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Potential Anti-Inflammatory Role of Activin A in Acute Coronary Syndromes

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OBJECTIVES We sought to investigate whether activin A could be involved in the immunopathogenesis of acute coronary syndromes.

BACKGROUND Inflammatory mechanisms seem to play a pathogenic role in atherosclerosis and acute coronary syndromes, but the actual mediators have not been fully identified. Activin A, a pleiotropic member of the transforming growth factor-beta cytokine family, has recently been suggested to play a role in inflammation.

METHODS We examined the role of activin A and its endogenous inhibitor follistatin in patients with stable (n = 26) and unstable angina (n = 20) and healthy control subjects (n = 20) by different experimental approaches.

RESULTS 1) Patients with stable angina had raised activin A concentrations, as assessed by protein levels in serum and messenger ribonucleic acid levels in peripheral blood mononuclear cells (PBMCs). 2) Although several activin A–related mediators were upregulated in PBMCs from patients with stable angina compared with controls (i.e., activin A and Smad3), no changes or even downregulation (i.e., Smad2) were seen in unstable disease. 3) The activin type II receptors, representing the primary ligand-binding proteins, were downregulated in unstable compared with stable angina. 4) Percutaneous coronary intervention induced a decrease in the activin A/follistatin ratio, suggesting downregulatory effects on activin A activity. 5) Although activin A dose-dependently suppressed the release of inflammatory cytokines from PBMCs in angina patients, an opposite effect was found in healthy controls.

CONCLUSIONS Our findings suggest an anti-inflammatory potential of activin A in angina patients, and such effects may be of particular relevance in unstable angina in which several of the activin parameters were downregulated. (J Am Coll Cardiol 2004;44:369–75) © 2004 by the American College of Cardiology Foundation

It is widely recognized that inflammatory mechanisms play a pathogenic role in coronary artery disease (CAD). In fact, recent research has suggested that inflammatory mediators play a causal role in several steps involved in the progression of atherosclerosis from local inflammation through plaque formation and rupture (1–3). However, these inflammatory mediators exert an array of biologic functions, and the identification and characterization of the different actors, as well as their relative importance, are not fulfilled.

Along with transforming growth factor (TGF)-beta and bone morphogenetic protein, activin A is a member of the TGF-beta superfamily (4). Although originally described as an inducer of follicle-stimulating hormone release, activin A has more recently been recognized as a multifunctional cytokine expressed in a wide range of tissues and cells with roles in regulation of wound repair, cell differentiation, apoptosis, and embryogenesis (4). Furthermore, growing evidence implicates activin A in the pathogenesis of inflammatory disorders such as rheumatoid arthritis, sepsis, and inflammatory bowel disease, possibly mediating anti-inflammatory net effects (5–7). Recent studies suggest that this cytokine could also be involved in atherogenesis by inhibiting foam cell formation and inducing differentiation of neointimal smooth muscle cells (8–10).

Based on its role in inflammation, in the present study, we attempted to further clarify the potential role of activin A in atherogenesis and acute coronary syndromes by use of several experimental approaches. First, we examined protein and messenger ribonucleic acid (mRNA) levels of activin A and its natural inhibitor follistatin (4) in peripheral blood from patients with stable and unstable angina and healthy control subjects. Second, we investigated the expression of the activin A signal transduction pathway involving the receptor-Smad system (4) in peripheral blood mononuclear cells (PBMCs) from these individuals. Finally, we examined the effect of activin A on the release of inflammatory cytokines suggested to be involved in atherogenesis and immune-mediated plaque destabilization.

METHODS

Patients and controls. Angina patients undergoing clinically indicated diagnostic coronary angiography in our

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Abbreviations and Acronyms

CAD = coronary artery disease
EIA = enzyme immunoassay
IL = interleukin
MIP = macrophage inflammatory protein
mRNA = messenger ribonucleic acid
PBMC = peripheral blood mononuclear cell
PCI = percutaneous coronary intervention
TGF = transforming growth factor
TNF = tumor necrosis factor

coronary care unit were consecutively recruited into the study (Table 1). All patients with unstable angina (n = 20) had experienced ischemic chest pain at rest within the preceding 48 h (i.e., Braunwald class IIIB), but with no evidence of myocardial necrosis by enzymatic criteria. Transient ST-T segment depression and/or T-wave inversion were present in all cases. All patients with stable angina (n = 26) had stable effort angina lasting longer than six months and a positive exercise test. Exclusion criteria were myocardial infarction or thrombolytic therapy in the previous month, electrocardiographic abnormalities invalidating ST-segment analyses, concomitant inflammatory diseases such as infections and autoimmune disorders and liver or kidney disease. Coronary angiography was performed by standard techniques within one to two days after admission, and the diagnosis of CAD was confirmed by at least one-vessel disease, defined as >75% narrowing of the luminal diameter, in all patients. Control subjects in the study were 20 gender- and age-matched healthy blood donors. Informed consent for participation in the study was obtained from all individuals.

