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LTBP-1 Blockade in Dioxin Receptor-Null Mouse Embryo Fibroblasts Decreases TGF-β Activity: Role of Extracellular Proteases Plasmin and Elastase

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Abstract In mouse embryonic fibroblasts (MEF) lacking dioxin receptor (AhR), high levels of latent transforming growth factor-β (TGF-β)-binding protein-1 (LTBP-1) correlated with increased TGF-β1 activity, an observation suggesting that LTBP-1 could contribute to maintain TGF-β1 levels. Here, using small interfering RNAs (siRNA), we have first analyzed if LTBP-1 expression affected TGF-β1 activity in MEF cells. We have then determined how LTBP-1 levels could alter the activity of extracellular proteases known to activate TGF-β1, and finally, whether protease inhibition could reduce TGF-β1 activation. LTBP-1 inhibition by siRNA in AhR−/− MEF decreased the amount of active TGF-β1 and reduced plasminogen activators (PA)/plasmin and elastase activities and thrombospondin-1 (TSP-1) expression, without significantly affecting their mRNA levels. On the contrary, LTBP-1 siRNA restored matrix metalloproteinase-2 (MMP-2) activity in AhR−/− MEF. Interestingly, whereas a TGF-β1 neutralizing antibody mimicked many of the LTBP-1 siRNA effects on extracellular proteases, addition of recombinant TGF-β1 protein increased proteases activity over basal levels in AhR−/− MEF. These proteases contributed to TGF-β activation since their specific inhibitors reduced active TGF-β levels in these cells. These results suggest that LTBP-1 contributes to TGF-β1 activation in MEF, possibly by influencing the activities of PA/plasmin, elastase, TSP-1, and MMP-2. TGF-β1, on the other hand, could be also involved in maintaining the activity of these extracellular proteases. Thus, LTBP-1 appears to play a role in TGF-β1 activation through a process involving extracellular protease activities, which, in turn, could be affected by TGF-β1 levels. J. Cell. Biochem. 97: 380–392, 2006.

Key words: dioxin receptor; LTBP-1; TGF-β1; PA/plasmin; elastase; transglutaminase-II; thrombospondin-1

Transforming growth factors β (TGF-β) are cytokines having a relevant role in cell proliferation and growth, development, matrix homeostasis, immunomodulation, and cancer [Massague, 2000; Koli et al., 2001; Lawrence, 2001; Rifkin, 2005]. TGF-βs are synthesized as latent complexes composed by a dimmer of TGF-β covalently bound to a dimmer of the propeptide LAP. After synthesis and before secretion, a furin convertase cleaves the propeptide between Arg278-Arg279, rendering a latent TGF-β non-covalently associated to LAP [Dubois et al., 1995]. In most cells, the efficient secretion of latent TGF-β requires its association to a third protein called latent TGF-β-binding protein (LTBP), thus forming the large latent TGF-β complex [Miyazono et al., 1993]. Whereas the carboxyl terminus of latent TGF-β-binding protein-1 (LTBP-1) binds latent

Abbreviations used: AhR, dioxin receptor; ECM, extracellular matrix; LTBP-1, latent TGF-β-binding protein-1; MEF, mouse embryo fibroblasts; MMP, matrix metalloproteinase; PA, plasminogen activators; TGase-II, transglutaminase-II; TGF-β, transforming growth factor-β; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; TSP-1, thrombospondin-1.

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TGF-β by forming a disulphide bound between its third 8-Cys domain and LAP [Saharinen and Keski-Oja, 2000], the amino terminus anchors the protein to the extracellular matrix (ECM) [Saharinen et al., 1999]. Binding of LTBP-1 to the ECM is accomplished by tissue transglutaminase (tTGase II) activity [Nunes et al., 1997; Verderio et al., 1999] following a mechanism that often involves fibrillin-1 [Isogai et al., 2003] or fibronectin [Dallas et al., 2005] as docking proteins. Once in the ECM, TGF-β is activated by several mechanisms that include proteolytic cleavage of LTBP-1 or LAP, release by thrombospondin-1 (TSP-1) or integrin $\alpha_v\beta_6$, modification of LAP by reactive oxygen species or acidification of the medium (reviewed in [Koli et al., 2001; Lawrence, 2001; Annes et al., 2003]). Activated TGF-β will bind to membrane-bound serine-threonine receptors and trigger a complex intracellular transduction pathway [Massague, 1998].

A relevant question in TGF-β biology is to understand its mechanisms of activation, and in particular, the action of proteases such as the plasminogen activators (PA)/plasmin system, elastase, matrix metalloproteinases (MMPs), and TSP-1. Previous studies have shown that proteolytic cleavage of LTBP-1 by osteoclasts releases latent TGF-β from ECM stores [Pedrozo et al., 1999; Dallas et al., 2002]; purified plasmin produces large amounts of latent [Taipale et al., 1995; Pedrozo et al., 1999; Dallas et al., 2002] and small amounts of active TGF-β [Pedrozo et al., 1999]; purified elastase proteolizes LTBP-1 and releases latent TGF-β from bone [Dallas et al., 2002], and epithelial cell matrix [Taipale et al., 1995]; MMP-2 and MMP-9 cleave soluble LTBP-1 [Dallas et al., 2002] and TSP-1 activates this cytokine in vitro [Schultz-Cherry and Murphy-Ullrich, 1993] and in vivo [Crawford et al., 1998]. The large latent TGF-β complex has been proposed as a sensor for TGF-β function in which LTBP is the localizer, LAP the detector and the cytokine the effector activating membrane receptors [Annes et al., 2003]. Therefore, it appears plausible that LTBP could have a role in proteolysis-mediated release and activation of TGF-β from the ECM [Miyazono et al., 1991; Koli et al., 2001].

The dioxin receptor (AhR) is a transcription factor that mediates the toxic and carcinogenic responses to polycyclic-aromatic hydrocarbons [Fernandez-Salguero et al., 1996; Gonzalez and Fernandez-Salguero, 1998; Shimizu et al., 2000]. In addition to this role in xenobiotic signaling, the AhR is also related to TGF-β function in absence of xenobiotics. Primary hepatocytes and mouse embryo fibroblasts (MEF) from AhR−/− mice had increased levels of total and active TGF-β that were associated to decreased proliferation and higher apoptosis numbers [Zaher et al., 1998; Elizondo et al., 2000; Santiago-Josefat et al., 2004]. Differential gene expression analysis in AhR+/+ and AhR−/− MEF revealed that the mutant cells had increased expression of LTBP-1 and that antibodies against this protein could partially lower their elevated TGF-β1 levels. Further, mRNA analysis showed no changes in LTBP-2, 3, and 4 in AhR−/− MEF with respect to wild-type cells, suggesting a specific role for this receptor in the control of LTBP-1 expression [Santiago-Josefat et al., 2004]. In vivo, the portal areas of AhR−/− livers had higher levels of LTBP-1 that co-localized with TGF-β1 and with collagen accumulation [Corchero et al., 2004], thus providing a plausible explanation for the portal fibrosis found in this animal model [Fernandez-Salguero et al., 1995; Peterson et al., 2000].

