ANG II increases TIMP-1 expression in rat aortic smooth muscle cells in vivo

GIOVANNA CASTOLDI,1,8 CIRA R. T. DI GIOIA,2* FEDERICO PIERUZZI,1 CRISTINA D’ORLANDO,1 WILLY M. M. VAN DE GREEF,3 GIUSEPPE BUSCA,1 GIOVANNI SPERTI,3 AND ANDREA STELLA1

1Unità Didattico Assistenziale Nefrocardiovascolare, Dipartimento di Medicina Clinica, Prevenzione e Biotecnologie Sanitarie, Università degli Studi di Milano-Bicocca 20052, Monza and Centro di Fisiologia Clinica e Iper tensione, Ospedale Maggiore, Instituto di Ricovero e Cura a Carattere Scienti, 20100 Milan; 2Dipartimento di Medicina Sperimentale e Patologia, Istituto di Anatomia Patologica, Università La Sapienza, 00161 Rome; and 3Istituto di Cardiologia, Università Cattolica, 00168 Rome, Italy

Submitted 12 November 2001; accepted in final form 30 September 2002

Castoldi, Giovanna, Cira R. T. di Gioia, Federico Pieruzzi, Cristina D’Orlando, Willy M. M. van de Greef, Giuseppe Busca, Giovanni Sperti, and Andrea Stella. ANG II increases TIMP-1 expression in rat aortic smooth muscle cells in vivo. Am J Physiol Heart Circ Physiol 284: H635–H643, 2003. First published October 10, 2002; 10.1152/ajpheart.00986.2001.—Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are involved in tissue remodeling processes. TIMP-1 is the main native inhibitor of MMPs and it contributes to the development of tissue fibrosis. It is known that ANG II plays a fundamental role in vascular remodeling. In this study, we investigated whether ANG II modulates TIMP-1 expression in rat aortic smooth muscle cells. In vitro, ANG II induces TIMP-1 mRNA expression in a dose-dependent manner. The maximal increase in TIMP-1 expression was present after 3 h of ANG II stimulation. The ANG II increase in TIMP-1 expression was mediated by the ANG type 1 receptors because it was blocked by losartan. The increase in TIMP-1 expression was present after the first ANG II treatment, whereas repeated treatments (3 and 5 times) did not modify TIMP-1 expression. In vivo, exogenous ANG II was administered to Sprague-Dawley rats (200 ng·kg⁻¹·min⁻¹·sc) for 6 and 25 days. Control rats received physiological saline. After treatment, systolic blood pressure was significantly higher (P < 0.01), whereas plasma renin activity was suppressed (P < 0.01), in ANG II-treated rats. ANG II increased TIMP-1 expression in the aorta of ANG II-treated rats both at the mRNA (P < 0.05) and protein levels as evaluated by Western blotting (P < 0.05) and/or immunohistochemistry. Neither histological modifications at the vascular wall nor differences in collagen content in the tunica media were present in both the ANG II- and saline-treated groups. Our data demonstrate that ANG II increases TIMP-1 expression in rat aortic smooth muscle cells. In vivo, both short- and long-term chronic ANG II treatments increase TIMP-1 expression in the rat aorta. TIMP-1 induction by ANG II in aortic smooth muscle cells occurs in the absence of histological changes at the vascular wall.

VASCULAR SMOOTH MUSCLE CELLS (VSMC) are involved in the pathogenesis of atherosclerosis, restenosis, and hypertension. In vivo, VSMC are surrounded by extracellular matrix proteins; in the normal vascular wall, they remain in a contractile state, whereas in response to injury they can assume a synthetic phenotype. VSMC may synthetize collagen, matrix proteins, and enzymes involved in extracellular matrix degradation, such as plasminogen activators and metalloproteinases and their inhibitors (19, 22, 26).

ANG II has multiple effects on VSMC. It increases matrix protein synthesis and has profibrotic effects (14, 15). In addition, in experimental models (9, 23), ANG II may modulate the expression of the matrix metalloproteinases (MMPs), the main extracellular matrix degradation enzymes, whose activity is in turn tightly regulated by endogenous tissue inhibitors.

