Changes in extracellular matrix composition regulate cyclooxygenase-2 (COX-2) expression in human mesangial cells

Matilde Alique 1,2*, Laura Calleros 1,2*, Alicia Luengo 1,2, Mercedes Griera 1,2, Miguel Ángel Íñiguez 3, Carmen Punzón 3, Manuel Fresno 3, Manuel Rodríguez-Puyol 1,2 and Diego Rodríguez-Puyol 2,4

1Departamento de Fisiología, 2IRSIN, and 4Nephrology Section of the “Príncipe de Asturias Hospital”, Alcalá University, Alcalá de Henares, 3Centro de Biología Molecular “Severo Ochoa”, CSIC-UAM, Universidad Autónoma, Madrid, Spain

*Both authors contributed equally to the study.

Running head: Extracellular matrix and COX-2

Correspondence and reprint request to:
Matilde Alique PhD
Atherothrombosis Research Unit
Mount Sinai School of Medicine
One Gustave L. Levy Place, Box 1030. Atran-Berg Building 6th Floor, Room 20,
New York, NY 10029.
Contact: 212-241-8484 (voice); 212-426-6962 (fax);
E-mail: matilde.alique@mssm.edu

Copyright © 2011 by the American Physiological Society.
Abstract

Glomerular diseases are characterized by a sustained synthesis and accumulation of abnormal extracellular matrix proteins, such as collagen type I. The extracellular matrix transmits information to cells through interactions with membrane components, which directly activate many intracellular signaling events. Moreover, accumulating evidence suggests that eicosanoids derived from COX-2 participate in a number of pathological processes in immune-mediated renal diseases and it is known that AKT may act through different transcription factors in the regulation of the COX-2 promoter. Present results show that progressive accumulation of collagen I in the extracellular medium induces a significant increase of COX-2 expression in human mesangial cells, resulting in an enhancement in PGE$_2$ production. COX-2 over-expression is due to increased COX-2 mRNA levels. The study of the mechanism implicated in COX-2 up-regulation by collagen I showed FAK activation. Furthermore, we observed that the activation of the PI3K/AKT pathway by collagen I, and collagen I-induced COX-2 over expression was abolished by PI3K and AKT inhibitors. Additionally, we showed that the CRE transcription factor is implicated. Finally, we studied COX-2 expression in an animal model, L-NAME hypertensive rats. In renal tissue and vascular walls, COX-2 and COL I content were up-regulated. In summary, our results provide evidence that collagen type I increases COX-2 expression via FAK/PI3K/AKT/CREB signaling pathway.

**Keywords:** Collagen type I, FAK, PI3K, AKT, CREB.
Introduction

Cyclooxygenase (COX) is known to exist in two isoforms, a constitutive isoform (COX-1), and an inducible isoform (COX-2) (40). Both are expressed in the human kidney, and COX-derived prostaglandins play a pivotal role in the maintenance of renal blood flow, glomerular filtration rate, and tubular sodium handling (12). COX-1 is found in the glomerulus and afferent arteriole, whereas COX-2 is expressed constitutively in the cells of the macula densa (MD) and in scattered cells in the cortical thick ascending limb (cTAL) immediately adjacent to the MD and in a subset of medullary interstitial cells near the papillary tip in normal adult kidney (18, 19). In the human kidney, COX-2 expression also has been reported to be present in podocytes, glomerular mesangial cells and arteriolar smooth muscle cells (28). COX-2 expression is also abundant in the lipid-laden medullary interstitial cells in the inner medulla and papilla (18, 19). Some investigators have reported that COX-2 may also be expressed in inner medullary collecting duct cells or intercalated cells in the renal cortex (11). Up-regulation of the COX-2 isoform has been implicated in a wide variety of pathological processes, mainly those characterized by inflammation, suggesting that activation of COX-2 might play an important role in the pathogenesis and progression of nephropathies (6) (7). As inflammation and fibrosis are closely linked, there has also been an increasing interest in the effect of COX-2 on modulation of fibrotic changes in renal disease. Prostanoid production via COX is increased in various renal diseases (30, 33, 37, 48), and may serve to offset the profibrotic influence of factors such as TGF-β and angiotensin II, which may also be elevated in glomerular disease (35, 39). Interestingly, evidence obtained in the past decade indicates that progression to advanced renal fibrosis requires the participation of inflammatory events such as COX-2 up-regulation (21, 44).

