The parathyroid hormone-related protein system and diabetic nephropathy outcome in streptozotocin-induced diabetes

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The pathophysiology of the diabetic kidney (e.g., hypertrophy, increased urinary albumin excretion (UAE)) is still ill-defined. Parathyroid hormone-related protein (PTHrP) is overexpressed in several nephropathies, but its role remains unclear. We evaluated the effect of high glucose on PTHrP and the PTH1 receptor (PTH1R) protein (by Western blot and immunohistochemistry) in the kidney of mice with streptozotocin-induced diabetes, and in several mouse renal cells *in vitro*. Diabetic mice showed a significantly increased renal expression of PTHrP and PTH1R proteins within 2–8 weeks from the onset of diabetes. These animals exhibited an intense immunostaining for both proteins in the renal tubules and glomeruli. Using transgenic mice overexpressing PTHrP targeted to the renal proximal tubule, we found a significant increase in the renal hypertrophy index and in UAE in these diabetic mice relative to their control littermates. Moreover, logistic regression analysis showed a significant association between both PTHrP and PTH1R protein levels and UAE in all diabetic mice throughout the study. High-glucose (25 mM) medium was found to increase PTHrP and PTH1R in tubuloepithelial cells, mesangial cells, and podocytes *in vitro*. Moreover, this increase in PTHrP (but not that of PTH1R) was inhibited by the AT_1 receptor antagonist losartan. Collectively, these results indicate that the renal PTHrP/PTH1R system is upregulated in streptozotozin-induced diabetes in mice, and appears to adversely affect the outcome of diabetic renal disease. Our findings also suggest that angiotensin II might have a role in the PTHrP upregulation in this condition.

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In diabetes mellitus, nephropathy – the most common single cause of end-stage renal disease in developed countries – occurs related to a poor glycemic balance.[1] Hypertrophy of mesangial and proximal tubular cells is an early hallmark of diabetes renal involvement.[2–4] In fact, because the tubulointerstitium comprises the bulk of the kidney, proximal tubular hypertrophy is quantitatively responsible for the largest component of renal enlargement in this condition.[2,3] Recent functional studies in the streptozotocin (STZ)-diabetic model have shown that, in early diabetes, kidney growth causes a primary increase in proximal tubule reabsorption, which is responsible for diabetic renal hyperfiltration.[5,6] This hyperfiltration state may contribute to proteinuria, structural renal lesions, and the progression to chronic renal failure.[2,4] As a matter of fact, several observations indicate that the development of diabetic glomerulosclerosis and tubulointerstitial fibrosis is always preceded by renal hypertrophy.[2] Interactions among several cytokines and hemodynamic agents are thought to be responsible for the renal changes occurring in diabetes.[2,7,8] However, the true molecular mechanisms of diabetic nephropathy remain to be fully characterized.

In the adult kidney, both parathyroid hormone-related protein (PTHrP) and the PTH1 receptor (PTH1R) are abundant throughout the renal parenchyma, including the intrarenal vasculature.[9–11] In the kidney, PTHrP appears to modulate renal plasma flow and glomerular filtration rate, and induces proliferative effects on both glomerular mesangial and tubuloepithelial cells.[12–18]

Renal PTHrP is overexpressed in several experimental nephropathies, including a rat model of tubulointerstitial scarring after protein overload, associated with the development of proteinuria.[12,19] Although PTHrP upregulation appears to be a common event associated with renal injury and repair, its pathophysiological significance remains to be clarified.[12] Moreover, no study has yet explored the putative involvement of PTHrP in the pathophysiology of the diabetic kidney.

In the present study, we initially assessed the expression and localization of PTHrP and PTH1R in the kidney of

STZ-induced diabetic mice. We then explored the functional consequences of chronic PTHrP overexpression in the diabetic kidney using transgenic mouse strains overexpressing PTHrP in the renal proximal tubule.[20] Furthermore, the effect of high-glucose medium on the expression of PTHrP and PTH1R was analyzed in three mouse kidney cell lines: tubuloepithelial MCT cells, mesangial cells, and conditionally immortalized podocytes.

RESULTS

Renal functional parameters in diabetic mice

Diabetic mice exhibited hyperglycemia and developed renal hypertrophy and increased urinary albumin excretion (UAE), consistent with previous findings using a similar time schedule in this model[4,8,21,22] (Figure 1a and b). In these mice, total plasma protein levels showed a tendency to decrease (Figure 1c).

