British Journal of Pharmacology (2007) 150, 424–433
 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Long-term treatment with TGFβ_1 impairs mechanotransduction in bovine aortic endothelial cells

M Watanabe, M Oike, Y Ohta and Y Ito

Department of Pharmacology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Background and purpose: Vascular endothelial cells play a role in the physiological response to mechanical stress. Transforming growth factor β_1 (TGFβ_1) induces morphological changes in endothelial cells, and this may alter their mechanosensitive responses. The aim of this study was to examine the effects of TGFβ_1 on hypotonic stress (HTS)-induced responses in bovine aortic endothelial cells (BAECs).
Experimental approach: Cultured BAECs were treated with 3 ng ml^{-1} TGFβ_1 for 24 h (24h-TGFβ_1) or 7 days (7d-TGFβ_1). Cytosolic actin fibres were stained with rhodamine-phalloidin. Intracellular Ca^{2+} concentration was measured using fura2. Tyrosine phosphorylation and RhoA expression were assessed by Western blotting. Expression of RhoA mRNA was assessed by real-time PCR.
Key results: BAECs developed pseudopod-like processes within 24 h and showed a fibroblast-like appearance after 7 days. HTS induced Ca^{2+} transients via endogenous ATP release in both control and 24h-TGFβ_1 BAECs but not in 7d-TGFβ_1 BAECs. We have previously shown that HTS-induced ATP release is mediated by sequential activation of RhoA and tyrosine kinases. The basal amount of membrane-bound RhoA was significantly lower in 7d-TGFβ_1 than in 24h-TGFβ_1 or control BAECs. HTS increased the membrane-bound RhoA to the same fractional level in 24h-TGFβ_1 and control BAECs, but its net maximal amount was significantly lower in 7d-TGFβ_1. HTS-induced downstream signals of RhoA activation, i.e. the tyrosine phosphorylation of FAK and paxillin, were markedly suppressed in 7d-TGFβ_1 BAECs.
Conclusions and Implications: These results indicate that long-term treatment with TGFβ_1 does not impair mechanoreception in BAECs but impairs mechanotransduction by affecting RhoA membrane translocation.
British Journal of Pharmacology (2007) **150**, 424–433. doi:10.1038/sj.bjp.0707123; published online 15 January 2007

Keywords: vascular endothelium; TGFβ_1; hypotonic stress; RhoA; ATP release; calcium; tyrosine kinase; mechanosensitivity

Abbreviations: BAECs, bovine aortic endothelial cells; FAK, focal adhesion kinase; FBS, foetal bovine serum; HTS, hypotonic stress; TGFβ_1, transforming growth factor β_1

Introduction

Transforming growth factor β_1 (TGFβ_1) is a multifunctional cytokine that regulates cellular growth and proliferation. TGFβ_1 is generated in the vascular wall, especially in injured vessels and atherosclerotic foci (McCaffrey, 2000). The vascular endothelium is one of the main targets of TGFβ_1, which is known to suppress proliferation (Frater-Schroder *et al*., 1986) and to induce morphological changes in endothelium (Arciniegas *et al*., 1992). However, the beneficial effect of TGFβ_1 on vascular function, especially on endothelial function, is still controversial. It has been hypothesized that TGFβ_1 is an atheroprotective cytokine that plays an important role in maintaining normal vascular structure (Grainger, 2004), because a neutralizing antibody for TGFβ_1 was shown to accelerate atherosclerosis (Mallat *et al*., 2001). In contrast, other groups have proposed that TGFβ_1 is essential for the development of atherosclerosis (Chen *et al*., 2003) and medial thickness after a balloon catheter-induced injury (Kanzaki *et al*., 1995).

Vascular endothelial cells have a major role in the physiological response to mechanical stress (Davies, 1995; Takahashi *et al*., 1997). We have previously shown that hypotonic stress (HTS) induces membrane translocation of small G protein RhoA and subsequent tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin (Hirakawa *et al*., 2004). Sequential activation of RhoA and tyrosine kinases then leads to ATP release and actin reorganization in bovine aortic endothelial cells (BAECs) (Koyama *et al*., 2001)

Correspondence: Dr M Oike, Department of Pharmacology, Graduate School of Medical Sciences, Kyushu University, 3-1-1, Maidashi, Fukuoka, 812-8582, Japan.
E-mail: moike@pharmaco.med.kyushu-u.ac.jp
Received 21 August 2006; revised 23 October 2006; accepted 23 November 2006; published online 15 January 2007

and human umbilical cord vein endothelial cells (Hirakawa *et al*., 2004). HTS-induced ATP release leads to Ca^{2+} transients (Oike *et al*., 2000) and subsequent nitric oxide (NO) production (Kimura *et al*., 2000). Mechanical stress also activates endothelial functions, such as gene regulation (Takahashi *et al*., 1997) and cell re-alignment, as a result of altered haemodynamics (Malek and Izumo, 1996). The morphological changes in endothelium induced by TGFβ_1 (Arciniegas *et al*., 1992) might modify the effects of hydrodynamic forces on the membrane of the endothelial cells and thereby affect their responsiveness to mechanical stimuli.

In previous studies it has been shown that TGFβ_1 induces actin fibre formation in vascular endothelial cells (Arciniegas *et al*., 1992; Clements *et al*., 2005). Clements *et al*. (2005) demonstrated that the TGFβ_1-induced actin reorganization in bovine pulmonary artery endothelial cells was partially owing to activation of the RhoA/Rho-kinase pathway. TGFβ_1 might therefore upregulate endothelial mechanosensitivity, a process in which RhoA activation has an essential role (Koyama *et al*., 2001; Hirakawa *et al*., 2004). In the present study, we attempted to clarify the effects of TGFβ_1 on mechanical stress-induced responses and intracellular signals in BAECs. Previous studies have focused mainly on the short-term effects of TGFβ_1 on endothelial cells (Ota *et al*., 2002; Saura *et al*., 2002; Clements *et al*., 2005), although the increased expression of TGFβ_1 messenger RNA (mRNA) persisted for 2 weeks in rabbit arteries injured with a balloon catheter (Kanzaki *et al*., 1995) and even for 25 months after angioplasty in human restenotic coronary artery lesions (Nikol *et al*., 1994). Therefore, we examined both short- and long-term effects of TGFβ_1 on endothelial mechanosensitivity. Our results revealed that long-term treatment with TGFβ_1 impairs endothelial mechanotransduction by an action on RhoA membrane translocation.