To date, little is known about the effects of ANG II on the endogenous tissue inhibitors of the MMP system (TIMPs) that is considered one of the major factors involved in tissue remodeling and in the development of tissue fibrosis (1, 28). Four types of TIMPs have been identified (2, 12). Among these, TIMP-1 is produced by different cell types, including most types of connective tissue cells and those involved in the inflammatory processes (18). It has been shown that TIMP-1 inhibits with different affinity all members of the collagenases,stromelysins, and gelatinases (8). In the tunica media, the constitutive expression of TIMP-1 plays an important role in vessel wall homeostasis (21). Overexpression of TIMP-1 retards vascular cell migration in the injured rat carotid artery (10). Besides the inhibition on matrix metalloproteinases, TIMP-1 has also some

*G. Castoldi and C. R. T. di Gioia contributed equally to this study.
Address for reprint requests and other correspondence: A. Stella, Università degli Studi di Milano-Bicocca, Dipartimento di Medicina Clinica, Prevenzione e Biotecnologie Sanitarie, Azienda Ospedaliera S. Gerardo di Monza, UDA Nefrocardiovascolare, Via Donizetti 106, 20052 Monza (MI), Italy (E-mail: andrea.stella@unimib.it).

http://www.ajpheart.org 0363-6135/03 $5.00 Copyright © 2003 the American Physiological Society

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MMP inhibitory-independent effects. In some cell types, as erythroid progenitors (4), TIMP-1 shows growth factor-like effects. In addition, a role of TIMP-1 in modulation of apoptosis processes in B cells (13) and mesangial cells (16) has been recently described.

It has been demonstrated that ANG II increases TIMP-1 expression in rat heart endothelial cells in culture (6), whereas the effect in vivo of ANG II on TIMP-1 expression in VSMC, which is the major source of extracellular matrix components implicated in the vascular remodeling process, is unknown.

Our experiments were done to verify in vitro whether ANG II modulates TIMP-1 mRNA expression in rat aortic smooth muscle cells and to investigate in vivo the effect of chronic ANG II administration on TIMP-1 expression in rat aortic smooth muscle cells in conscious rats.

Fig. 1. A: time course of ANG II-induced increase in tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) mRNA expression [mRNA TIMP-1/GAPDH, in arbitrary units (au)] in cultured rat aortic smooth muscle cells. Bar graphs indicate the means ± SE calculated on four separate experiments after quantification of the Northern blot signal. Inset: example of Northern blot showing ANG II-induced (5 × 10⁻⁶ M) TIMP-1 mRNA expression at 1, 3, 8, and 24 h and the corresponding serum-free (SF) conditions. *P < 0.05 vs. SF. B: dose-response relationship of the ANG II-induced increase in TIMP-1 mRNA expression (mRNA TIMP-1/GAPDH, au) in cultured rat aortic smooth muscle cells. Inset: example of Northern blot showing the increase in TIMP-1 mRNA expression after a 3-h treatment with increasing doses of ANG II in cultured rat aortic smooth muscle cells. Data are means ± SE calculated on three separate experiments. *P < 0.05 vs. SF.

Fig. 2. A: effect of the ANG type 1 (AT₁) receptor blocker losartan (Los) on ANG II-induced increase in TIMP-1 mRNA expression (TIMP-1/GAPDH mRNA, au). Bar graphs indicate the means ± SE calculated on three separate experiments after the quantification of the Northern signal. Inset: example of Northern blot showing the effect of the AT₁ receptor blocker losartan, on ANG II-induced TIMP-1 mRNA increase after a 3-h treatment in cultured rat aortic smooth muscle cells. *P < 0.01 vs. SF; †P < 0.01 vs. ANG II. B: effect of ANG II in cultured rat aortic smooth muscle cells after 1, 3, and 5 repeated treatments on TIMP-1 mRNA expression (TIMP-1/GAPDH mRNA, au). Bar graphs indicate the means ± SE calculated on three separate experiments after the quantification of the Northern signal. Inset: example of Northern blot showing the effect of the ANG II after one, three, and five repeated treatments in cultured rat aortic smooth muscle cells. *P < 0.01 vs. SF.
ANG II (5–10 M) or the same volume of vehicle was added to control cultures. In ANG type 1 (AT 1) receptor blocker experiments, losartan (1 × 10⁻⁶ M) was added to ANG II (5 × 10⁻⁸ M) at the same time, and the cells were incubated for 3 h. To evaluate the effect of repeated ANG II treatments on TIMP-1 expression, cells were treated one, three, and five consecutive times with ANG II (5 × 10⁻⁸ M) for 4 h of treatment.