In this study, we have analyzed whether the increase in TGF-β1 activity in AhR−/− MEF could be related to changes in the activity of extracellular proteases known to activate this cytokine such as the PA/plasmin system and elastase, TSP-1, and MMP2. Since LTBP-1 is considered to contribute to TGF-β1 activation, we have also determined by siRNA if LTBP-1 levels affect TGF-β activity and proteases activation. Our data suggest that LTBP-1 could contribute to TGFβ activation in AhR−/− MEF through a mechanism involving extracellular proteases known to activate this cytokine. These results support a role for LTBP-1 not only as a localizer but also in the activation of TGF-β in primary mouse fibroblasts.

MATERIALS AND METHODS

Chemicals and Reagents

Dicer siRNA generation kit was purchased from Gene Therapy Systems, Inc., Plasmin substrate Spectrozyme PL was obtained from American Diagnostica, Inc., *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide, biotinylated cadaverine, *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (MeOSuc), aprotinin, amiloride, anti-β-Actin, streptavidin-HRP, *o*-phenylenediamine,

and human recombinant TGF-β1 were from Sigma-Aldrich. Taq DNA polymerase and M-MLV reverse transcriptase were from Ecogen and from Ambion, respectively. SuperScript II RNase H was purchased from Invitrogen. SYBR Green I was obtained from Molecular Probes and QTaq DNA Polymerase Mix from Becton-Dickinson. Dulbecco's-modified Eagle's medium (D-MEM), OPTI-MEM, cell media supplements and fetal bovine serum (FBS) were obtained from BioWhittaker. Mouse anti-rabbit IgG-HRP was obtained from Pierce. Anti-TGF-β 1D-11 neutralizing antibody was from R&D Systems and its neutralizing activity was titrated as indicated below. Anti-TSP-1 antibodies for Western immunobloting (clone D4.6) and to neutralize TSP-1 activity (clone A4.1) were from NeoMarkers. Rabbit anti-LTBP-1 "hinge" antibody was a generous gift from Dr. Sarah Dallas and its specificity has been described previously [Dallas et al., 2000].

Cell Culture

AhR-null and wild-type control mice of the same genetic background (C57BL6/N × 129/sV) were produced as previously reported [Fernandez-Salguero et al., 1995] and used following the Regulation for Animal Care and Use established by the University of Extremadura. MEF were obtained from 14.5-day *post-coitum* embryos [Santiago-Josefat et al., 2004] and cultured in D-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. MEF from the second passage were used in all the experiments. MEF-conditioned media were routinely obtained from cultures maintained at confluence for 48 h in complete D-MEM. To determine TGF-β levels and MMP activities, MEF were grown to confluence and then transferred to serum-free OPTI-MEM for 48 h. Human recombinant TGF-β1 protein was used at 10 ng/ml whereas anti-TGF-β antibody was added at 1 μg/ml (see below). Mink lung epithelial Mv1Lu cells were grown in minimum essential medium (Earle's salts) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate at 37°C in a 5% CO_2 atmosphere.

Reverse Transcription, PCR, and Real-Time PCR

Total RNA was isolated with the RNeasy kit from Qiagen following the manufacturer's instructions. Aliquots of 1 μg total RNA were reverse transcribed at 42°C for 60 min using oligo(dT) priming and M-MLV reverse transcriptase. PCR amplification for a 852 bp fragment of the *Elastase* cDNA (GenBank NM008605) was carried out using the primers forward 5′-GATGGCAAAGGTGGTACACT-3′ and reverse 5′-GGTGACACGACGGAACAGGG-3′; for a 258 bp fragment of the *urokinase Plasminogen Activator* cDNA (*uPA*, GenBank NM008873) the primers were forward 5′-TGCCCAAGGAAATTCCAGGG-3′ and reverse 5′-GCCAATCTGCACATAGCACC-3′; for a 273 bp of the *tissue Plasminogen Activator* cDNA (*tPA*, GenBank NM008872) the primers were forward 5′-GGGAGGTTCAGAAGAGGAGCCCGGT-3′ and reverse 5′-GCGTTTCCCTACAAATCCATCAGGG-3′, and for a 478 bp of the *β-Actin* cDNA (GenBank NM007393) the primers were forward 5′-GGTCAGAAGGACTCCTATGTGG-3′ and reverse 5′-TCCCTCTCAGCTGTGGTGGT-3′. Amplification was carried out for 30 cycles in 50 μl reaction mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM each primer, 2.5 U Taq polymerase, and 3 μl of each reverse transcription reaction as template. Cycling conditions were: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. PCR products were visualized in agarose gels stained with ethidium-bromide.

For real-time PCR, 10 μg total RNA were reverse transcribed for 2 h at 42°C using 2.5 μM random hexamers and 150 U of SuperScript II RNase H. RNA was degraded by the addition of NaOH and by 10 min incubation at 70°C. After neutralization with HCl, cDNAs were ethanol-precipitated and dissolved in DEPC water. Real-time PCR was carried out using SYBR Green I, QTaq DNA Polymerase Mix, and 5 μl of each RT reaction in a DNA Engine Opticon 2 System (MJ Research) as described [Guo et al., 2004]. A single RNA sample per treatment was used to give four measurements per gene per experimental condition. *β-Actin* amplification of the same cDNAs was used as internal standard. Primers for *tPA* and *β-Actin* were as above whereas for *uPA* the following sequences were used: forward 5′-TGAGCTGGGGCCGAGGATG-3′ and reverse 5′-TGGGCAGCACAGCAGAGGAT-3′. In negative controls, in which no template was added to the *β-Actin* primers, no PCR products were detected after 40 cycles.

PCR conditions were: initial denaturation at 95°C for 10 min followed by denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. Average cycle thresholds (CT) for the *uPA* and *tPA* genes and for the *β-Actin* reference were obtained using the Opticon Monitor software version 2.02.

LTBP-1 siRNA Synthesis and Transfection

LTBP-1 mRNA expression was downregulated in MEF cultures by transient transfection with siRNAs synthesized in vitro using the Dicer siRNA generation kit, following the manufacturer's instructions. Briefly, a 5′ region of 623 bp of the mouse LTBP-1 mRNA was selected using the method of Elbashir et al. [2002]. An aliquot of 1 μg total RNA was reverse transcribed and amplified by PCR as indicated above using the primers forward 5′-GCGTAATACGACTCACTATAGGGAGATACAGCCGGAGCTCAACAGCC-3′ and reverse 5′-GCGTAATACGACTCACTATAGGGAGAGGCTTGAGGGTCAAGGTCATC-3′. This cDNA fragment was the template to synthesize by in vitro transcription a double-stranded RNA (dsRNA) using T7 RNA polymerase (annealing sites underlined) and NTPs. The dsRNA obtained was used to generate siRNAs by digestion with recombinant Dicer enzyme: 1 μg dsRNA, 1 mM ATP, 2.5 mM $MgCl_2$, 1 U Dicer in a final volume of 10 μl. The reaction was incubated overnight at 37°C and the products analyzed by agarose gel electrophoresis. 21-mer siRNAs were purified by column chromatography and quantitated. AhR−/− MEF were transfected with siRNAs in 35 mm plates at 60%−70% confluence. Gene Silencer transfection reagent was prepared by diluting 3.5 μl reagent with 25 μl serum-free medium. siRNA solution was prepared by mixing 10 μl diluent in 15 μl serum free medium. Both solutions were mixed, incubated at room temperature for 5 min and added to the cultures. When cultures reached confluence (e.g., 24−36 h post-transfection), fresh medium was added and cultures maintained for 48 additional hours.