Methods

Cell culture

Bovine aortae were obtained from the local slaughter house. BAECs were scraped off with a razor blade and cultured in Dulbecco's modified essential medium supplemented with 10% foetal bovine serum. The cells were seeded on collagen-coated dishes or embedded in a three-dimensional collagen gel lattice in the experiment shown in Figure 4b, as described previously (Kimura *et al*., 2002). The cells were confirmed to be endothelial cells by determining their specific uptake of acetylated low-density lipoprotein (data not shown). Cells were stored at −80°C before use after two-step subculture. The present study was performed with BAECs from 10 aortae.

Treatments with TGFβ$_1$ and anti-TGFβ$_1$ antibody

Effects of TGFβ_1 were examined after incubating non-confluent cells (at approximately 70% confluence) with culture medium containing 0.1, 1 or 3 ng ml^{-1} TGFβ_1 for various periods. Effects of the neutralizing antibody for TGFβ_1 were examined by using culture medium that was preincubated with 3 ng ml^{-1} TGFβ_1 and 300 ng ml^{-1} anti-TGFβ_1 antibody for 1 h at room temperature.

Immunological staining of actin stress fibres

Actin stress fibres were stained using rhodamine-conjugated phalloidin as described previously (Koyama *et al*., 2001). Microscopic images were captured with a fluorescence digital microscope camera (VB-6000, Keyence Corp, Osaka, Japan) connected to a fluorescence microscope (Eclipse E600, Nikon, Tokyo, Japan).

Luciferase-luciferin bioluminescence assay

The extracellular ATP concentration ($[ATP]_o$) was measured by using luciferase-luciferin bioluminescence. Cells were seeded on 96-well plates at densities of 500 and 2000 cells per well for control and TGFβ_1-treated, respectively, and cultured for 7 days in the absence or presence of 3 ng ml^{-1} TGFβ_1. After careful removal of the culture medium, we added 50 μl of isotonic or hypotonic Krebs solution containing 10 mg ml^{-1} luciferase-luciferin to each well, and counted the light emitted for10 min by a cooled CCD luminescence detection system (FAS-1000, Toyobo, Osaka, Japan). The luminescence obtained was converted into $[ATP]_o$ using standard curves determined in the same solutions to avoid possible artifacts of ionic composition on the luciferin bioluminescence. Because TGFβ_1 inhibits endothelial proliferation (Heimark *et al*., 1986), $[ATP]_o$ was further converted into ATP release per cell by manually counting the number of cells after each measurement.

Measurement of $[Ca^{2+}]_i$

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured from non-confluent BAECs with fura-2 by using an Attofluor digital fluorescence microscopy system (Atto Instruments, Rockville, MD, USA), as described previously (Koyama *et al*., 2001). For the statistical analysis of the Ca^{2+} responses, percentage of cells on a coverslip was calculated and regarded as one data point.

Western blot analysis of RhoA and tyrosine phosphorylation

RhoA and tyrosine phosphorylation were assessed with enhanced chemiluminescence Western blotting using anti-RhoA and anti-phosphotyrosine antibodies, respectively. For the assessment of the membrane translocation of RhoA activation, cell lysate was centrifuged for 1 h at 100 000 *g*, and the pellet and the supernatant were harvested as membrane and cytosolic fractions, respectively.

In each experiment, the bands were detected with a chemiluminescence system (SuperSignal West Dura, Pierce Co., Rockford, IL, USA) and analysed with FAS-1000.

RT-PCR and real-time PCR analysis of RhoA mRNA expression

Expressions of RhoA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were examined by reverse transcriptase–polymerase chain reaction (RT-PCR) and

real-time PCR. Cellular total RNA was extracted from control and 7d-TGFβ_1 BAECs using a commercial kit (RNAqueous 4PCR kit, Ambion, Austin, TX, USA), and converted to first-strand complementary DNA (cDNA) using reverse transcriptase (Superscript II, Invitrogen Carlsbad, CA, USA). Qualitative RT-PCR was then performed for 32 cycles with a thermal cycler (PC-8000, Astec, Fukuoka, Japan) in the reaction mixture of template cDNA, primers and Ready-To-Go RT-PCR beads (GE Healthcare Life Sciences, Piscataway, NJ, USA). The resulting PCR products were analysed by use of agarose gel electrophoresis, after which the cDNA bands were excised and extracted with a spin column (Quantum Prep, Bio-Rad, Hercules, CA, USA) as standards for real-time PCR.

Real-time PCR was performed for a quantitative analysis of mRNA expression. First-strand cDNA was mixed with primers and a reaction reagent (Full Velocity SYBR Green QPCR Master Mix, Stratagene, La Jolla, CA, USA), and a real-time PCR was performed with MX3000P (Stratagene) to obtain the threshold cycle numbers (C_t) at which the amplified fluorescent PCR products become detectable. The C_t values obtained for RhoA were normalized to those for GAPDH.

The same sets of primers were used for qualitative RT-PCR and real-time PCR: 5′-ctggttgggaacaagaagga-3′ (forward) and 5′-caaaaacctccctcactcca-3′ (reverse) for RhoA (expected product size; 175 bp), and 5′-ttcaacggcacagtcaagg-3′ (forward) and 5′-acatactcagcaccagcatcac-3′ (reverse) for GAPDH (expected product size; 118 bp).

Materials

Anti-RhoA antibody was purchased from Cytoskeleton (Denver, CO, USA). Anti-phosphotyrosine antibody (clone PY20) was purchased from Exalpha Biologicals (Watertown, MA, USA). Anti-TGFβ_1 and anti β-actin (clone AC-15) antibodies were purchased from Sigma (St Louis, MO, USA). Rhodamine-conjugated phalloidin and fura 2-AM were purchased from Molecular Probes (Eugene, OR, USA). Luciferase-luciferin was purchased form Wako Pure