In Vivo Experiments

The experiments were performed in 49 conscious male 12-wk-old Sprague-Dawley rats [200–250 g body wt (BW)]. Animals were individually housed in metabolic cages in a temperature-controlled room with a 12:12-h light-dark cycle for the whole experimental periods and allowed to acclimate to the metabolic cages and the experimental procedures. Rats had free access to a standard rat chow and tap water. Systolic blood pressure (SBP, mmHg), heart rate (HR, beats/min), and BW (g) were measured three times a week by the same investigator who was unaware of the specific treatment. SBP and HR were assessed by the tail-cuff method (average of 6 recordings).

To evaluate the effect of the exogenous administration of ANG II, rats were subcutaneously implanted with osmotic minipumps under pentobarbital sodium anesthesia (40 mg/kg ip) to receive either ANG II at the dose of 200 ng·kg⁻¹·min⁻¹ (treated groups) or physiological saline (control groups).

One group of 23 rats (n = 11, ANG II treated; n = 12, saline treated) underwent this protocol, and osmotic minipumps (Alzet 2001; Alzet, Palo Alto, CA) delivered ANG II or physiological saline for 6 days. At the end of this period, the rats were euthanized.

Another group of 12 rats (n = 6, ANG II treated; n = 6, saline treated) underwent the same protocol. The osmotic minipumps (Alzet 2001) delivered ANG II or saline for 6 days, but the animals were euthanized 3 wk after the ANG II withdrawal.

To evaluate the effect of longer ANG II infusion, a group of 14 rats (n = 8, ANG II treated; n = 6, saline treated) were subcutaneously implanted with osmotic minipumps (Alzet 2004) to receive ANG II or physiological saline for 25 days. The rats were euthanized at the end of this period.
At the end of the experimental periods, all rats were decapitated and trunk blood was collected to measure plasma renin activity (PRA; AI ng·ml⁻¹·h⁻¹) by radioimmunoassay. Aortas were immediately excised. For gene and protein expression studies, the media were separated from the adventitia and endothelium, snap-frozen in liquid nitrogen, and stored at −80°C. Aortic TIMP-1 mRNA expression was evaluated by Northern blot analysis and the TIMP-1 protein level was evaluated by Western blotting and/or immunohistochemistry. An aortic ring was fixed with 10% formalin for histological and morphometric analysis. For immunohistochemical study, an aortic ring was put in a cryomold with OCT compound (Tissue-Tek, Sakura; Zoeterwoude, The Netherlands). The rings were snap-frozen first in isopentane that was previously chilled in liquid nitrogen and again in liquid nitrogen and stored at −80°C until analysis.

Total RNA Extraction and Northern Blot Analysis

Total RNA was extracted from cell cultures and from each aorta according to the guanidium-thiocyanate method used by Chomczynski and Sacchi (5). RNA was quantified on a spectrophotometer determining absorbance at 260 nm, and its integrity was confirmed on an agarose gel stained with ethidium bromide. For Northern blot analyses, 20 μg of RNA were loaded on formaldehyde-agarose (1%) gel, separated by electrophoresis (20), and vacuum blotted (VacuGene, Pharmacia; Uppsala, Sweden) onto a nylon membrane (GeneBind 45, Pharmacia). Blots were hybridized with ³²P-labeled rat TIMP-1 cDNA probe (kind gift from Dr. L. Schaefer, University of Muenster, Muenster, Germany) in 0.5 M sodium phosphate and 7% SDS at 60°C and washed twice at 60°C in 2× sodium chloride/sodium citrate and 1% SDS. The same blots were hybridized with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. Blots were exposed to an Instant Imager (Canberra Packard Electronic Autoradiography Instrument) for direct acquisition of data. TIMP-1 expression was normalized to the housekeeping GAPDH gene expression and was reported in arbitrary units (au).

