Journal of Cellular Biochemistry 90:23–32 (2003)

Pitavastatin Alters the Expression of Thrombotic and Fibrinolytic Proteins in Human Vascular Cells

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Abstract In addition to lowering blood lipids, clinical benefits of 3-hydroxy-3-methylglutaryl coenzyme A (HMG Co-A; EC 1.1.1.34) reductase inhibitors may derive from altered vascular function favoring fibrinolysis over thrombosis. We examined effects of pitavastatin (NK-104), a relatively novel and long acting statin, on expression of tissue factor (TF) in human monocytes (U-937), plasminogen activator inhibitor-1 (PAI-1), and tissue-type plasminogen activator (t-PA) in human aortic smooth muscle cells (SMC) and human umbilical vein endothelial cells (HUVEC). In monocytes, pitavastatin reduced expression of TF protein induced by lipopolysaccharide (LPS) and oxidized low-density lipoprotein (OxLDL). Similarly, pitavastatin also reduced expression of TF mRNA induced by LPS. Pitavastatin reduced PAI-1 antigen released from HUVEC under basal, OxLDL-, or tumor necrosis factor-alpha (TNF-α)-stimulated conditions. Reductions of PAI-1 mRNA expression correlated with decreased PAI-1 antigen secretion and PAI-1 activity as assessed by fibrin–agarose zymography. In addition, pitavastatin decreased PAI-1 antigen released from OxLDL-treated and untreated SMC. Conversely, pitavastatin enhanced t-PA mRNA expression and t-PA antigen secretion in untreated OxLDL-, and TNF-α-treated HUVEC and untreated SMC. Finally, pitavastatin increased t-PA activity as assessed by fibrin–agarose zymography. Our findings demonstrate that pitavastatin may alter arterial homeostasis favoring fibrinolysis over thrombosis, thereby reducing risk for thrombi at sites of unstable plaques. J. Cell. Biochem. 90: 23–32, 2003.

Key words: endothelial cell; monocyte; smooth muscle; statin; thrombosis; fibrinolysis

Elevated serum cholesterol is a long-recognized major risk factor for atherosclerotic coronary artery disease [Kannel et al., 1971; Kannel, 1995]. In recent years, HMG-CoA reductase inhibitors, commonly called statins, have gained wide use for reducing low-density lipoprotein cholesterol (LDL-C) [Jacobson et al., 1998; Gotto, 2001]. In addition to their cholesterol-lowering effect, clinical studies of statins have reported reduced indices of cardiovascular disease independent of the ability of statins to decrease LDL-C [Scandinavian Simvastatin Survival Study Group, 1994; Shepherd et al., 1995; Sacks et al., 1996]. The finding that statins impart cardiovascular benefits, separate from their cholesterol lowering action, has prompted studies of statin effects on vascular inflammation, plaque stability, platelet and endothelial cell function, smooth muscle cell proliferation, and downstream modulation of the HMG-CoA reductase metabolic pathway,

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Grant sponsor: Sponsored Research Agreement from Kowa Company, Ltd., Tokyo, Japan; Grant sponsor: Physiology and Morphology Group, Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ.

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Received 24 April 2003; Accepted 22 May 2003

DOI 10.1002/jcb.10602

among others [reviewed in Rosenson and Tangney, 1998; Laufs and Liao, 2000; Takemoto and Liao, 2001].

Pitavastatin, also known as NK-104, (CAS 147526-32-7; monocalcium bis [(3R, 5S, 6E)-7-(2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl)-3,5-dihydroxy-6-heptenoate]) is a recently developed HMG-CoA reductase inhibitor that significantly reduces serum total cholesterol, LDL-C, and triglycerides while modestly raising high density lipoprotein-cholesterol (HDL-C) at dosages as little as one-fifth that reported for atorvastatin [Kajinami et al., 2000; Saito et al., 2002]. Pitavastatin also lowers serum apolipoproteins B, CII, CIII, and E while raising apolipoproteins AI and AII [Kajinami et al., 2000]. In the balloon-injury model of the rabbit carotid artery, pitavastatin reduces injury-induced neointimal smooth muscle cell growth and extracellular matrix thickening [Kitahara et al., 1998] and in the Watanabe heritable hyperlipidemic rabbit, lesion surface area and progression are reduced [Suzuki et al., 2000]. Pitavastatin produces long-lasting (over 6 h) inhibition of liver sterol synthesis [Aoki et al., 1997] and its bioavailability in humans is high with a circulating half-life just over 13 h. The drug undergoes very slight modification by the cytochrome P450 enzyme system [Fujino et al., 1999], and it is eliminated from the body through the bile with drug predominantly intact [Fujino et al., 1999; Kojima et al., 1999].