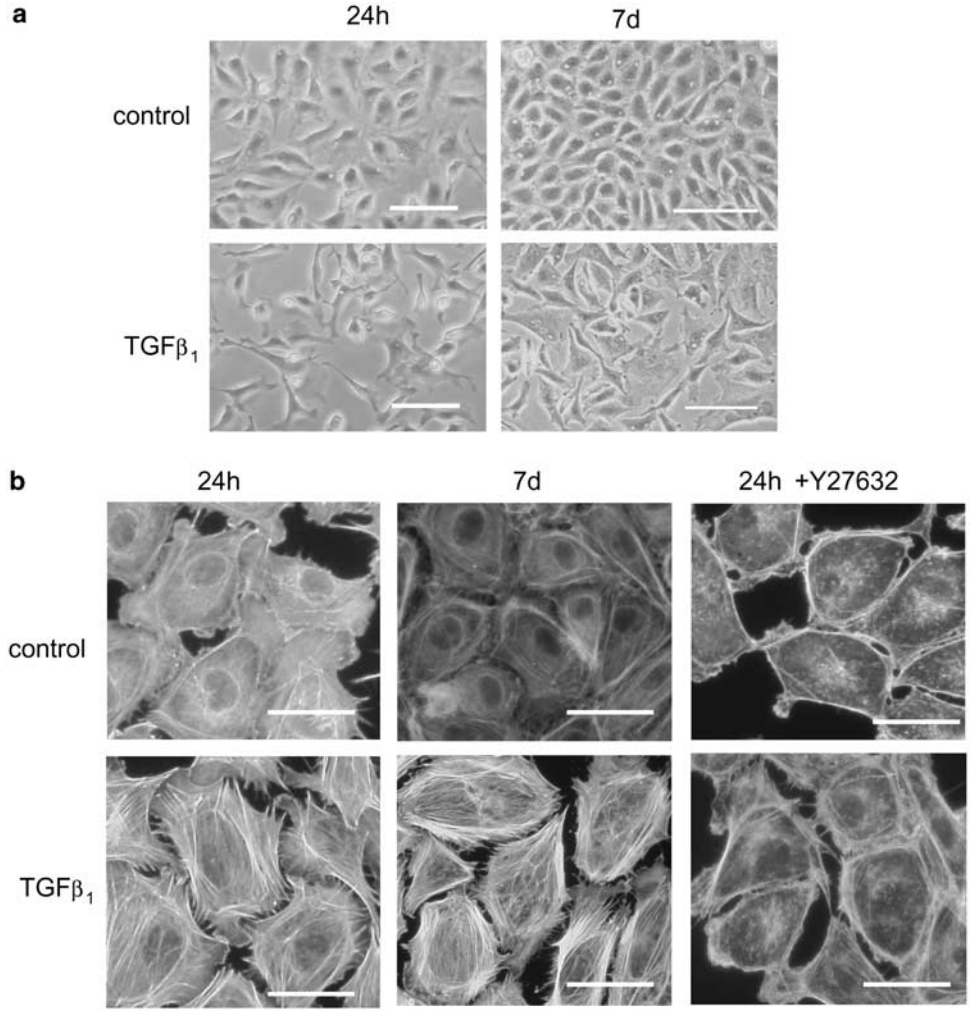

Figure 1 TGFβ_1-induced morphological changes in BAECs. (**a**) Cells were seeded on culture plates, and cultured for 24 h or 7 days in the absence (control) or presence of 3 ng ml^{-1} TGFβ_1. Transillumination microscopic pictures are shown. Scale bars, 50 μm. (**b**) Actin fibres were visualized with rhodamine-phalloidin in control and 3 ng ml^{-1} TGFβ_1-treated BAECs after culturing for 24 h or 7days. Effects of 10 μM Y27632, a Rho-kinase inhibitor, are also depicted. Scale bars, 20 μm.

