ONLINE SUPPLEMENT

Increased Renin Production in Mice with Deletion of PPARγ in Juxtaglomerular Cells

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SUPPLEMENTAL METHODS

The standard techniques used in our labs and applied in this study are described in details as follows:

Isolation of single juxtaglomerular cells- Please see Supplemental References.[1,2] In short, one male mouse (6-8 wk old) was killed by cervical dislocation. The kidneys were extirpated, decapsulated, and minced with a razor blade at 4°C. The minced tissue was incubated under gentle stirring for 90 min at 37°C in 50 ml Buffer I [(in mmol/L): 130 NaCl, 5 KCl, 2 $CaCl_2$, 10 glucose, 20 sucrose, and 10 Tris, pH 7.4] supplemented with 0.25% trypsin (Sigma) and 0.05% collagenase A (Boehringer). Next the tissue was sieved through a 22.4-μm screen. The sieved cells were collected, washed, and resuspended in 4 ml of Buffer I. The cell suspension was divided into two tubes each containing 30 ml 30% isosmotic Percoll (Pharmacia) in Buffer I and centrifuged at 25,000 *g* for 30 minutes (4°C). Single large granular cells (JG cells) were transferred through modified patch pipettes to 50 μL of guanidinium-thiocyanate solution. Ten micrograms of yeast tRNA was added as a carrier, and total RNA was isolated followed by reverse transcription and PCR amplification for 60 cycles (for better visualisation, Figure 2C) or 40 cycles (for screening for renin or PPARγ expression, Figures 2E and S1E).

Isolation of native juxtaglomerular (JG) cell cultures- Please see Supplemental References.[2] Primary cultures from one RC-PPARγ$^{wt/wt}$ and one RC-PPARγ$^{fl/fl}$ mouse were prepared in parallel. In general, the protocol described above was used till centrifugation in Percoll/Buffer I. Four cellular layers with different specific renin activities were obtained. The cellular layer (density = 1.07 g/mL) with the highest specific renin activity was used for culture. Cells were incubated on 24-well plates for 1 day before subsequent RNA isolation.

Immunohistochemistry- Please see Supplemental References.[3] In short, kidneys from adult mice were fixed by perfusion with 4% paraformaldehyde and embedded in paraffin. Immunolabeling was performed on 5-μm sections incubated with chicken anti-renin IgG (diluted 1:200; Davids Biotechnologie GmbH, Regensburg, Germany) and mouse anti-αSMA IgG (diluted 1:100; Beckman Coulter-Immunotech, Krefeld, Germany).

Protein extraction and Western blot- Please see Supplemental References.[2,4,5] In short, samples were homogenized and lysed in protein lysis buffer (10 mmol/L TRIS, 1% SDS, 1% Nonidet P-40, 5 mmol/L Pefablock). Total cellular protein concentration was determined with Micro Protein Determination Kit (Sigma). Samples were boiled in Laemmli buffer for 5 minutes and loaded on 10% SDS-polyacrylamide gels. After electrophoresis proteins were transferred to nitrocellulose membranes (BioRad) in transfer buffer (48 mmol/L TRIS, 39 mmol/L glycine, 0.037% SDS, 20% methanol). The membranes were blocked, then incubated with 1:500-diluted rabbit polyclonal anti-PPARγ (Cell Signaling) or 1:1000-diluted mouse anti-β-actin antibody. Bound antibody was detected with horseradish peroxidase-conjugated anti-rabbit IgG (DiaNova) followed by ECL detection (Santa Cruz) and exposure to Kodak Biomax MS film (Kodak).