Renal fibrosis is the common final outcome of many clinical conditions, both inflammatory and non-inflammatory, that lead to chronic renal failure (10). It is characterized by a progressive substitution of cellular elements by extracellular matrix (ECM) proteins, as a consequence of an imbalance in the rate of proliferation, necrosis, and apoptosis of the cells (20), as well as the loss of the normal equilibrium between the synthesis and degradation of ECM (27, 50). Glomerular mesangial cells seem to play a key
role in the genesis of glomerular sclerosis (16). Main components of the glomerular basement membrane (GBM) and glomerular interstitium are collagens, proteoglycans, laminin, and nidogen (entactin) (22, 23).

In normal conditions, collagen IV seems to be the most relevant glomerular collagen, acting as an important anchorage substrate for many cell types, and the regulation of collagen IV synthesis and degradation plays an important role in cell function, growth, migration, and organ remodeling (18). In pathological conditions, such as hypertension or diabetes, characterized by an augmented profibrotic cytokines at the local level (26, 41), an increased ECM protein synthesis takes place at glomerular level, accompanied by qualitative changes in its composition (51, 52). Thus, in some glomerular diseases, a progressive accumulation of interstitial collagen I (39) and a loss of basement membrane integrity, with decreased content of laminin and collagen IV, have been described (46).

Changes in ECM composition may induce significant changes in cell phenotype. ECM proteins, through the interaction with transmembrane proteins that act as receptors (36), may modify the growth pattern of the cells (29), protect them from apoptosis (15), induce changes in the synthesis of cellular autacoids (17, 34) or modify the cellular content of proteins with well-defined biological activity (9), with the subsequent changes in cell biology and organ structure and function.

As mentioned before, the relationship between COX-2 and renal fibrosis have only been studied to analyze the role of the enzyme in the protection against glomerular sclerosis (1) and tubulo-interstitial scarring (42), or in the development of glomerular and tubulo-interstitial injury (2). However, the possibility that the very fibrosis may act as a regulatory mechanism of COX-2 expression has not been previously evaluated. Positive or negative feed-back, regarding the enzyme expression, could be established in renal diseases with fibrosis, and this fact could have relevant consequences in the development of disease. In the present study, we hypothesized that changes in ECM composition could play a role in the regulation of COX-2 expression in human mesangial cells (HMC), and we devoted part of the experiments to analyze the mechanisms involved in this modulation.
Experimental procedures

Drugs and reagents, human mesangial cell (HMC) culture, protein and RNA analysis:
Supplementary information is available at AJP-Cell Physiology’s website.

Experimental Design: Studies were performed in cells cultured on a thin film of collagen type I (COL I) or collagen type IV (COL IV). For this, culture plates were incubated for 16 h at 4°C in a solution of 12.5 μg/ml COL I or COL IV in bicarbonate buffer (15 mM Na₂CO₃ and 35 mM NaHCO₃ [pH 9]), to allow the formation of a thin film of collagen, as described (24). Then, the remaining collagen was discarded and Petri dishes were washed with Hanks’ balanced salt solution to restore the pH. Trypsinized cells were seeded onto the COL I- or COL IV-coated plates. Cells were grown for the indicated times in RPMI 1640 supplemented with 10% FBS. In the studies with soluble collagens, cells were grown to 90% confluency during 3 days and they were treated with 12.5 μg/ml COL I or COL IV. For inhibition experiments, cells were pre-incubated with the corresponding inhibitor for 1 h and continuously exposed to soluble COL I or COL IV plus the inhibitor.

Determination of PGE₂ formation: The cultured medium of HMC treated as described in the figures legend was collected and diluted 2 times. PGE₂ concentrations in the medium were determined in triplicate using a commercially available enzyme immunoabsorbent assay (EIA) kit (Cayman Chemical Company, Ann Arbor, MI), following the manufacturer's protocol. The assay was performed in a total volume of 150 μl, with the following components being added in 50 μl volumes: standards or biological samples, enzymatic tracer and specific antiserum. After overnight incubation at 4°C, the plates were washed, and 200 μl Ellman's reagent was added into each well. After 1–3 h, the absorbance at 414 nm of each well was measured. A standard curve, with values ranging from 50 to 0.39 pg/ml, was used to evaluate the concentrations. The reliable limit of quantification for PGE2 was 15 pg/ml, and the coefficient of variation was less than 14% within the calibration range (15–1000 pg/ml). Results were calculated by using the nonlinear regression of a four-parameter logistic model. Each experiment was performed five times.
**Transient Transfection Experiments:** Cells were plated in six-well plates 24 h before transfection, and incubated at 37°C until they were 60 - 80% confluent. Then, cells were incubated for 8 h at 37°C with 2ml Opti-MEM (Invitrogen, CA, USA) containing complexes of 5 μg LipofectAMINE (Invitrogen, CA, USA), 1.0 μg human COX-2 promoter luciferase constructs reporter plasmids, and 0.1 μg renilla luciferase reporter as an internal control. Transfected cells were next incubated with complete growth medium for 16 h and then they were serum-starved for 10 h before the cells were treated. Finally, firefly luciferase activity of the COX-2 reporter was measured with a Lumat LB9506 luminometer (Berthold Technologies, Herts, UK) and normalized against the renilla luciferase activity by using the dual-luciferase reporter assay system (Promega, Madison, WI, USA). Luciferase activity is represented as relative luciferase units of firefly per renilla.