Thrombosis and fibrinolysis are regulated by the physiological balance of prothrombotic and profibrinolytic proteins. Three key proteins involved in this balance are tissue factor (TF), plasminogen activator inhibitor-1 (PAI-1), and tissue-type plasminogen activator (t-PA). TF is a membrane-bound, 47-kDa glycoprotein that initiates the extrinsic coagulation cascade leading to thrombin formation and fibrin deposition [Taubman et al., 1997]. TF is constitutively produced in the adventitia but only minimally within the intima-media of the normal arterial wall [Wilcox et al., 1989]. However, it is readily induced across the wall by tissue injury [Marmur et al., 1993]. TF gene expression in monocytes/macrophages can be induced by inflammatory mediators [Edgington et al., 1991; Mackman et al., 1991] and TF and fibrin have been co-localized with macrophages within coronary plaques obtained from patients with unstable angina, suggesting a role for TF in thrombogenicity of atheroma [Kaikita et al., 1997]. PAI-1 and t-PA are the key proteins for balancing the fibrinolytic system in vivo [Edelberg et al., 1994]. Vascular disorders associated with increased thrombosis (such as coronary heart disease and deep-vein thrombosis) correlate with increased PAI-1 antigen and activity; decreased t-PA activity has been associated with coronary heart disease [Juhan-Vague and Alessi, 1993; Aznar and Estelles, 1994; Wiman, 1995]. Thus, clinical benefit should derive from interventions reversing this PAI-1 and t-PA profile.

We have examined in vitro effects of pitavastatin on prothrombotic and profibrinolytic proteins (TF, PAI-1, and t-PA). This is the first study that has examined three major thrombogenic proteins in human vascular cells in response to a long-lasting, highly potent statin.

MATERIALS AND METHODS

Reagents

Cell culture media, endothelial cell growth supplement (ECGS), 2% ITS (insulin, transferrin, selenious acid), glutamine, penicillin, and streptomycin were purchased from GIBCO (Carlsbad, CA). Mouse TNF-α was purchased from Roche (Indianapolis, IN). Pitavastatin was obtained from the Pharmaceutical Division, Kowa Company Ltd., (Tokyo, Japan). OxLDL was prepared from normal human plasma with a 12-h oxidation period as previously described [Han et al., 2001]. Anti- human TF, PAI-1, t-PA, uPA, and plasmin-free human glu-plasminogen were purchased from American Diagnostica (Greenwich, CT). ECL detection reagents for Western blots were purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). The ^{32}P-labeled cDNA probes were prepared with a random labeling kit from Fermentas, Inc. (Hoover, MD). All chemicals were from Sigma (St. Louis, MO).

Cell Culture

U-937 cells, a human monocytic cell line were obtained from American Type Culture Collection and grown in RPMI 1640 medium containing 10% fetal calf serum, 2 mmol/L glutamine, penicillin and streptomycin (50 μg/ml of each). At the time of experiments, $2.5–5.0 \times 10^6$ cells were transferred to 60-mm dishes, acclimated for 2 h before treatment. Human aortic SMC (passage 5 from ATCC) and HUVEC (passage 2 or 3) cultures were prepared as described

previously [Nicholson et al., 1996] and the media conditions were adjusted at the time of experiments as described below. For optimal cell adhesion and viability, experimental treatments of SMC and HUVEC did not exceed 20 h and at the time of harvest cultures were examined for equal confluence.

Analyses of TF, PAI-1, and t-PA Proteins by Western Blotting

U-937 monocytes were used for all TF experiments and whole cell lysates were prepared as detailed previously [Han et al., 2001]. Protein contents of supernatants were measured by the Lowry method [Lowry et al., 1951]. Equal amounts of supernatant protein were separated in 10 or 12% SDS–PAGE, then transferred to a nitrocellulose membrane. All blocking steps and incubations were done at room temperature. Membranes were blocked with a solution of 5% non-fat milk in Tris buffered saline (TBS) for 1 h, then first incubated for 1 h with rabbit polyclonal anti-human TF (1 μg/ml) in 3% non-fat milk/TBS, followed by serial washings (3×15 min) with 0.1% Tween 20/TBS. The membrane was re-incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:3,000 final dilution) in 3% milk/TBS, then washed three times as above. Immune-positive bands were detected with an ECL-Plus chemiluminescence kit following the manufacturers guidelines and developed on Kodak Bio Max MR X-ray film. Autoradiograms were semi-quantitatively assessed by scanning with an Agfa ArcusII flatbed scanner linked to a Macintosh G4 computer running Adobe Photoshop 6.0 with densitometric analysis of the images using Scion Image Beta 4.0.2 software.

