

Decreased Nitric Oxide Synthesis in Human Endothelial Cells Cultured on Type I Collagen

L. González-Santiago, S. López-Ongil, M. Rodríguez-Puyol, D. Rodríguez-Puyol

Abstract—Endothelial dysfunction, considered as a defective vascular dilatation after certain stimuli, is characteristic of different pathological conditions, such as hypertension, atherosclerosis, or diabetes. A decreased synthesis or an increased degradation of nitric oxide (NO) has been postulated as the mechanism responsible for this alteration. The present experiments were designed to test the hypothesis that the presence of an abnormal extracellular matrix in vessel walls could be responsible for the decreased NO synthesis observed in these pathological conditions. Experiments were performed in cultured human umbilical vein endothelial cells (HUVECs) grown on type IV (Col. IV) or type I (Col. I) collagen. Cells seeded on Col. I showed decreased nitrite synthesis, nitric oxide synthase activity, eNOS protein content, and eNOS mRNA expression when compared with cells grown on Col. IV. Moreover, cells grown on Col. I failed to respond to glucose oxidase activation of the eNOS system. In both cases, the changes in the eNOS mRNA expression seemed to depend on the modulation of eNOS promoter activity. The downregulation of eNOS induced by Col. I was blocked by D6Y, a peptide that interferes with the Col. I-dependent signals through integrins, as well as by specific anti-integrin antibodies. Moreover, a decreased activation of integrin-linked kinase (ILK) may explain the effects observed in Col. I-cultured cells because the activity of this kinase was decreased in these cells and ILK modulation prevented the Col. I-induced changes in HUVECs. Taken together, these findings may contribute to explaining the basis of endothelial dysfunction in some vascular diseases. (*Circ Res.* 2002;90:539-545.)

Key Words: endothelial dysfunction ■ nitric oxide ■ extracellular matrix ■ integrins ■ integrin-linked kinase

JOURNAL OF THE AMERICAN HEART ASSOCIATION

Vascular endothelial cells play a significant role in the regulation of multiple vascular functions, including the modulation of vascular tone and the maintenance of an equilibrium between adherence and nonadherence of circulating cells.¹ The failure in these homeostatic functions is usually referred to as endothelial dysfunction and it characterizes different pathological conditions, such as hypertension, atherosclerosis, hypercholesterolemia, or diabetes.²⁻⁵ The most widely studied characteristic of this endothelial dysfunction is a defective vascular vasodilatation after acetylcholine infusion, as a consequence of a decreased synthesis or an increased degradation of nitric oxide (NO).⁶⁻⁸

The mechanisms underlying these alterations in the NO system have been extensively studied.⁹ Alterations in the substrate for the nitric oxide synthase (NOS) enzyme, changes in endothelial NOS (eNOS) expression/structure, a defective eNOS activation, or a decreased tetrahydrobiopterin availability have been proposed as the mechanisms responsible for the impaired activation of the NO system.⁹⁻¹³ More recently, it has been demonstrated that reactive oxygen intermediates (ROI) inactivate NO, and it is now generally accepted that an increased local ROI synthesis, with the subsequent increased degradation of NO, could account for the NO deficiency observed in some clinical disorders.¹⁴⁻¹⁶

However, other possible explanations for the NO deficit have not been adequately explored. For instance, it could be proposed that the presence of an abnormal extracellular matrix (ECM) in the vessel walls could be responsible for the decreased NO synthesis detected in pathological conditions. Two arguments support this hypothesis. First, changes in the composition of the ECM are detected in vascular diseases, with an increasing proportion of abnormal collagens such as collagen I and III.¹⁷⁻¹⁹ Second, it is a well-known fact that ECM proteins regulate cell phenotype, mainly by maintaining the equilibrium between proliferation and apoptosis,²⁰⁻²² but also by modifying other aspects of cell function, such as contractility^{23,24} or even protein synthesis.²⁵ In this context, it could be proposed that other aspects of cell function, such as eNOS activity, may be linked to the presence of an abnormal ECM.

These ECM proteins interact with cells through transmembrane heterodimeric proteins called integrins that are formed by 2 subunits, α and β chains, depending on the cell type.²⁶⁻²⁸ Different combinations of integrin subunits on the cell surface allow cells to recognize and respond to a variety of matrix proteins under different physiological and pathological conditions. As a consequence of the interaction ECM

Original received August 13, 2001; revision received January 25, 2002; accepted January 25, 2002.