PA/Plasmin and Elastase Activity Assays

AhR+/+ and AhR−/− MEF were grown to confluence in serum-containing medium. Cells were then treated for 48 h with transfection reagents (Mock), 1μg LTBP-1 siRNA, 1 μg/ml anti-TGF-β, or 10 ng/ml recombinant TGF-β1. Conditioned media were recovered, normalized to the same number of cells and used for enzyme activity assays. PA/plasmin activity was determined by an amidolytic assay using the initial-rate method (initial velocity of the reaction) in a reaction mixture containing 50 mM Tris-HCl pH 7.4, 105 μM of the plasmin-specific substrate Spectrozyme PL (H-D-norleucyl-hexahydrotyrosol-lysine-*p*-nitroanilide diacetate), and 200 μl conditioned medium. The amount of product formed (*p*-nitroanilide) was measured at 405 nm and 25°C. Elastase activity was determined at 410 nm and 25°C by the initial-rate method in a reaction mixture containing 100 mM Tris-HCl pH 8.0, 293 μM substrate (*N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide), and 200 μl conditioned medium.

In Situ tTGase II Activity

tTGase II activity was measured by the method of Ladd et al. [2003]. Briefly, AhR+/+ and AhR−/− MEF were grown and maintained at post-confluence for 48 h. During the last 2 h of culturing, 2 mM biotinylated cadaverine was added and conditioned media harvested and normalized to the same number of cells. For the in situ ELISA, 96-well plates were coated overnight at 4°C with 50 or 75 μl conditioned medium in 100 mM Tris-HCl pH 8.3. After washing in 100 mM Tris-HCl pH 8.3, plates were blocked at room temperature for 1 h in PBS containing 5% BSA, 0.01% SDS, and 0.01% Tween 20. Plates were washed and samples incubated at room temperature for 4 h with streptavidin-HRP in PBS containing 1% BSA and 0.01% Tween 20. Following additional washing, 0.36 mg/ml *o*-phenylenediamine dihydrochloride in citrate-phosphate buffer was added and incubation continued for 10 min at room temperature. Reactions were stopped by the addition of 150 μl 1.5 N HCl. tTGase II activity was determined from the increase in absorbance at 492 nm.

Immunoblotting

Cells were washed with PBS and lysed at 4°C in a buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenyl-methyl sulfonyl fluoride, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT, 10 mM β-glycerophosphate, and 4 μg/ml complete protease inhibitor cocktail. Lysates were centrifuged at 15,000*g* for 15 min at 4°C and protein concentration determined in the supernatants

using the Coomassie Plus protein assay reagent and BSA as standard. Aliquots of 15 μg protein were denatured, separated on 8% SDS–PAGE gels, and transferred to nitrocellulose membranes. Membranes were blocked for 2 h at room temperature in TBS-T (50 mM Tris-HCl pH 7.5, 10 mM NaCl, 0.5% Tween 20) containing 5% non-fat milk and incubated overnight at 4°C with the corresponding primary antibodies. After washing in TBS-T, blots were incubated with the HRP-coupled secondary antibody for 1 h at RT. Following additional washing in TBS-T, the SuperSignal chemiluminescence substrate was added and the blots were exposed and developed using a Molecular Imager FX System (Bio-Rad).

Bioassay for TGF-β Activity

Confluent AhR+/+ and AhR−/− MEF were switched to serum-free OPTI-MEM medium for 48 h. Conditioned media were recovered, centrifuged at 500*g* for 15 min at 4°C and the supernatants supplemented with 0.1 mg/ml bovine serum albumin and 0.57 mM phenylmethyl sulfonyl fluoride. Mv1Lu cells were seeded in 24-well plates at 2.5×10^4 cells/well and washed twice with OPTI-MEM. A volume of conditioned medium equivalent to the same number of MEF cells was added to Mv1Lu and culture continued for 24 h. In some experiments, 1 μg/ml neutralizing anti-TGF-β antibody was added to the conditioned media at the time of incubation with the Mv1Lu cells. Two hours to the end of the 24 h incubation period, 0.5 μCi [^{3}H]-methyl-thymidine (esp. act. 7 Ci/mmol) was added to label DNA synthesis. Mv1Lu cells were fixed for 1 h in 1 ml methanol:acetic acid (3:1), washed in 80% methanol, trypsinized in 0.05% trypsin, and solubilized in 1% SDS. The amount of radiactivity incorporated was measured using a Beckman LS-3801 liquid scintillation counter. To analyze the contribution of LTBP-1 to TGF-β activation, AhR−/− MEF were transfected with siRNA as indicated above and conditioned medium collected and used in the bioassay. The neutralizing activity of the anti-TGF-β antibody was previously determined from a titration curve made with conditioned medium from AhR−/− MEF (data not shown). The IC_{50} for neutralization corresponded to 0.06 μg/ml antibody, and based on this result, a 1 μg/ml concentration of anti-TGF-β antibody was used in all the experiments.

Gelatin Zymography for MMP-2 and MMP-9

AhR+/+ and AhR−/− MEF cultures were grown to confluence and media changed to serum-free OPTI-MEM for 48 h. Conditioned media were collected and MMP activity determined by a gelatinolytic assay. After centrifugation at 500*g* for 15 min at 4°C, a volume of medium equivalent to the same number of cells was mixed with non-reducing Laemmli's sample buffer (62.2 mM Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 0.025% bromophenol blue) and applied to 8% SDS–PAGE gels polymerized in presence of 1% gelatin. After electrophoresis, gels were extensively washed in 2.5% Triton X-100 to eliminate SDS and to allow protein reconstitution. MMP activity was developed by incubating the gels for 16 h at 37°C in reaction buffer (50 mM Tris-HCl pH 6.8, 150 mM NaCl, 5 mM $CaCl_2$, and 0.05% sodium azide). MMPs were visualized by staining the gels in Coomassie G-250 solution. To determine the contribution of LTBP-1 to MMP activity, AhR−/− MEF were previously transfected with siRNA as indicated above.

Treatment with Protease Inhibitors

Confluent AhR−/− MEF were treated for 48 h in serum-free OPTI-MEM with protease inhibitors specific for elastase (100 μM MeOSuc, *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone), TSP-1 (1 μg/ml neutralizing A4.1 antibody), plasmin (30 μM aprotinin), or uPA (100 μM amiloride). Conditioned media were colleted and used in a TGF-β bioassay as indicated above.