PAI-1 and t-PA proteins in HUVEC- and SMC-conditioned media were assessed by Western blotting. Equal volumes of conditioned media were loaded on 10% SDS–PAGE for PAI-1 analysis. A 500 μl aliquot from each culture was used to enrich t-PA antigen by immunoprecipitation overnight at 4°C, then separated by 10% SDS–PAGE. Sample buffer for t-PA was supplemented with β-mercaptoethanol, but omitted for PAI-1 samples. Primary antibodies used were mouse monoclonal anti-human PAI-1 antibody and goat anti-human melanoma t-PA antibody, each prepared in 1% milk/TBS at a working concentration of 5 μg/ml. Secondary antibodies were HRP-conjugated goat anti-mouse IgG (for PAI-1) and HRP-conjugated rabbit anti-goat IgG (for t-PA) at 1:3,000 final concentrations in 1% milk/TBS. Immunoblots in the results section are representative of replicate experiments.

Northern Analyses For Expression of TF, PAI-1, and t-PA mRNA

Cells were lysed in RNAzol^{TM}, total RNA and poly (A+) RNA (purified from 100 μg of total RNA) were prepared as previously described [Han et al., 2001]. Fifteen to twenty micrograms of total RNA or poly A^+ RNA were loaded in a 2% formaldehyde–1% agarose gel, separated by electrophoresis, then transferred by capillary action overnight to a Zeta-Probe membrane in $10\times$ SSC. The blot was UV cross-linked, then pre-hybridized with Hybrisol-I^{TM} for 30 min before adding cDNA probe for TF, PAI-1, or t-PA, randomly labeled with [^{32}P] dCTP. Preparations of the PAI-1 and t-PA probes were detailed earlier [Etingin et al., 1991]; the TF probe was generated by reverse transcription-polymerase chain reaction according to the published sequence. The sequences of primers were cactaagtcaggagattgg (6392–6410) and tctccaggtaaggtgtg (6591–6575), respectively [Mackman et al., 1989]. Probes were hybridized to the membrane overnight at 43°C, followed by a 20 min wash in $0.2\times$ SSC and 0.2% SDS at 55°C. Autoradiographic images of the blots were prepared on X-ray film and semi-quantitatively evaluated as described above. Northern blots for TF mRNA were re-probed for GAPDH mRNA [Han et al., 2001]; for PAI-1 and t-PA mRNA blots, the 18S and 28S rRNA bands stained with ethidium bromide were localized by UV illumination; the 18S is shown. Northern blots shown in the results section are representative of replicate experiments.

Analyses of Biological Activity of PAI-1 and t-PA Proteins

Zymography for PAI-1 and t-PA was performed on conditioned media from HUVEC and SMC, based upon fibrin–agarose indicator film methods [Loskutoff and Schleef, 1988; Essig et al., 1998]. For t-PA activity, aliquots of conditioned media (35 μl from HUVEC and 5 μl from SMC) were mixed with sample buffer, not boiled, then run on a 10% SDS–PAGE gel. The polyacrylamide gel was laid atop a 1.5 mm fibrin–agarose (0.75%) gel containing 25 μg of plasmin-free human glu-plasminogen, 5 U of bovine thrombin, and 2.5 ml of freshly prepared

plasminogen-depleted human fibrinogen (10 mg/ml). The zymogram was developed overnight at 37°C in a humidified container. Bands of t-PA activity appeared as clear zones of fibrin lysis. The agarose gel was transferred to a gel bonding film, dried, and stained with Coomasie blue. PAI-1 activity was detected by reverse zymography using 40 μl aliquots of conditioned media with the addition of uPA (0.5 U/ml final concentration) to the fibrin–agarose underlay. The SDS–PAGE/fibrin–agarose gel sandwich was incubated at 37°C for 1–2 h; zones of PAI-1 activity remain opaque while the agar gel clears. After transferring and drying the agarose gel on bonding film, it was stained with amido black.

RESULTS

Pitavastatin Inhibits TF Production in U-937 Monocytes

U-937 monocytes were stimulated with LPS and harvested between 2 and 24 h to ascertain the time frame for induction of TF protein. Western blotting revealed the first increase of TF antigen occurring 5 h after LPS treatment (up 75%), peaking at 2.5-fold control TF levels by 16 h, and sustained to at least 24 h (data not shown). Subsequent experiments evaluated TF expression between 16 and 24 h after treatment. Pitavastatin (1, 5, and 10 μmol/L) inhibited LPS-induction of TF antigen by 30–60% in a dose-dependent manner (Fig. 1A). We next tested whether pitavastatin could reduce TF antigen levels in monocytes challenged with OxLDL, another stimulator of TF production in U-937 monocytes [Ohsawa et al., 2000]. As seen in Figure 1B, TF antigen levels were increased similarly by OxLDL treatment (25, 50, or 100 μg/ml). Pitavastatin reduced TF antigen levels by 50% in both control and OxLDL-treated monocytes.

