AngII to stimulate the upregulation of p27^{Kip1} on podocytes (Figure 4B, right panel).

Furthermore, PTHrP(1–36) was as effective as TGF-β₁ in inducing p27^{Kip1} upregulation. Interestingly, no additive effect was observed when cells were stimulated with both PTHrP and TGF-β₁. These results raise the question that these two proteins might share a common mechanism for the stimulation of p27^{Kip1} (Figure 4C).

The stimulatory effect of PTHrP(1–36) on the expression of p27^{Kip1} as well as in podocyte hypertrophy is mediated by TGF-β₁

On podocytes transfected with PTHrP siRNA (Figure 5A), we observed that, whilst both HG and AngII were unable to stimulate the expression of p27^{Kip1} protein (Figure 5B), TGF-β₁ was still able to upregulate p27^{Kip1} protein to values not different from those of the control cells (Figure 5C). The same result was obtained by p27^{Kip1} immunofluorescence in the above-mentioned conditions (Figure 6). These results suggest that TGF-β₁ might mediate the upregulation of p27^{Kip1} induced by PTHrP(1–36). To confirm this hypothesis, podocytes were transfected with TGF-β₁ siRNA and then stimulated with PTHrP(1–36) (Figure 7). In this condition, PTHrP(1–36) was able to induce neither p27^{Kip1} upregulation nor podocyte hypertrophy (Figure 7B and C).

The expression of renal TGF-β₁ and p27^{Kip1} are constitutively upregulated in a transgenic mice strain characterized by renal PTHrP overexpression

Previously, we observed that PTHrP transgenic mice, besides showing PTHrP overexpression in the proximal tubule, also display a remarkable immunostaining for PTHrP in the glomeruli (data not shown). Of note is the fact that the expressions of TGF-β₁ and p27^{Kip1} proteins are upregulated in the diabetic glomeruli [7]. To analyse the potential role of PTHrP on the whole animal, the renal expression of TGF-β₁, TβIR and p27^{Kip1} was analysed in transgenic PTHrP-overexpressing mice as well as in their control littermates with and without the induction of experimental diabetes.