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DOI: 10.1161/01.RES.0000012445.68979.9D

integrins, intracellular kinases are activated and complex responses develop, inducing profound changes in cell function. The best known kinases involved in cell responses after matrix-cell interaction include a group of tyrosine kinases, such as the focal adhesion kinase (FAK), and serine-threonine kinases, such as integrin linked kinase (ILK).^{27–29}

Taking into account this evidence, our complete hypothesis was that the presence of increased amounts of abnormal collagens could induce phenotypic changes in endothelial cells, with subsequent modifications in the pattern of NO synthesis and the maintenance of endothelial dysfunction. Integrins and perhaps ILK could be involved in the genesis of this response.

Materials and Methods

Materials

M-199, fetal calf serum, trypsin-EDTA (0.02%), L-glutamine, HEPES, and antibiotics were from Bio-Whittaker (Walkersville, Md). The nitrocellulose filter and Dowex AG 50WX-8 resin (100 to 200 mesh) were from Bio-Rad. The DNA-labeling kit (Rediprime), nylon membranes (Hybond N), and deoxy-^[32P]cytidine triphosphate were from Amersham. L-^[3H]arginine (40 to 70 Ci/mmol) was from NEN. The BCA assay kit, the goat anti-mouse HRP conjugated antibody, and the Supersignal detection system were from Pierce (Rockford, Ill). X-OMAT films were from Kodak. Myelin basic protein was from Gibco. Lipofectamine reagent and OptiMem media were from Life Technologies. The following antibodies were used: anti-eNOS (monoclonal; Transduction Labs, Lexington, Ky); anti-ILK (polyclonal; Upstate Biotechnology, Lake Placid, NY); and specific anti-integrins antibodies (Dr Sanchez Madrid, Hospital de la Princesa, Madrid, Spain). D6Y peptide (Asp-Gly-Gly-Arg-Tyr-Tyr) was supplied by Neosystem (Strasbourg, France). The other reagents were from Sigma Chemical, unless otherwise specified.

Cell Culture

Human endothelial cells from the umbilical vein (HUVECs) were cultured as described.³⁰ Cells were seeded on 0.2% gelatin-coated culture plates at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Individual clones were established and subcloned to obtain pure cell populations and subsequently characterized. Cells were fed every 2 days with M-199 medium supplemented with 20% fetal calf serum, 100 U/mL penicillin, 2 mmol/L L-glutamine, 20 mmol/L HEPES, and 300 µg/mL endothelial growth factor. Cells were passaged every 5 to 7 days with trypsin-EDTA and cultured on type IV (Col. IV) or type I (Col. I) collagen-coated dishes (12.5 µg/mL) for all the experiments.²⁴ Studies were routinely performed on confluent monolayers at passages 2 to 4. Toxicity was evaluated in each experimental condition by the trypan blue dye exclusion method and LDH measurement.

Nitrite Determination

Nitrite synthesis was evaluated by a fluorometric assay.³¹ The medium used in these experiments did not contain phenol red. At the end of the incubations, cell supernatants were mixed with freshly prepared light-protected 2,3-diaminonaphthalene (0.05 mg/mL in 0.62 mol/L HCl) and incubated for 10 minutes at room temperature. The reaction was stopped with 2.8 mol/L NaOH and the fluorescent signal was measured with excitation and emission wavelengths of 365 and 450 nm, respectively. The fluorescent signal was compared with the values given by nitrite standards (purity >98%) freshly prepared and dissolved in phenol red-deficient M-199 medium.

Measurement of L-^[3H] Citrulline Formation

The assay was performed as described previously with L-^[3H]arginine purified by anion-exchange chromatography.³² Briefly, cells were washed and maintained for 3 hours in medium containing purified L-^[3H]arginine (6×10⁵ cpm/well) at 37°C. The reaction was

stopped by the addition of ice-cold trichloroacetic acid. Samples were extracted 5 times with 2 mL of water-saturated ether, vacuum lyophilized, resuspended in 2 mL of Buffer A (1 mmol/L EDTA, 20 mmol/L HEPES, pH 5.5), and applied to 2-mL wet bed volumes of Dowex AG 50WX-8 (Li⁺ form), followed by 2 mL of sterile water. L-^[3H]citrulline was quantitated in the 4-mL column effluent by using a scintillation counter. Cells with or without 500 µmol/L L-NAME were tested in parallel in each assay, and the difference between both values was considered as the specifically NOS-dependent citrulline formation. Additionally, the uptake of extracellular L-^[3H]arginine by cells grown in both collagens was determined in medium devoid of L-arginine following addition of L-^[3H]arginine (0.1 µCi/mL) for 60 minutes.³²

Western Blot Analysis of eNOS and ILK Content

HUVECs were washed and homogenized in 1 mL of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mmol/L NaCl, 1 mmol/L vanadate, 10 µg/mL leupeptin, 50 mmol/L Tris-HCl, pH 7.2) to obtain total proteins. The homogenate was centrifuged, and the supernatant was collected. The protein concentration was determined by the BCA assay. Proteins were separated on SDS-PAGE 7% gels (30 µg protein/lane) and transferred onto nitrocellulose membrane at 20 V overnight. The blots were blocked in 5% (wt/vol) nonfat dry milk in TTBS (10 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, 0.05% Tween 20) for 1 hour, incubated with 1:2500 dilution of anti-eNOS or 1:10 000 dilution of anti-ILK, washed in TTBS, and re-incubated with 200-fold-diluted goat anti-mouse HRP-conjugated antibody. Incubations with antibodies were performed at room temperature for 1 hour. The immunoreactive bands were visualized with the Supersignal detection system after exposure to X-OMAT UV film. Commercial molecular weight standards were electrophoresed in parallel.