RESULTS

LTBP-1 Downregulation in AhR−/− MEF Decreased Levels of Active TGF-β and Increased MMP-2 Activity

In a previous work, we have shown that AhR−/− MEF had increased LTBP-1 expression that was coincident with higher TGF-β1 activity and decreased MMP-2 levels. Since anti-LTBP-1 hinge antibody could partially block these effects [Santiago-Josefat et al., 2004], we suggested that LTBP-1 could be involved in maintaining TGF-β1 and MMP-2 activities. To analyze this possibility, we have downregulated LTBP-1 expression in AhR−/− MEF by siRNA. As determined by Western immunobloting and quantitation of several

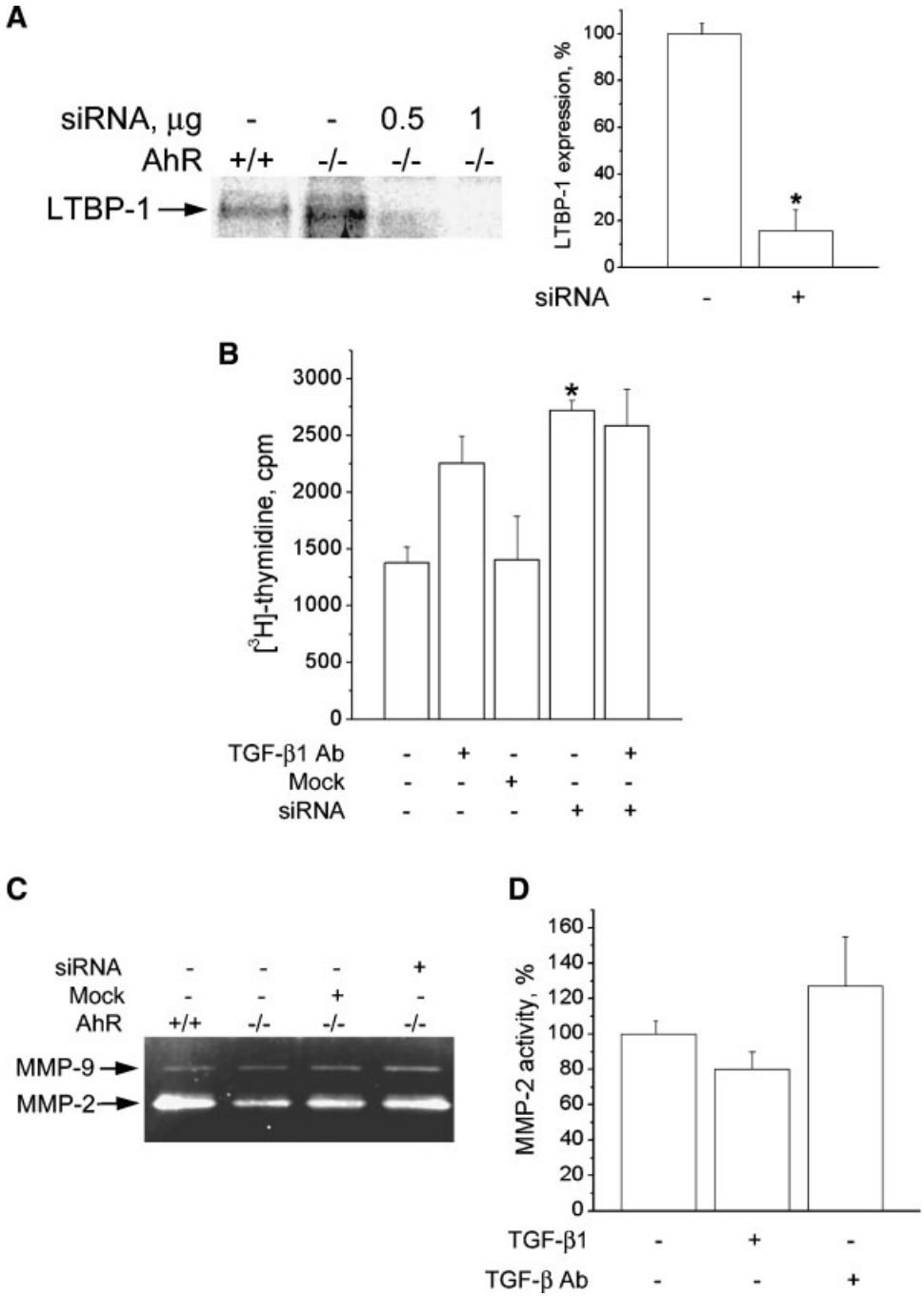

Fig. 1. Transfection of LTBP-1 siRNA in AhR−/− MEF decreases active TGF-β and increases MMP-2 activity. After transfection with siRNA for LTBP-1, AhR+/+, and AhR−/−MEF were grown to confluence and maintained in culture for an additional 48 h. **A**: LTBP-1 siRNA markedly reduced LTBP-1 protein levels in AhR−/− MEF as shown by Western immunoblotting of cell total extracts. Quantitation of three different experiments is shown on the right panel. *The difference is statistically significant at $P < 0.05$ (ANOVA). **B**: AhR−/− MEF were transfected with LTBP-1 siRNA or transfection reagents (Mock) and grown to confluence. Parallel confluent AhR−/− MEF cultures were left untreated or treated with 1 µg/ml neutralizing TGF-β antibody (TGF-β1 Ab). Media were replaced by serum-free OPTI-MEM, which was collected 48 h later. Mv1Lu cells were plated, grown in the MEF conditioned media and used for a TGF-β bioassay. *The addition of LTBP-1 siRNA produced a statistically significant increase in Mv1Lu proliferation ($P < 0.05$, ANOVA). **C**: MMP-2 and MMP-9 activities were determined by gelatin zymography in AhR+/+ and AhR−/− OPTI-MEM-conditioned media. AhR−/− MEF were transfected with transfection reagents (Mock) or LTBP-1 siRNA. **D**: To determine the effect of TGF-β on MMP-2 activity, gelatin zymography was also performed in AhR−/− MEF treated with 10 ng recombinant TGF-β1 (TGF-β1) or with 1 µg/ml neutralizing anti-TGF-β antibody (TGF-β1 Ab). Quantitation was performed in polyacrylamide gels as those shown in panel C by subtracting background from signal at each experimental condition. The results from three different experiments are shown. Data represent mean ± SE.

experiments, transfection of AhR−/− MEF with 1 μg LTBP-1 siRNA could downregulate LTBP-1 protein to levels below 15%–20% those present in untransfected AhR−/− cultures (Fig. 1A). When AhR−/− MEF were transfected with LTBP-1 siRNA and the conditioned media used for a TGF-β bioassay, an increase in Mv1Lu proliferation was observed that matched that obtained by the addition of a TGF-β neutralizing antibody (Fig. 1B), thus indicating that downregulation of LTBP-1 expression could decrease the levels of active TGF-β. Regarding MMPs, AhR−/− MEF had lower MMP-2 activity than AhR+/+ (Fig. 1C and [Santiago-Josefat et al., 2004]). The addition of siRNA for LTBP-1 increased MMP-2 activity to levels close to that present in AhR+/+ cultures (Fig. 1C). To address if this effect of LTBP-1 siRNA on MMP-2 could be mediated, at least partly, by a decrease in TGF-β activity, treatments with recombinant TGF-β1 protein and anti-TGF-β antibody were performed (Fig. 1D). Exogenous TGF-β1 produced a slight decrease in MMP-2 activity whereas a TGF-β neutralizing antibody moderately increased MMP-2 over basal AhR−/− levels. No significant differences were observed under the same experimental conditions for MMP-9 (not shown). These effects of TGF-β on MMP-2 activity, although modest, were reproducible among cultures and suggested that LTBP-1 could be modulating MMP-2 activity through TGF-β.