Expression and localization of PTHrP and PTH1R in the diabetic mouse kidney

A single band corresponding to an apparent molecular weight of 18 kDa was detected in the mouse kidney by PTHrP antiserum C6, consistent with our recent findings[20,23,24] (Figure 2, left). A similar band was observed in W256 tumor extracts using this C-terminal antiserum, in agreement with previous results using an N-terminal PTHrP antibody.[25] This molecular weight is similar to that reported for the predominant PTHrP form isolated from other human and animal tumors overexpressing this protein,[26–28] and it is likely to correspond to the single full-length PTHrP isoform described in the mouse.[9] Moreover, a significant increase in this 18-kDa band was observed in the mouse kidney 2 weeks after diabetes induction (Figure 3). Using immunoblotting with antiserum C6, an increase in a single protein band with a similar apparent molecular weight was also found in a rat model of acute renal failure.[24]

In the mouse kidney, PTH1R was detected with the antibody Ab-VII as a major 90-kDa band, consistent with our findings in the rat kidney[23,24] (Figure 2, right). This apparent molecular weight is consistent with that reported for the glycosylated receptor isolated from various tissues.[29] This antibody also revealed a minor band corresponding to 66 kDa in mouse kidney extracts (Figure 2, right), which might be the native nonglycosylated form of PTH1R.[29] As preliminary experiments showed that this minor band changed in accordance with the major band, only the results of densitometric analysis of the latter are shown below. Two weeks after diabetes induction, a significant increase in the PTH1R protein expression was found in the diabetic mouse kidney (Figure 4). Renal levels of PTHrP and the PTH1R protein peaked within 4–6 weeks of diabetes, decreasing

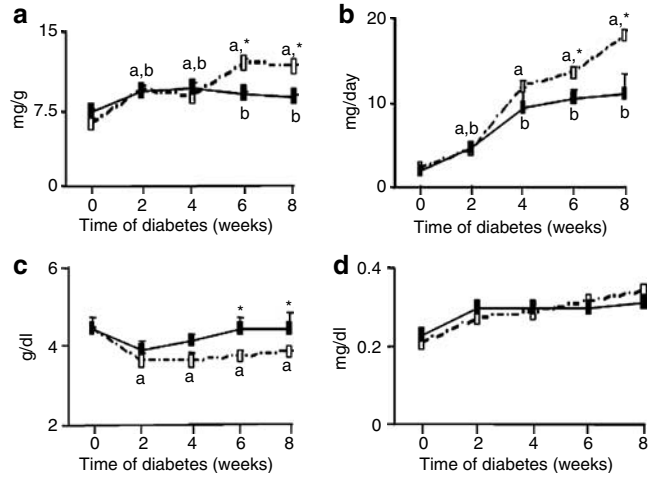

Figure 1 | Renal functional parameters in experimental diabetes. Index of renal hypertrophy (**a**), UAE (**b**), total plasma proteins (**c**), and plasma creatinine (**d**) were evaluated at different time periods after diabetes development in PTHrP-overexpressing mice (□) and their normal littermates (■). $^{a}P<0.05$, diabetic vs nondiabetic (at time '0') mice overexpressing PTHrP; $^{b}P<0.05$, diabetic vs nondiabetic normal littermates; $^{*}P<0.05$, diabetic PTHrP-overexpressing mice vs diabetic littermates.

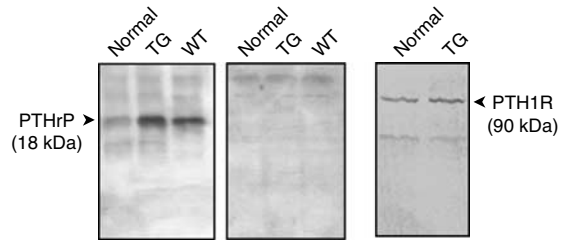

Figure 2 | Protein expression of PTHrP and PTH1R in the kidney of PTHrP-overexpressing mice and their normal littermates. Representative autoradiograms corresponding to Western blot analysis of both PTHrP (using antiserum C6) (left) and PTH1R (using antibody Ab-VII) (right) in the kidney of normal and PTHrP-overexpressing transgenic mice (TG) in basal conditions. A minor 66-kDa band was detected with the latter antibody. Walker 256 (W256) tumor protein extracts (WT) were used as a positive control for PTHrP. Antiserum C6 was replaced by preimmune serum, as a negative control for PTHrP (middle panel).

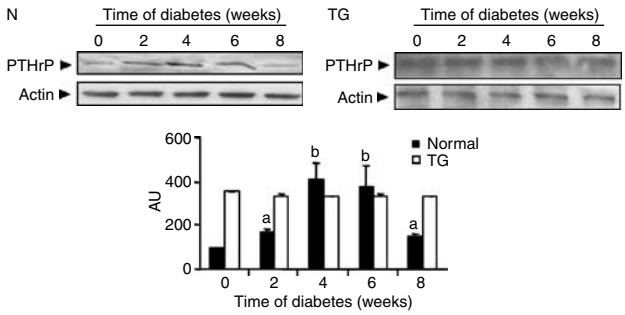

Figure 3 | Renal PTHrP protein expression in experimental diabetes. Total protein was isolated from the mouse kidney at different time periods after the development of diabetes. Autoradiograms and densitometric analysis corresponding to changes in PTHrP protein by Western blot (using antiserum C6) in PTHrP-overexpressing mice (□) and their normal littermates (■) are shown. PTHrP protein levels were corrected to those of actin. AU: arbitrary units. $^{a}P<0.05$; $^{b}P<0.01$, diabetic vs the corresponding nondiabetic controls. Renal PTHrP protein levels in PTHrP-overexpressing mice were significantly ($P<0.05$) higher than in their normal (nondiabetic) littermates and unchanged by diabetes for the time period studied.

thereafter, but remained over control values throughout the study. Moreover, PTHrP levels were two-fold higher in the renal cortex than in renal medulla in diabetic mice ($P<0.05$; $n=6$), but were similar in both cortex and medulla in nondiabetic mice (not shown).