RNA Isolation and Northern Blot

Total cellular RNA was isolated by the guanidinium lysis method. For Northern analysis, 10 µg samples of RNA were electrophoresed in 1% agarose gels and transferred to nylon filters.³³ After the filters were dried and UV cross-linked, they were blocked by incubation at 42°C in a prehybridization solution (50% formamide, 5X Denhardt's solution, 5X SSPE, 0.5% SDS, and 100 to 200 µg Salmon sperm DNA solution) and probed overnight at 42°C with ³²P-labeled probes. Filters were then washed in medium stringency conditions and exposed to X-OMAT film. The cDNA probe used in this study was human eNOS (4.1-kb fragment) from Dr S. Lamas (Consejo Superior de Investigaciones Científicas, Spain). Filters were stripped and reprobed with a house-keeping gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH, or 28S). The densitometric analysis of the films was performed with an Apple scanner and appropriate software (NIH image from the National Institutes of Health).

ILK Kinase Assays

ILK kinase activity was determined in cell extracts by *in vitro* immunoprecipitation kinase assay as described by Dr S. Dedhar.³⁴ Myelin basic protein (MBP) was used as a substrate for ILK, and phosphorylated proteins were electrophoresed on 12% SDS/PAGE gels. [³²P]-ATP was used as the phosphate donor in the kinase assay, and [³²P]-MBP was detected by autoradiography of the gels.

Transient Transfection Assays

Cells were grown on Col. I or Col. IV (60% to 70% confluent) and preincubated in OptiMem media for 15 minutes at 37°C. The cDNA plasmids (1 µg) and a plasmid containing *Renilla* luciferase (0.1 µg) were mixed with Lipofectamine (5 µL/well) and incubated for 15 minutes at 37°C. The lipid-coated DNA was then added to each well containing 2 mL of OptiMem media and incubated for 8 hours. At the end of this period, media were removed and replaced with complete media for 24 hours, after which HUVECs were lysed. Extracts were then used for measurement of the protein concentration (BCA assay) and luciferase activity, which was measured at

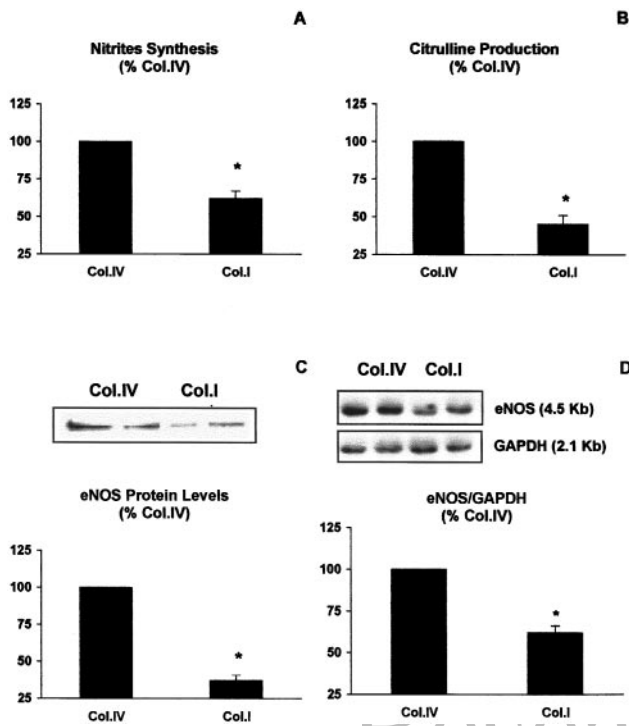


Figure 1. Effect of the presence of collagen type I on the nitric oxide system in basal conditions. HUVECs were cultured to confluence on type IV (Col. IV) or type I (Col. I) collagen and studied after 24 hours of serum deprivation. Nitrite concentrations (A), eNOS activity measured as citrulline production (B), eNOS protein content (C), and eNOS mRNA expression (D) were measured. C and D, Representative experiment and the statistical analysis of the densitometric measurements. Results are expressed as a percentage of the collagen IV values and are the mean \pm SEM of a variable number of experiments (15 for A, 6 for B, and 8 for the other panels). * $P < 0.05$ vs Col. IV.

least 3 times in duplicate using a Berthold luminometer. All data were normalized as relative light units/ μ g protein. The reporter vector pRL was used as a technical control.