LTBP-1 siRNA Decreased PA/Plasmin and Elastase Activities and TSP-1 Expression in AhR−/− MEF

A possible explanation for the increased TGF-β1 activity found in AhR−/− MEF could be that these cells also had increased activity of extracellular proteases that activate this cytokine. As shown in Figure 2, PA/plasmin activity was higher in AhR−/− than in AhR+/+ MEF and treatment of mutant cells with LTBP-1 siRNA decreased PA/plasmin activity to levels similar to those found in wild-type cells. In addition, recombinant TGF-β1 increased PA/plasmin activity above basal AhR−/− levels whereas neutralizing anti-TGF-β antibody decreased this activity below AhR−/− levels. Thus, LTBP-1 could contribute to higher levels of PA/plasmin activity in AhR−/− MEF through increased TGF-β activity. To determine if these effects were related to changes in the expression of plasminogen activators tPA and uPA, their

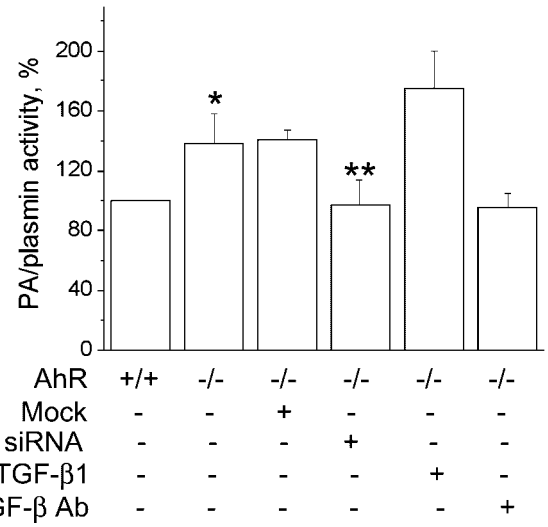

Fig. 2. Amidolytic assay for PA/plasmin activity in AhR−/− MEF. Confluent AhR−/− MEF were left untreated or transfected with LTBP-1 siRNA or transfection reagents (Mock) and cultured 48 h postconfluence. Parallel confluent cultures were treated with 10 ng/ml recombinant TGF-β1 or 1 μg/ml neutralizing anti-TGF-β antibody for 48 h. Untreated AhR+/+ MEF were also used under the same conditions. Conditioned media were harvested and PA/plasmin activity determined using the spectrozyme PL amidolytic assay. *The difference between AhR+/+ and AhR−/− MEF is statistically significant at $P < 0.05$ (ANOVA). **The difference between AhR−/− and AhR−/− transfected with LTBP-1 siRNA is statistically significant at $P < 0.05$ (ANOVA). Values are shown considering PA/plasmin activity in AhR+/+ as 100%. Results from four different experiments are presented.

mRNA levels were analyzed by real-time PCR (Fig. 3A). It was found that *tPA* and *uPA* mRNA levels were similar between AhR−/− and AhR+/+ MEF, thus indicating that increased PA/plasmin activity in mutant cells was not due to increased expression of PAs. In addition, transfection of LTBP-1 siRNA in AhR−/− MEF did not significantly alter *uPA* and *tPA* mRNA expression nor did treatments with TGF-β1 protein or anti-TGF-β antibody (Fig. 3B). Thus, increased PA/plasmin activity in AhR−/− MEF appeared to be associated to TGF-β activity rather than to changes in the expression of uPA and tPA.

Elastase activity was also increased in AhR−/− with respect to AhR+/+ MEFs (Fig. 4A). Although with a less pronounced effect than that observed for PA/plasmin, the addition of LTBP-1 siRNA decreased elastase activity below basal AhR−/− levels. Similarly to the data shown above, recombinant TGF-β1 protein markedly increased while neutralizing anti-TGF-β antibody decreased elastase activity