RNA interference, transient transfection and luciferase assay- Please see Supplemental References.[2,5] In short, ready-to-use double-stranded siRNAs targeting human PPARγ was synthesized by Dharmacon (ON-TARGETplus siRNA SMARTpool L-003436-00). One hundred nanomol double-stranded non-targeting siControl siRNA (Dharmacon, used as control), or PPARγ-specific siRNA (siPPARγ) together with mPPREmPal3 firefly luciferase reporter vector (0.2 μg) and plasmid encoding *Renilla* luciferase (pRL-0 vector, 0.01 μg;

Promega) were transfected into Calu-6 cells using Dharmafect 2 transfection reagent (Dharmacon) according to the manufacturer's protocol. Cells were harvested 72 hours after transfection. Relative luciferase activity (RLA) was calculated as firefly luciferase to renilla luciferase ratio.

Isolated perfused kidney and plasma renin concentration (PRC)- Please see Supplemental References.[6,7] In short, knockout mice and littermates (25-30 g body weight, males) were used as kidney donors. The animals were anaesthetized with an intraperitoneal injection of 12 mg/kg xylazine (RompunR, Bayer, Germany) and 80 mg/kg ketamine-HCl (Curamed, Germany), the abdominal aorta was cannulated, the right kidney was excised, placed in a thermostated moistening chamber and perfused at constant pressure (90 mmHg). Using an electronic feedback control, perfusion pressure was changed and held constant in a pressure range between 40 and 140 mmHg. Finally, the renal vein was cannulated and the venous effluent was collected for determination of renin activity and venous blood flow.
The basic perfusion medium consisted of a modified Krebs-Henseleit solution supplemented with 6 g/100 mL bovine serum albumin and with freshly washed human red blood cells (10 % hematocrit). Renin activity in the venous effluent was determined by radioimmunoassay (Byk & DiaSorin Diagnostics, Germany). Renin secretion rates were calculated as the product of the renin activity and the venous flow rate [mL/min*g kidney weight]. Vascular resistance was calculated as pressure/flow ratio [mmHg*min*g kidney weight/mL]. For determination of PRC in vivo, blood samples were collected through mandibular bleeding. Plasma samples were then incubated for 1.5 hours at 37°C with plasma from bilaterally nephrectomized male rats as renin substrate. The generated angiotensin I [$ng*mL^{-1}*h^{-1}$] was determined by radioimmunoassay.

RNA isolation, Reverse Transcription and Quantitative Light Cycler PCR- Please see Supplemental References.[2,5] In short, total RNA was isolated using Qiagen RNeasy Spin Columns. Standard protocol for reverse transcription was used.
Real time RT-PCR was performed with Light Cycler System (Roche, Mannheim, Germany). All PCR experiments were done using the Light Cycler-FastStart DNA Master SYBR Green I kit provided by Roche Molecular Biochemicals (Mannheim, Germany). Each reaction (20 µL) contained 2 µL cDNA, 3.0 mmol/L $MgCl_2$, 1 pmol of each primer, and 2 µL of Fast Starter Mix (containing buffer, dNTPs, Sybr Green and Taq polymerase). The amplification program consisted of 1 cycle of 95° with 10-minute hold ("hot start") followed by 40 cycles of 15 s 95°C, 8 s 58°C, and 16 s 72°C. Amplification was followed by melting curve analysis (increasing the temperature of the reaction up to 95°C, by 0.1°C/s, starting at 50°C for 15s) to verify the correctness of the amplified product. The melting curves were converted to display the first negative derivative (-d*F*/d*T*) versus the temperature. This is an indication of the purity of the products, in which one melting point (*T*m) is indicative for one product, while more melting points indicate the presence of more amplicons, e.g. by non-specific binding of one or both primers. To verify the accuracy of the amplification PCR products were further analyzed on ethidium bromide stained 2% agarose gel. The expression of renin, PPARγ, β1 and β2 adrenoreceptors was quantified relative to ribosomal protein L32. For this purpose, a standard calibration curve was made. The Roche software uses the second derivative maximum method to calculate the fractional cycle numbers where the fluorescence rises above background. The crossing point (*C*p) is the point at which the rate of change of fluorescence is fastest. For the standard curve Cps are plotted versus log concentration for the standards. This standard curve is used to estimate the concentration of each sample.
The efficiency of the PCR was calculated using the formula: Eff = 10^{-1}/slope.