Blood sampling protocol. Peripheral venous blood was drawn into pyrogen-free tubes without additives (serum) or with ethylenediamide tetra-acetic acid as an anticoagulant (plasma). The tubes were immediately immersed in melting ice and centrifuged at 1,500 *g* for 10 min within 20 min (plasma) or allowed to clot before centrifugation (serum). All samples were stored at −80°C and thawed less than three times.

Cell culture experiments. The PBMCs (2×10^6 cells/ml), obtained from heparinized blood by Isopaque-Ficoll gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway), were incubated in 96-well trays (Costar, Cambridge, Massachusetts) in medium alone (RPMI 1640 with 2 mmol/l L-glutamine [Gibco, Paisley, United Kingdom] supplemented with 10% fetal calf serum) or stimulated with different concentrations of activin A (R&D Systems, Minneapolis, Minnesota). Cell-free supernatants were harvested after 18 h and stored at −80°C. Activin A levels were also examined in platelet-rich plasma activated by the thrombin receptor agonist SFLLRN (11). Endotoxin levels in all media, buffers, and stimulants were <10 pg/ml (limulus amoebocyte lysate test; BioWhittaker, Walkersville, Maryland).

Table 1. Characteristics of the Study Group

	Unstable Angina (n = 20)	Stable Angina (n = 30)
Age (yrs)	58 ± 13	58 ± 9
Gender (females/males)	2/18	5/26
Smokers (%)	31	32
Medication (%)		
Beta-blockers	94	91
Aspirin	94	91
Statins*	65	71
Calcium antagonists	24	11
Warfarin	12	14

*Hydroxymethylglutaryl coenzyme A reductase inhibitors. Data are presented as the mean value ± SD or percentage.

Real-time quantitative reverse transcription polymerase chain reaction. Total RNA was extracted from PBMCs using RNeasy columns (Qiagen, Hilden, Germany), subjected to DNase I treatment (RQI DNase; Promega, Madison, Wisconsin) and stored at −80°C. Primers were designed using the Primer Express software version 1.5 (Applied Biosystems, Foster City, California) (Table 2). Quantification of mRNA was performed using the ABI Prism 7000 (Applied Biosystems) (12). SyBr green assays (Table 2) were performed using 300 nmol/l sense and anti-sense primers (12). Gene expression of the housekeeping gene beta-actin (Applied Biosystems) was used for normalization.

Enzyme immunoassay (EIA). Interleukin (IL)-6, IL-8, IL-10, macrophage inflammatory protein (MIP)-1-alpha, monocyte chemoattractant protein-1, TGF-beta, and tumor necrosis factor (TNF)-alpha were measured by EIA (R&D Systems). Activin A was analyzed by EIA from Serotec (Oxford, United Kingdom; serum with pretreatment of samples according to the manufacturer's description) and R&D Systems (plasma). Follistatin levels were measured by EIAs provided by R&D Systems.

Statistical analysis. When comparing three groups of individuals, one-way analysis of variance was followed by the Scheffé post hoc test for statistical significance. For comparisons within the same individuals, the Wilcoxon signed-rank test was used. Probability values (two-sided) were considered significant at value of <0.05.

RESULTS

Circulating levels of activin A and follistatin. As shown in Figure 1A, both patients with stable (n = 26) or unstable angina (n = 20) had significantly increased serum levels of activin A, compared with healthy controls (n = 20), with no significant differences between the two groups of CAD patients. In contrast, we found no differences in plasma levels of follistatin between these three groups of individuals (Fig. 1B). Notably, although activin A levels in the patient group as a whole were significantly correlated with serum levels of C-reactive protein ($r = 0.32$, $p < 0.05$), no such correlation was found for follistatin ($r = 0.14$, $p = 0.49$).

