Supplementary Data

Supplementary Materials and Methods

Experimental model of CCN2(IV)-induced vascular damage

Mice received a single intraperitoneal injection of CCN2(IV) at the dose of 2.5 ng/g of body weight, dissolved in saline, and were studied after 24 h. The *in vivo* CCN2(IV) dose used in this study (2.5 ng/g of total weight mice) is based on *in vitro* experiments in vascular smooth muscle cells, showing that 10 ng/ml CCN2(IV) activates the NF-κB pathway (Fig. 7). Moreover, we have previously described that 10 ng/ml CCN2(IV) activates the NF-κB pathway and EGFR signaling in renal cells (3, 4). There are few studies evaluating CCN2 serum concentrations in cardiovascular pathologies. Several studies have measured the circulating or urinary levels of the whole molecule of CCN2, the N-terminal fragment or the C-terminal fragment. However, there is some controversy between studies. In this sense, the urinary CCN2 excretion in chronic renal diseases varied largely (1- to 100-fold increase) across the studies, as reviewed in (1). In cardiac patients, plasma CCN2 concentrations higher than 80 ng/ml were an independent predictor of reduced right ventricular function (evaluated with an antibody that recognized the C-terminal module) (2). Therefore, we could speculate that our *in vitro* doses of 10–50 ng/ml are around circulating concentrations observed in pathological conditions.