Protein Extraction and Western Analysis

Total proteins were extracted from aortic tissues and homogenized in the presence of EDTA and protease inhibitors (10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mg/ml antipain). Protein samples were then separated by 12% SDS-PAGE and transferred onto Hybond-ECL membranes (Amersham). These were saturated with 5% nonfat milk in PBS and 0.1% Tween 20 for 1 h at room temperature and then incubated with a mouse anti-human TIMP-1 monoclonal antibody (dilution 1:2,000) (rabbit α-actin antibody, Sigma) was used tonormalize the immunoreactivity of TIMP-1 in each sample. Densitometric analysis of signals was performed with an Imaging Densitom...
eter (Bio-Rad). Data are expressed (in au) as the TIMP-1-to-α-actin ratio.

**Histological, Morphometric, and Immunohistochemical Analysis**

For histomorphology, aortic samples were fixed with 10% neutral-buffered formalin and subsequently processed and embedded in paraffin wax. Aortic histological sections (5 μm thick) were cut, stained with routine hematoxylin and eosin stain, and studied with light microscopy (Leica).

Collagen deposition in the tunica media was quantified microscopically using a computerized imaging analysis system (IAS 2000, Delta Sistemi; Rome, Italy) from images taken at ×40 magnification (Leica Light Microscopy) by paraffin-embedded aortic sections stained with Sirius red. Collagen deposition in the tunica media was calculated as follows: area of medial collagen content/area of tunica media, and the value was expressed as a percentage. For immunohistochemistry, aortic samples in OCT compound were snap-frozen in isopentane previously chilled in liquid nitrogen and then in liquid nitrogen. The specimens were stored at −80°C until analysis. Four-micrometer-thick sections were treated with 3% hydrogen peroxide for 20 min and then incubated for 30 min at room temperature with a mouse anti-human TIMP-1 monoclonal antibody (dilution 1:50) (Chemicon). The avidin-biotin peroxidase complex was used to label the primary antibody. The reaction product was detected using 3,3-diaminobenzidine. Control experiments were accomplished by omitting the primary antibody.

**Statistical Analysis**

Data are presented as means ± SE. Data from the cell experiments were assessed with the use of factorial analysis of variance, followed by the Fisher’s protected least-significant difference procedure for post hoc comparisons. Differences between ANG II- and saline-treated groups for SBP, HR, BW, PRA, TIMP-1 mRNA and protein expression, and collagen content in the tunica media were analyzed by unpaired Student’s t-test. Differences between means were considered significant at $P < 0.05$.

**RESULTS**

**In Vitro Experiments**

In quiescent VSMC, ANG II (5 × 10⁻⁸ M) induced a time-dependent increase in TIMP-1 mRNA expression. A significant increase in the TIMP-1 mRNA signal compared with the unstimulated control cultures was present at 1 h ($P < 0.05$, $n = 4$), maximal at 3 h ($P < 0.01$, $n = 4$), remained over the control SF values at 8 h, and returned to control SF values at 24 h (Fig. 1A).

ANG II induced a dose-dependent increase in TIMP-1 mRNA expression (Fig. 1B), with a maximal effect at a concentration of 5 × 10⁻⁹ M ($P < 0.05$, $n = 3$). The block of AT₁ receptors with losartan at a concentration of 1 × 10⁻⁶ M abolished the ANG II-induced increase in TIMP-1 mRNA expression ($P < 0.01$, $n = 3$) (Fig. 2A).

Repeated treatments to the cells with ANG II did not induce TIMP-1 mRNA expression. In fact, ANG II treatment determined an increase in TIMP-1 mRNA expression only after the single dose ($P < 0.05$, $n = 3$) but not after three and five repeated treatments (Fig. 2B).