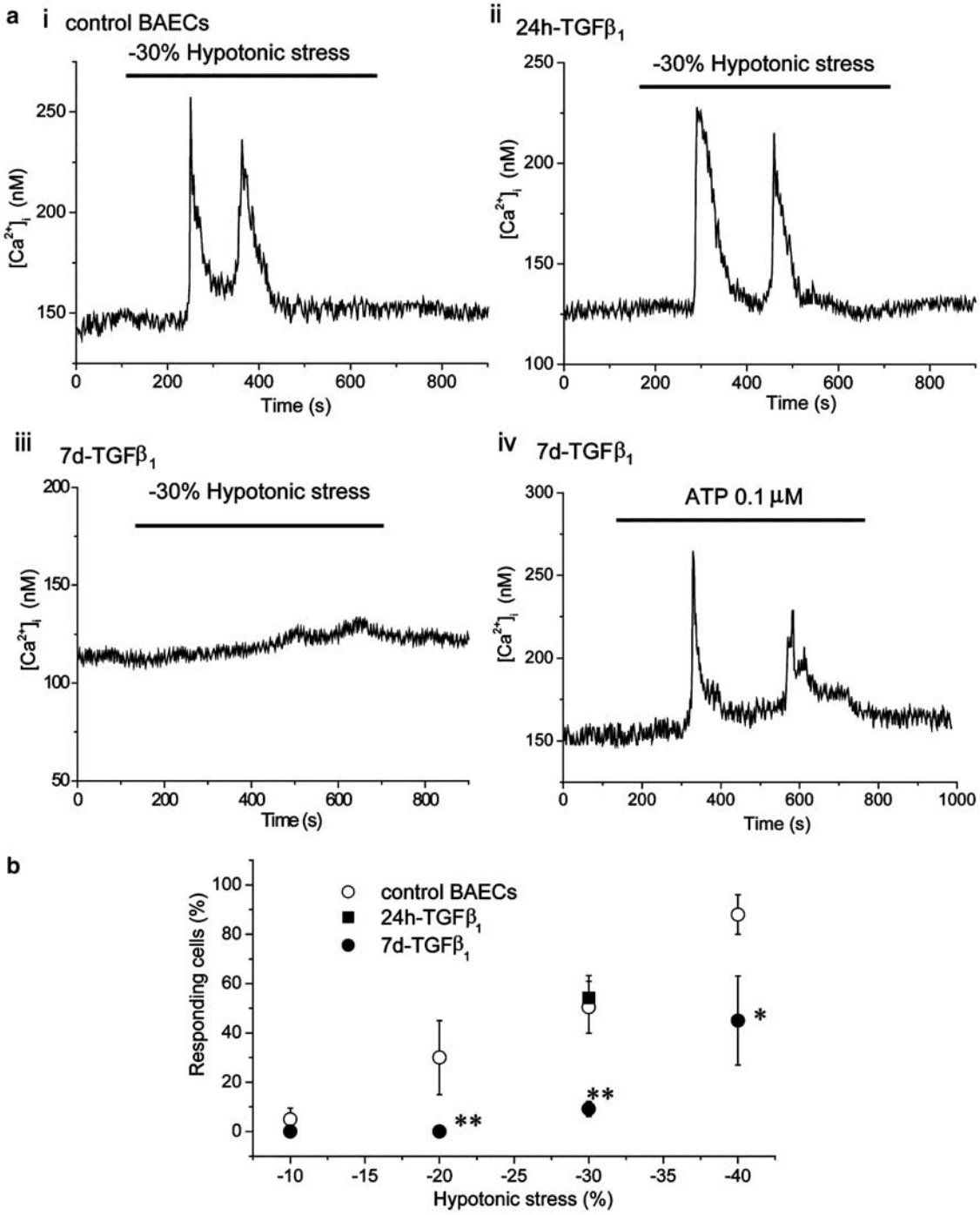

Figure 2 Ca^{2+} transients in control and TGFβ_1-treated BAECs. (**a**) BAECs were seeded on coverslips and cultured for 7 days. Control cells were left untreated (**i**). Cells to which 3ng ml^{-1} TGFβ_1 was applied at day 6 and cultured for subsequent 24 h are denoted as 24 h-TGFβ_1 (**ii**). Cells that were cultured in the presence of 3 ng ml^{-1} TGFβ_1 for 7 days are denoted as 7d-TGFβ_1 (**iii, iv**). The effects of HTS (−30%; **i–iii**) and ATP (0.1 μM; **iv**) on $[Ca^{2+}]_i$ were then examined. Representative Ca^{2+} traces are shown. (**b**) Hypotonicity-dependence of the HTS-induced Ca^{2+} transients. The percentage of cells showing HTS-induced Ca^{2+} transients in a microscopic field was calculated. Each point was obtained from six measurements; vertical lines shown s.e.m. $^{**}P<0.01$, $^{*}P<0.05$ vs control.

Chemicals (Osaka, Japan). All other reagents including TGFβ_1 were purchased from Sigma.

Solutions

Krebs solution used in the present study contained (mM); NaCl 132.4, KCl 5.9, $CaCl_2$ 1.5, $MgCl_2$ 1.2, glucose 11.5, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid 11.5, and had a pH of 7.4 (adjusted with NaOH). Hypotonic solutions were made by adding appropriate amounts of distilled water to normal Krebs solution. We have previously confirmed that the reduction of the ionic concentrations did not influence the $[Ca^{2+}]_i$ responses (Oike *et al*., 2000).

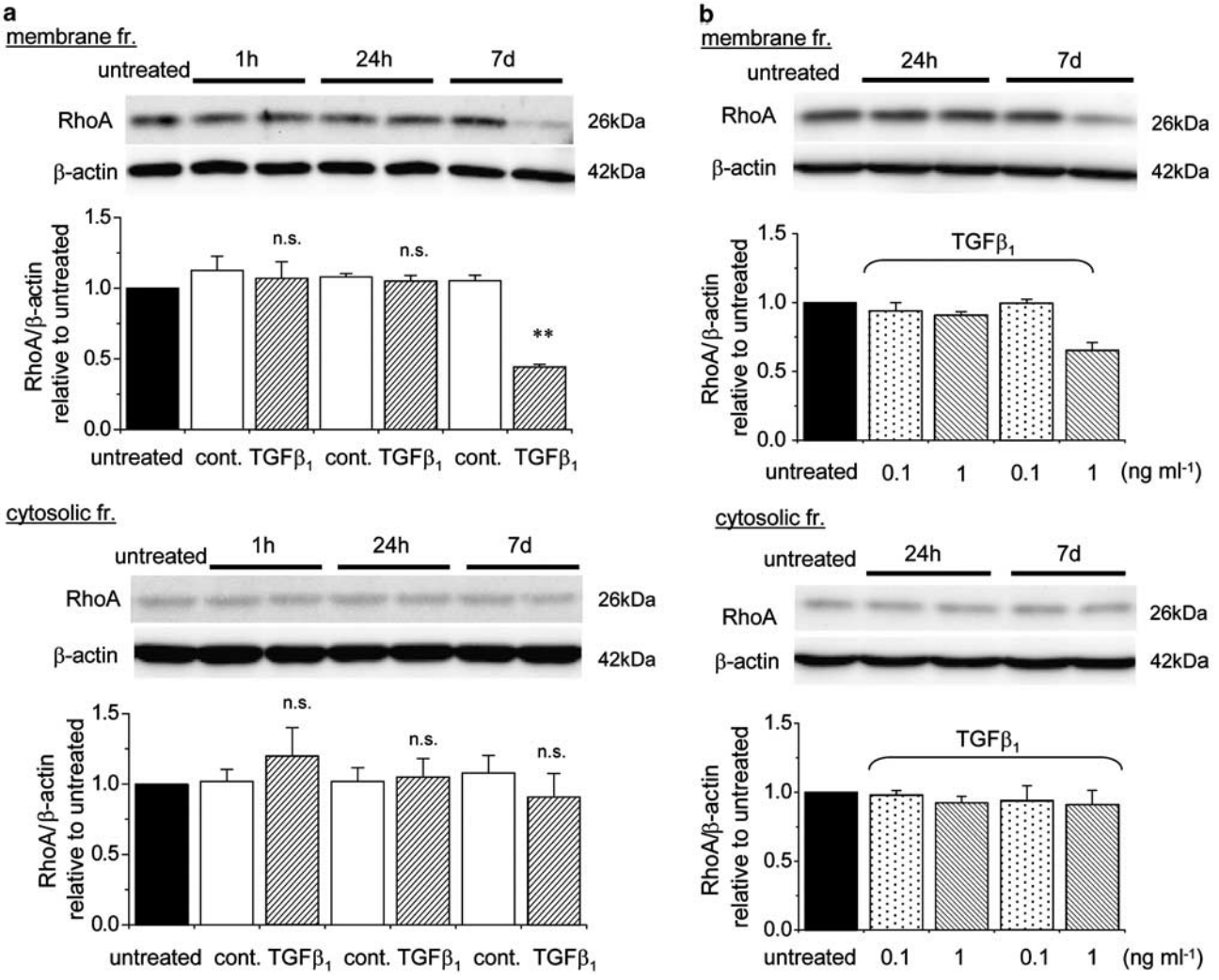

Figure 3 Effects of TGFβ_1 on the expression of RhoA in BAECs in isotonic conditions. (**a**) BAECs were seeded on culture plates and cultured for 7 days under various conditions. Untreated cells were cultured for 7days with a medium change at day 4. Other cells were cultured for 7 days with an additional medium exchange without (denoted as cont.) or with 3 ng ml^{-1} TGFβ_1 6 h after seeding (denoted as 7d) or 1 or 24 h before cell lysis (denoted as 1 and 24 h, respectively). Cell lysate was then centrifuged at 100 000 *g* for 1 h, and pellet and supernatant were collected as membrane (upper panel) and cytosolic (lower panel) fractions, respectively. RhoA/β-actin ratio was determined densitometrically and expressed relative to control values in untreated cells. Note that RhoA in the membrane fraction but not in the cytosolic fraction was decreased only in 7d-TGFβ_1. **$P<0.01$ vs control. ns, $P>0.05$ vs control. (**b**) A similar analysis was performed with 0.1 and 1 ng ml^{-1} TGFβ_1. Note the reduced expression of RhoA in the membrane fraction with 1 ng ml^{-1} TGFβ_1.