Baseline TF levels in untreated U-937 monocytes were lowered by pitavastatin, however, this was not attributable to cell death. We found no detrimental effect of 10 μmol/L pitavastatin on cell viability as determined by trypan blue exclusion. The percentage of dead cells in control vs. pitavastatin-treated cultures were $3.1\% \pm 1.6$ vs. $3.6\% \pm 1.0$, respectively ($P = 0.6$, unpaired t-test). To determine if TF breakdown was accentuated by pitavastatin, U-937 cells were treated first with LPS for 24 h to induce TF expression. Protein synthesis was halted with

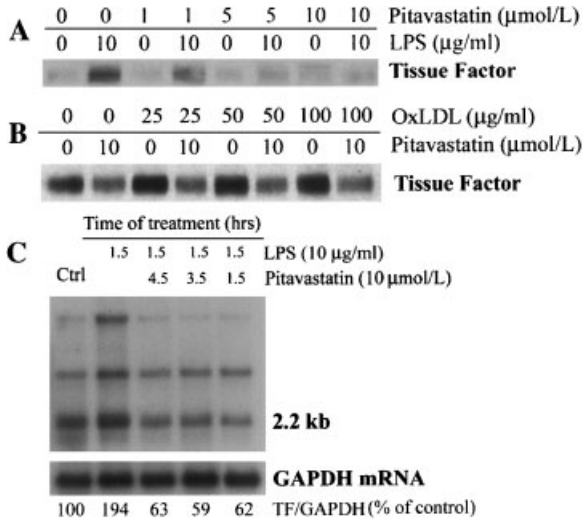

Fig. 1. **A**: Pitavastatin inhibits monocyte expression of TF induced by LPS. U-937 cells in 60 mm dishes ($2.5–5 \times 10^6$) were treated with increasing concentrations of pitavastatin in the presence or absence of LPS (10 μg/ml) added at the same time and incubated for 24 h. Protein was extracted and 40 μg of whole cell lysate protein was separated by gel electrophoresis. TF (47 kDa) was detected by Western blot as described in Materials and Methods. **B**: Pitavastatin inhibits TF production in monocytes stimulated by OxLDL. Cells were treated with the indicated concentrations of OxLDL and pitavastatin (10 μmol/L) at the same time, and then incubated 20 h. Protein was extracted and analyzed by Western blot as in 1A. **C**: Pitavastatin decreases LPS-stimulated TF mRNA expression in monocytes. U-937 cells were cultured in 100 mm dishes with complete RPMI medium and treated with (or without, control) LPS (10 μg/ml) for 1.5 h. Pitavastatin (10 μmol/L) was added at the same time or prior to the addition of LPS as indicated. After treatment, cells were collected and lysed by addition of RNAzol™ (1 ml). Total RNA was extracted and used to isolate Poly (A^+) RNA. mRNA for TF and GAPDH were analyzed by Northern blot as described in Materials and Methods.

cycloheximide (CHX; 1.0 μg/ml), and then 15 min later pitavastatin was added (final concentrations 1, 5, and 10 μmol/L). The cells were harvested 6.5 h later, i.e., approximately twice the 3.7 h half-life for TF in human monocytes [Hamik et al., 1999]. We observed no differences of TF levels with or without pitavastatin after CHX, suggesting that pitavastatin down-regulated TF antigen production rather than decreasing TF protein stability (data not shown).

Finally, we examined whether TF translocation from the cytosol to the plasma membrane was affected by pitavastatin. Western blots were performed on lysates prepared in a two-step sequence as described [Han et al., 2001] to assess membrane-associated and cytosolic TF protein. We found that TF protein was almost totally associated with the plasma membrane preparation, indicating that pitavastatin did

not alter translocation of TF from the cytosol to the membrane (data not shown).

To determine whether pitavastatin modified TF mRNA expression, Northern analyses were performed on U-937 monocytes. LPS induced the expression of TF mRNA (twofold) at 1.5 h after treatment, consistent with the time frame reported by others [Hall et al., 1999]. The increased expression of TF was blocked by pretreatment or concurrent treatment with pitavastatin (Fig. 1C).