Table 2. Characteristics of the Real-Time Polymerase Chain Reaction Assays Used in the Study

Target	Sequence (5′→3′)	Acc. Nr.
Activin $beta_A$	(+)-GCAGAAATGAATGAACTTATGGA	M13436
	(−)-GTCTTCCTGGCTGTTCCTGACT	
ActRIA	(+)-CGAGACGTGGAGTATGGCACTA	Z22534
	(−)-CGAATGATCCAATAAATCTGCTAAAGT	
ActRIB	(+)-GTGGGCACCCAAGGGAA	NM_004302
	(−)-GCTATGGCACACATGCCATT	
ActRIIA	(+)-TTGGCAGAAACATGCTGGAA	M93415
	(−)-ATCTGGGTAATTCTTTCACCTACACA	
ActRIIB	(+)-GCACCATCGAGCTCGTGAA	X77533
	(−)-CCACACACTCCTGCCTATCGT	
Smad2	(+)-CCTCTGGATGACTATACTCACTCCATT	NM_005901
	(−)-GGAGGTGGCGTTTCTGGAA	
Smad3	(+)-TCCCACCAGGATGCAACCT	NM_005902
	(−)-GCACATTCGGGTCAACTGGTA	
Smad7	(+)-AGCCGACTCTGCGAACTAGAGT	NM_005904
	(−)-GACAGTCTGCAGTTGGTTTGAGA	

This table shows the sequence of primers used in the real-time polymerase chain reaction assays.
(+) = forward primers; (−) = reverse primers; Acc. Nr. = GenBank accession number; Act = activin; R = receptor.

Measurement in serum samples could potentially be influenced by release from platelets during ex vivo coagulation. However, we found no release of activin A or follistatin from SFLLRN-stimulated platelets in either patients or controls. It has previously been reported that heparin induces a rapid increase in serum levels of activin A and follistatin (13), but importantly, all blood samples were collected before heparin administration.

Gene expression of activin $beta_A$ and follistatin in PBMCs. Activin A is a homodimer of two activin $beta_A$ subunits (4), and we next examined the gene expression of activin $beta_A$ and follistatin in PBMCs from 15 patients with stable angina, 15 patients with unstable angina, and 10 healthy controls. Although activin $beta_A$ gene expression was significantly upregulated in PBMCs from patients with stable angina, no such increase was found in those with unstable disease (Fig. 2A). Thus, while serum activin A levels were raised in both stable and unstable angina patients, only those with stable disease had increased expression of this cytokine in PBMCs. This apparent discrepancy most probably reflects that cells other than PBMCs, such as macrophages, endothelial cells, mast cells, fibroblasts, and vascular smooth muscle cells, are major contributors to serum levels of activin A (4,6). As for follistatin expression, no differences were found between patients and controls or between the two patient groups (data not shown).

Expression of activin A receptors and Smads in PBMCs. We next examined the gene expression of activin A receptors (type I and II; Table 2) and Smads in PBMCs from these 15 patients with unstable angina, 15 patients with stable angina, and 10 healthy controls. The signal transduction pathway involving the receptor-Smad system is highly similar for the TGF-beta family members, including activin A (4,14), and notably, unstable angina patients had decreased gene expression of the activin type II, IIA, and IIB receptors, representing the primary ligand-binding proteins (4,14), compared with those with stable disease (Figs. 2B and 2C). Moreover, as shown in Figure 3, a similar pattern with decreased levels in unstable compared with stable angina was also seen for the gene expression of the intracellular proteins Smad2 and Smad3, which bind to the activin A heterocomplex upon ligand binding, representing the principal transducer of signals from the receptors (14). In fact, although Smad2 expression was significantly decreased in unstable angina patients compared with healthy controls, Smad3 expression was increased in stable angina patients compared with healthy individuals (Fig. 3), sug-