PCR-based detection of recombined PPARγ transcript was performed exactly as described by He et al.[8] (please see Supplemental References)

Renin allele-specific TaqMan assay

Trizol (Invitrogen) and the Purelink™ RNA Mini Kit (Invitrogen) with on-column DNase treatment was used to extract kidney RNA. For cDNA synthesis, 500 ng of total RNA was reverse transcribed using Superscript III (Invitrogen) in a total reaction volume of 20 µl. The cDNA was diluted 40X and 9 µl was used for quantitative real-time PCR. Taqman® assays were run using primer-probe sets and master mix from Applied Biosystems- Ren1 (Assay ID- Mm02342888_gH), Ren2 (Assay ID- Mm00651435_mH), and β-Actin (Part #- 4352933E). The specificity of Ren1 and Ren2 probes was tested using cloned cDNAs for each gene.

Primers

	forward	reverse
renin	5'-atgaaggggtgtctgtggggtc-3'	5'-atgtcggggagggtgggcacctg-3'
ribosomal protein L32	5'-ttaagcgaaactggcggaaac-3'	5'-ttgttgctcccataaccgatg-3'
PPARγ	5'-ccagctctacaacaggcc-3'	5'-gcttcaatcggatggttc-3'
β1 adrenoreceptor	5'-gctcaccaacctcttcatca-3'	5'-acagcacatctaccgaagtc-3'
β2 adrenoreceptor	5'-gagcgactacaaaccgtcac-3'	5'-tgggtggcacggtaccagtg-3'

The renin primers listed above amplify the product of both *Ren1* and *Ren2*.

SUPPLEMENTAL RESULTS

Ren-cre and PPARγ-floxed mice were generated after recombination of the targeted loci in 129 stem cells and were maintained in C57Bl/6 background. Strain 129 has two (*Ren1d* and *Ren2*), while strain C57Bl/6 has one (*Ren1c*) renin gene(s). Therefore the cre allele is accompanied by *Ren2* gene (Figure S1A). Thus, mice heterozygous for the targeted renin locus which we used for the experiments (genotype RC) harbor one copy of each *Ren1c* and *Ren2*. This genetic background is the reason why we did not use genotype $RR^{fl/fl}$ as littermate control - it has two copies of *Ren1c* and none of *Ren2*.

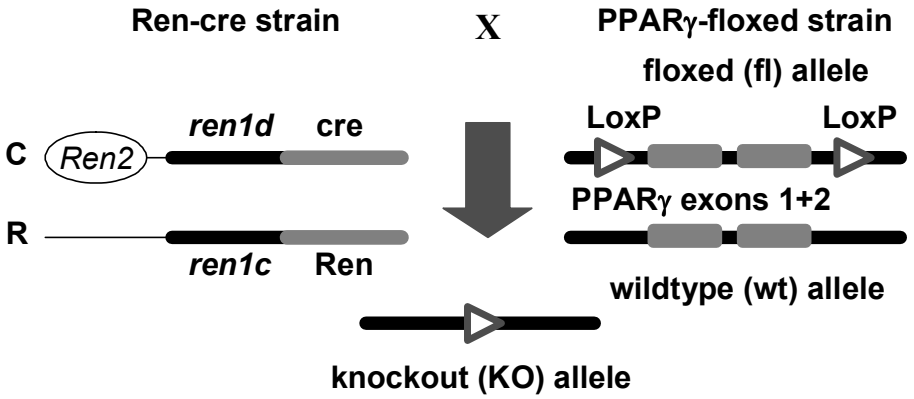
The functional significance of the gene duplication of renin in mice is not well understood. In addition to the kidney, *Ren2* is also strongly expressed in the submandibular gland. *Ren1d* and *Ren1c* are expressed predominantly in the kidney, thus corresponding to the general renin expression pattern observed in rats and humans.