**siRNA transfection:** To deplete AKT protein with specific siRNA oligonucleotides, experiments were performed as described (4). Cells were seeded in 6-well plates the day before transfection. Cells were incubated in 1 ml OPTIMEM with 100nM Signal-Silencer™ AKT siRNA, which targets AKT1 and AKT2 (catalog number 6211, Cell Signaling Technology, Beverly, MA, USA) or Silencer™ negative control#1 siRNA (Ambion, Inc. Austin, TX, USA), using LipofectAMINE. After 24 h incubation, 1 ml of medium containing FBS was added. Transfected cells were next incubated for 24 h and then they were serum-starved for 10 h before the cells were treated, as reflected in the figures legend. Cells were harvested 24 h later.

**Studies in animals:** Twelve-week-old male Wistar rats were housed in a pathogen-free, temperature-controlled room (22 ± 2°C). Food and water were available ad libitum. They were treated with L-NAME (20mg/kg/day) or water, and blood pressure was measured by a tail cuff sphygmanometer (14). After 4 weeks, they were anesthetized with halothane, and kidneys and aortas were removed and shock-frozen immediately for Western blot analysis. The investigation conforms to the Guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH...
publication No. 85-23, revised 1996) and was supervised and approved by the veterinary authority of the animal facilities from the Universidad de Alcalá.

**Data analysis and statistical procedures:** Results are reported as means ± SEM from at least three independent experiments, some of them performed in duplicate or triplicate. Statistical differences were tested by using non-parametric unpaired (Mann-Whitney, Wilcoxon, or Friedman test, as needed). A p value < 0.05 was considered significant.
Results

Changes in COX-2 protein content and mRNA expression in HMC in contact with different collagen types.

HMC grown on COL IV for up to 72 h exhibited a progressive decrease of COX-2 protein content (Figure 1A), similar to the one observed in plastic cultured cells. In fact, COX-2 protein expression was similar in plastic and COL IV cultured cells, at the different times tested (data not shown). The decrease in COX-2 was not observed in cells grown on COL I, and the levels of the protein were significantly higher than in the COL IV grown cells. Differences were maximal at 72 h (Figure 1A). As a consequence of this difference, the synthesis of PGE2 after 72 h on collagens was higher when the culture substrate was COL I (Figure 1B). No differences were observed in the protein content of COX-1, as a consequence of the culture substrate (results shown in the Figure 1 of “Supplemental data”). Interestingly, the differences on COX-2 expression were also observed when different collagen proportions were used to plate the cells. As shown in figure 1C, COX-2 protein content increased when COL I proportion augmented. The differences in COX-2 were also observed when plastic plated cells were incubated with soluble collagens. COL IV did not modify the COX-2 protein content in HMC-treated for 72 h, whereas COL I induced a significant increase of the protein that was maximal at 8 h, and remained for 48 h (Figure 1D). Similar effects were observed when cells grown in COL IV were incubated with soluble COL I (results shown in Figure 2 of “Supplemental data”).

To analyze the mechanism involved in the changes in the protein content, the expression of COX-2 mRNA was examined in plastic plated HMC treated for 24 h with both soluble collagen types. As shown in figure 2A, significant differences in the COX-2 mRNA expression were observed between both collagens. Furthermore, cells transiently transfected with P2-1900-LUC, a construct of the human COX-2 gen promoter linked to a reporter system, showed an increased luciferase activity when incubated with COL I, up to similar levels as cells treated with IL-1β, a strong inducer of COX-2 (Figure 2B). No changes in the transcriptional activity of the promoter were observed in cells treated with soluble COL IV.
Thus, the changes in the steady state COX-2 mRNA levels seem to be the consequence of modifications in the transcriptional activity of its promoter.