The following plasmids were used: human eNOS promoter cDNA supplied by Dr C. Lowenstein (Johns Hopkins University, Baltimore, Md); and human ILK (ILK-WT) and ILK kinase-deficient form as a dominant negative mutant (DN-ILK) cDNAs provided by Dr S. Dedhar (University of British Columbia, Canada).

Statistical Analysis

The results shown are the mean \pm SEM of a variable number of experiments (see the Figure legends). Nonparametric statistics was used when $n < 10$ (the Wilcoxon test for 2 groups of values and the Friedman test for more than 2 groups). Parametric statistics was used in the other cases (the paired Student's t test for 2 groups of values or nested ANOVA for more than 2 groups), after assessing the distribution normality. A value $P < 0.05$ was considered statistically significant.

Results

HUVECs Cultured on Col. I Synthesize Less NO in Basal and Stimulated Conditions

As can be observed in Figure 1, the presence of Col. I as the culture substrate inhibits NO synthesis in basal conditions. In comparison to Col. IV, cells grown on Col. I showed a decreased synthesis of nitrites, the final product of NO degradation (Figure 1A), probably as a consequence of the

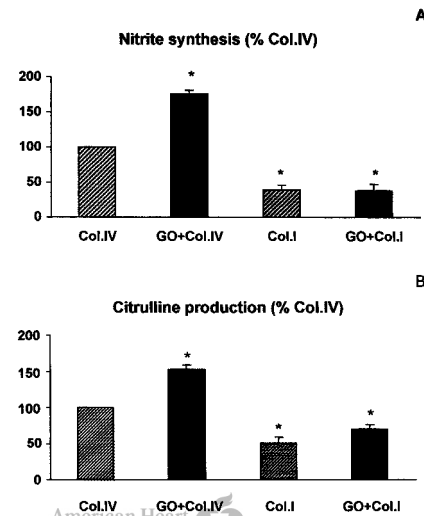


Figure 2. Effect of the presence of collagen type I on the nitric oxide system in cells incubated with glucose oxidase (GO). HUVECs were cultured to confluence on type IV (Col. IV) and type I (Col. I) collagen and incubated for 24 hours with GO (1 mU/mL) in the absence of serum. Nitrite concentrations (A) and eNOS activity measured as citrulline production (B) were measured. Results are expressed as a percentage of the collagen IV values and are the mean \pm SEM of a variable number of experiments (15 for A and 6 for B). * $P < 0.05$ vs Col. IV.

reduction in eNOS activity, measured as citrulline production (Figure 1B). No statistically significant differences in arginine uptake were detected in cells grown on both collagens (Col. IV: $3.3 \pm 0.2 \times 10^4$ cpm/well; Col. I: $3.7 \pm 0.3 \times 10^4$ cpm/well; $n = 3$). Similar results were obtained when eNOS protein content and its mRNA expression were tested: the protein and the mRNA levels were significantly lower when the culture substrate was Col. I (Figures 1C and 1D, respectively). Cell density, measured as protein content, was similar with both types of collagen.

Although these results were obtained in confluent HUVECs after 24 hours of incubation in serum-free medium, the decreased nitrite synthesis appeared as early as 24 hours of cell contact with ECM proteins (Col. IV: 100%; Col. I: $68 \pm 2\%$; $n = 8$, $P < 0.05$). Also in this case, cell density was comparable with both collagen types (protein measurement).

Comparable results to those obtained in basal conditions were obtained after cell stimulation by reactive oxygen species. Glucose oxidase (GO), a well-known hydrogen peroxide-generating system, induced a significant increase in nitrite synthesis (Figure 2A), as well as a significantly higher citrulline production (Figure 2B) in cells grown on Col. IV, whereas GO failed to stimulate NO synthesis in Col. I-grown cells (Figures 2A and 2B). Parallel changes were observed in eNOS mRNA expression, which was stimulated by GO when the culture was performed on Col. IV, whereas no effect on the enzyme expression was observed in Col. I-grown cells (Figure 3). These differences cannot be attributed to different antioxidant properties of the cells grown on each matrix, as cell processing of exogenous hydrogen peroxide was comparable in both cases (data not shown). GO did not elicit significant cell toxicity, as evaluated by the trypan blue exclusion method and LDH measurement.