We obtained total 544 animals from breeding of Ren-cre with PPARγ-floxed mice. The nine possible genotypes were represented at Mendelian ratios as expected from the genotypes of the breeder pairs.

There was no difference between kidney-to-body weight ratios (mean±SD) between littermate control (RC-$PPAR\gamma^{wt/wt}$) and JG-specific PPARγ knockout (RC-$PPAR\gamma^{fl/fl}$) mice (6.98±0.6 versus 7.09±0.93 mg/g, n=5 in each group, p=0.44) indicating that renal growth was not affected in the knockout animals.

Supplemental Figure S1

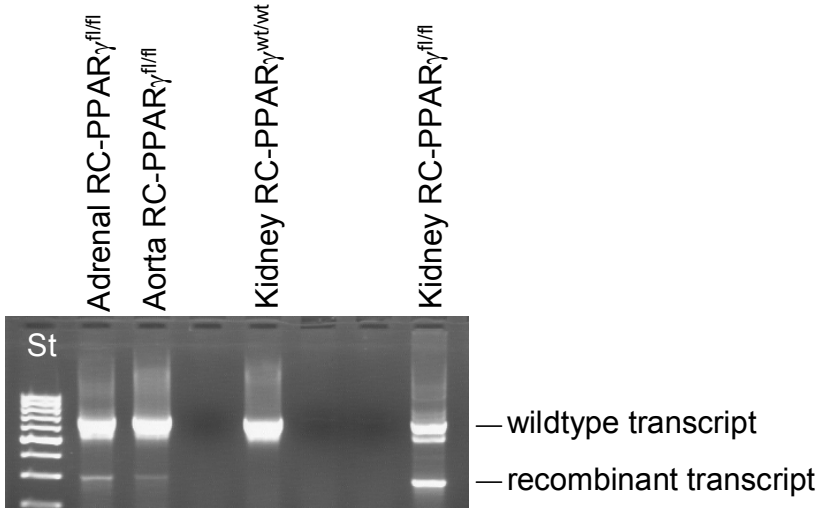
A.



RC-PPARγ$^{wt/fl}$ X RC-PPARγ$^{wt/fl}$

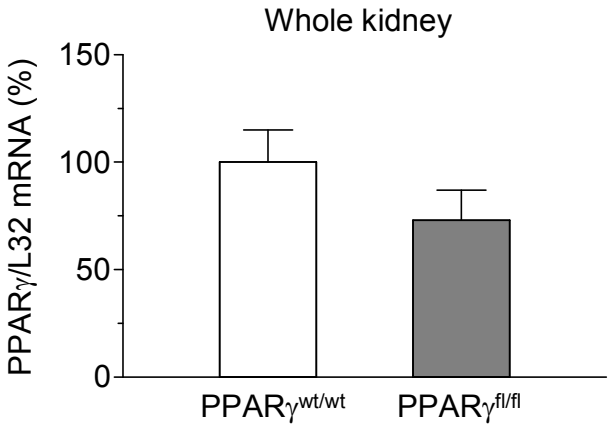
RC-PPARγ$^{wt/wt}$
RC-PPARγ$^{wt/fl}$
RC-PPARγ$^{fl/fl}$
CC-PPARγ$^{wt/wt}$
CC-PPARγ$^{wt/fl}$
CC-PPARγ$^{fl/fl}$
RR-PPARγ$^{wt/wt}$
RR-PPARγ$^{wt/fl}$
RR-PPARγ$^{fl/fl}$

B.