**In Vivo Experiments**

**Effects of chronic short-term ANG II infusion.** Figure 3 shows the in vivo effects of chronic short-term ANG II administration on SBP, PRA, and TIMP-1 expression. After 6 days of treatment, SBP was significantly higher in the ANG II-treated rats compared with the corresponding control animals. PRA in ANG II-treated animals for 6 days was significantly and markedly lower than PRA of saline-treated rats, indicating that ANG II administration, by increasing the circulating level of ANG II, was effective in suppressing renin release. TIMP-1 mRNA expression resulted significantly higher in ANG II-treated rats than in saline-treated rats. The increase in TIMP-1 mRNA expression was accompanied by a significant increase in the TIMP-1 mRNA expression only after the single dose ($P < 0.05$, $n = 4$) but not after three and five repeated treatments (Fig. 3).

**Effects obtained 3 wk after the withdrawal of ANG II administration (6 days) in rats. A: SBP (mmHg); B: PRA (ANG I ng·ml⁻¹·h⁻¹); C: TIMP-1 mRNA expression (TIMP-1/GAPDH mRNA, au) in saline ($n = 6$) and ANG II-treated rats ($n = 6$). Inset: example of Northern blot showing TIMP-1 mRNA expression in both groups of rats. Data are means ± SE.**
protein level (Fig. 3B), indicating that in the aortic smooth muscle cells the ANG II induction in TIMP-1 mRNA caused an increase in its transduction into the protein.

At the end of the treatment period, BW and HR in the saline-treated group were higher than in ANG II-treated group (302.3 ± 4.4 vs. 283.7 ± 4.0 g, P < 0.01; 412.9 ± 9.2 vs. 380.7 ± 14.1 beats/min, P = 0.06, respectively).

The increase in TIMP-1 protein expression in aortas of ANG II-treated rats was also demonstrated by immunohistochemistry (Fig. 4A). In ANG II-treated rats, aortic smooth muscle cells showed a stronger immunostaining of TIMP-1 than aortic smooth muscle cells of saline-treated rats. As shown in Fig. 4B, the administration of ANG II caused no histological modification at the vascular wall. No differences in collagen content (Fig. 4C), calculated as the fibrosis percentage in the tunica media, were present between saline- and ANG II-treated rats (%fibrosis: 13.1 ± 1.4 vs. 13.4 ± 1.2, P = 0.88).

Effects of ANG II infusion withdrawal. Figure 5 shows the results obtained 3 wk after the withdrawal of ANG II administration (6 days) on SBP, PRA, and TIMP-1 mRNA expression. As expected, after 6 days of ANG II infusion, the increase in blood pressure was similar to that observed in both ANG II groups treated for 6 and 25 days (data not shown); then blood pressure, after ANG II withdrawal, progressively decreased to values similar to the corresponding saline-treated group. At the end of the treatment period, BW and HR were similar in the saline- and ANG II-treated groups (408.0 ± 9.4 vs. 413.0 ± 14.6 g, P = 0.78; 401.7 ± 14.1 vs. 435.7 ± 20.0 beats/min, P = 0.19, respectively).

Immunohistochemistry demonstrated no differences in TIMP-1 protein expression in aortas between saline- and ANG II-treated rats after ANG II withdrawal (Fig. 6A). Neither histological modification (Fig. 6B) nor differences in collagen content (Fig. 6C) were observed between saline- and ANG II-treated rats after ANG II withdrawal (%fibrosis: 19.1 ± 1.6 vs. 15.0 ± 2, P = 0.13).

Effects of chronic long-term ANG II infusion. Figure 7 shows the in vivo effects of chronic long-term ANG II administration on SBP, PRA, and TIMP-1 mRNA ex-
expression. As expected, after 25 days of treatment, SBP was significantly higher in the ANG II-treated rats compared with the corresponding control animals, whereas PRA in ANG II-treated animals was significantly and markedly lower than PRA of saline-treated rats. TIMP-1 mRNA expression resulted significantly higher in ANG II- than saline-treated rats.

At the end of the treatment period, BW and HR in the saline and ANG II-treated groups were similar (391.3 ± 9.3 vs. 391.8 ± 15.3 g, P = 0.98; 417.2 ± 9.9 vs. 414.9 ± 22.8 beats/min, P = 0.93, respectively).