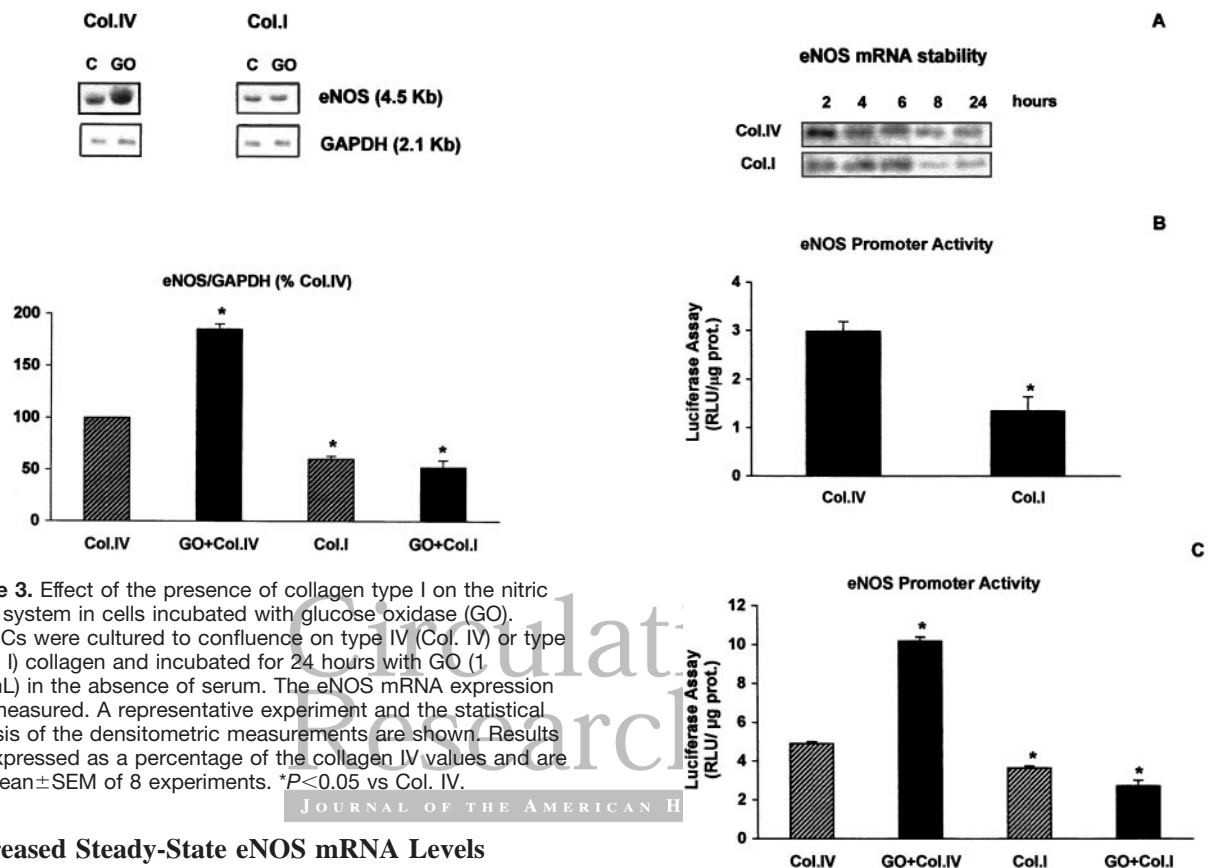


Figure 3. Effect of the presence of collagen type I on the nitric oxide system in cells incubated with glucose oxidase (GO). HUVECs were cultured to confluence on type IV (Col. IV) or type I (Col. I) collagen and incubated for 24 hours with GO (1 mU/mL) in the absence of serum. The eNOS mRNA expression was measured. A representative experiment and the statistical analysis of the densitometric measurements are shown. Results are expressed as a percentage of the collagen IV values and are the mean \pm SEM of 8 experiments. * $P<0.05$ vs Col. IV.

Decreased Steady-State eNOS mRNA Levels Detected in HUVECs Grown on Col. I Is Consequence of Decreased Promoter Activity

In order to analyze the origin of the changes in the mRNA expression detected in the presence of Col. I, gene expression was studied in cells treated with actinomycin. As shown in Figure 4A, the Col. I-induced alterations in eNOS expression were not due to changes in the mRNA stability. In contrast, the eNOS promoter activity was decreased in HUVECs grown on Col. I when compared with cells grown on Col. IV (Figure 4B). The lack of stimulation of eNOS mRNA expression observed in Col. I-grown cells treated with GO was also mainly due to the absence of response of the eNOS promoter (Figure 4C).