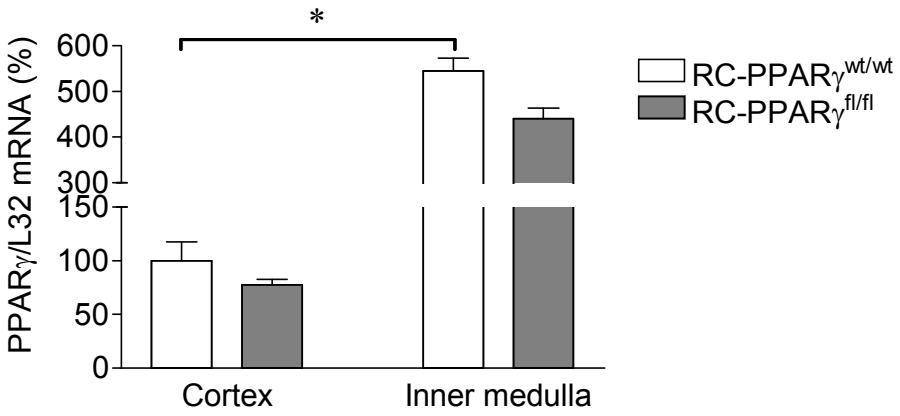


Supplemental Figure S1

C.



D.



E.

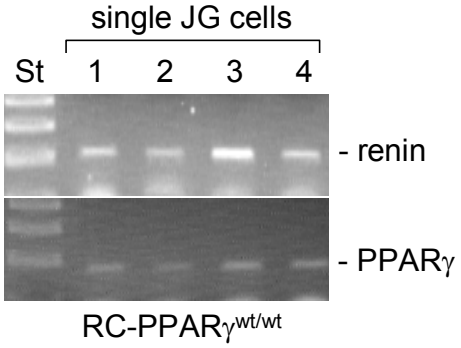


Figure S1

A. Generation of double transgenic mice with targeted disruption of PPARγ allele in JG cells. Breeding scheme- transgenic mice expressing cre recombinase from the endogenous renin locus were crossed with a second transgenic strain in which loxP sites flank exons 1 and 2 of the PPARγ gene. When recombination occurs the PPARγ gene is silenced. Crossing of double heterozygote parents produces nine possible genotypic variants in the progeny that are labeled according to the following scheme: R and C (for renin and cre respectively) refer to the alleles of the renin locus, while superscripted wt and fl (for wildtype and floxed respectively) refer to the alleles of the PPARγ locus. RC-PPARγ$^{wt/wt}$ - littermate control, RC-PPARγ$^{fl/fl}$ - juxtaglomerular (JG) specific PPARγ knockout;

B. PPARγ recombination in aortas and adrenals of individual RC-PPARγ$^{fl/fl}$ mice. Kidney cDNAs isolated from RC-PPARγ$^{wt/wt}$ and RC-PPARγ$^{fl/fl}$ mice were amplified as negative and positive controls respectively, St- length standard;

C. PPARγ mRNA abundance in kidneys of RC-PPARγ$^{wt/wt}$ (n=5) and RC-PPARγ$^{fl/fl}$ (n=6) mice. PPARγ and ribosomal L32 (internal control) mRNA levels were quantified by real-time RT-PCR;

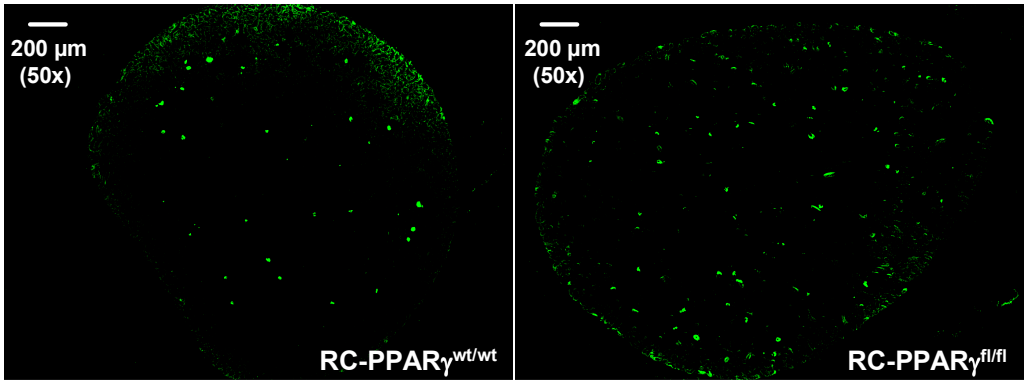
D. PPARγ mRNA abundance in renal cortex and inner medulla of RC-PPARγ$^{wt/wt}$ (n=4) and RC-PPARγ$^{fl/fl}$ (n=6) mice. PPARγ and ribosomal L32 (internal control) mRNA levels were quantified by real-time RT-PCR. *$P < 0.05$.