In the kidney of nondiabetic animals, PTHrP immunostaining (using antiserum C6) was present in the tubular epithelium, whereas PTH1R staining was mainly observed in the basolateral membrane of tubular cells (Figure 5b and d). No positivity for either PTHrP or PTH1R was detected within the glomerulus in these mice. By contrast, 6 weeks after diabetes induction, the mouse kidney – showing a normal morphology – displayed an intense staining in most glomeruli for both PTHrP ($17\pm1/20$ glomeruli, $n=5$) and PTH1R ($15\pm1/20$ glomeruli, $n=5$) (Figure 5c and e). In addition, proximal and distal tubules (identified by morphological criteria) of diabetic mice showed a remarkable nuclear immunolocalization of PTHrP not observed in the nondiabetic animals (Figure 5b and c). Moreover, by using double staining, an intense PTHrP immunofluorescence was found in the tubular cell nucleus in the mouse diabetic, but not nondiabetic, kidney with either PTHrP antiserum used, C6 (not shown) or C13 (Figure 6). No nuclear staining was observed for PTH1R in these mice (Figure 6).

Outcome of diabetic nephropathy in PTHrP-overexpressing mice

To explore the functional consequences of PTHrP upregulation in the diabetic mouse kidney, we used transgenic mice overexpressing PTHrP in the renal proximal tubule.[20] In these animals, the high renal PTHrP protein levels were unchanged by diabetes (Figure 3). However, a diabetes-related increase in renal PTH1R protein occurred in these mice, although it was lower than that in their diabetic littermates (Figure 3). Diabetes in PTHrP-overexpressing mice was also associated with positive staining for both PTHrP and PTH1R in the glomerulus, and for PTHrP in the tubular cell nucleus (not shown).

After diabetes induction, PTHrP-overexpressing mice progressively developed a significant increase in the renal hypertrophy index and UAE, and had lower total plasma protein values, compared to their normal littermates (Figure 1a–c). No changes in plasma creatinine occurred in diabetic mice throughout the study (Figure 1d).

Using logistic regression analysis, renal PTHrP protein levels were highly associated with UAE values in all diabetic mice included in the study ($P<0.01$; $n=49$). Similarly, a significant association ($P<0.01$) was found between the renal PTH1R levels and UAE in these mice (Table 1).

The effect of high glucose on PTHrP and the PTH1R protein in mouse kidney cells

We next examined the effect of high glucose on the expression of PTHrP and PTH1R in several renal cell lines *in vitro*. Similar to findings in whole mouse kidney extracts, a single band corresponding to 18 kDa was detected by antiserum C6 in the three cell lines studied (Figure 7, left). In addition, a single 66-kDa band was observed with the antibody Ab-IV in these cell lines (Figure 7, right). Differences in the specificity between this antibody and antibody Ab-VII might account for their relative efficiency in detecting either the 66-kDa or the 90-kDa band as observed herein *in vitro* and *in vivo*, respectively.[20,23,24]

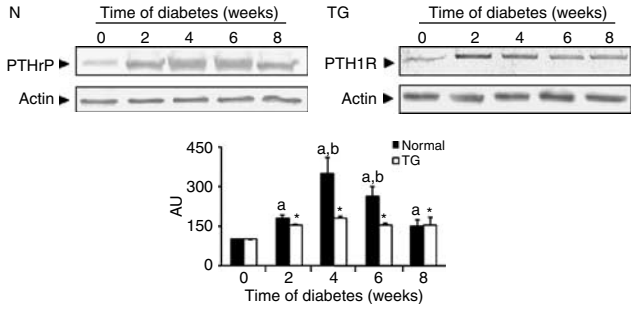

Figure 4 | Renal PTH1R protein expression in experimental diabetes. Total protein was isolated from the mouse kidney at different time periods after the development of diabetes. Autoradiograms and densitometric analysis corresponding to changes in the PTH1R protein by Western blot (using antibody Ab-VII) in PTHrP-overexpressing mice (□) and their normal littermates (■) are shown. The PTH1R protein levels were corrected to those of actin. AU: arbitrary units. $^{a}P<0.05$; $^{*}P<0.01$, diabetic vs the corresponding nondiabetic controls; $^{b}P<0.05$, diabetic PTHrP-overexpressing mice vs diabetic littermates.