Pitavastatin Reduces PAI-1 Release, mRNA Expression, and Activity in HUVEC and SMC

The effect of pitavastatin on PAI-1 production and activity was assessed in HUVEC and human aortic SMC. PAI-1 is a secreted protein, and therefore, we detected PAI-1 antigen from conditioned medium by Western blot. Figure 2A shows that PAI-1 release by HUVEC in 2% ITS-containing media (serum-free) was boosted twentyfold by TNF-α (10 ng/ml) and fivefold by OxLDL (50 μg/ml). Pitavastatin (5 μmol/L) reduced the TNF-α- and OxLDL-induced release of PAI-1 by 30 and 38%, respectively.

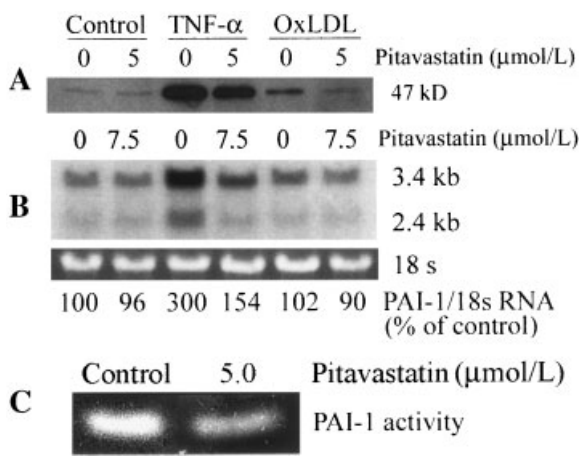

Fig. 2. **A**: Pitavastatin reduces OxLDL- and cytokine-induced PAI-1 release by HUVEC. HUVEC medium was changed to serum-free 2% ITS (without ECGS and heparin) prior to the addition of pitavastatin; 2 h later OxLDL (50 μg/ml) or TNF-α (10 ng/ml) was added for an additional 24 h. Culture medium was collected and equal aliquots used for Western blot of released PAI-1 antigen (47 kDa). Both TF (Fig. 1) and PAI-1 are 47 kDa proteins. **B**: Effect of pitavastatin on expression of PAI-1 mRNA in HUVEC. Serum supplementation of the medium was reduced to 7.5% 2.5 h before addition of pitavastatin; 4 h later TNFα (5 ng/ml) or OxLDL (100 μg/ml) was added. Cells were harvested after 17 h and lysed to extract total RNA. Loading was normalized (as indicated) to the UV ethidium image of the 18s rRNA bands. **C**: Pitavastatin reduces PAI-1 activity in HUVEC conditioned media. Serum supplementation of the medium was reduced to 5% immediately before treatment with pitavastatin. Conditioned medium was collected after 16 h; equal aliquots were used for reverse zymography as described in Materials and Methods. Areas of PAI-1 activity appear opaque on a clearer agar background. An inverted gel image is shown for clarity.

Northern analyses were performed to determine if pitavastatin reduced PAI-1 mRNA in HUVEC. Figure 2B shows that in HUVEC, TNF-α induced a threefold increase of both the 3.4 and 2.4 kb species of PAI-1 mRNA. Pretreatment with pitavastatin reduced by 45 and 60% the TNF-α induction of the 3.4 and 2.4 kb species, respectively. In HUVEC treated with OxLDL, PAI-1 mRNA of the 3.4 kb band which was modestly reduced 10% by pitavastatin. Pitavastatin also decreased PAI-1 activity of conditioned medium of normal HUVEC as evaluated by reverse zymography (Fig. 2C).

Similarly, pitavastatin decreased PAI-1 expression in SMC. In the absence of serum, PAI-1 was below detectable levels for the antibody, but with SMC in low serum, pitavastatin reduced PAI-1 antigen by 35% (Fig. 3A). In the presence of OxLDL (50 μg/ml), SMC secreted more PAI-1 antigen (up 25%), which was inhibited by pitavastatin (Fig. 3B). However, pitavastatin did not appreciably alter PAI-1 mRNA expression in SMC (Fig. 3C). Reverse zymography did, however, show reduced activity of PAI-1 in a dose-dependent manner in conditioned medium from SMC treated with pitavastatin (Fig. 3D). Finally, reverse zymography showed that PAI-1 activity of SMC increased in the presence of OxLDL and was significantly reduced by pitavastatin (Fig. 3E).

Pitavastatin Enhances t-PA Protein, mRNA Expression, and Activity in HUVEC and SMC

t-PA antigen was modestly increased (15%) in the conditioned media of untreated HUVEC by pitavastatin (Fig. 4A). Both TNF-α (5 ng/ml) and OxLDL (100 μg/ml) also suppressed t-PA antigen release by HUVEC. Pitavastatin reversed the effects of TNF-α and OxLDL by raising t-PA antigen levels twofold or more than untreated control levels (Fig. 4A). Changes of t-PA message expression seen in the Northern blot in control and OxLDL-treated HUVEC (Fig. 4B) mirrored those in the Western blot (Fig. 4A) whereas t-PA message expression after TNF-α treatment was essentially unaltered. Zymography of conditioned media showed pitavastatin increased t-PA activity (Fig. 4C). Importantly, pitavastatin increased t-PA activity in the presence of OxLDL (Fig. 4D).