Interaction of Col. I With Integrins Seems to Play a Significant Role in the Genesis of the Changes Observed in Nitrite Synthesis in Basal Conditions

In order to test the possible role played by the interaction of Col. I with specific membrane proteins of the integrin family on the changes observed in nitrite synthesis, Asp-Gly-Gly-Arg-Tyr-Tyr (D6Y), an oligopeptide that blocks Col. I-dependent effects, but not adherence, in some cell types,³⁵ was used. Results are included in the Figure 5. Incubation with D6Y completely abolished the decreased NO synthesis elicited by Col. I (Figure 5A). In accordance, the reduction in the eNOS mRNA expression induced by Col. I was also inhibited by the incubation with D6Y (Figure 5B). To better analyze the role of integrins, cells were incubated with some anti-integrin antibodies and nitrite synthesis was measured. Results are shown in Figure 6: both the anti- α_1 and anti- β_1

Figure 4. Mechanisms involved in the effect of type I collagen on the eNOS mRNA expression. A, Stability of eNOS mRNA. HUVECs were cultured to confluence on type IV (Col. IV) or type I (Col. I) collagen and incubated with actinomycin D for variable periods of time (2 to 24 hours). eNOS mRNA expression was measured by Northern blot. A representative experiment is shown (n=3). B, eNOS promoter activity in basal conditions. HUVECs were cultured on type IV (Col. IV) or type I (Col. I) collagen and transfected as described in Materials and Methods (eNOS promoter-luciferase, *Renilla*-luciferase). Results are expressed as relative light units/ μ g protein and are the mean \pm SEM of 6 experiments. * $P<0.05$ vs Col. IV. C, eNOS promoter activity after glucose oxidase (GO) treatment. HUVECs were cultured on type IV (Col. IV) or type I (Col. I) collagen, transfected as described in Materials and Methods (eNOS promoter-luciferase, *Renilla*-luciferase), and incubated with GO (1 mU/mL). Results are expressed as relative light units/ μ g protein and are the mean \pm SEM of 6 experiments. * $P<0.05$ vs Col. IV.

antibodies prevented the effect observed in the presence of Col. I.

ILK Seems to Play a Significant Role in the Decreased NO Synthesis Observed in Cells Grown on Col. I

In order to evaluate the importance of ILK as a mediator of the decreased NO synthesis observed in cells cultured on Col. I, ILK kinase activity in these cells was measured. The results are included in Figure 7A. ILK kinase activity was significantly decreased in cells grown on Col. I, an effect that was not due to a decreased protein content. The inhibitory effect of an anti- β_1 integrin antibody is also shown in this same panel. The link between the decreased ILK kinase activity

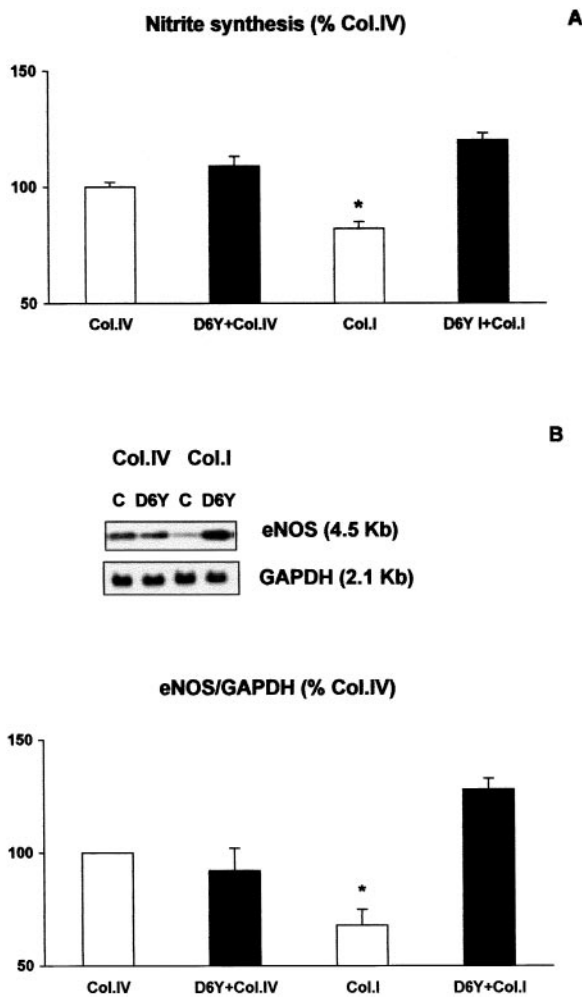


Figure 5. Modulation of collagen I-induced effects on the NO system in basal conditions by a peptide that blocks the interaction of collagen type I with integrins (D6Y). HUVECs were cultured to confluence on type IV (Col. IV) or type I (Col. I) collagen and incubated for 24 hours with 50 μ mol/L D6Y (Asp-Gly-Gly-Arg-Tyr-Tyr). Nitrite concentrations (A) and eNOS mRNA expression (B) were measured. B, Representative experiment and the statistical analysis of the densitometric measurements. Results are expressed as a percentage of the collagen IV values and are the mean \pm SEM of a variable number of experiments (15 for A and 8 for B). * $P < 0.05$ vs the other groups.

and the reduced activity of the eNOS promoter was demonstrated by double transfection experiments. As shown in Figure 7B, cell transfection with wild-type ILK prevented the decreased eNOS promoter activity induced by Col. I culture, whereas cell transfection of Col. IV-grown cells with a dominant negative ILK elicited an inhibitory effect comparable to that of Col. I. Both transfections significantly modulated the measured ILK kinase activity (Figure 7B, bottom half).