Statistical analysis

Data are expressed as mean$\pm$s.e.m. values. Statistical significance was examined with Student's unpaired *t*-test. A probability below 0.05 ($P<0.05$) was considered as significantly different.

Results

Morphological changes induced by TGFβ_1 in BAECs

Control BAECs grown on a culture plate showed a cobblestone-like appearance (Figure 1a). In the presence of 3 ng ml^{-1} TGFβ_1 in the culture medium, pseudopod-like processes developed in 24 h and the cells showed a fibroblast-like appearance after 7days (Figure 1a). Because the cytoskeletal architecture is the main determinant of cell morphology, we firstly examined the effects of TGFβ_1 on actin cytoskeleton in BAECs. Control BAECs showed only trace levels of actin cytoskeleton, but TGFβ_1 induced dense actin formation in 24 h, which persisted for at least 7 days (Figure 1b). Y27632, a Rho-kinase inhibitor, markedly suppressed the formation of actin fibres both in the presence and absence of TGFβ_1 (Figure 1b).

HTS-induced, ATP-mediated Ca^{2+} transients in TGFβ_1-treated BAECs

To investigate whether the endothelial responses were affected by the TGFβ_1-induced morphological changes we then examined Ca^{2+} transients and ATP-release induced by HTS in control and TGFβ_1-treated BAECs. The previously described (Oike *et al*., 2000), HTS (−30%)-evoked Ca^{2+}

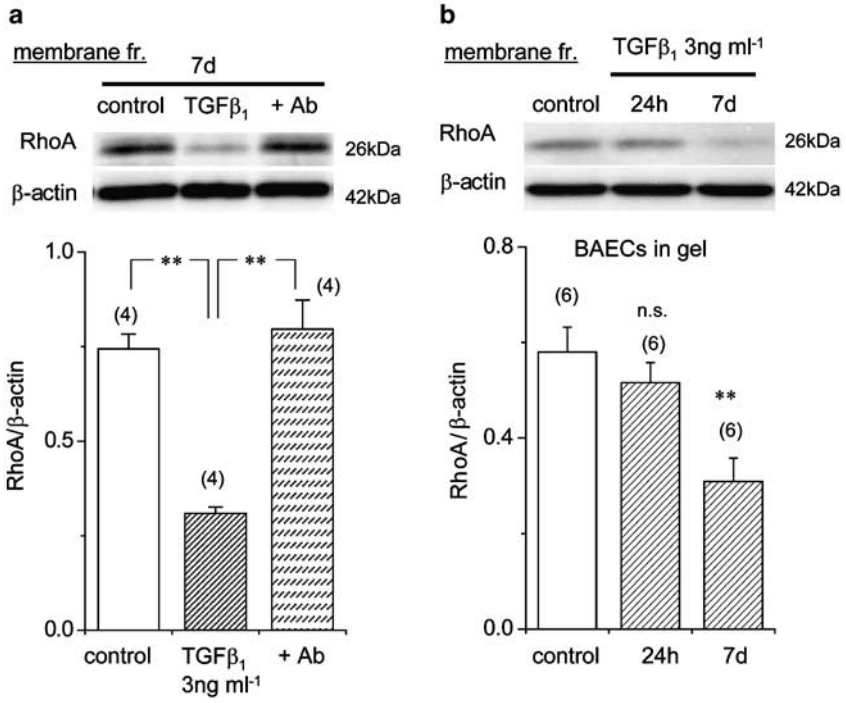

Figure 4 Effects of a TGFβ_1 antibody and collagen gel on TGFβ_1-induced reduction of membrane-bound RhoA. (**a**) Cells were cultured for 7days with 3 ng ml^{-1} TGFβ_1 in the presence or absence of 300 ng ml^{-1} anti-TGFβ_1. Control cells were left untreated for 7days. $^{**}P<0.01$. (**b**) BAECs were embedded in collagen gel lattice, and cultured for 7 days. Culture medium was replaced with 3 ng ml^{-1} TGFβ_1-containing medium at day 6 or 0 and incubated for 24 h or 7 days, respectively. Control gels were left untreated. BAECs embedded in collagen gels were quickly frozen and homogenized. Cell lysates were then centrifuged and the membrane fraction was prepared as described in the Methods. $^{**}P<0.01$ vs control.

oscillations in control BAECs (Figure 2a–i) also occurred in cells pretreated with TGFβ_1 for 24 h (24h-TGFβ_1 BAECs, Figure 2a–ii). The percentage of cells that showed Ca^{2+} oscillations in a microscopic field in response to −30% HTS was not significantly different in the cell populations, that is, 50.7±6.2% ($n=12$) in control cells and 54.1±9.1% ($n=6$) in 24 h-TGFβ_1 BAECs, respectively (Figure 2b). In contrast, the HTS-induced Ca^{2+} oscillations in cells pretreated with TGFβ_1 for 7days (7d-TGFβ_1 BAECs) were markedly suppressed (Figure 2a–iii), and only observed in 9.2±3.0% ($n=6$) of 7d-TGFβ_1 BAECs ($P<0.01$ vs control, Figure 2b). A significant suppression of HTS-induced Ca^{2+} oscillations in 7d-TGFβ_1 BAECs was also observed at −20 and −40% HTS (Figure 2b), but was not observed in the cells that were treated with a low concentration of TGFβ_1 (0.1 ng ml^{-1}, data not shown).