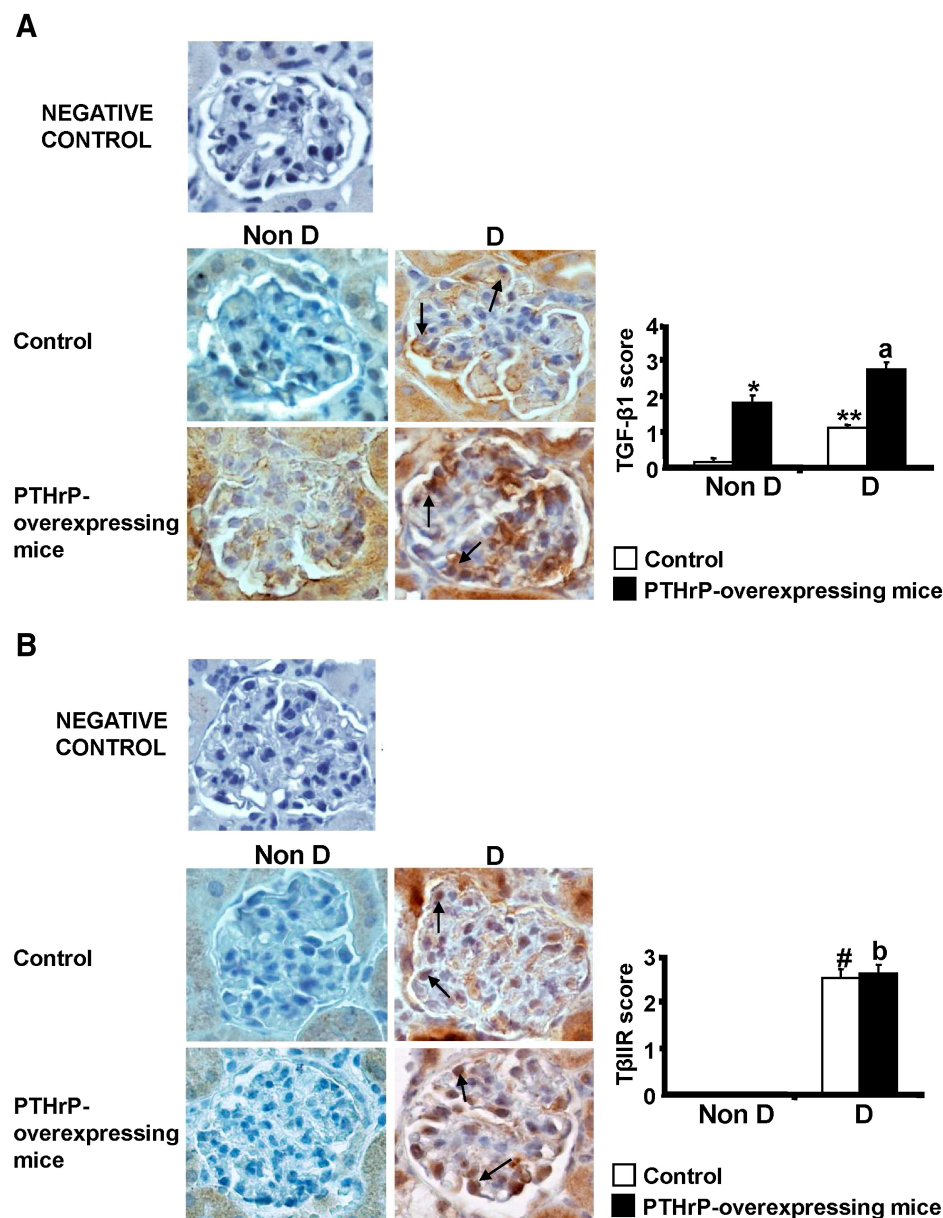


Fig. 8. Changes in the expression of both TGF- β_1 and its receptor T β IIR in transgenic PTHrP-overexpressing mice and in their control littermates with and without diabetes (6 weeks). Immunohistochemical staining for TGF- β_1 (**A**) and T β IIR (**B**) (original magnification, $\times 200$) in non-diabetic (Non D) and in diabetic mice (D) shows an increased staining of TGF- β_1 in the glomerulus of the PTHrP-overexpressing mice non-diabetic compared with their corresponding controls (**A**). Diabetic condition induced a significant increase of TGF- β_1 in both types of animals. This increase was higher in PTHrP-overexpressing mice compared to control diabetic mice. T β IIR staining (**B**) shows increased glomerular T β IIR expression in diabetic mice compared to non-diabetic mice. Quantification of TGF- β_1 as well as T β IIR was performed as described in the Materials and methods section. Incubation without primary antibody anti-TGF- β_1 or anti-T β IIR (right panel in **A** or **B**, respectively) was included as negative control. Data are shown as the mean \pm SEM. * $P < 0.01$ or ** $\#P < 0.05$ vs non-diabetic control mice; ^{a,b} $P < 0.01$ vs non-diabetic PTHrP-overexpressing mice.

We first observed that glomerular cells, including podocytes, from control diabetic mice compared to control non-diabetic mice, had a significant increase in the expression of TGF- β_1 , T β IIR (Figure 8A and B) and p27^{Kip1} (Figure 9).

Interestingly, PTHrP-overexpressing mice display a constitutive upregulation of both TGF- β_1 (Figure 8A) and p27^{Kip1} (Figure 9). After the induction of diabetes, these mice showed a further increase in TGF- β_1 expression to higher values than those of their corresponding controls (Figure 8A), without changes in p27^{Kip1} expression

(Figure 9). Furthermore, diabetic PTHrP-overexpressing mice showed an increase in the expression of the T β IIR which was not statistically different from those of their corresponding controls (Figure 8B).