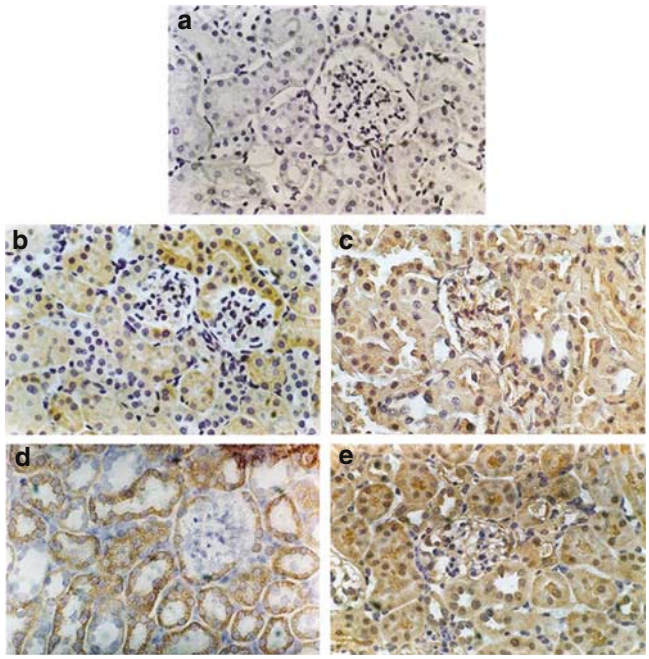

Figure 5 | Immunostaining for PTHrP and PTH1R in the kidney of diabetic mice at 6 weeks of diabetes. Kidney sections of representative nondiabetic (**b**, **d**) and diabetic (**c**, **e**) animals, showing immunolocalization of PTHrP (by using antiserum C6) (**b**, **c**) or PTH1R (by using antibody Ab-VII) (**d**, **e**) in the glomerulus of diabetic mice are shown. (**a**) Negative control without primary antibody. Original magnification $\times 250$.

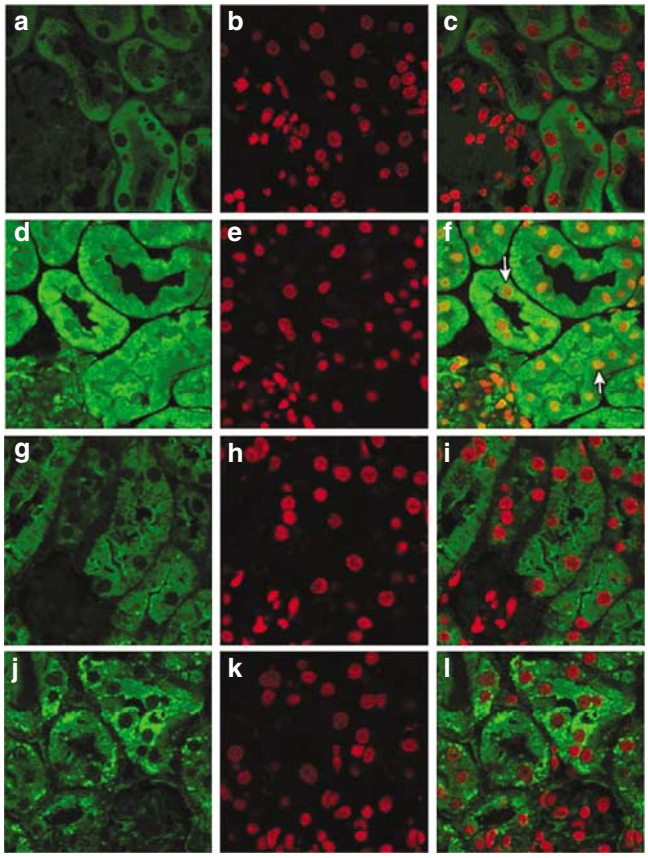

Figure 6 | PTHrP, but not PTH1R, localizes into the nucleus of the renal tubules in the mouse kidney at 6 weeks of diabetes. Kidney sections of representative nondiabetic (**a**, **g**) and diabetic (**d**, **j**) mice showing immunostaining for either PTHrP (using antiserum C13) (**a**, **d**) or PTH1R (using antibody Ab-IV) (**g**, **j**) are shown. Immunolocalization of both PTHrP and PTH1R was assessed by double staining, for the nucleus (with propidium iodide; red) (**b**, **e**, **h**, **k**), and a fluorescein isothiocyanate-labeled IgG (green) for either PTHrP (**a**, **d**) or PTH1R (**g**, **j**) detection. The overlaid images in red and green yielding an orange tone confirmed the presence of intense PTHrP fluorescence in the nucleus of tubular cells in the mouse diabetic kidney (**f**), but not in the nondiabetic mice (**c**). Similar results were obtained with antiserum C6 (data not shown). By using this maneuver, no nuclear staining was observed for PTH1R in either nondiabetic (**g**, **i**) or diabetic mice (**j**, **l**).

Table 1 | Association between proteinuria and the PTHrP/PTH1R system, and other biological parameters in diabetic mice

Variable	OR	CI (95%)
a. Univariate analysis		
PTH1R	5.8	1.8–17.8
PTHrP	6.2	2.2–17.3
Diuresis	4.1	1.4–12.1
Creatinine	3.4	1.2–9.4
Weight	1.1	0.4–3.2
Cholesterol	0.5	0.2–1.3
Glucose	2.2	0.8–5.3
b. Multivariate logistic regression analysis		
PTH1R	16.8	2.3–124.9
PTHrP	6.7	1.0–43.5
Diuresis	9.0	1.3–64
Constant	0.06	

CI, confidence interval; PTH1R, parathyroid hormone 1 receptor; PTHrP, parathyroid hormone-related protein; OR, odds ratio.