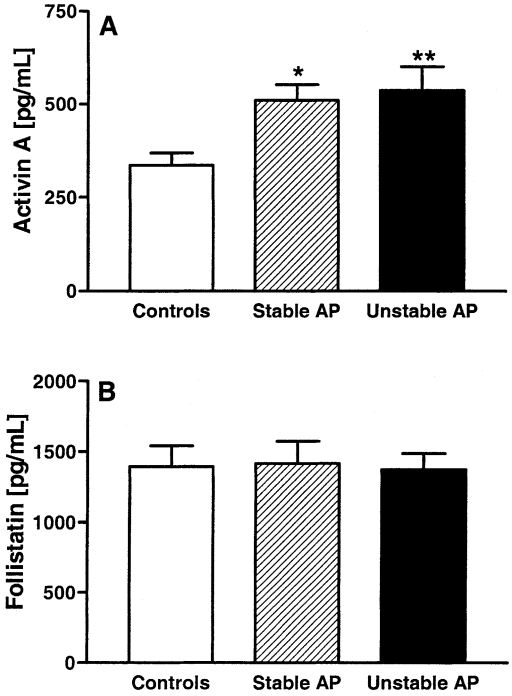

Figure 1. **(A)** Serum levels of activin A and **(B)** plasma levels of follistatin in 20 patients with unstable angina pectoris (AP), 26 patients with stable AP, and 20 healthy controls. Data are presented as the mean value ± SEM. *$p < 0.05$ and **$p < 0.01$ versus healthy controls.

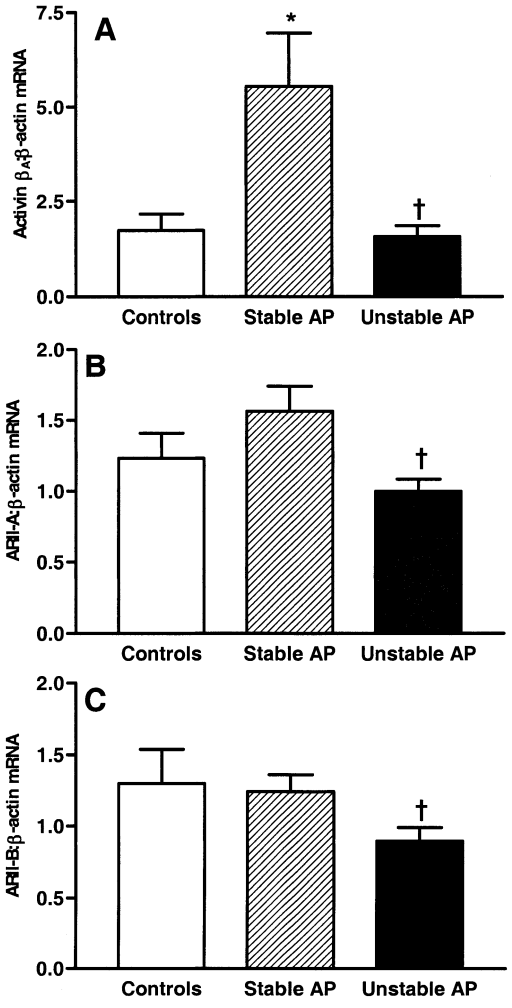

Figure 2. Gene expression of the activin A subunit activin beta$_A$ **(A)** and the activin A receptor II type A (ARIIA) **(B)** and type B (ARIIB) **(C)** in peripheral blood mononuclear cells from 15 patients with unstable angina pectoris (AP), 15 patients with stable AP, and 10 healthy controls. The messenger ribonucleic acid (mRNA) levels were quantified using real-time reverse transcription polymerase chain reaction, and data are presented relative to the gene expression of beta-actin (mean ± SEM). *$p < 0.05$ versus healthy controls. †$p < 0.05$ versus stable AP.