Discussion

The present experiments demonstrate a decreased activity of the eNOS system in cells cultured on Col. I with respect to those seeded on Col. IV. In fact, these cells express less eNOS mRNA, show decreased levels of the protein and a reduction in its activity, and synthesize less nitrites, the stable

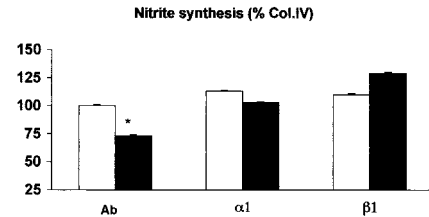


Figure 6. Modulation of collagen I-induced effects on nitrite synthesis in basal conditions by anti-integrin antibodies. HUVECs were cultured to confluence on type IV (open bars) or type I (closed bars) collagen and incubated for 24 hours with specific anti- $\alpha 1$ and - $\beta 1$ integrin antibodies. Nitrite concentrations were measured. Data from cells incubated with nonimmune IgG (Ab) are provided as controls (they were comparable to basal data). Results are the mean \pm SEM of 8 experiments. * $P < 0.05$ vs the other groups.

metabolite of NO. Considering these findings together, and taking into account that the quantitative differences between the parameters tested were comparable, it can be suggested that both collagens induce different steady-state eNOS mRNA levels, with the subsequent changes in protein content and enzyme activity. Differences in cell confluence have been reported as causing changes in eNOS mRNA expression,³⁶ but cell confluence was similar with both collagen types. Moreover, no significant cell toxicity was detectable in the cells.

Additional information may be obtained from the above mentioned results. First, the differences observed in the eNOS system in the culture conditions selected appeared after 24 hours of cell contact with the substrate and remained for a long period of time. Thus, they were not a transient phenomenon disappearing with time. Second, they took place not only in basal conditions but also after stimulation. Although eNOS is constitutively expressed, both in vivo and in vitro studies have made it clear that its expression is subject to modest, but probably important, degrees of regulation.⁹ Hydrogen peroxide is one of the factors that upregulates eNOS mRNA expression,³⁷ and GO failed to elicit this effect in Col. I-seeded cells. Third, the differences in the steady-state mRNA levels in both collagen cultures, in basal conditions as well as after GO treatment, seem to be the consequence of changes in the promoter activity of the gene encoding for eNOS and not in its stability.

These experimental findings support the initial hypothesis. As a consequence of the presence of increasing amounts of abnormal collagen in the vessel walls in some pathological situations, endothelial cells interact not only with the normal Col. IV of the basal membrane, but also with other newly synthesized collagen types. A progressive and sustained downregulation of the eNOS promoter activity takes place, and endothelial cells form less amounts of NO in basal conditions and after stimuli. However, this in vivo extrapolation of the in vitro data requires specific experiments for confirmation. Additionally, other questions such as the pattern of response of eNOS to acute stimuli, like acetylcholine, in cells grown on both collagen types must be answered in forthcoming experimental designs.

The mechanisms involved in the differential response to both collagens have been explored at 2 levels. First, the