**COL I-dependent COX-2 up-regulation is mediated by FAK and PI3K/AKT activation.**

HMC incubated with soluble COL I exhibited a rapid and sustained increase in FAK tyrosine 397 phosphorylation, which was not observed in cells treated with COL IV (Figure 3A). Pretreatment of HMC with 10 µM PP2, a selective inhibitor of FAK activation, completely decreased COL I-induced COX-2 induction (Figure 3B). Similarly, significant differences were observed in AKT phosphorylation at Ser473 when HMC were treated with both types of collagen. As shown in Figure 4A, the treatment with COL I induced a sustained increase in the cellular content of the phosphorylated protein with respect to the COL IV treated cells. The pharmacological blockade of PI3K or AKT inhibited the COL I induced up-regulation of COX-2 protein content (Figure 4B), an effect that was also observed when cells were depleted from AKT with a specific siRNA (Figure 4C). The increased AKT phosphorylation induced by COL I was prevented by cell incubation with the inhibitor of FAK activation, PP2 (Figure 4D). Additionally, the possible involvement of the FAK/PI3K/AKT signaling pathway in the collagen type I induced increase of COX-2 expression was analyzed by measuring the transcriptional activity of the COX-2 promoter in the presence of specific inhibitors. As shown in Figure 5, HMC pretreatment with the pharmacological antagonists of PI3K, AKT, or FAK inhibited the COL I-induced COX-2 increased promoter activity.

**COL I-dependent COX-2 up-regulation is mediated by the transcription factor CREB.**

To identify the region in the COX-2 promoter responsible for COL I-mediated activation, HMC were transfected with different constructs of the human COX-2 promoter, linked to the luciferase reporter system. As shown figure 6A, progressive serial deletions of the promoter did not modify the COL I-induced promoter activity even in the shorter construct P2-192 containing the proximal pNFAT site and a CRE site of response to transcription factors. However, the specific mutation of this CRE site in the responsive p192 promoter (P2-192 CRE-MUT) completely abrogated the stimulatory effect of COL I. To
further confirm the involvement of CRE transcription factor in COL I-induced COX-2 expression, we examined CREB activation by analyzing its phosphorylation. An increased and sustained CREB phosphorylation on Ser 133 was detected in cells incubated with COL I, with respect to cells incubated with COL IV (Figure 6B). This CREB phosphorylation was prevented by addition of the FAK or AKT inhibitors to the cells (Figure 6C).

“\textit{In vivo}” relationship between COL I and COX-2 tissue content

In order to analyze the physiological relevance of the changes observed in cells, some studies were performed in hypertensive animals. Rats were treated for 4 weeks with the NO-synthase inhibitor L-NAME (Blood pressure after 4 weeks of L-NAME: 230 ± 12* mmHg. Blood pressure in control rats: 142 ± 10 mmHg; *p < 0.05), and the renal and vascular protein content of COL I and COX-2 was simultaneously analyzed. The results obtained are shown in Figure 7, with panel A including the renal data and panel B the vascular data. In both cases, a significantly increased content of both proteins was observed. Moreover, these two parameters showed a highly significant statistical correlation.
Discussion

Here, we demonstrated that COL I induces an increased expression of COX-2 in HMC, with respect to plastic or COL IV cultured cells. This up-regulation was observed not only when cells were cultured on COL I covered plates, but also when soluble COL I was added to cells cultured on plastic or COL IV. As a consequence of this over-expression, HMC cultured on COL I synthesized increased amounts of PGE2. The changes observed in the COX-2 protein content in HMC in contact with COL I seem to be the consequence of increased mRNA steady state levels that were due, at least in part, to increased promoter activity. Similar results were previously published by Cho and cols in a cellular line of macrophages (8), and by Broom and cols in embryonic intestinal epithelial cells (3), but this is the first description of this effect in mesenchimal cells such as HMC.

Simultaneous studies were performed, in collagen-plated cells and in plastic-plated cells incubated with soluble collagens, and in both cases results were comparable. Studies in collagen-plated cells could better reproduce the pathophysiological conditions in damaged glomeruli, in which chronic changes in extracellular matrix composition would modify the expression of COX-2 in mesangial cells. However, to analyze the initial events that trigger COX-2 overexpression, studies in collagen-plated cells could be inadequate. Immediately after plating, differences in adhesion or proliferation could exist, and the degree of confluence or the percentages of cells in the different phases of cellular cycle could differ in both collagens. In these conditions, the changes observed could not be the consequence of the different collagen composition. Thus, after demonstrating that changes in COX-2 expression were similar in collagen-plated and collagen incubated cells, we selected this later experimental approach to analyze the early cellular events that take place after collagen interaction.