E. PPARγ mRNA is expressed in native JG cells of RC-PPARγ$^{wt/wt}$ mice. Single-cell RT-PCR with renin (upper panel) or PPARγ (lower panel) specific primers of total RNA extracted from four different JG cells of RC-PPARγ$^{wt/wt}$ mice. St- length standard.

Recombination was detected in aortas and adrenals of individual knockout animals (five and two, respectively of ten RC-PPARγ$^{fl/fl}$ mice tested) (Figure S1B) reflecting the developmental activity of renin promoter. PPARγ mRNA abundance tended to be lower in total kidney, cortex or medulla preparations of RC-PPARγ$^{fl/fl}$ as compared to RC-PPARγ$^{wt/wt}$ mice (Figure S1C,D). Our results confirmed previous studies which demonstrated that PPARγ is stronger expressed in medulla than in cortex (Figure S1D).[9,10]

Supplemental Figure S2

A.



B.

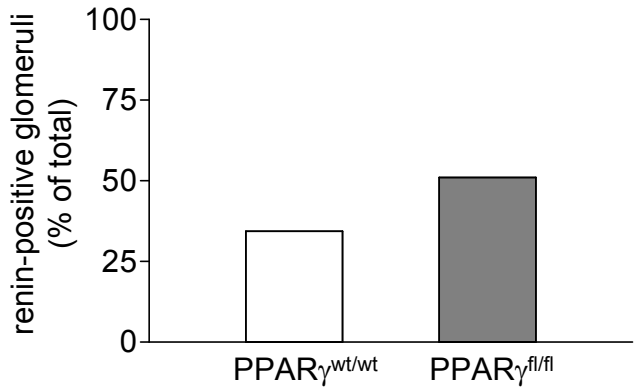
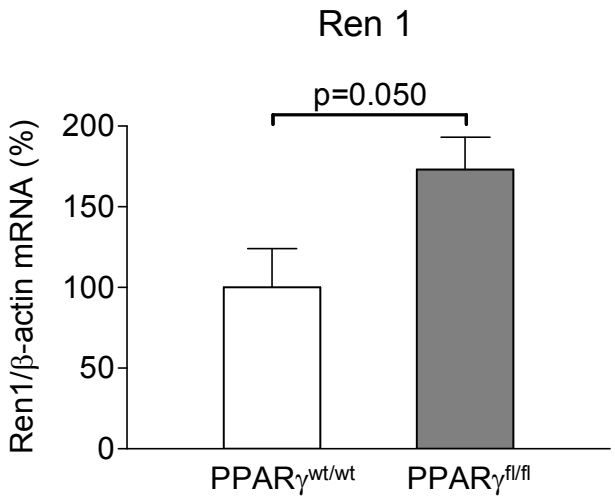


Figure S2
A. Immunohistochemical staining for renin of whole kidney cortical slices of RC-PPARγ$^{wt/wt}$ and RC-PPARγ$^{fl/fl}$ mice;
B. Number of renin-positive glomeruli in kidney slices of RC-PPARγ$^{wt/wt}$ and RC-PPARγ$^{fl/fl}$ mice presented as percent of total glomeruli.