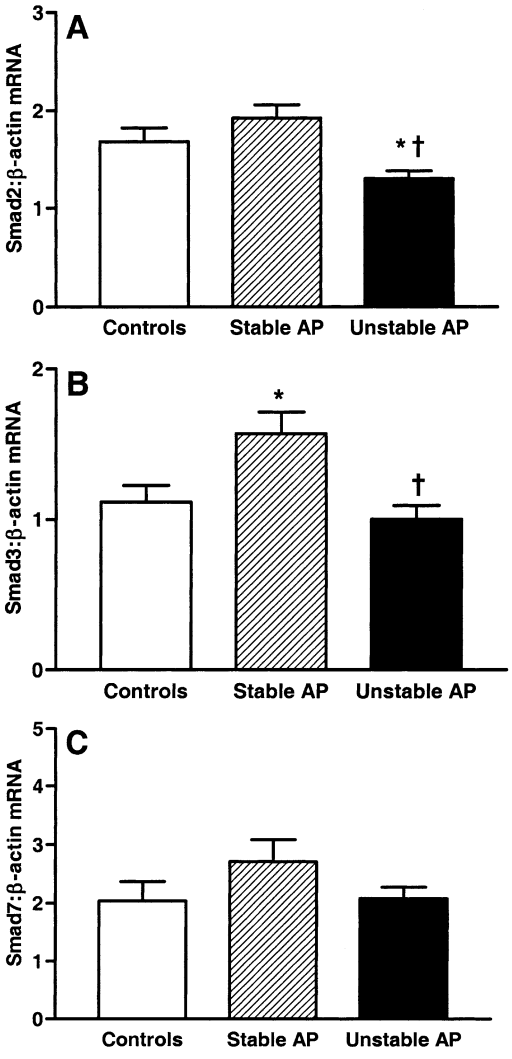

Figure 3. Gene expression of Smad2 **(A)**, Smad3 **(B)**, and Smad7 **(C)** in peripheral blood mononuclear cells from 15 patients with unstable angina pectoris (AP), 15 patients with stable AP, and 10 healthy controls. The messenger ribonucleic acid (mRNA) levels were quantified using real-time quantitative reverse transcription polymerase chain reaction, and data are presented relative to the gene expression of beta-actin (mean ± SEM). *$p < 0.05$ versus healthy controls. †$p < 0.05$ versus stable AP.

gesting that the Smad system may be differently or even oppositely regulated in stable and unstable disease. In contrast, no differences were found in mRNA levels of the inhibitory Smad7 (Fig. 3C) or the activin type I receptors (data not shown) between either unstable and stable angina patients or between patients and controls.

Effect of percutaneous coronary intervention (PCI) on activin A and follistatin levels. To examine the expression pattern of activin A and follistatin upon inflammatory stimulation in vivo, we next examined plasma levels of activin A and follistatin in 14 patients with stable angina and 13 patients with unstable angina before and 48 h after PCI. Heparin has been shown to induce a rapid increase in both activin A and follistatin levels (13), and indeed, immediately after PCI there was an early (i.e., within 1 h) rise in these cytokines, returning to baseline levels within 4 h; as for activin A, there was a subsequent late decrease in the concentration, reaching significantly lower levels than baseline after 48 h (Fig. 4). In contrast, after returning to baseline levels within 4 h, follistatin increased, reaching significantly higher levels than baseline at 48 h after PCI (Fig. 4). We found a similar pattern in both stable and unstable angina patients, although the decrease in activin A did not reach statistical significance in those with unstable disease (Fig. 4).

Effects of activin A on cytokine levels in PBMCs. In contrast to several reports on inflammatory cytokines, activin A levels seem to be equal (serum) or even lower (PBMCs) in unstable compared stable angina patients. To map any possible pathogenic consequences of decreased activin A activity with respect to plaque rupture, we next examined the effect of activin A on the release of inflam-

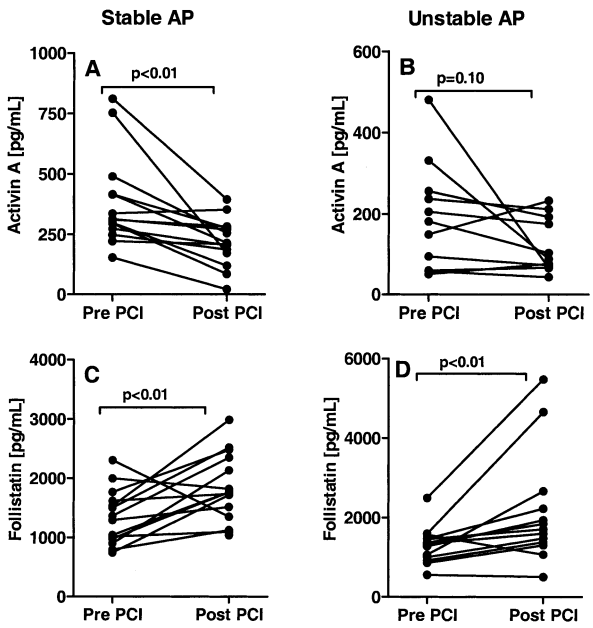

Figure 4. Plasma levels of activin A and follistatin in 14 patients with stable angina pectoris (AP) **(A and C)** and 13 patients with unstable AP **(B and D)** before (Pre) and 48 h after (Post) percutaneous coronary intervention (PCI). Activin A levels were only measured in 11 of the patients with unstable AP.