We have previously demonstrated that HTS-induced Ca^{2+} oscillations are mainly owing to endogenous ATP release (Oike *et al*., 2000). In control cells, −30% HTS induced a release of 41.3±7.3 amol ATP per cell ($n=7$) in 10 min, a value that is significantly higher than that in isotonic solution (20.5±3.1 amol per cell, $n=7$, $P<0.01$ vs HTS). In contrast, the HTS-induced release of ATP was completely abolished in 7d-TGFβ_1 BAECs (isotonic, 19.0±6.5 amol per cell; HTS, 19.8±8.0 amol per cell in 10 min, $n=6$ for each condition, $P>0.05$), although exogenous ATP, applied at a threshold concentration of 0.1 μM, still induced Ca^{2+} oscillations in these cells (Figure 2a–iv).

Effects of TGFβ$_1$ on membrane translocation of RhoA

As the Rho-kinase inhibitor Y27632 inhibited the TGFβ_1-induced formation of actin fibres (Figure 1b), we next examined the effects of TGFβ_1 on RhoA activation in BAECs.

As shown in Figure 3a, the expression level of RhoA in the membrane fraction was not affected by 3 ng ml^{-1} TGFβ_1 after 24 h, but significantly reduced after 7 days. In contrast, the cytosolic expression of RhoA did not differ between control and TGFβ_1-treated BAECs (Figure 3a). TGFβ_1-induced inhibition of membrane-bound RhoA was concentration-dependent, and a significant inhibition was observed with 1 ng ml^{-1} TGFβ_1 (Figure 3b). This inhibition of RhoA membrane translocation in 7d-TGFβ_1 BAECs was completely reversed by addition of the neutralizing antibody for TGFβ_1 (Figure 4a). Furthermore, the reduction of membrane-bound RhoA after long-term treatment with TGFβ_1 was also observed in cells embedded in three-dimensional collagen gels (Figure 4b).

We then examined the effects of HTS on the membrane translocation of RhoA in control and 7d-TGFβ_1 BAECs. In control BAECs HTS (−30%) induced a transient increase in membrane-bound RhoA that was maximal after 2 min (Figure 5a). A similar HTS-induced increase in membrane-bound RhoA was also observed in 7d-TGFβ_1 BAECs and this occurred with a similar time course (Figure 5a). When the data were normalized to the level observed under isotonic conditions in control cells, the maximal increase was found to be significantly lower in 7d-TGFβ_1 BAEC than in control cells (Figure 5b–i). In contrast, when the data obtained with

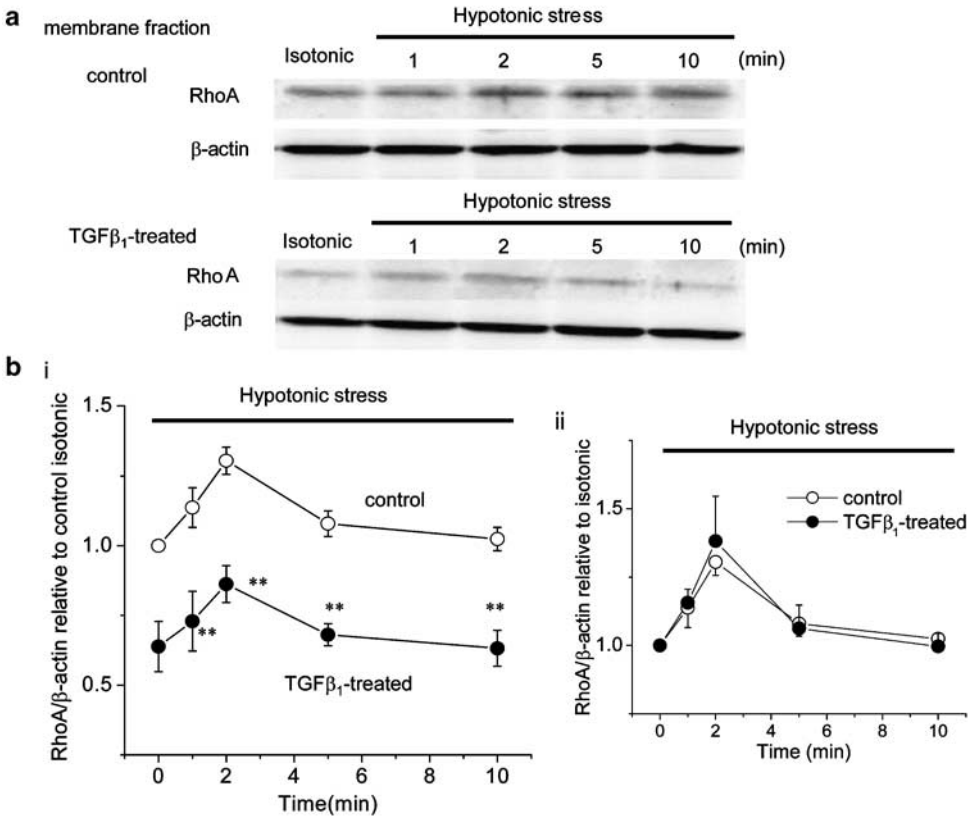

Figure 5 Effects of TGFβ_1 on HTS (−30%)-induced RhoA membrane translocation in BAECs. BAECs were cultured in the presence or absence of 3 ng ml^{-1} TGFβ_1 for 7 days. Expression of RhoA as well as β-actin in the membrane fraction was then determined by Western blotting. Representative band images are shown in (**a**). Densitometric analysis of the RhoA band relative to β-actin band is shown in (**b**). Band densities in 7d-TGFβ_1 BAECs expressed are relative to those in control isotonic conditions in (**i**) and to 7d-TGFβ_1 isotonic conditions in (**ii**). Each point is the mean of four results and vertical lines show s.e.m. $^{**}P<0.01$ vs control.

7d-TGFβ_1 BAECs were normalized to their own isotonic level, the maximal increase was comparable between the control and 7d-TGFβ_1 cells (Figure 5b–ii).

Effects of TGFβ_1 *on the expression of RhoA mRNA in BAECs*

Next we examined the effects of TGFβ_1 on the expression level of RhoA mRNA. RhoA mRNA, assessed by RT-PCR, was detected in both control and 7d-TGFβ_1 BAECs (Figure 6a). A quantitative analysis with real-time PCR revealed that the expression of RhoA mRNA relative to that of housekeeping GAPDH was not different between control and 7d-TGFβ_1 BAECs (Figure 6b).