We have used the number of renin-positive glomeruli as a rough estimate for renin expression. Previous 3D reconstruction studies of our group have demonstrated that practically all glomeruli (> 95%) in the adult mouse kidney are renin-positive (K. Machura and A. Kurtz, personal communication).[3] Therefore the number of renin-positive glomeruli per slide would statistically reflect the number of renin-expressing cells in the single afferent arteriole.

Supplemental Figure S3

A.



B.

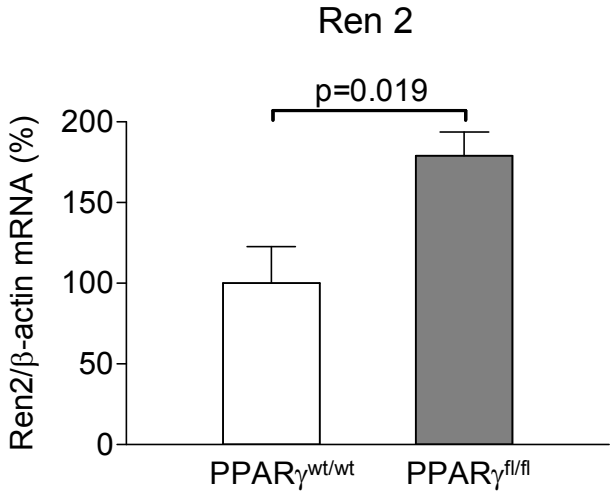


Figure S3
Ren1 and Ren2 allele specific TaqMan assays (A. and B. respectively) in kidneys of RC-PPAR$\gamma^{wt/wt}$ (n=4) and RC-PPAR$\gamma^{fl/fl}$ (n=5) mice.

Supplemental Figure S4

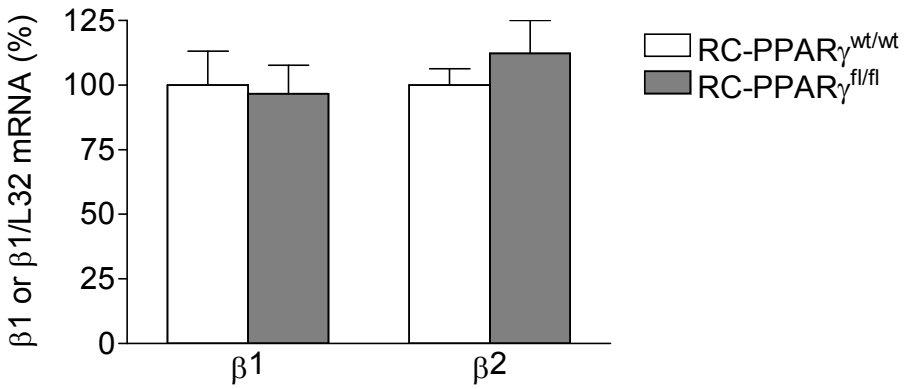


Figure S4
Beta-adrenoreceptor (β1 and β2) mRNA abundance in kidney cortex of RC-PPARγ$^{wt/wt}$ (n=4) and RC-PPARγ$^{fl/fl}$ (n=6) mice. β1, β2 and ribosomal L32 (internal control) mRNA levels were quantified by real-time RT-PCR.

Renal sympathetic nerves and circulating catecholamines acting through beta-adrenoreceptors (β1 and possibly β2) located on the JG cell membrane are important for the regulation of renin expression in vivo.[11] Moreover a very recent study demonstrated that PPARγ regulates the β2 adrenoreceptor expression in the vasculature.[12] However, renal β1/β2 adrenoreceptor expression was not different between RC-PPARγ$^{wt/wt}$ and RC-PPARγ$^{fl/fl}$ mice implying that the adrenergic-dependent regulation of renin production is not affected by the local PPARγ deficit (Figure S4).

SUPPLEMENTAL REFERENCES

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