matory cytokines, suggested to be involved in plaque destabilization (3), in PBMCs from seven patients with stable angina, six patients with unstable angina, and six healthy controls. Several significant findings were revealed (Fig. 5). First, activin A induced a significant and dose-dependent decrease in the release of the inflammatory cytokines IL-6, IL-8, and MIP-1-alpha, but not TNF-alpha, in both stable and unstable angina patients. In contrast to this suppressive effect, activin A dose-dependently increased the release of IL-6, TNF-alpha, and MIP-1-alpha from PBMCs in healthy controls, with a particularly enhancing effect on MIP-1-alpha (~10-fold increase). Finally, in contrast to the effects on inflammatory cytokines, activin A had no effect on TGF-beta or IL-10 in either patients or controls (data not shown), indicating that the suppressive effects of activin A on inflammatory cytokines in unstable angina patients do not involve regulation of these typically anti-inflammatory cytokines.

DISCUSSION

Activin A and follistatin have previously been reported to be expressed in human vascular tissue at different stages of atherosclerosis (9). In the present study, we extend these findings by showing raised activin A concentrations as assessed by protein levels in serum and mRNA levels in PBMCs in patients with stable angina. However, whereas numerous reports on inflammatory cytokines have demonstrated the highest levels in those with unstable disease (11,15–18), this was not seen for activin A and related parameters. In fact, although several of these parameters were upregulated in stable angina patients compared with healthy controls (i.e., activin $beta_A$ and Smad3), no changes or even downregulation (i.e., Smad2) were seen in unstable disease. Moreover, the activin type II receptors, representing the primary ligand-binding proteins, were downregulated in unstable compared with stable angina. Although we found a similar pattern of responses to activin A in PBMCs from both stable and unstable angina patients, the different and even opposite regulation of several activin-related parameters in these two groups of angina patients suggest that this system may be of importance in the progression from stable to unstable disease. Interestingly, the TGF-beta type II receptor has been reported to be profoundly decreased in advanced atherosclerotic lesions (19), and it is tempting to hypothesize that also dysregulated expression of activin A and its receptors, being a related member of the TGF-beta superfamily, could contribute to atherogenesis and plaque destabilization.

Activin A has been suggested to promote plaque stabilization by inhibiting foam cell formation through regulating scavenger receptor mRNA expression and by inducing a contractile, nonproliferative phenotype in cultured smooth muscle cells (8,9). Herein we show that activin A dose-dependently attenuated the release of IL-6, IL-8, and MIP-1-alpha in PBMCs from CAD patients, suggesting that an "inadequate rise" in activin A could contribute to an inappropriate inflammatory response in these patients. Moreover, all these cytokines are expressed within atherosclerotic plaques, with particularly high levels in advanced lesions (2,3,20,21), and may enhance plaque rupture through various mechanisms such as promotion of matrix degradation and apoptosis (3,13,21). Interestingly, TGF-beta has recently been shown to inhibit chemokine expression through Smad-related pathways (22), and similar mechanisms could also be operating in the activin A–mediated inhibition of inflammatory cytokines. Whatever the mechanisms, our findings with markedly suppressive effects of activin A on inflammatory cytokines in CAD patients, along with decreased or inadequately raised levels of several activin A–related mediators in unstable angina, further support a potential role for activin A in the pathogenesis of plaque destabilization.

A major finding of the present study was that in contrast to the anti-inflammatory effects of activin A in PBMCs from CAD patients, this cytokine markedly enhanced the release of inflammatory cytokines in PBMCs from healthy controls. There is a growing appreciation of the importance of activin A as a modulator of immune function, showing both inflammatory and anti-inflammatory effects (6,23). Thus, activin A has been reported to inhibit IL-1 and IL-6 activity by both inhibiting their production and antagonizing their action (23,24). On the other hand, activin A has been found to enhance IL-6 and IL-8 expression during pregnancy (25) and to increase IL-6 levels in monocytes (26), and our findings further underscore the dichotomy between pro- and anti-inflammatory actions of this cytokine. Similar pleiotropic effects have also been reported for