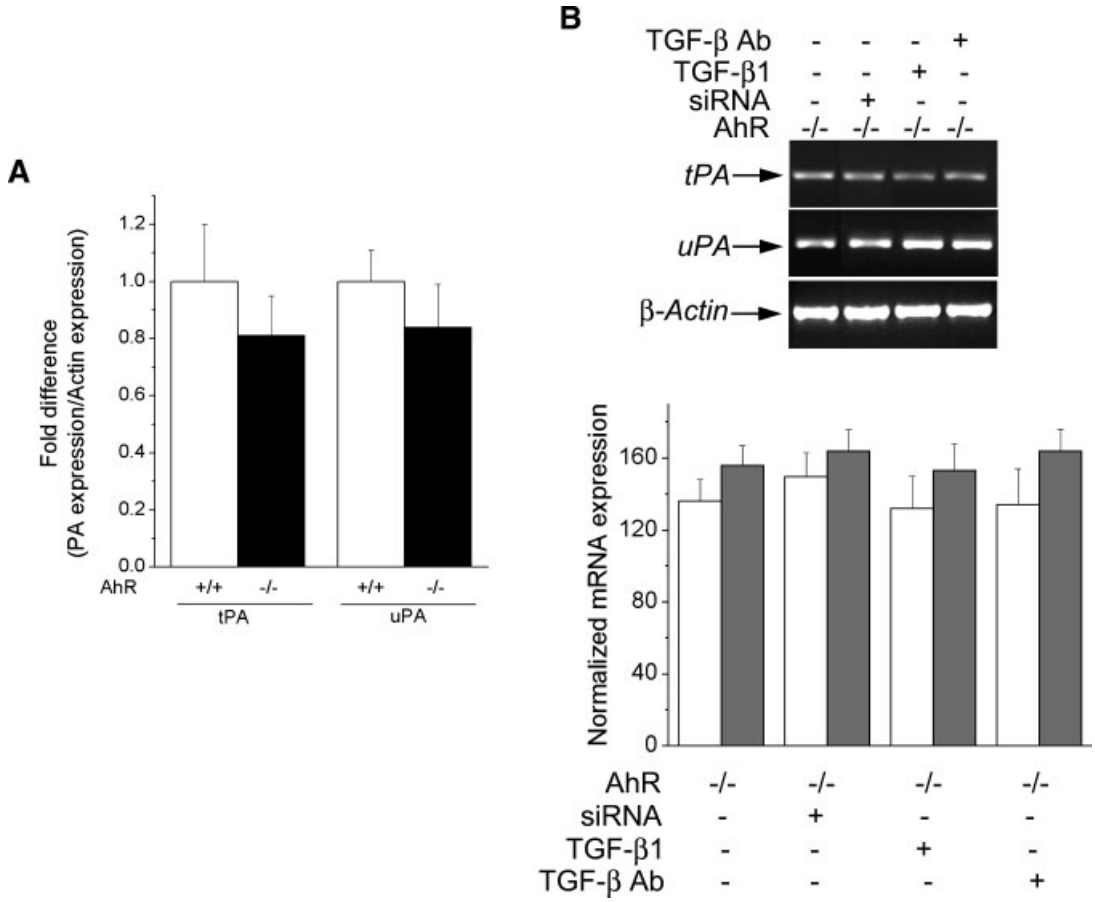

Fig. 3. *uPA* and *tPA* plasminogen activators mRNA levels in AhR+/+ and AhR−/− MEF. **A**: Basal mRNA expression for *tPA* and *uPA* was analyzed by real-time PCR in 48 h post-confluent MEF cultures. **B**: Confluent AhR−/− cells were left untreated, transfected with LTBP-1 siRNA or treated with 10 ng/ml recombinant TGF-β1 or 1 μg/ml neutralizing anti-TGF-β antibody for 48 h, and PA mRNA expression determined by semi-quantitative RT-PCR. Background was subtracted and *tPA* (clear bars) and *uPA* (gray bars) expression normalized by *β-Actin*. Data correspond to mean ± SE from duplicates of two cultures.

(Fig. 4A). With respect to *elastase* mRNA expression, no significant differences were found between AhR−/− and AhR+/+ MEF. Treatment with recombinant TGF-β1 readily increased *elastase* mRNA levels whereas anti-TGF-β antibody or LTBP-1 siRNA had no significant effect (Fig. 4B). These data indicated that increased elastase activity in AhR−/− MEF could be also associated to high levels of TGF-β1 activity.

TSP-1 is an additional protein involved in TGF-β activation. TSP-1 protein expression was increased in AhR−/− with respect to AhR+/+ MEF as could be observed by a representative Western immunobloting (Fig. 5A). Transfection of LTBP-1 siRNA in AhR−/− decreased TSP-1 protein to levels similar to those found in AhR+/+ MEF (Fig. 5A). In contrast, addition of recombinant TGF-β1 protein or anti-TGF-β antibody had only a slight effect on TPS-1 protein (Fig. 5B). Thus, since modulation of TGF-β levels by recombinant protein or antibody had a less pronounced effect on TSP-1 than transfection of LTBP-1 siRNA, it could be possible that LTBP-1 affects TSP-1 levels independently of TGF-β.

tTGase Levels were Similar en AhR+/+ and AhR−/− MEF. The ability of LTBP-1 to localize TGF-β in the ECM is regulated by tTGase II. To determine if increased levels of LTBP-1 and TGF-β in AhR−/− MEF could be related to increased tTGase II activity, we have performed the experiments shown in Figure 6. It could be observed that tTGase II activity was similar in AhR−/− and AhR+/+ MEF. However, LTBP-1 downregulation by siRNA in AhR−/− cells reproducibly increased this enzymatic activity over basal levels. Treatment with

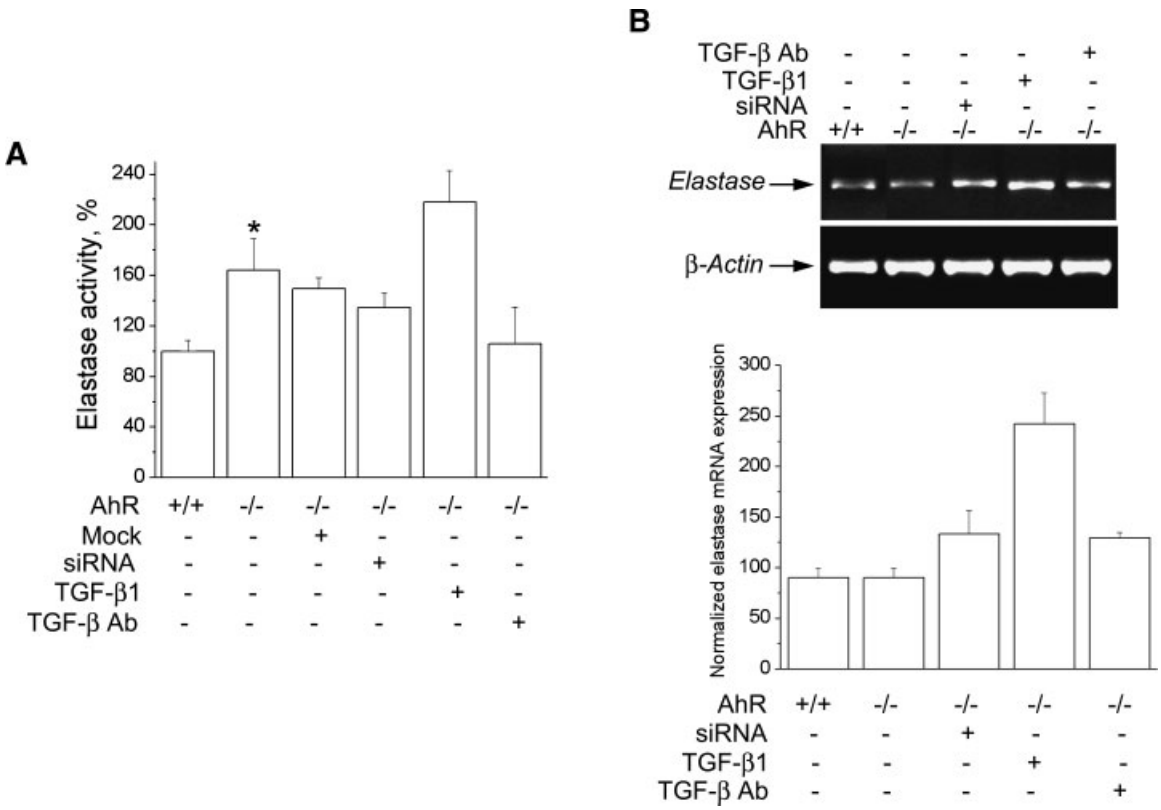

Fig. 4. Elastase activity is increased in AhR−/− MEF. **A**: Confluent AhR−/− MEF growing in complete medium were treated with transfection reagent (Mock), LTBP-1 siRNA, 10 ng recombinant TGF-β1, or 1 μg/ml neutralizing anti-TGF-β antibody for 48 h and elastase activity determined using *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide as specific substrate. Results from at least four different experiments are shown. *The difference is statistically significant at $P < 0.05$ (ANOVA). **B**: *Elastase* mRNA expression was determined in 48 h post-confluent MEF cultures by semi-quantitative RT-PCR. Data were normalized by *β-Actin* expression as indicated in the legend for Figure 3. Data correspond to mean ± SE from two experiments.

recombinant TGF-β1 protein or neutralizing anti-TGF-β antibody did not significantly affect tTGase II activity.