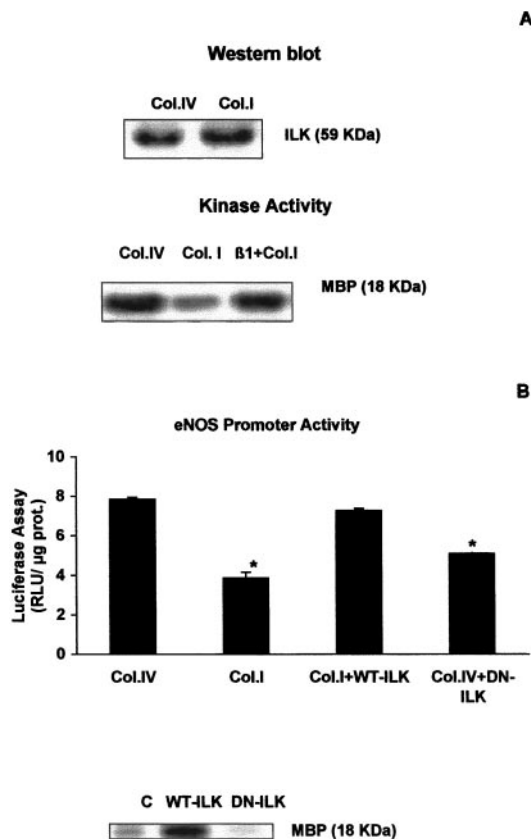


Figure 7. Role of integrin-linked kinase (ILK) on the NO system in basal conditions. A, ILK activity. HUVECs were cultured to confluence on type IV (Col. IV) or type I (Col. I) collagen and ILK content (top half) and activity (bottom half) were measured after 24 hours (myelin basic protein, MBP, was used as the kinase substrate). A representative experiment ($n=4$), including the effect of a specific anti- β_1 antibody, is shown. B, eNOS promoter activity after ILK modulation. HUVECs were cultured on type IV (Col. IV) or type I (Col. I) collagen, and transfected as described in Materials and Methods. Plasmids containing eNOS promoter-luciferase and *Renilla*-luciferase constructions were used as reporters. Vector plasmids (control) and plasmids containing ILK (WT-ILK) or a kinase-deficient ILK (DN-ILK) were used to modulate the activity of ILK (see bottom half of this panel). Results (top half of the panel) are expressed as relative light units/ μ g protein and are the mean \pm SEM of 6 experiments. * $P<0.05$ vs the other groups.

possible involvement of integrins has been tested. Second, the importance of ILK has been analyzed. Integrins act as membrane receptors to specific peptidic domains of extracellular matrix proteins.^{26–28} Two of the members of the integrin family named $\alpha_1\beta_1$ and $\alpha_2\beta_1$ act as receptors for collagens and are present in endothelial cells.³⁸ Two strategies were selected to evaluate the role of integrins in the genesis of the Col. I-dependent changes in the eNOS system. First, cells were incubated with D6Y. A previous report has demonstrated that this peptide blocks the Col. I-dependent activation in some cell types, without modifying cell adhesion.³⁵ Second, adherent cells were treated with specific anti- α_1 and anti- β_1 antibodies. In both cases, no significant cell detachment occurred but the Col. I-dependent inhibition of NO synthesis was prevented. These results support the role of integrins in the transmission of the message generated by Col. I. How-

ever, a specific experimental design to assess the relative importance of the different integrins was not performed.

In the last years, an increasing amount of data has emerged that supports the role of ILK in the intracellular signal system coupled to integrin activation.^{38–40} To evaluate a possible role for this protein in the differences detected between both collagens, we tested (1) the presence of ILK in HUVECs, (2) the activity of this protein in cells grown on Col. IV or Col. I, and (3) the modifications of eNOS promoter activity after modulation of ILK. Col. I-cultured cells showed a decreased ILK activity that was reversed by anti- β_1 antibody, when compared with cells grown on Col. IV. This finding suggests that the decreased ILK activity could be linked to the decreased eNOS promoter activity previously described, but it does not demonstrate a cause-effect relationship between both phenomena. Transfections with wild-type and kinase-deficient ILK confirmed this relationship. By diminishing ILK activity in cells grown on Col. IV, the kinase-deficient ILK transfection induced a reduction in eNOS promoter activity. On the other hand, by increasing ILK activity, wild-type ILK transfection abolished the inhibitory effect of Col. I on eNOS promoter activity. These results strongly suggest that a sustained downregulation of ILK activity could account for the decreased eNOS mRNA expression observed in cells cultured on Col. I.

Future studies must evaluate the mechanisms responsible for the ILK-dependent modulation of the eNOS promoter activity. Interestingly, a link between ILK and AP-1 has been recently described,^{39,40} and this transcription factor seems to play a significant role in the modulation of eNOS mRNA expression by different stimuli.^{37,41}

In summary, HUVECs cultured on Col. I synthesize less NO, probably as a consequence of a decreased expression of eNOS mRNA, when compared with Col. IV-grown cells. This effect is observed not only in basal conditions but also after GO stimulation. The decreased synthesis observed in basal conditions seems to depend on integrin-linked signals, probably through the modulation of ILK activity. The pathological relevance of this finding, which could contribute to explaining the basis of endothelial dysfunction in some vascular diseases, must be assessed in forthcoming *in vivo* experimental designs.

Acknowledgments

This work was supported by the Comisión Interministerial de Ciencia y Tecnología (CICYT), SAF1998-0054, and by the Ministerio de Ciencia y Tecnología (MCYT), SAF2001-0395. Laura González-Santiago was funded by the Ministerio de Educación y Cultura (MEC) and Susana López-Ongil by the Comunidad Autónoma de Madrid (CAM).

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