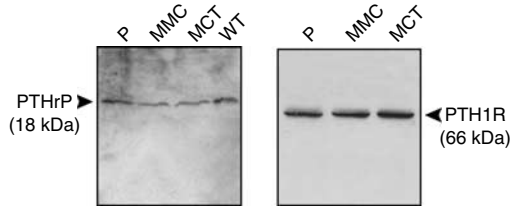

Figure 7 | Western blot analysis of PTHrP and PTH1R in mouse renal cell lines. Representative autoradiograms corresponding to Western blot analysis of both PTHrP (using antiserum C6) (left) and PTH1R (using antibody Ab-IV) (right) in mouse kidney cell lines in basal conditions: podocytes (P), mesangial cells (MMC), and cortical tubule cells (MCT). Walker 256 (W256) tumor protein extracts (WT) were used as a positive control for PTHrP.

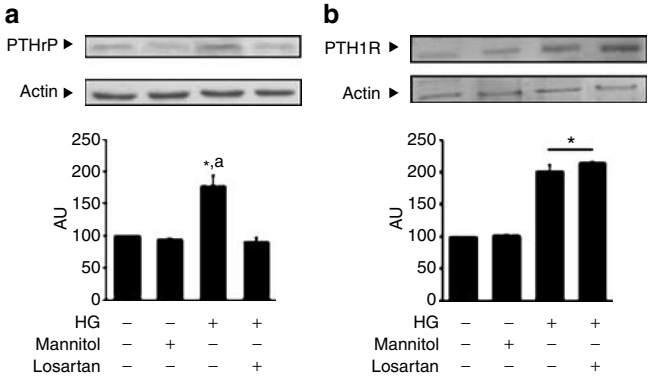

Figure 8 | Induction of PTHrP and the PTH1R protein expression by high glucose in MCT cells. Cells were cultured with or without high-glucose (25 mM) medium (or the same concentration of mannitol), in the presence or absence of 10 μM losartan, for 24 h. Autoradiograms and densitometric analysis corresponding to changes in PTHrP (**a**) and the PTH1R (**b**) protein levels by Western blot (using antiserum C6 and antibody Ab-IV, respectively) are shown. PTHrP and the PTH1R protein levels were corrected to those of actin ($n = 4$). HG: high glucose; AU: arbitrary units. *$P < 0.05$, compared to normal glucose medium; $^{a}P < 0.05$, compared to HG medium + losartan.

High glucose induced an upregulation of PTHrP and PTH1R protein at 24 h in MCT cells (Figure 8) and in both glomerular cell lines (Figures 9 and 10). Losartan abolished this PTHrP protein overexpression only in MCT cells and podocytes (Figures 8–10), whereas it failed to affect the PTH1R protein upregulation by high glucose in all cells studied (Figures 8–10).

DISCUSSION

Renal PTHrP and angiotensin-converting enzyme genes are upregulated in several experimental nephropathies.[12,16,18,19,23,24,30] Recently, we found that AT_1 receptor blockade in rats with acute renal failure was associated with improved tubular damage and renal function related to a lack of PTHrP upregulation.[23,24] In renal tubuloepithelial cells,

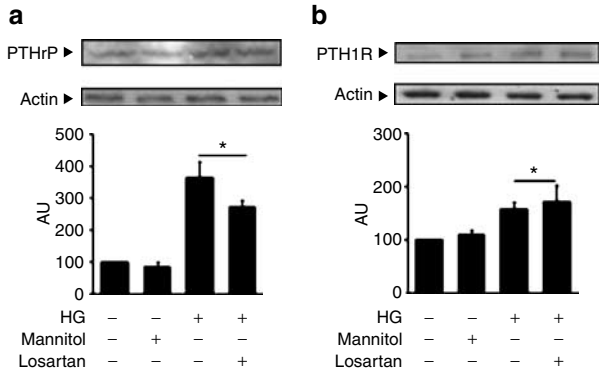

Figure 9 | Induction of PTHrP and the PTH1R expression by high glucose in mouse mesangial cells. Cells were cultured with or without high-glucose (25 mM) medium (or the same concentration of mannitol), in the presence or absence of 10 μM losartan, for 24 h. Autoradiograms and densitometric analysis corresponding to changes in PTHrP (**a**) and the PTH1R (**b**) protein levels by Western blot (using antiserum C6 and antibody Ab-IV, respectively) are shown. PTHrP and the PTH1R protein levels were corrected to those of actin ($n = 4$). HG: high glucose; AU: arbitrary units. *$P < 0.05$, compared to normal-glucose medium.