The mechanisms involved in the COX-2 modulation by COL I were explored with a systematic approach that included a) the measurement of the activity of proteins that are implied in the signal transmission of extracellular matrix protein stimuli to cells, and b) the consequences of the blockade of these proteins on COX-2 expression. FAK, PI3K and AKT are some of the proteins activated after the
interaction of collagen with transmembrane integrins (38, 43). HMC treated with soluble COL I showed
an increased phosphorylation of FAK and AKT, a fact that implies protein activation (31, 47). The
pharmacological blockade of the activity of the three proteins, as well as the cellular depletion of AKT
with a siRNA, inhibited the COL I dependent COX-2 up-regulation, both at the protein level and in the
promoter activity, supporting the relevance of these proteins in the COX-2 modulation under the proposed
experimental conditions. Finally, to better establish the relationships between FAK and AKT, AKT
phosphorylation was analyzed in the presence of FAK blockade. The results found suggest that FAK
activation precedes the phosphorylation of AKT.

To gain further insight into the intracellular mechanisms that mediate the COL I-dependent COX-2
up-regulation, cells were transfected with plasmids containing serial deletions of the COX-2 promoter
(25), and the luciferase activity was analyzed in the presence of both kinds of collagens. The results
demonstrated that the transcription factors NFκB, NF-IL6 and NFAT, involved in the modulation of the
COX-2 promoter activity in some cell types (13, 32), were not involved in the genesis of the differences
between COL I and COL IV, as the deletion of their response elements in the promoter region did not
modify these differences. Only the deletion of a fragment containing response elements to NFAT and
CREB made changes in the luciferase activity after incubation with both collagens were similar. The
relevance of the CREB was supported by the fact that a) phosphorylation of CREB increased in cells
incubated with COL I, b) this phosphorylation was abolished by FAK and AKT blockade, and c) the
directed mutation of the CREB response element in the COX-2 promoter region (25) completely
abolished the luciferase changes induced by COL I.

Considering the data previously discussed, a possible putative model of COL I-induced COX-2
over-expression in HMC can be envisaged (Figure 7). Not every possible mechanism involved has been
analyzed and, thus, the possibility that alternative mechanisms could also be involved exists. In this
regard, in intestinal epithelial cells, Broom and cols. (3, 13) demonstrated that the coordinate activation of
two transmembrane proteins, CD47 and alpha2beta1 integrin, with the subsequent stimulation of
PKCalpha, the small GTPase ras, and NFkB, seem to mediate the COX-2 up-regulation induced by COL I. In contrast, Cho and cols. (8) described that, for the same stimulus and effect but in a different cellular line, macrophages, FAK, PI3K and CREB may be involved, but also p38 kinase, extracellular signal-regulated kinase (ERK 1/2) and CCAAT/enhancer binding protein.

A critical point concerning the present findings is the in vivo relevance of the observed effects in cells. The fact that variable mixtures of collagens modify the pattern of expression of COX-2 with a progressive increase as COL I proportion augments supports the importance of the findings. In fact, in pathophysiological conditions in vivo, cells are not expected to come in contact with just one collagen type, but with different extracellular matrix proteins, both normal and abnormal. Interestingly, we found that, in animals with an increased COL I expression in kidney and vessel walls, such as hypertensive rats, the COX-2 expression parallels the changes in COL I tissue content, pointing to a possible relationship between these two phenomena. Further studies have to be carried out to definitively proof this relationship. The pathophysiological consequences of the increased COX-2 content when COL I is over-expressed have not been evaluated in our experiments. Specifically designed experiments in which the functional and structural consequences of the COX-2 blockade were analyzed, in a context characterized by COL I augmentation, ought to be performed. Previous reports have shown contradictory consequences of the administration of COX-2 inhibitors in renal diseases characterized by the accumulation of extracellular matrix. In some cases, the pharmacological blockade of COX-2 leads to a decrease in renal damage (5, 45) whereas in other COX-2 over-expression is critical for the progression of the disease (5, 49). Thus, the modulation of the enzyme by COL I could be interpreted as a protective or a harmful mechanism. Careful experiments that analyze simultaneously the relationship between COL I and COX-2, and the consequences of the inhibition of the latter, must be performed to clarify the mechanisms of progression of renal damage in chronic renal diseases.

In summary, we reported here the first description of COX-2 up-regulation by extracellular matrix proteins, particularly the proportion between collagens I and IV, in HMC, and we describe part of
the mechanisms involved in the development of this effect (Figure 8). Moreover, by analyzing an experimental model of hypertension characterized by an increased renal and vascular accumulation of COL I, we point to the possibility that this in vitro up-regulation could also take place in vivo. Additional experiments are needed to better understand the pathophysiological consequences of these changes.