Immunohistochemistry demonstrated an increase in TIMP-1 protein expression in aortas of ANG II-treated rats (Fig. 8A). In ANG II-treated rats, aortic smooth muscle cells showed a stronger immunostaining of TIMP-1 than aortic smooth muscle cells of saline-treated rats. The administration of ANG II did not cause any histological modification at the vascular wall (Fig. 8B). No differences in collagen content were present between saline- and ANG II-treated rats (%fibrosis: 17.2 ± 1.3 vs. 15.0 ± 1.3, P = 0.12) (Fig. 8C).

DISCUSSION

Our data demonstrate that ANG II increases TIMP-1 expression in rat aortic smooth muscle cells.

In vitro, the ANG II-induced increase in TIMP-1 mRNA expression in VSMC is time and dose dependent, and it is mediated by AT₁ receptors because losartan blocks the increase in TIMP-1 expression in response to ANG II stimulation.

Repeated ANG II treatments to the cells in culture demonstrate that this increase occurs after a single stimulation but not after repeated ANG II treatments. These results may be explained by the downregulation of ANG II responsiveness to repeated applications of ANG II in VSMC. In these conditions, it has been described that ANG II downregulates its receptors, and VSMC develop an attenuation of responsiveness to ANG II through complex intracellular mechanisms implying G protein signaling pathways (24).

Our in vivo data demonstrate that short- (6 days) and long-term (25 days) chronic ANG II administrations increase TIMP-1 expression in rat aortic smooth muscle cells both at the mRNA and protein levels. The results obtained by the immunohistochemical staining demonstrate that the increase in TIMP-1 expression is mainly located in the cytoplasm of smooth muscle cells in the aorta of ANG II-treated rats. In vivo the modulation of TIMP-1 expression in the rat aorta was time related to ANG II administration. In fact, ANG II administration for 6 days induced TIMP-1 expression and this increase was evident even after a longer ANG II administration (25 days). In addition, the evidence that TIMP-1 expression was not increased after ANG II withdrawal further demonstrates the role of ANG II on TIMP-1 expression at the vascular wall.

In our experimental conditions, no differences in the medial collagen content, evaluated by morphometric analysis, were present in the aorta of ANG II-treated compared with saline-treated rats both after 6 days, as it has been described (7), and 25 days of continuous ANG II administration. These results suggest that the ANG II-dependent increase in TIMP-1 expression in the aortic smooth muscle cells occurs early, before the development of histological changes at the vascular wall.

The possibility that the increase in arterial pressure caused by chronic ANG II administration might have contributed in modulating the TIMP-1 induction cannot be excluded, although it seems unlikely because the effect of ANG II on TIMP-1 expression was first observed in VSMC in vitro, a condition in which hemodynamic and humoral influences are absent.

Increasing evidence indicates that TIMP-1 is implicated in the remodeling process leading to fibrosis (28). In spontaneously hypertensive rats, the reduced expression of TIMP-1 in the heart is associated to the regression of myocardial fibrosis (27), and recently it...
has also been suggested that TIMP-1 might be a potential marker of fibrosis in human hypertension (17).

ANG II plays an important role in the remodeling processes, and it contributes to atherosclerosis not only through its hemodynamic effects but also through its several proinflammatory (3, 11, 25) and profibrotic actions (14, 15). In particular, in the early phase of atherosclerosis processes, smooth muscle cells in the tunica media progressively change phenotype, losing the contractile properties and assuming the synthetic phenotype. Because TIMP-1 plays a fundamental role in tissue remodeling processes, the long-lasting ANG II induction on TIMP-1 in VSMC could contribute to the unfavorable long-term effects of ANG II at the vascular wall.

In summary, our data, by demonstrating in vivo that the increase in TIMP-1 expression induced by ANG II in VSMC occurs in absence of histological changes at the vascular wall, suggest that this effect might be involved in the early phase of the remodeling processes. It is possible to speculate that this link between ANG II and TIMP-1 in VSMC may represent a further intracellular pathway involved in the early phase of the vascular remodeling process caused by ANG II at the vascular wall level.

The authors acknowledge Rossana Rosati for excellent histological technical assistance and Delta Sistemi (Rome, Italy) for allowing us to use the computerized imaging analysis system (IAS 2000).

REFERENCES
7. Diep QN, El Mabrouk M, Cohn JS, Endemann D, Amiri F, Virdis A, Fritsch Neves M, and Schiffrin EL. Structure,