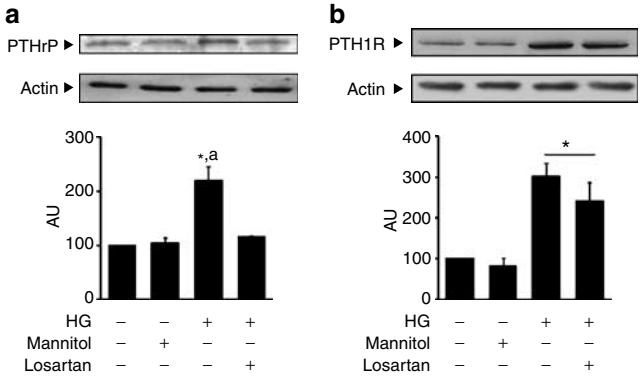

Figure 10 | Induction of PTHrP and the PTH1R expression by high glucose (25 mm) in mouse podocyte cells. Cells were cultured with or without high-glucose (25 mM) medium (or the same concentration of mannitol), in the presence or absence of 10 μM losartan, for 24 h. Autoradiograms and densitometric analysis corresponding to changes in PTHrP (**a**) and the PTH1R (**b**) protein levels by Western blot (using antiserum C6 and antibody Ab-IV, respectively) are shown. PTHrP and the PTH1R protein levels were corrected to those of actin ($n = 4$). HG: high glucose; AU: arbitrary units. *$P < 0.05$, compared to normal-glucose medium; $^{a}P < 0.05$, compared to HG medium + losartan.

angiotensin II (Ang II) interaction with AT_1 receptors activates mitogen-activated protein kinase and the transcription factor cAMP-responsive element binding protein, leading to increased PTHrP expression.[24] Thus, PTHrP appears to exert a reciprocal control on, or recapitulate, some Ang II effects in the damaged kidney.

We presently used the STZ model, which is considered as the 'work horse' for experimental studies in diabetic nephropathy,[31,32] a condition associated with angiotensin-converting enzyme and mitogen-activated protein kinase activation.[2,6] Consistent with previous findings in this model,[4,8,21,22] diabetic mice developed renal hypertrophy and an increase in UAE throughout this study. We found that both PTHrP and the PTH1R protein were upregulated in the diabetic mouse kidney. However, this increase in PTH1R was partly blunted in PTHrP-overexpressing mice, suggesting that homologous downregulation of this receptor, as observed in these mice after acute renal failure,[20] may counteract in part the PTH1R upregulation in the diabetic kidney.

An intense immunostaining for both PTHrP and PTH1R within the glomerular capillary tuft was found in the mouse kidney but only in diabetic mice. The presence of PTHrP has been previously disclosed in the glomerulus in frozen and paraffin-embedded renal sections in rats and rabbits.[10,16,19] Glomerular PTH1R mRNA has also been found by *in situ* hybridization in frozen sections from normal rat glomeruli.[33] Thus, the immunohistochemistry method used might require a high protein expression of PTHrP and PTH1R – as that found in diabetic animals – to be detected.

We found that diabetic mice, in contrast to nondiabetic animals, had a remarkable nuclear staining for PTHrP in the renal tubules. The mechanism of PTHrP internalization into the nucleus of tubular cells is currently unknown. The present findings, however, make it unlikely that such mechanism involves PTH1R, but rather the nuclear localization signal domain in the 88–106 region of the PTHrP molecule.[34,35] The antisera used in our immunolocalization studies, recognizing two different C- and N-terminal epitopes, seem to be similarly efficient in detecting full-length PTHrP (containing nuclear localization signal domain) in the nucleus. Consistent with the PTHrP/PTH1R system upregulation in experimental diabetic nephropathy, high glucose was found to stimulate this system in glomerular and tubuloepithelial cells *in vitro*.

Collectively, our present findings indicate that hyperglycemia should be added to the increasing list of renal pathophysiological conditions associated with an altered PTHrP/PTH1R system. These results also suggest that PTHrP could act in an autocrine/paracrine as well as an intracrine fashion in the diabetic mouse kidney.

In the present study, Ang II activation, which is known to occur in the diabetic kidney,[2,7] might have triggered the renal PTHrP/PTH1R system, as observed in acute renal failure.[24] In fact, losartan inhibited the high-glucose induction of PTHrP in both tubuloepithelial MCT and mouse glomerular cells. However, our findings also suggest that an AT_1-independent mechanism might be responsible for the PTH1R upregulation induced by high glucose in these cells. In this regard, Ang II infusion into normal rats increased PTHrP and the PTH1R immunostaining in the renal parenchyma, but this vasoactive agent failed to affect the PTH1R protein in rat mesangial and MCT cells *in vitro*.[23] Thus, definite conclusions about the putative role of Ang II or other unidentified hyperglycemia-related factors in the PTH1R expression in the mouse diabetic kidney cannot be made at present.

Our findings demonstrate that diabetic PTHrP-overexpressing mice, in comparison to their control littermates, have increased renal hypertrophy. Although the mechanisms

of diabetic renal hypertrophy are still ill-defined, Ang II and transforming growth factor-β have important roles in this condition.[2] Transforming growth factor-β is upregulated by high glucose and can transform a mitogenic stimulus into hypertrophy in tubuloepithelial cells.[36–39] Of note, PTHrP is a mitogen for proximal tubular cells, and has shown to act as a downstream effector of transforming growth factor-β at least in some cell systems.[17,40–42] As recently suggested by Clemens *et al.*,[9] PTHrP also appears to be an important regulatory factor of glomerular hemodynamics, and might participate in the pathophysiology of glomerular hyperfiltration. Collectively, these data strongly suggest that PTHrP could be involved in the mechanisms of renal hypertrophy in diabetes.