**Disclosure**

All the authors declared no competing interests.
This study was supported by grant ISCIII-RETIC REDinREN/RD06/0001, by grants Fundación Mutua Madrileña (FMM2008-004) and Proyecto CAM-UAH (CCG08-UAH/BIO-4237) to MA, by grant Ministerio de Educación (SAF 2007 623471) to MRP, by grant Fondo de Investigaciones Sanitarias (FISS-PI070695) to DRP and by grants Ministerio de Ciencia e Innovación (SAF2007-61716; BFU2007-62659/BMC), ISCIII-RETIC RED RECAVA RD06/0014/1013, Comunidad Autónoma de Madrid, (S-SAL-0159/2006), CAM-UAM (CCG08-UAM/BIO-4299), European Union (EICOSANOX LSH-CT-2004-005033) to MF and MAI.


activity, which in turn attenuates the fibrotic response to TGF-beta2 by impeding CTGF expression.


Fig. 1. Changes in COX-2 protein expression and PGE2 production in human mesangial cells (HMC) in contact with different collagen types. (A) COX-2 protein expression in HMC grown on plates coated with collagen I (COL I) and collagen IV (COL IV), for the indicated times. Expression of COX-2 protein was analyzed by western blot (a characteristic western blot is shown). Equal protein loading was confirmed by probing with an α-actin antibody. The graphs present densitometric band analysis normalized to α-actin. The data represent means ± SEM of five independent experiments. *p < 0.01 versus COL IV. (B) PGE2 synthesis by HMC grown on plates coated with both collagen types for 48 hours. PGE2 in the medium was determined in triplicate in five separate experiments. *p < 0.01 vs COL IV. (C) COX-2 protein expression in HMC grown on plates coated with different proportions of COLI/COL IV for 72 h. Expression of COX-2 protein was analyzed by western blot (a characteristic western blot is shown). Equal protein loading was confirmed by probing with an α-actin antibody. The graphs present densitometric band analysis normalized to α-actin. The data represent means ± SEM of five independent experiments. *p < 0.01 versus 100 % COL IV. (D) COX-2 protein expression in HMC grown on plastic plates, and incubated with both kinds of soluble collagens (12.5 µg/ml) for the indicated times. Expression of COX-2 protein was analyzed by western blot (a characteristic western blot is shown). Equal protein loading was confirmed by probing with an α-actin antibody. The graphs present densitometric band analysis normalized to α-actin. The data represent means ± SEM of five independent experiments. *p < 0.01 versus COL IV at time 0.

Fig. 2. Changes in COX-2 mRNA expression and COX-2 transcriptional activity in human mesangial cells (HMC) in contact with different collagen types. (A) COX-2 mRNA expression in HMC grown on plastic, and incubated with soluble collagen I (COL I, 12.5 µg/ml) and collagen IV (COL
IV, 12.5 µg/ml), for 24 h. Expression of COX-2 mRNA was analyzed by RT-PCR (a characteristic PCR is shown). Equal mRNA loading was confirmed by coamplification of 18S mRNA. The graphs present densitometric band analysis normalized to 18S RNA. The data represent means ± SEM of four independent experiments. *p < 0.01 versus COL IV. (B) COX-2 promoter activity in HMC grown on plastic, and incubated with soluble collagen I (COL I, 12.5 µg/ml) and collagen IV (COL IV, 12.5 µg/ml), for 24 h. HMC were transiently transfected with the P2-1900 COX-2 promoter construct and pRL-TK-Renilla, and luciferase and renilla activities were assayed after incubations. Luciferase activity was normalized with renilla activity and concentration of proteins. IL-1β (10 ng/ml) was used as a positive control. The data represent means ± SEM of four independent experiments (triplicates), and are expressed as fold induction with respect to control values (untreated cells). *p<0.01 versus control, #p<0.01 versus COL IV.