Protease Inhibitors Decreased Active TGF-β Levels in AhR−/− MEF

The results suggested that PA/plasmin, elastase and TSP-1 activities could have a role in maintaining increased TGF-β activation in AhR−/− MEF. To address this issue, AhR−/− MEF were grown in presence of specific inhibitors for these enzymes and changes in active TGF-β determined by a bioassay (Fig. 7). Addition of elastase inhibitor MeOSuc and anti-TSP-1 A4.1 neutralizing antibody partially restored Mv1Lu proliferation, indicating that, to some extent, both enzymes contributed to TGF-β activation. Interestingly, specific inhibitors for either plasmin (aprotinin) or uPA (amiloride) produced a marked increase in Mv1Lu proliferation, indicating not only that PA/plasmin is relevant for TGF-β activation in AhR−/− MEF, but also that both components of the system, plasmin and plasminogen activator urokinase, were involved.

DISCUSSION

One of the less known issues in TGF-β biology is its mechanism of activation in the extracellular environment. Recent studies have suggested that LTBP-1 is not only a localization signal for TGF-β but also a component of its activation mechanism, thus providing experimental support for the proposed sensor model of TGF-β activation [Annes et al., 2003, 2004]. The AhR is functionally related to TGF-β since primary hepatocytes [Zaher et al., 1998] and MEF [Elizondo et al., 2000; Santiago-Josefat et al., 2004] from AhR-null mice had increased activation of TGF-β that probably contributes to their lower proliferation potential. The AhR is also related to LTBP because AhR−/− MEF specifically overexpressed the LTBP-1 isoform

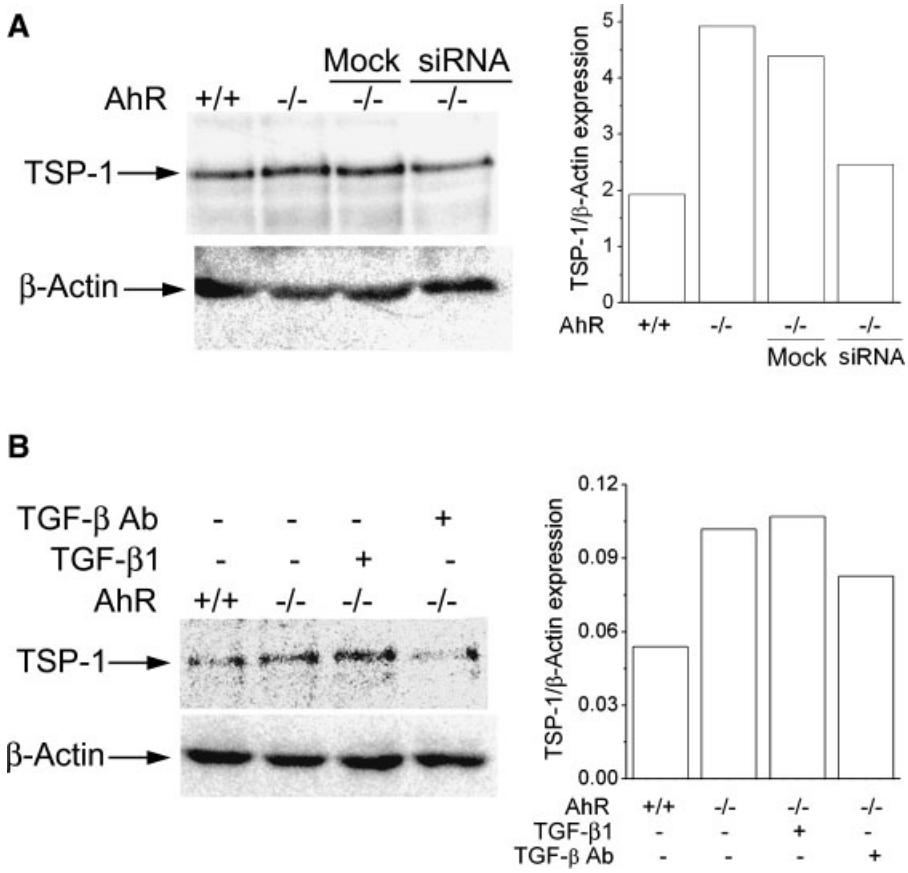

Fig. 5. AhR−/− MEFs have increased TSP-1 expression that can be downregulated by LTBP-1 siRNA. **A**: Confluent AhR−/− MEF were transfected with LTBP-1 siRNA or transfection reagent (Mock) for 48 h, and TSP-1 expression determined by Western immunobloting. β-Actin expression was also analyzed to check for equal protein loading. Quantitation of a representative experiment is shown on the right panel. **B**: Confluent AhR−/− MEF were left untreated or treated with 10 ng recombinant TGF-β1 or 1 μg/ml neutralizing anti-TGF-β antibody for 48 h, and TSP-1 levels determined by Western immunobloting. TSP-1 protein expression in AhR+/+ MEF was also analyzed. β-Actin was used to check for equal loading. Quantitation of a representative Western blot is shown on the right panel. The experiments were performed in three different cultures.

[Santiago-Josefat et al., 2004]. In vivo, AhR-null mouse liver had portal fibrosis [Fernandez-Salguero et al., 1995; Peterson et al., 2000] with overexpression and co-localization of LTBP-1 and TGF-β1 [Corchero et al., 2004]. Considering these previous results, we have analyzed if LTBP-1 could have a role in TGF-β activation and whether or not the absence of AhR could affect the activity of proteases known to activate this cytokine. Among the mechanisms described for TGF-β activation [Annes et al., 2003, 2004; Rifkin, 2005], we have studied changes in proteases such as PA/plasmin and elastase and TSP-1. Since LTBP-1 is linked to the ECM by tTGase II, we have also determined changes in tTGase II activity between AhR+/+ and AhR−/− MEF.

In parallel to increased levels of active TGF-β1, AhR−/− MEF had elevated PA/plasmin, elastase, and TSP-1 activities. The PA/plasmin system has been proposed to participate in the release of LTBP-1-bound latent TGF-β from bone matrix stores in osteoclasts [Dallas et al., 2002] and chondrocytes [Pedrozo et al., 1999]. Plasmin is specifically produced from plasminogen in the extracellular medium by the PAs uPA and tPA. It is known that uPA directly, or via plasmin, can activate or release ECM-bound growth factors such as TGF-β [Tkachuk et al., 1996] and bFGF [Saksela and Rifkin, 1990]. In agreement to these observations, the fact that inhibitors of plasmin (aprotinin) and uPA (amiloride) restored Mv1Lu proliferation suggest that both enzymes of the PA/plasmin system participate in maintaining elevated TGF-β activity in AhR−/− MEF. Purified elastase released latent TGF-β from the ECM of epithelial and endothelial cells [Taipale et al.,