References

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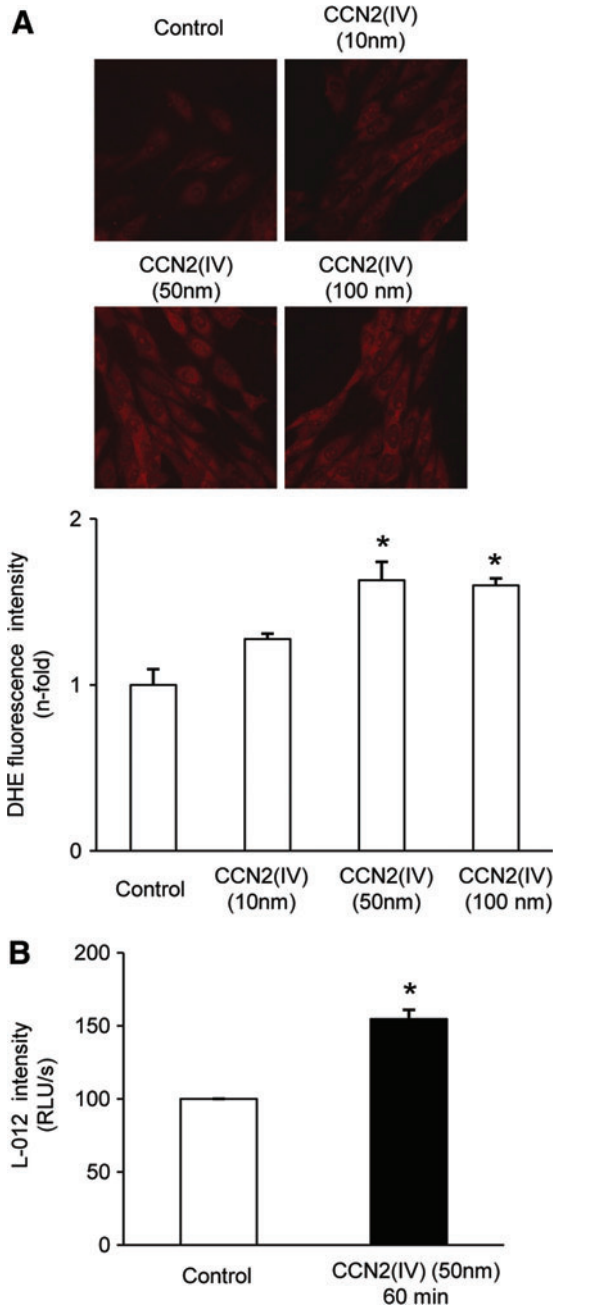
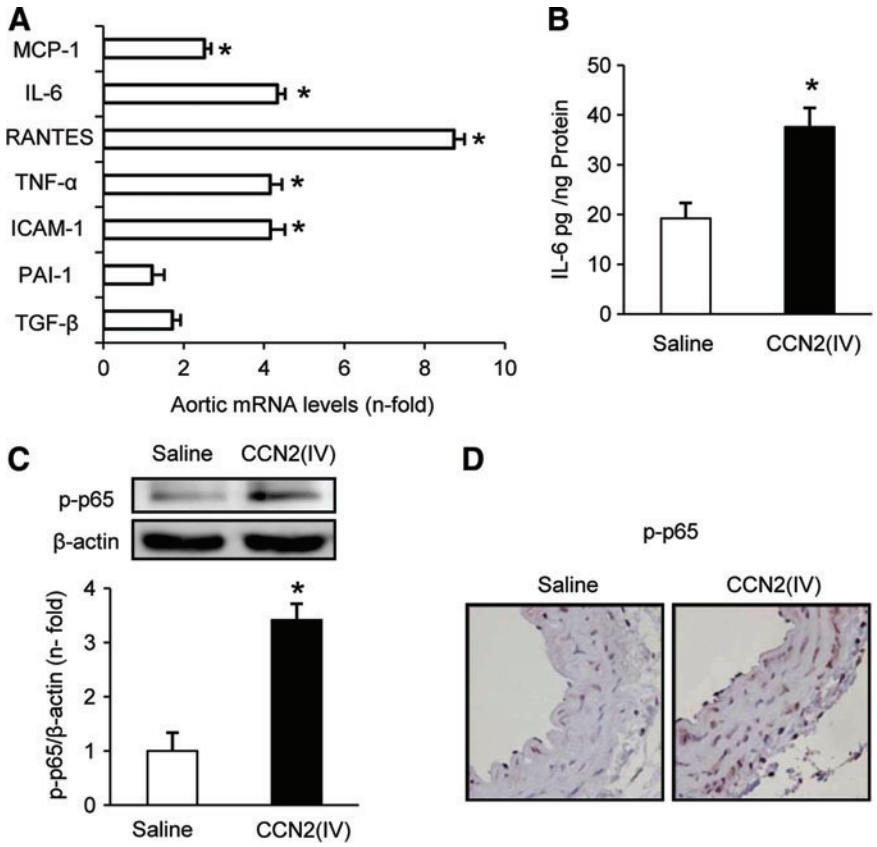
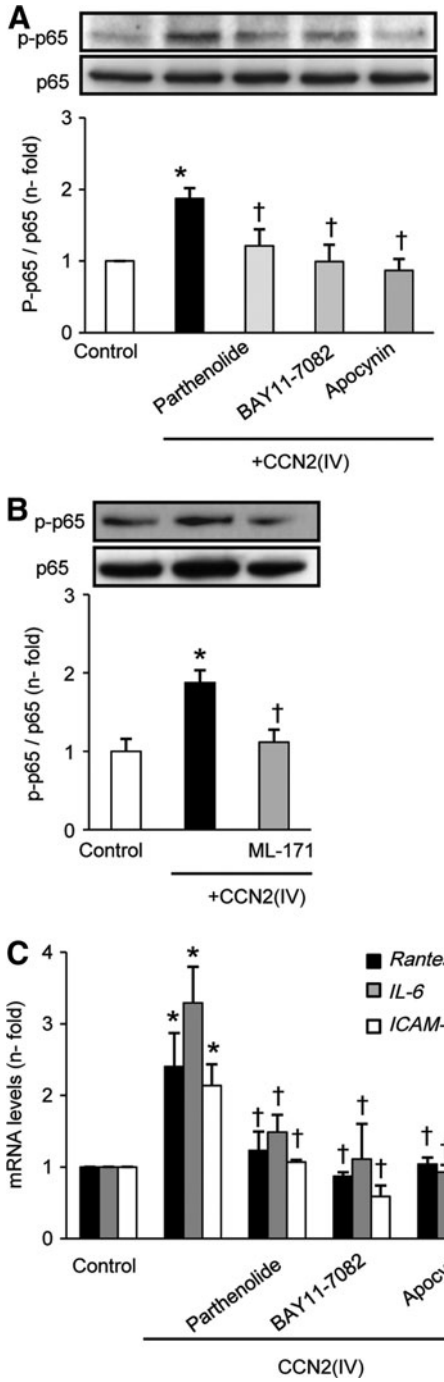