We also found that diabetic PTHrP-overexpressing mice have higher UAE and lower total plasma protein levels than their diabetic control littermates. Furthermore, there was a six-fold increase in the risk of developing proteinuria in those mice with the higher PTHrP and PTH1R levels, according to the logistic regression analysis. PTHrP is a potent vasorelaxant and can interact with vasoactive factors affecting glomerular hemodynamics and permeability.[9,10,13,14] These effects seem to occur partly through interaction with PTH1R and subsequent activation of the nitric oxide/cyclic guanosine monophosphate pathway.[10] In addition, PTHrP has proinflammatory/profibrogenic features in some cells.[43–45] Interestingly, an increase in nitric oxide and various proinflammatory cytokines appear to play critical roles in the diabetic kidney.[1,46,47]

It is thus tempting to speculate that any of these mechanisms would contribute to the relationship between the PTHrP/PTH1R system and proteinuria in this diabetic model. In any case, our data indicate that changes in the renal PTHrP/PTH1R system have predictive value on the early development of proteinuria in mice. Although the STZ model has limitations for assessing long-term histomorphological changes in the diabetic kidney,[31,32] our findings might have pathophysiological implications, as the amount of proteinuria is a reliable predictor of diabetic nephropathy.[48,49]

In conclusion, the present results demonstrate that the renal PTHrP/PTH1R system is upregulated during STZ-induced diabetes in mice, and this upregulation appears to affect adversely the outcome of diabetic renal disease. Our findings also suggest that Ang II might have a role in PTHrP upregulation in this setting.

MATERIALS AND METHODS

Experimental procedure

We used two types of mice, normal CD-1 mice and PTHrP-overexpressing mice, generously supplied by Professor AF Stewart and Dr A García-Ocaña (University of Pittsburg School of Medicine, Pittsburg, PA, USA).[20] The renal specificity of the transgene was conferred by the γ-glutamyl transpeptidase-l promoter, mainly expressed in the renal proximal tubule. PTHrP-overexpressing mice were generated by breeding two types of transgenic mice: one containing a γ-glutamyl transpeptidase-l promoter fragment upstream of a tetracycline transactivator fusion protein – which functions as a strong transcription activator; and the other with a PTHrP complementary DNA placed under the control of a tetracycline operator construct. Transgene-bearing founders were continuously outbred to normal CD-1 mice to generate hemizygotes. Genotyping of these mice was performed by tail DNA PCR, as described.[20] In all of the experiments described below, adult mice (4–8 months old) were used, and the results obtained with PTHrP-overexpressing transgenic mice were compared with those using their corresponding normal littermates.[20,50] All studies were performed with the approval of and in accordance with guidelines established by Institutional Animal Care and Use Committees at the University of Alcalá and Fundación Jiménez Díaz.

Mice were housed in a temperature-controlled room ($21 \pm 2^{\circ}$C) on a 14/10 h light/dark cycle under pathogen-free conditions and with free access to food and water. Diabetes was induced by three consecutive daily intraperitoneal injections of STZ (Sigma, St Louis, MO, USA), 65 mg/kg body weight in citrate buffer, pH 4.5 (vehicle). This is a previously reported model of early diabetic nephropathy characterized by renal hypertrophy and increased UAE during the first month of diabetes.[21,22] After the last STZ injection, induction of diabetes was confirmed by measurement of blood glucose levels. Forty-nine diabetic animals (25 PTHrP-overexpressing mice and 24 normal littermates), with blood glucose >300 mg/dl, were included in the study. Animals were killed under ether anesthesia 2, 4, 6, and 8 weeks following the development of diabetes ($n = 6$–7, at each time period). Another group ($n = 6$–10) of weight-matched PTHrP-overexpressing mice or their corresponding normal littermates received the same volume of vehicle and were used as nondiabetic controls.

Animals were individually housed in metabolic cages with free access to tap water, and 24-h urine was collected for protein measurement by the sulfosalicylic acid method, as previously reported.[51] Blood was taken by cardiac puncture under ether anesthesia, and plasma glucose was determined by the glucose oxidase technique.[52] One kidney of each animal was removed, weighed, frozen in liquid nitrogen, and stored at -80°C for subsequent total protein extraction. In some cases, the kidneys were dissected, the capsule removed, and the cortex – separated from the medulla – was frozen as described. The remaining kidney of each animal was fixed in 4% buffered p-formaldehyde for morphological and immunohistochemistry studies. The degree of renal hypertrophy was expressed as an index, the ratio of kidney weight to total body weight.

Histology and immunohistochemistry

Fixed renal tissue sections were dehydrated by graded ethanols and xylene, and then embedded in paraffin. Paraffin-embedded tissue sections (4 μm) were stained with hematoxylin. PTHrP immunostaining was performed using rabbit polyclonal anti-PTHrP antiserum C6, recognizing the highly conserved C-terminal epitope 107–111 in the intact PTHrP molecule,[17,53] as described.[23,24] For the PTH1R staining, we tested two well-characterized affinity-purified polyclonal antibodies to this receptor: one recognizing its extracellular domain (Ab-VII; Covance, Berkeley, CA, USA), which has been extensively used in previous studies;[18–20,23] and antibody Ab-IV (Covance), directed against the region 248–262 in the PTH1R molecule, contributing to receptor activation.[20,54] We found that both antibodies had similar sensitivity to detect this receptor in the mouse kidney, by immunohistochemistry. Some kidney samples were incubated without primary antibody or with nonimmune rabbit serum as negative controls.