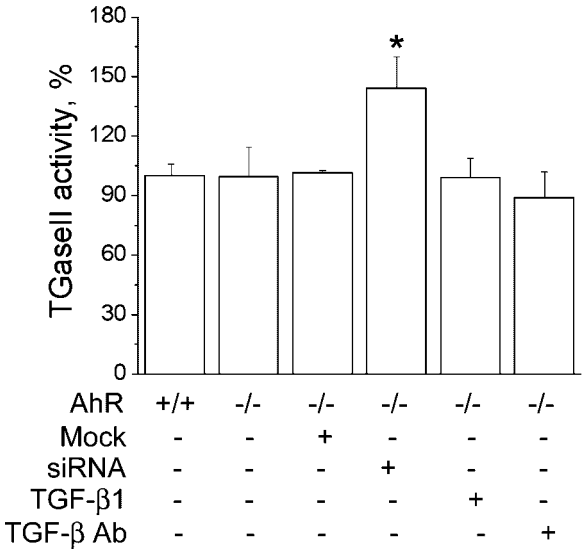

Fig. 6. tTGase II activity has similar levels in AhR+/+ and AhR−/− MEF. Confluent cultures of AhR−/− MEF were treated with transfection reagent (Mock), LTBP-1 siRNA, 10 ng recombinant TGF-β1, or 1 μg/ml neutralizing anti-TGF-β antibody for 48 h. Post-confluent AhR+/+ MEF were also analyzed. tTGase II activity was determined in the conditioned media by in situ ELISA. The experiment was done in octuplicate samples in at least three MEF preparations. Data are shown as mean ± SD. *The difference is statistically significant at $P < 0.05$ (ANOVA).

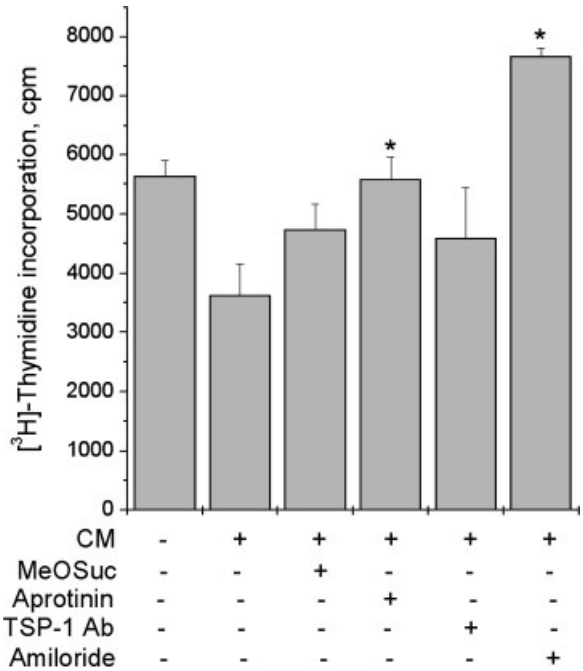

Fig. 7. Protease inhibitors decrease TGF-β levels in AhR−/− MEF. Confluent AhR−/− MEF were treated in serum-free OPTI-MEM for 48 h with protease inhibitors for elastase (100 μM MeOSuc), TSP-1 (1 μg/ml neutralizing A4.1 antibody), plasmin (30 μM aprotinin), or uPA (100 μM amiloride). Conditioned media were colleted and added to Mv1Lu cultures, and a TGF-β bioassay performed as indicated in the Methods. *The difference with respect to CM from AhR−/− MEF is statistically significant at $P < 0.05$.

1995], but it was unable to produce the active form of the cytokine. Interestingly, the elastase-specific inhibitor MeOSuc partially restored Mv1Lu proliferation, suggesting that elastase also participates in TGF-β activation in AhR−/− MEF. In addition, since plasmin generated from plasminogen by uPA and tPA can activate elastase [Mignatti and Rifkin, 1996], and considering that AhR−/− conditioned medium was sensitive to the plasmin inhibitor aprotinin, it is possible that the increase in elastase activity in AhR−/− MEF derives, at least in part, from plasmin activity. Another matrix-associated protein that activates TGF-β is TSP-1, which exerts its role by interacting with LAP [Murphy-Ullrich and Poczatek, 2000]. TSP-1 expression was increased in AhR−/− MEF and a neutralizing antibody partly restored Mv1Lu proliferation, indicating that this protein could be involved in TGF-β activation. Interestingly, however, MMP-2, which also contributes to TGF-β activation [Baramova et al., 1997; Yu and Stamenkovic, 2000] had lower levels in AhR−/− than in AhR+/+, a result showing that this protease may not have a significant contribution to TGF-β activation in AhR-null MEF. Because decreasing TGFβ levels by LTBP-1 siRNA or anti-TGF-β antibody increased MMP-2 activity, it is possible that high TGF-β levels could be inhibiting this protease. In agreement, previous studies have shown that TGF-β inhibits MMP-2 activity not only in cell culture [Wang and Hirschberg, 2003] but also in animal models [Chernajovsky et al., 1997].

siRNA represents a powerful tool to analyze protein function in vitro and in vivo [Hannon, 2002; Sioud, 2004]. Recombinant Dicer has been shown to efficiently release siRNAs from dsRNA [Myers et al., 2003]. LTBP-1 protein expression was downregulated in AhR−/− MEF transfected with siRNA and, in parallel, active TGF-β decreased and MMP-2 activity increased over basal levels. Further, LTBP-1 siRNA consistently decreased PA/plasmin and elastase activities, and TSP-1 overexpression in AhR−/− MEF. A possible explanation for this observation is that TGF-β could be involved in maintaining the activity of these enzymes. Experiments performed in presence of recombinant TGF-β1 or neutralizing antibody against

this cytokine revealed that this could be the case since increasing TGF-β increased enzyme activities and decreasing TGF-β decreased enzyme activities. On the other hand, increased PA/plasmin activity in AhR−/− MEF was not associated to higher uPA and tPA mRNA levels, suggesting that the effect of TGF-β on this system did not affect regulation of gene expression. On the contrary, recombinant TGF-β1 consistently increased *elastase* mRNA, an effect that could contribute to the marked increase in elastase activity induced by this cytokine. tTGase II is a major enzyme involved in linking LTBP-1 to the ECM [Nunes et al., 1997]. In AhR−/− MEF, however, increased LTBP-1 protein was not followed by a parallel increase in tTGase II activity. This result could be explained based on previous observations showing that the correlation between tTGase II activity and LTBP-1 depends on the length of culture. Thus, whereas a positive correlation could be observed in short-term culture, in long-term culture (over 48 h, similar to this study) increased tTGase II activity did not correlate with LTBP-1 binding to the ECM [Verderio et al., 1999].

In summary, we have found that AhR−/− MEF had increased levels of enzymes involved in the activation of TGF-β such as PA/plasmin, elastase, and TSP-1. LTBP-1 appears to contribute to TGF-β activation since LTBP-1 downregulation by siRNA decreased the levels of active cytokine. Decreasing LTBP-1 protein by siRNA had similar effects on PA/plasmin, elastase, and TSP-1 activities than neutralizing antibodies against TGF-β, which suggested that this cytokine could have a role in maintaining their activity. The functional interaction between the PA/plasmin system, elastase, TSP-1, TGF-β, and LTBP-1 could be relevant for the association found between fibrosis, LTBP-1, and TGF-β in the portal areas of AhR−/− mice liver.

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