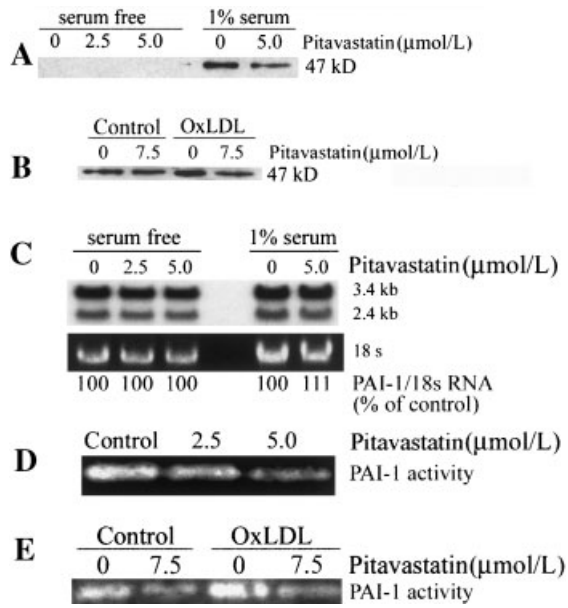

Fig. 3. **A**: Pitavastatin reduces basal PAI-1 release by human aortic SMC. Confluent SMC were changed to serum-free or 1% serum supplemented medium (without ECGS) 2 h before treatment with pitavastatin. Conditioned media was harvested after incubating for 19 h. Equal aliquots were used for gel electrophoresis and Western blots. **B**: Pitavastatin reduces OxLDL-induced PAI-1 release. Serum was reduced to 1% 1 h before adding pitavastatin. Cells were treated with OxLDL (50 μg/ml) 4 h later and incubated for 15 h. Western analysis was done on equal aliquots of conditioned medium. **C**: Northern analyses of PAI-1 mRNA expression in SMC treated with pitavastatin. At the time of experiments, serum supplementation of the medium was reduced as shown 2 h before treatment with pitavastatin. Cells were harvested after incubating for 19 h. Loading was normalized (as indicated) to the UV ethidium image of the 18s rRNA bands. **D**: Pitavastatin decreases PAI-1 activity in SMC conditioned medium. Serum supplementation of medium was reduced to 0.5% 1 h before treatment with pitavastatin; conditioned medium was collected after 16 h. Equal aliquots were used for reverse zymography as described. Areas of PAI-1 activity appear opaque with a clearer agar background. An inverted gel image is shown for clarity. **E**: Pitavastatin decreases PAI-1 activity in the conditioned medium of OxLDL-treated (50 μg/ml) SMC. Serum was reduced to 1% 2 h before pitavastatin treatment. Conditioned medium was collected after 19 h. Reverse zymograph was developed as above.

In SMC, release of t-PA antigen to the conditioned media showed a dose-dependent stimulation by pitavastatin under serum-free and low serum conditions (Fig. 5A). Induction of t-PA message mirrored increases of t-PA antigen as seen in the accompanying Northern blot (Fig. 5B), up 70% when SMC were in low serum rather than serum free medium. Finally, t-PA activity as assessed by zymography was increased by pitavastatin, both with and without OxLDL (Fig. 5C).