Discussion

PTHrP upregulation has been demonstrated in several experimental nephropathies such as nephrotoxic and ischaemic

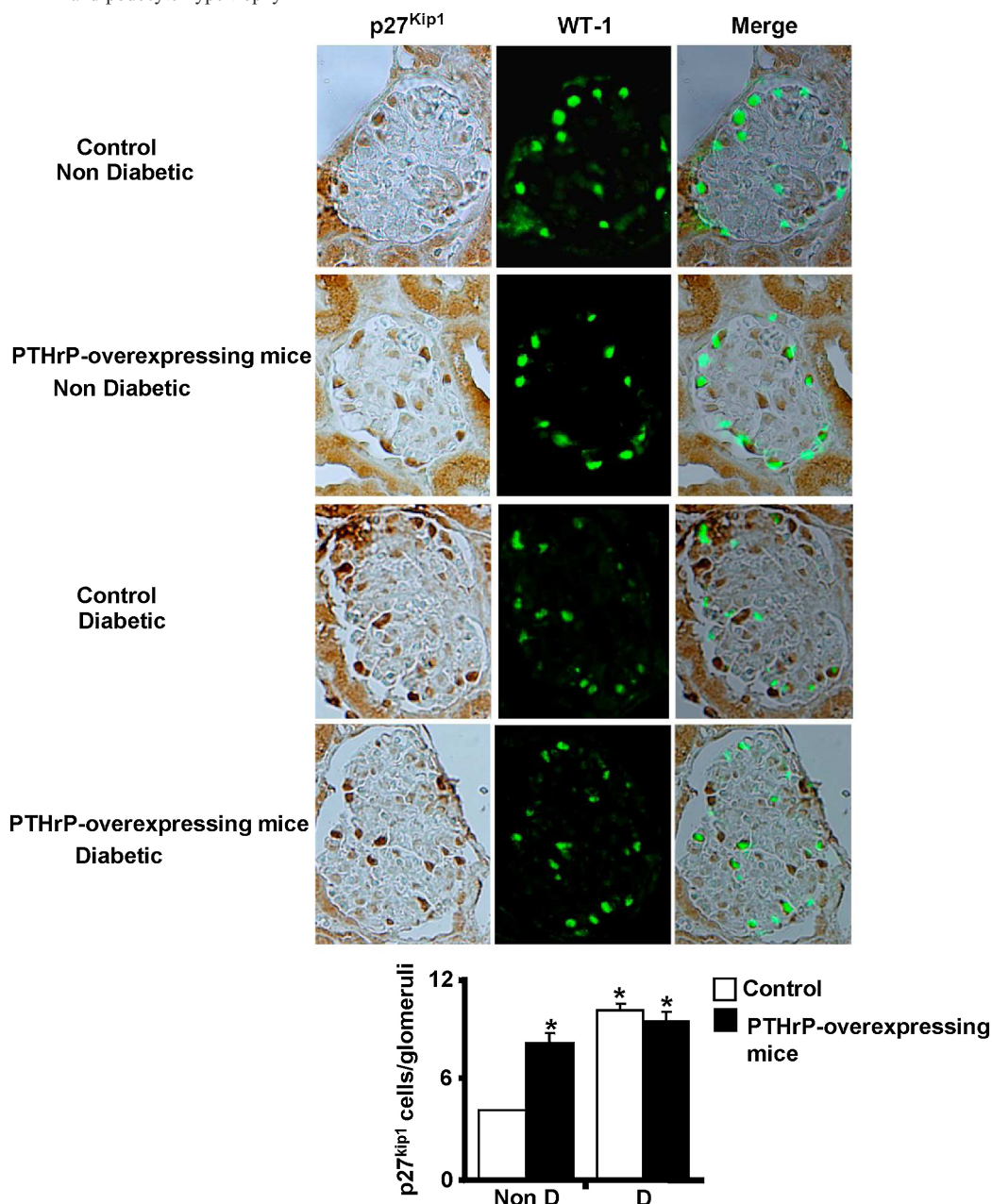


Fig. 9. Changes in the expression of p27^{Kip1} protein in the kidney of transgenic PTHrP-overexpressing mice and in their control littermates with and without the induction of experimental diabetes (6 weeks). A significant increase of p27^{Kip1} was observed with the induction of diabetes in the control mice. Interestingly, these increased levels of p27^{Kip1} were observed not only in the diabetic PTHrP-overexpressing mice, but also in non-diabetic PTHrP-overexpressing mice. To identify p27^{Kip1}-stained podocytes, an immunostaining in consecutive tissue sections for that protein, as well as for WT-1 (as a podocyte marker), was performed in all mice. The podocyte contribution to this increase of p27^{Kip1} was confirmed by overlaying images (merge). Original magnification, ×200. Data are shown as the mean ± SEM. *P < 0.01 vs non-diabetic control mice.

mic acute renal failure [12,16,27] and, more recently, DN in rodents [14]. However, to our knowledge, this is the first study supporting a role for PTHrP on the mechanisms of HG-induced podocyte hypertrophy (Figure 10).

It is well documented that HG, in the absence of other exogenous growth factors, is able to induce hypertrophy in mesangial cells as well as in podocytes [2]. More recently, Xu *et al.* [7] found that p27^{Kip1} mRNA and proteins were increased in podocytes exposed to HG media and in STZ-induced diabetic glomeruli. Interestingly,

these authors also found that this increment in p27^{Kip1} was abrogated by AngII receptor blocker treatment [7]. These findings clearly suggest that the blockade of the AngII actions promotes the attenuation of podocyte and glomerular hypertrophy.