Fig. 3. Role of focal adhesion kinase (FAK) in the changes in COX-2 expression induced by collagens. (A) Human mesangial cells (HMC) were treated with soluble collagen I (COL I, 12.5 µg/ml) and soluble collagen IV (COL IV, 12.5 µg/ml) for the indicated times, and FAK phosphorylation was assessed by western blot (a representative blot is shown). Equal protein loading was confirmed by probing with anti-total FAK. The graphs present densitometric band analysis normalized to total-FAK. The data represent means ± SEM of five independent experiments. *p < 0.01 versus COL IV. (B) Effect of pre-incubation with the FAK inhibitor PP2 (10 µM, 1h) on the COX-2 modulation induced by soluble collagens (12.5 µg/ml, 24 h). Expression of COX-2 protein was analyzed by western blot (a characteristic western blot is shown). Equal protein loading was confirmed by probing with anti- α-actin antibody. The graphs present densitometric band analysis normalized to α-actin. The data represent means ± SEM of four independent experiments. *p < 0.01 versus COL IV, #p<0.01 versus COL I.
Fig. 4. Role of PI3K/AKT pathway in the changes in COX-2 expression induced by collagens. (A) HMC were treated with soluble collagen I (COL I, 12.5 μg/ml) and soluble collagen IV (COL IV, 12.5 μg/ml) for the indicated times, and AKT phosphorylation was assessed by western blot (a representative blot is shown). Equal protein loading was confirmed by probing with anti-total AKT. The graphs present densitometric band analysis normalized to total-AKT. The data represent means ± SEM of five independent experiments. *p < 0.01 versus COL IV. (B) Effect of pre-incubation with the PI3K inhibitor, LY294002, (20 μM, 1h) and AKT inhibitor (30 μM, 1h) on the COX-2 modulation induced by soluble collagens (12.5 μg/ml, 24 h). Expression of COX-2 protein was analyzed by western blot (a characteristic western blot is shown). Equal protein loading was confirmed by probing with anti-α-actin antibody. The graphs present densitometric band analysis normalized to α-actin. The data represent means ± SEM of four independent experiments. *p < 0.01 versus COL IV, #p < 0.01 versus COL I. (C) Effect of AKT depletion on the COX-2 modulation induced by soluble collagens (12.5 μg/ml, 24 h). Cells were depleted of AKT with a specific siRNA, and a scrambled RNA (Sc) was used as control. Expression of COX-2 and AKT proteins was analyzed by western blot (a characteristic western blot is shown). Equal protein loading was confirmed by probing with anti-α-actin antibody. The graphs present densitometric band analysis normalized to α-actin. The data represent means ± SEM of three independent experiments. *p < 0.01 versus COL IV, #p < 0.01 versus COL I. (D) Effect of pre-incubation with the FAK inhibitor PP2 (10 μM, 1h) on the AKT phosphorylation (P-AKT) induced by soluble collagens (12.5 μg/ml, 24 h). Equal protein loading was confirmed by probing with anti-total AKT antibody. The graphs present densitometric band analysis normalized to total-AKT. The data represent means ± SEM of four independent experiments. *p < 0.01 versus COL IV, #p < 0.01 versus COL I.

Fig. 5. Role of focal adhesion kinase (FAK) and PI3K/AKT in the changes in COX-2 transcriptional activity induced by collagens. Human mesangial cells (HMC) were transiently transfected with the
human COX-2 promoter luciferase construct P2-1900 and pRL-TK-Renilla. Then, cells were pre-
incubated with the FAK inhibitor, PP2 (10 μM, 1h), the PI3K inhibitor, LY294002 (20 μM, 1h) and the
AKT inhibitor (30 μM, 1h), treated with soluble collagen I (COL I, 12.5 μg/ml) or collagen IV (COL IV,
12.5 μg/ml) for 24 hours, and assayed for luciferase and renilla activity. Luciferase activity was
normalized with renilla activity and concentration of proteins. The data represent means ± SEM of four
independent experiments (triplicates), and are expressed as fold induction with respect to control values
(untreated cells). *p < 0.01 versus COL IV, #p < 0.01 versus COL I.

Fig. 6. Analysis of the transcription factors involved in the changes in COX-2 transcriptional
activity induced by collagens. (A) Human mesangial cells (HMC) were transiently transfected with
human COX-2 promoter luciferase constructs P2-1900-LUC, P2-274-LUC, P2-192-LUC or with the P2-
192-LUC construct with the binding site for CRE mutated (P2-192 CRE-MUT) and pRL-TK-Renilla.
Then, they were treated with soluble collagen I (COL I, 12.5 μg/ml) or collagen IV (COL IV, 12.5 μg/ml)
for 24 hours, and assayed for luciferase and renilla activity. Luciferase activity was normalized with
renilla activity and concentration of proteins. The data represent means ± SEM of four independent
experiments (triplicates), and are expressed as fold induction with respect to control values (untreated
cells). *p < 0.01 versus control and COL IV. (B) HMC were treated with soluble COL I (12.5 μg/ml) and
soluble COL IV (12.5 μg/ml) for the indicated times, and CREB phosphorylation was assessed by
western blot (a representative blot is shown). Equal protein loading was confirmed by probing with anti-
α-actin. The graphs present densitometric band analysis normalized to α-actin. The data represent means
± SEM of five independent experiments. *p < 0.01 versus COL IV. (C) Effect of pre-incubation with the
FAK inhibitor PP2 (10 μM, 1h) and AKT inhibitor (30 μM, 1h) on the CREB phosphorylation induced by
soluble collagens (12.5 μg/ml, 24 h). Expression of CREB phosphorylation was assessed by western blot
(a representative blot is shown). Equal protein loading was confirmed by probing with anti- α-actin
antibody. The graphs present densitometric band analysis normalized to α-actin. The data represent means ± SEM of four independent experiments. *p < 0.01 versus COL IV, #p<0.01 versus COL I.