HTS-induced tyrosine phosphorylation in TGFβ_1*-treated BAECs*

HTS-induced tyrosine phosphorylation of FAK and paxillin, a downstream response of RhoA/Rho-kinase activation (Hirakawa *et al*., 2004), was determined in control and 7d-TGFβ_1 BAECs. In control BAECs, HTS (−30%) induced a transient tyrosine phosphorylation of 125 and 68 kDa (Figure 7), which we have previously identified as FAK and paxillin, respectively (Hirakawa *et al*., 2004). In contrast, the tyrosine phosphorylation of these proteins by −30% HTS was significantly reduced in 7d-TGFβ_1 BAECs, whereas their levels under isotonic conditions were not different from the values in control cells (Figure 7).

Discussion and conclusions

The initial aim of this study was to investigate whether the TGFβ_1-induced morphological changes affect endothelial mechanosensitivity. We observed that treatment of BAECs with TGFβ_1 for 7 days inhibited mechanosensitive ATP release and the concomitant Ca^{2+} transients (Figure 2). Ca^{2+} transients could still be evoked in these cells by addition of exogenous ATP (0.1 μM), indicating that their Ca^{2+} responsiveness was intact. In contrast, TGFβ_1 did not affect HTS-induced Ca^{2+} responses after 24 h, whereas morphological changes and increased actin fibre formation were already apparent (Figure 1). It is therefore unlikely that the inhibition of the mechanosensitive Ca^{2+} transients in 7d-TGFβ_1 BAECs is a consequence of TGFβ_1-induced morphological changes that, in turn, could alter the profile of mechanical forces on the cell surface.

Membrane translocation of RhoA has been regarded as a hallmark of the activation of RhoA (Kranenburg *et al*., 1997). We observed that TGFβ_1 induced a marked concentration-dependent reduction of membrane-bound RhoA under

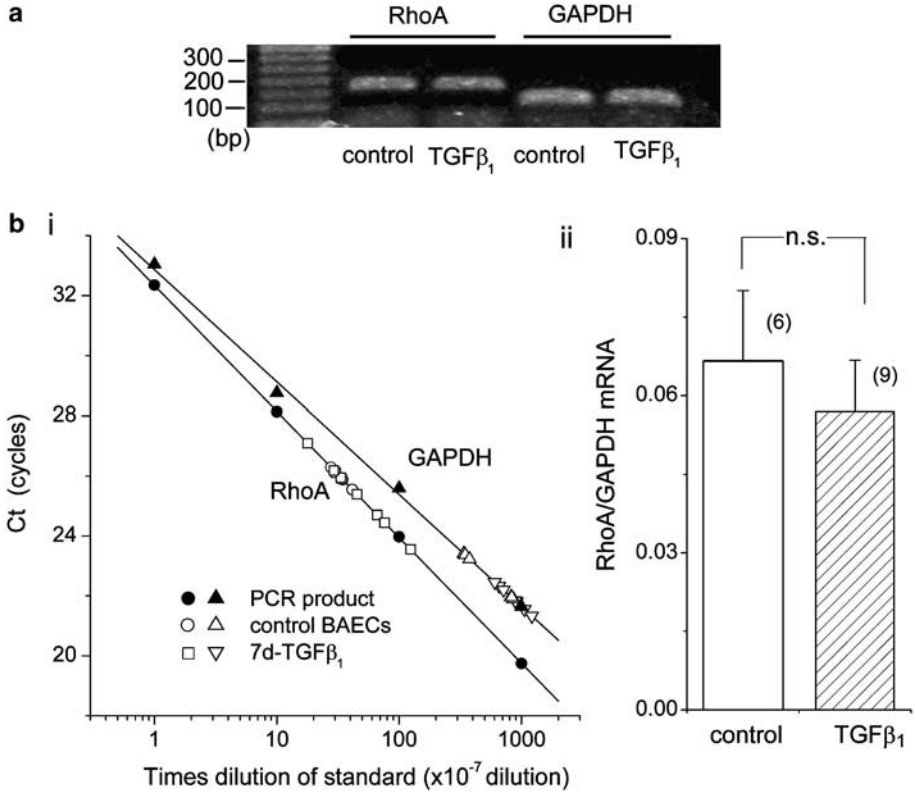

Figure 6 Expression of RhoA mRNA in control and TGFβ_1-treated BAECs. (**a**) Total RNA was prepared from confluent BAECs that were untreated or treated with 3 ng ml^{-1} TGFβ_1 for 7 days. PCR products after 32 cycles of amplification were electrophoresed. Housekeeping GAPDH is also shown as an internal control. (**b**) Quantitative analysis of RhoA mRNA expression with real-time PCR. Control RT-PCR products in (**a**) were purified and used as standards after serial dilution. We confirmed the validity of this assay by examining the linear relationship between the degree of dilution of these standards and the detection thresholds (C_t) for RhoA and GAPDH, and also confirmed that the samples were on these lines (**i**, open symbols). C_t for RhoA mRNA is expressed relative to that for GAPDH mRNA, and compared between control and TGFβ_1-treated BAECs (**ii**). ns $P>0.05$. Numbers in parentheses represent the number of RNA preparations.

isotonic conditions after 7 days but not after 24 h (Figure 3). It has been shown that activation of RhoA by TGFβ_1 is transient in endothelial and non-endothelial cells and declines within a few hours (Bhowmick *et al*., 2003; Clements *et al*., 2005). However, we were unable to detect an acute, TGFβ_1-induced increase in membrane translocation of RhoA (Figure 3a). At present, no studies have been carried out on the chronic effects of TGFβ_1 on RhoA. Hence, the present study is the first to show that long-term treatment with TGFβ_1 suppresses membrane-bound RhoA in BAECs. As the expression of RhoA mRNA was similar in control and 7d-TGFβ_1 BAECs (Figure 6), we hypothesize that the reduction of RhoA in 7d-TGFβ_1 BAECs was due to an increased extrusion of this protein from the cells. This could also explain the absence of acute effects of TGFβ_1 on RhoA expression, as time is required for this extrusion process to occur.