The number of glomeruli showing capillary staining for both PTHrP and PTH1R per 20 glomeruli without selection was counted for each mouse.[18] Histological evaluations were performed by two independent observers in a blinded fashion. The final score was the mean of the two evaluations.

To assess putative changes in the localization pattern of PTHrP and PTH1R in the diabetic mouse kidney, renal tissue samples from diabetic and nondiabetic mice were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 30 min at 37°C. After blocking with 1.5% normal goat serum, the samples were incubated overnight at 4°C with the following primary antibodies: PTHrP antiserum C6 and PTHrP antiserum C13 recognizing an N-terminal epitope in the intact PTHrP molecule and also the PTHrP (1–36) fragment,[53] each at 1:100 dilution; and the PTH1R antibody Ab-IV, at 1:250 dilution. Then, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma), at 1:200 dilution, was added for 30 min. After washing, samples were counterstained with propidium iodide to detect cell nuclei, and mounted with Mowiol (Calbiochem, San Diego, CA, USA). Immunofluorescence analysis was performed with a Leica DM-IRB confocal microscope.[24]

Cell cultures

We used three well-established mouse kidney cell lines – cortical tubule MCT cells, mesangial cells, and podocytes – to evaluate the effect of high glucose on the PTHrP/PTH1R system *in vitro*. MCT cells were grown in RPMI 1640 with 10% fetal bovine serum and antibiotics in 5% CO_2 at 37°C, as described.[23] Mouse mesangial cells (ATCC CRL-1927) were cultured in Dulbecco's modified Eagle's medium and Ham's F12 medium (3:1, v/v) with 5% fetal bovine serum.[55]

Conditionally immortalized mouse podocytes (a generous gift from Dr Herman Pavenstädt, University Hospital of Freiburg, Freiburg, Germany) were cultured as reported.[56,57] Podocytes were cultured on type I collagen (Sigma) and grown in RPMI 1640 medium with 5% fetal bovine serum and antibiotics, supplemented with 10 U/ml recombinant interferon-γ to enhance T-antigen expression, at 33°C (permissive conditions). To induce differentiation, podocytes were maintained on type I collagen at 37°C without interferon-γ (nonpermissive conditions).

Cells between passages 10 and 16 were used in all experiments. MCT and mesangial cells were serum-depleted, and podocytes were switched to 1% fetal bovine serum-containing medium for 24 h before experiments. The cells were then treated with 25 mM glucose (or mannitol) for 24 h,[58,59] in the presence or absence of the AT_1 receptor blocker losartan (MSD, Madrid, Spain).[23,24]

Western blot analysis of PTHrP and PTH1R

Proteins were isolated from either mouse kidney homogenates or cultured cells, using a standard procedure (TriReagent; Molecular Research Center, Cincinnati, OH, USA). Fifteen micrograms of protein from total kidney (or kidney cortex) or cultured renal cells, estimated by the Bradford's method,[60] were separated on 10–15% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked using 100 mM Tris, 150 mM NaCl, pH 7.5, and 0.1% Tween 20 (TTBS) with 5% bovine serum albumin (PTHrP) or 5% defatted milk (PTH1R). They were then incubated overnight at 4°C with PTHrP antiserum C6, at 1/2000 dilution, as reported,[20,23,24] and the anti-PTH1R antibodies Ab-VII or Ab-IV, at 1/200 and 1/1000 dilution, respectively, in TTBS. In the kidney, Ab-VII antibody detects mainly a 90-kDa band,[23,24] whereas a single 66-kDa band was revealed with the Ab-IV antibody,[20] in agreement with the manufacturer's information (website: www.crpinc.com). Walker 256 (W256) tumor protein extracts were used as a positive control for PTHrP.[25] An anti-actin antibody (Sigma), at 1:500 dilution, was used as a loading control. Following incubation with biotinylated anti-rabbit IgG (or peroxidase-conjugated anti-rabbit IgG), bands were detected with 3,3′-diaminobenzidine (DAKO, Glostrup, Denmark) or ECL chemiluminescence (Amersham, Buckinghamshire, UK). Blots were analyzed by densitometric scanning. Densitometric values were normalized against those of actin.

Statistical analysis

All results are expressed as mean ± s.e.m. Statistical significance ($P < 0.05$) was assessed by Kruskal–Wallis test or Mann–Whitney test, when appropriate. To assess the association between PTHrP and the PTH1R protein levels (by Western analysis) and UAE, quantitative values corresponding to all diabetic animals included throughout the study ($n = 49$) were converted to qualitative parameters by grouping the upper – over the median – and the lower – under the median – values. Then, univariate and multivariate logistic regression analysis was performed. The SPSS 9.0 statistical package (SPSS Inc., Chicago, IL, USA) was used.

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