In this regard, it is interesting to mention that, in a previous study, we observed that the renal PTHrP system was upregulated in STZ-induced diabetes by a mechanism which involved AngII [14]. Furthermore, these results supported the notion that PTHrP also played a role in the

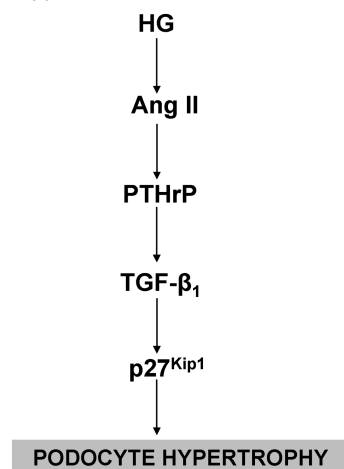


Fig. 10. Summary figure describing the suggested main mechanism by which PTHrP induces podocyte hypertrophy.

mechanism of renal hypertrophy in diabetes [14]. Thus, in the present study, we evaluate the role of PTHrP on HG-induced hypertrophy on podocytes. For this purpose, we analysed the hypertrophic response of HG on podocytes where the action or the expression of PTHrP was previously abolished. We first observed that HG-induced podocyte hypertrophy was prevented in cells pre-incubated with PTHrP-neutralizing antibodies. To rule out the possibility that this result might be unspecific, we also used a specific PTHrP mRNA silencer that was able to inhibit more than 70% of the PTHrP protein expression, in accordance with the manufacturer's target goal. This condition was also effective in preventing HG-induced podocyte hypertrophy. Taken together, these results show that PTHrP might participate in the hypertrophy signalling triggered by HG.

In order to analyse the mechanism by which PTHrP is able to induce podocyte hypertrophy, we studied the potential effect of PTHrP on the expression of TGF- β_1 and its receptor T β IIIR. We also observed that pre-incubation with PTHrP-neutralizing antibodies completely abolished HG-induced TGF- β_1 expression. These data suggest that PTHrP might be considered as a new factor capable of stimulating TGF- β_1 expression.

We then studied the direct effect of PTHrP(1–36) on podocyte hypertrophy *in vitro*. We found that PTHrP(1–36) was able to induce podocyte hypertrophy in a fashion similar to HG. Moreover, this peptide was able to induce podocyte hypertrophy in a dose-dependent manner.