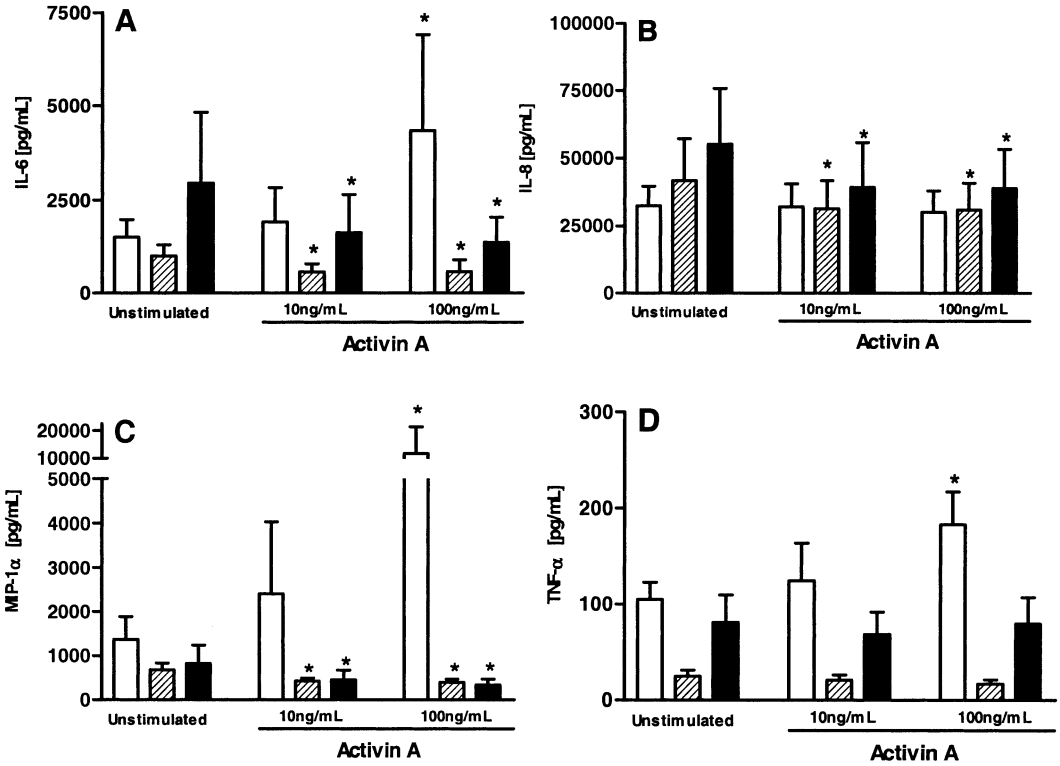

Figure 5. The effect of activin A (10 and 100 ng/ml) on the protein levels of interleukin (IL)-6 **(A)**, IL-8 **(B)**, macrophage inflammatory protein (MIP)-1-alpha **(C)**, and tumor necrosis alpha (TNF)-alpha **(D)** in peripheral blood mononuclear cells supernatants from six patients with unstable angina pectoris (AP) **(solid bars)**, seven patients with stable AP **(striped bars)**, and six healthy controls **(open bars)** after culturing for 18 h. Data are given as the mean value ± SEM. *$p < 0.05$ versus unstimulated cells.

TGF-beta, the prototypical cytokine in the TGF-beta superfamily (27). The reason for these different responses in PBMCs from angina patients and healthy controls is unclear at present, but may involve several factors such as a different degree of pre-activation, different expression pattern of the activin A receptors and Smads, and changes in levels of co-activators and co-repressors, as well as other transcriptional factors (14). Nevertheless, these findings underscore that caution is needed when interpreting the relevance of studies in healthy individuals with respect to CAD and similar disorders.

Several observations have linked the activin A/follistatin system to the acute-phase response during inflammation (6,23). The rapid rise of activin A during such responses has been suggested to induce anti-inflammatory effects both locally at the site of the injury or infection and at peripheral sites such as the liver, attempting to prevent an inappropriate inflammatory response (23). The findings of the present study may suggest an anti-inflammatory potential of this cytokine also in CAD patients with marked downregulatory effects on several inflammatory cytokines in mononuclear cells from these patients. Such effects may be of particular relevance in unstable angina patients in whom several of the activin parameters were downregulated. In addition to its previously demonstrated role in foam cell inhibition and smooth muscle cell stabilization, our findings suggest that activin A could represent a new therapeutic target in CAD and acute coronary syndromes.

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