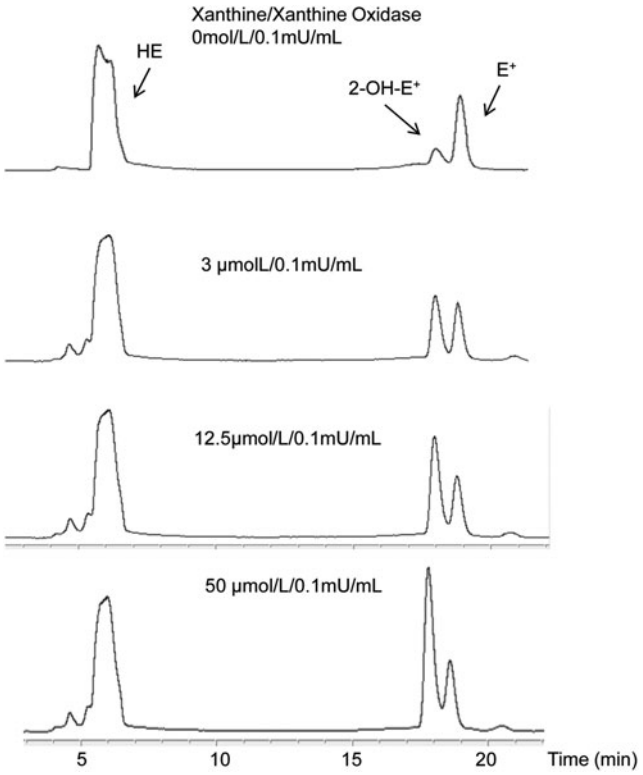
SUPPLEMENTARY FIG. S1. CCN2(IV) induced $O_2^{\bullet -}$ production in endothelial cells. (A) Concentration–response curve of $O_2^{\bullet -}$ production in ECs treated with CCN2(IV) (10–100 ng/ml) and representative fluorescent confocal photomicrographs of superoxide anion ($O_2^{\bullet -}$), measured as DHE staining production. **(B)** $O_2^{\bullet -}$ production in EC measured as luminescence of L-012. Data expressed as mean ± SEM of fold-change over control of at least three independent experiments. *$p < 0.05$ *versus* control. DHE, dihydroethidium; ECs, endothelial cells.



SUPPLEMENTARY FIG. S2. CCN2(IV) administration into normal mice increases inflammation. C57BL/6 mice received a single i.p. injection of recombinant CCN2(IV) (2.5 ng/g body weight) or saline and were sacrificed after 24 h. **(A)** Gene expression was evaluated by real-time PCR in aortic samples from saline and CCN2(IV)-injected mice and expressed as n-fold of increase *versus* saline mice, considered as 1. **(B)** Aortic IL-6 protein levels were detected by ELISA. **(C, D)** Levels of phosphorylated p65 NF-κB subunit (p-p65) were evaluated by Western blot and by immunostaining in paraffin-embedded aortic sections from saline and CCN2(IV)-injected mice. Data are expressed as mean ± SEM of fold increase over saline of 8–10 animals per group. $*p < 0.05$ *versus* saline.



SUPPLEMENTARY FIG. S3. CCN2(IV) activates NF-κB *via* Nox1 in vascular smooth muscle cells. Cells were preincubated during 60 min with apocynin (0.3 m*M*), parthenolide (1 μM), BAY11-7082 (1 μM), or ML-171 (1 μM) before treatment with 50 ng/ml CCN2(IV) for 60 min **(A, B)** or 6 h **(C). (A, B)** A representative Western blot and the quantification of phosphorylated-p65 NF-κB (p-p65) protein levels expressed as mean ± SEM of fold-change over control of eight **(A)** and four **(B)** independent experiments. $^{*}p < 0.05$ *versus* control. $^{\dagger}p < 0.05$ *versus* CCN2(IV). As loading control total p65 was used. **(C)** Gene expression levels determined by real-time PCR and expressed as n-fold of increase *versus* saline mice, considered as 1. Data were expressed as mean ± SEM of fold-change over control of five independent experiments. $^{*}p < 0.05$ *versus* control. $^{\dagger}p < 0.05$ *versus* CCN2(IV).



SUPPLEMENTARY FIG. S4. HPLC analysis of DHE fluorescence. The reaction of dihydroethidium with O_2 yields a very specific product, 2-OH-E^+, which can be detected by HPLC analysis. Xanthine/xanthine oxidase system was used to evaluate the generation of 2-OH-E^+. 2-OH-E^+, 2-hydroxyethidium