We further analysed the mechanism by which PTHrP(1–36) induces podocyte hypertrophy. First, we observed that PTHrP(1–36) did not affect podocyte apoptosis. Then, we studied the expression of several positive as well as negative cell cycle regulatory proteins. We found that, whilst PTHrP(1–36) was able to stimulate cyclin D₁ and thus promote podocytes to enter into G₁, it also downregulates cyclin E, hence blocking the cell cycle later in G₁. We then studied the negative cell cycle regulatory protein p27^{Kip1} which plays a key role in diabetic

cell hypertrophy by preventing the activation of cyclin E activity and arresting the cell cycle later in G₁ [6]. Herein, we observed that PTHrP(1–36) induces p27^{Kip1} upregulation to a degree similar to that of AngII and TGF- β_1 . Moreover, we found that the pharmacological blockade of PTH1R inhibited the p27^{Kip1} upregulation induced by both HG and AngII. Taken together, these data suggest that PTHrP might mediate the hypertrophic signalling acting in an autocrine/intracrine fashion through the PTH1R receptor.

To discern the mechanism involved in the stimulation of p27^{Kip1} induced by both PTHrP and TGF- β_1 , we performed two experimental approaches. First, we found that the use of a PTHrP siRNA inhibited the ability of HG and AngII to stimulate the upregulation of p27^{Kip1}, albeit it could not prevent the TGF- β_1 upregulation of this protein. Secondly, on TGF- β_1 siRNA transfected podocytes, PTHrP(1–36) failed to induce both p27^{Kip1} overexpression and hypertrophy. Collectively, these results suggest that TGF- β_1 mediates both p27^{Kip1} upregulation and the hypertrophy response induced by PTHrP in HG conditions.

We then decided to analyse the potential implication of PTHrP on the expression of TGF- β_1 , T β IIIR and p27^{Kip1} on glomerular cells *in vivo*. To this end, we studied a transgenic mice strain characterized by renal PTHrP overexpression [14,24]. Interestingly, we observed that the glomerular expression of both TGF- β_1 and p27^{Kip1} are constitutively upregulated in PTHrP-overexpressing mice. As previously described, the latter was not accompanied by renal hypertrophy [14].

This result seems plausible since the hypertrophic mechanism requires the entry into the cell cycle and subsequent arrest at the G₁/S interphase. Several studies have demonstrated that, in glomerular cells grown in ambient HG, initially, self-limited proliferation occurs due to the generation of HG-induced growth factors, followed by cell cycle arrest in G₁ due to the expression of factors that block the checkpoint G₁/S interphase and undergo cellular hypertrophy [6,30,31]. Of considerable interest is the fact that previous studies on PTHrP-overexpressing mice have revealed the constitutive upregulation of various proinflammatory mediators [19], including the vascular endothelial growth factor-1 [32] without evidence of kidney damage in the absence of renal insult. In any case, collectively, our data strongly suggest that PTHrP might participate in the upregulation of glomerular TGF- β_1 and p27^{Kip1}.

As previously observed, control diabetic animals showed a significant increase in the expression of p27^{Kip1}, TGF- β_1 and T β IIIR. Although both types of diabetic animals display a similar degree of T β IIIR expression, diabetic PTHrP-overexpressing mice showed a higher expression of TGF- β_1 which might explain the increase in the renal hypertrophy index described in these animals. Herein, though we could not detect a higher glomerular p27^{Kip1} expression in these animals, it is known that the upregulation of T β IIIR may contribute to the increased sensitivity of renal cells to the action of TGF- β_1 [29]. The relative contribution of these factors on the hypertrophic actions of PTHrP awaits further study.

Conclusion

In conclusion, these results suggest that PTHrP participates in the hypertrophic signalling triggered by HG on podocytes. In this condition, AngII induces the upregulation of PTHrP, which in turn might induce both TGF- β_1 and p27^{Kip1} expression and thereby promotes the hypertrophy of podocytes. These findings also provide insights into the renal protective effects of AngII antagonists in DN, opening new ways for intervention.

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Conflict of interest statement. None declared.

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