Fig. 7. In vivo analysis of the relationships between collagen I (COL I) and COX-2 protein content. The protein content of COX-2 and COL I were simultaneously evaluated by western blot (a representative western blot is shown in each case) in kidneys (panel A) and aortas (panel B) from male Wistar rats treated with L-NAME (20 mg/kg/day, 4 weeks). Equal protein loading was confirmed by probing with anti-α-actin antibody. In the upper part of each panel, the densitometric band analysis normalized to α-actin is given. The data represent means ± SEM of eight independent animals. *p < 0.01 versus control animals. In the lower part of the panels, the correlation between the two proteins analyzed is shown.

Fig. 8. Schematic diagram of the signaling pathways involved in collagen I-induced COX-2 expression in human mesangial cells. Collagen I increases COX-2 expression by phosphorylation and therefore, activation of focal adhesion kinase (FAK), PI3K/AKT, and CREB pathways, with the subsequently enhanced COX-2 promoter activity, leading to increased COX-2 mRNA and protein contents. Sites of action of inhibitors are shown.

Fig. 1S. Changes in COX-1 protein expression in human mesangial cells (HMC) in contact with different collagen types. (A) COX-1 protein expression in HMC grown on plates coated with collagen I (COL I) and collagen IV (COL IV), for the indicated times. Expression of COX-1 protein was analyzed by western blot (a characteristic western blot is shown). Equal protein loading was confirmed by probing with an α-actin antibody. The graphs present densitometric band analysis normalized to α-actin. The data represent means ± SEM of five independent experiments. (B) COX-1 protein expression in HMC grown on plates coated with different proportions of COLI/COL IV for 72 h. Expression of COX-1 protein was analyzed by western blot (a characteristic western blot is shown). Equal protein loading was confirmed by
probing with an α-actin antibody. The graphs present densitometric band analysis normalized to α-actin. The data represent means ± SEM of five independent experiments.

Fig. 2S. Changes in COX-2 protein expression in human mesangial cells (HMC) cultured on collagen IV (COL IV) and incubated with soluble collagen I (COL I). HMC were grown on COL IV-coated plates, and incubated with soluble COL I (12.5 μg/ml) for the indicated times. Expression of COX-2 protein was analyzed by western blot (a characteristic western blot is shown). Equal protein loading was confirmed by probing with an α-actin antibody. The graphs present densitometric band analysis normalized to α-actin. The data represent means ± SEM of five independent experiments. *p < 0.01 versus COL IV at time 0.

Fig. 3S. Changes in P-CREB transcription factor levels in human mesangial cells (HMC) in contact with different collagen types. HMC were grown on plastic and treated with soluble COL I (12.5 μg/ml) or soluble COL IV (12.5 μg/ml) for 2h and Phospho-CREB (P-CREB) was analyzed by immunofluorescence staining. Magnifications 40X. In each case a representative of four independent experiments is shown. Control: untreated cells. dBcAMP (0.1 mM, 2 hours) was used as a positive control.
Figure 1

A

<table>
<thead>
<tr>
<th></th>
<th>COL I</th>
<th>COL IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

COX-2

α-ACTIN

B

C

<table>
<thead>
<tr>
<th>% COL I</th>
<th>0</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>% COL IV</td>
<td>100</td>
<td>75</td>
<td>50</td>
<td>25</td>
<td>0</td>
</tr>
</tbody>
</table>

COX-2

α-ACTIN

D

<table>
<thead>
<tr>
<th></th>
<th>COL I</th>
<th>COL IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

COX-2

α-ACTIN
Figure 2
Figure 3

**A**

<table>
<thead>
<tr>
<th>COLLAGEN</th>
<th>I</th>
<th>IV</th>
<th>I</th>
<th>IV</th>
<th>I</th>
<th>IV</th>
<th>I</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIME (hours)</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-FAK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total FAK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>COL</th>
<th>PP2</th>
<th>COX-2</th>
<th>α-ACTIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**COX-2 expression**

<table>
<thead>
<tr>
<th>COL</th>
<th>PP2</th>
<th>COX-2 (ratio COX-2/α-actin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>PP2</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>IV</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>PP2</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

* * *
Luciferase activity (Arbitrary units)

Figure 5
Figure 6
Figure 7
COLLAGEN I

MEMBRANE

CYTOPLASM

FAK

PP2

PI3K

LY294002

AKT INHIBITOR

siRNA AKT

AKT

CREB

CRE

COX-2

PGE2

NUCLEUS

Figure 8