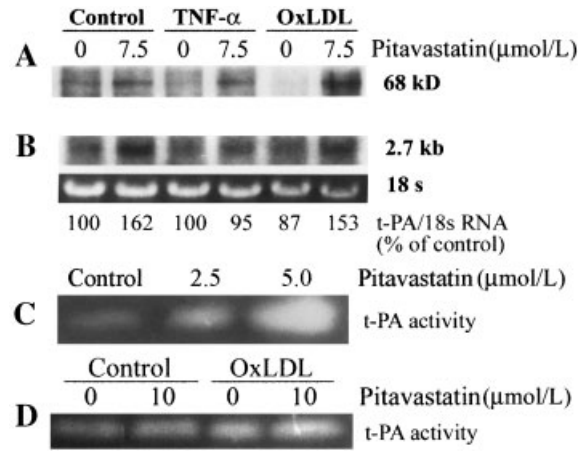

Fig. 4. **A**: Pitavastatin increases t-PA antigen release by HUVEC. Cells were treated as described for Figure 2B. Released t-PA in conditioned media was first immunoprecipitated followed by Western blot analysis (band locates at 68 kDa). **B**: Pitavastatin increases t-PA mRNA expression in HUVEC. Cells were treated as described for Figure 2B. Loading was normalized (as indicated) to the UV ethidium image of the 18s rRNA bands. **C**: Pitavastatin increases t-PA activity from condition media of HUVEC. Serum supplementation was reduced to 5% 4 h before pitavastatin treatment. Conditioned medium was collected after 16 h. Equal aliquots were used for zymography with clear bands of lysis indicating t-PA activity. **D**: Pitavastatin elevates t-PA activity in the presence of OxLDL. Serum supplementation of medium was reduced to 7.5%; cells were treated with OxLDL (100 μg/ml) and conditioned medium collected as described for Figure 2B.

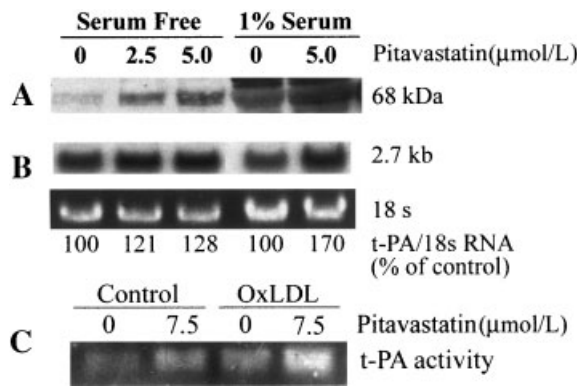

Fig. 5. **A**: Increased release of t-PA antigen by pitavastatin-treated SMC. Cells were changed to medium with serum reduced as shown, experimental conditions were as described for Figure 3C. Cells were harvested and conditioned medium collected after 19 h. To determine t-PA antigen, equal aliquots of conditioned media were prepared directly by SDS–PAGE and Western analysis. **B**: Northern analyses of t-PA mRNA expression. Cells were treated as described for Figure 3C. Equal aliquots of total RNA were analyzed. Loading was normalized (as indicated) to the UV ethidium image of the 18s rRNA bands. **C**: Pitavastatin elevates t-PA activity in the presence of OxLDL (50 μg/ml). SMC were treated as described in Figure 3B above. Equal aliquots were used for zymography with clear bands of lysis indicating t-PA activity.

DISCUSSION

Expression of pro-thrombotic mediators by cells within atheroma poses a risk of thrombosis if plaque integrity is compromised. As such, atherosclerotic lesions are vulnerable sites constantly primed for thrombus formation in the event of endothelial damage or plaque disruption. Recent studies on the "lipid-independent" properties of statin drugs describe a range of actions that favor fibrinolysis and plaque stability while reducing thrombogenicity, inflammation, lesion progression, and clinical events [Libby, 2000; Gotto, 2001; Takemoto and Liao, 2001]. In this study, we investigated the capability of pitavastatin, a novel and metabolically long-acting statin, to modulate expression and production of key proteins responsible for balancing activity of the thrombosis and fibrinolysis.

Our data demonstrate for the first time that an HMG-CoA reductase inhibitor (pitavastatin) will inhibit expression of TF antigen in monocytes stimulated by exposure to OxLDL. Furthermore, we demonstrate that pitavastatin reduces TF antigen expression by LPS-stimulated monocytes. Monocytes/macrophages play a key role in atherosclerosis through accumulation of cholesteryl esters, foam cell development [Steinberg et al., 1989; Hamilton et al., 1999], and their potential to destabilize plaque via expression of active metalloproteases [Katsuda et al., 1994; Galis et al., 1995]. Activated monocytes/macrophages also have the potential to promote coagulation by abundant TF production expressed in both the lipid core and foam cells that surround the core of atheroma [Wilcox et al., 1989; Taubman et al., 1997]. Monocytes/macrophages show increased expression of TF antigen upon exposure to OxLDL [Steinberg et al., 1989; Hamilton et al., 1999]. Thus, any reduction of TF expression in monocytes/macrophages exposed to OxLDL or inflammatory mediators should lower the risk of thrombosis, particularly at unstable atheroma. In cholesterol-fed rabbits, fluvastatin has also been shown to reduce TF expression, attenuate macrophage accumulation in experimental lesions [Baetta et al., 2002], and inhibit platelet adherence in perfused aortic segments [Camera et al., 2002].

Fluvastatin inhibits TF expression in vascular cells by a mechanism requiring geranylgeranylated proteins [Colli et al., 1991]. Pitavastatin inhibits osteopontin expression in rat aortic SMC by the same mechanism [Takemoto et al., 2001]. Pitavastatin also activates PPARα via statin-induced inhibition of the Rho-signaling pathway [Martin et al., 2001]. Although we did not specifically assess the effects of pitavastatin on isoprenoid synthesis, it is likely that pitavastatin inhibits the mevalonate pathway and isoprenylation of small G proteins in U-937 monocytes, HUVEC, and human aortic SMC by a similar mechanism [Martin et al., 2001; Takemoto et al., 2001].

Endothelial and smooth muscle cells also contribute to hemostasis and fibrinolysis by balancing production of t-PA and PAI-1. Our data showing decreased PAI-1 production by pitavastatin treatment of HUVEC and human SMC are consistent with the findings of others [Essig et al., 1998; Bourcier and Libby, 2000; Wiesbauer et al., 2002]. Importantly, we show that pitavastatin inhibits the increase in PAI-1 expression and decrease in t-PA expression associated with OxLDL, which has been shown to disrupt the fibrinolytic balance [Mertens and Holvoet, 2001]. We found that pretreatment of HUVEC with pitavastatin reduced TNF-α-induced PAI-1 antigen secretion, PAI-1 mRNA expression, and attenuated PAI-1 activity (Fig. 2). In SMC, pitavastatin decreased both basal PAI-1 antigen and activity, and PAI-1 antigen and activity stimulated by OxLDL. However, under the conditions and time frame that we evaluated, pitavastatin did not alter PAI-1 mRNA expression (Fig. 3). Basal secretion of t-PA antigen in HUVEC was lower in the presence of OxLDL [Beaudeux et al., 2001] and TNF-α (Fig. 4). Pitavastatin restored t-PA antigen to levels greater than control in addition to raising t-PA antigen activity as determined by zymography. Similar effects have been reported with simvastatin [Bourcier and Libby, 2000] and lovastatin [Essig et al., 1998]. In SMC, pitavastatin raised t-PA antigen and activity. However, an increase in t-PA mRNA was only observed when SMC were cultured in low serum, but not serum free conditions (Fig. 5).

Similar to our findings, Wiesbauer et al. [2002] reported that PAI-1 antigen was decreased and t-PA antigen increased in human umbilical vein SMC and HUVEC by five other statins. Essig et al. [1998] reported statin-induced changes of key proteins regulating fibrinolysis in rat aortic endothelial cells. They showed increases of t-PA

antigen mRNA and activity induced by lovastatin and decreased PAI-1 expression and activity. Mevalonate and geranylgeranyl pyrophosphate (GGPP), but not farnesyl pyrophosphate, inhibited the capability of lovastatin to augment t-PA activity and protein expression while decreasing PAI-1 activity [Essig et al., 1998]. C3 exoenzyme, which blocks isoprenylation of Rho proteins, induced the same effects as lovastatin on PAI-1 and t-PA. In addition, cytochalasin D (which disrupts actin filaments) mimicked the effect of lovastatin's on t-PA secretion in a dose-dependent fashion [Essig et al., 1998]. Pretreatment of SMCs with simvastatin reduced the PDGF-induced increase of PAI-1 [Bourcier and Libby, 2000]. Similarly, pretreatment of endothelial cells with simvastatin reduced PAI-1 induction by both PDGF and TGF-β1, while increasing t-PA antigen secretion. In SMC, simvastatin's inhibitory effect could be overridden with mevalonate and GGPP. Simvastatin inhibited expression of PAI-1 through a mechanism involving reduced PAI-1 promoter activity and gene transcription [Bourcier and Libby, 2000].

In summary, we demonstrate that pitavastatin inhibits OxLDL-induced TF production in monocytic cells and PAI-1 expression by both endothelial and vascular SMC. Conversely, pitavastatin increased basal release of t-PA antigen from vascular SMC and HUVEC and increased expression of t-PA in cells treated with either OxLDL or TNF-α. Pitavastatin decreased biological activity of PAI-1 and increased t-PA activity. Thus, pitavastatin may alter the local fibrinolytic balance toward increased fibrinolysis, reducing the risk of thrombosis and clinically significant cardiovascular events.

ACKNOWLEDGMENTS

This study was funded by a Sponsored Research Agreement from Kowa Company, Ltd., Tokyo, Japan awarded to Drs. D.P. Hajjar and A.M. Gotto, Jr. Dr. Yokoyama is a Senior Researcher with Kowa Co. Technical assistance by Yalina Disla, Jessica Hodin, and Matt Cheifetz is gratefully acknowledged. We also appreciate the methodological advice we received from Dr. Andrew Jacovina regarding the t-PA and PAI-1 activity assays. Dr. Markle was supported by a sabbatical leave from the Physiology and Morphology Group, Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ.

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