HTS-induced membrane translocation of RhoA showed a parallel downward shift in 7d-TGFβ_1 BAECs compared to control cells (Figure 5b–i). As its fractional increase was identical in control and 7d-TGFβ_1 BAECs (Figure 5b–ii), this was due to the lower initial level of membrane-bound RhoA. Mechanical stresses are sensed at the cell surface of endothelial cells and converted into intracellular signals, including RhoA and tyrosine kinases (Koyama *et al*., 2001). It has been suggested that some transmembrane molecules are endothelial mechanosensors, for example, heparan sulphate proteoglycan (Florian *et al*., 2003), molecular complexes including PECAM-1 (Tzima *et al*., 2005) and integrins (Shyy and Chien, 2002). The present results indicate that these molecules, in spite of the marked morphological alterations in the 7d-TGFβ_1 BAECs, are still able to sense HTS. They also imply that the inhibitory effects of TGFβ_1 on HTS-induced responses are caused by impaired mechanotransduction rather than impaired mechanoreception. In the endothelial cell line ECV38, it has been shown that RhoA is localized in caveolae-enriched membrane fractions (Gingras *et al*., 1998). Also in cardiac myocytes, shear stress-induced activation of RhoA and Rac1 was found to be initiated in caveolae (Kawamura *et al*., 2003). Therefore, although further studies are needed, we propose that long-term treatment of BAECs with TGFβ_1 affects some function(s) of caveolae involved in RhoA activation.

The HTS-induced tyrosine phosphorylation of FAK and paxillin, which is a downstream signal of RhoA activation in endothelium (Hirakawa *et al*., 2004), was also impaired in 7d-TGFβ_1 BAECs (Figure 7). However, unlike with RhoA, the basal levels of tyrosine-phosphorylated FAK and paxillin did not differ between control and 7d-TGFβ_1 BAECs. Thus, we propose that the primary pathogenesis of the impaired HTS-

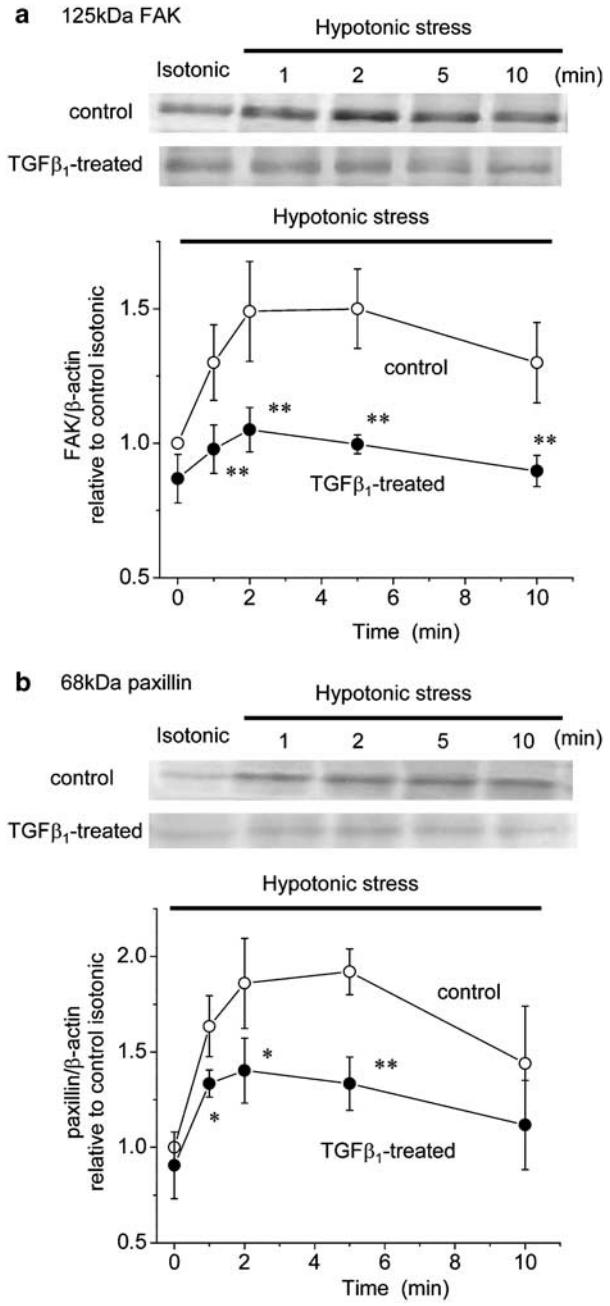

Figure 7 Effects of TGFβ_1 on HTS-induced tyrosine phosphorylation of FAK (**a**) and paxillin (**b**) in BAECs. Control and 7d-TGFβ_1 BAECs were exposed to −30% HTS. Tyrosine phosphorylation of 125 and 68 kDa proteins was then determined by Western blotting. Upper panels show the representative band images, and the lower panels show the densitometric analysis of the bands ($n=4$; vertical lines shown s.e.m.), which are expressed as phosphotyrosine/β-actin ratios relative to the control cells in isotonic solution. $^{**}P<0.01$ vs control.

induced responses in 7d-TGFβ_1 BAECs was the reduced basal level of membrane-bound RhoA.

Vascular endothelial cells generate NO to regulate vascular tone and this process is mainly Ca^{2+}-dependent (Moncada *et al*., 1988). Also, we have previously shown that ATP-mediated Ca^{2+} transients lead to NO production in BAECs (Kimura *et al*., 2000). The present results, therefore, suggest that mechanical stress-induced, NO-mediated autoregulation of vascular tone may be impaired in endothelial cells that have been chronically exposed to TGFβ_1. Increased secretion of TGFβ_1 and expression of its receptor have been observed in injured vessels, atherosclerotic lesions and post-angioplasty restenotic lesions (Grainger, 2004; McCaffrey, 2000). As the increased action of TGFβ_1 in these lesions was found to persist for up to 25 months (Nikol *et al*., 1994), we believe that the chronic effects of TGFβ_1 observed in the present study are pathophysiologically more relevant than its acute actions that appear within a few hours.

In conclusion, the data from the present study indicate that long-term treatment with TGFβ_1 impairs the transduction of mechanical stress-evoked signals in BAECs owing to an inhibitory effect on RhoA activation, rather than the mechanosensing machinery of these cells.

Acknowledgements

We thank Dr Guy Droogmans for his comments on the manuscript. This study was supported in part by a Grant-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Sciences and Technology, Japan (16590197).

Conflict of interest

The authors state no conflict of interest.

References

Arciniegas E, Sutton AB, Allen TD, Schor AM (1992). Transforming growth factor β1 promotes the differentiation of endothelial cells into smooth muscle-like cells *in vitro*. *J Cell Sci* **103**: 521–529.

Bhowmick NA, Ghiassi M, Aakre M, Brown K, Singh V, Moses HL (2003). TGF-β-induced RhoA and p160ROCK activation is involved in the inhibition of Cdc25A with resultant cell-cycle arrest. *Proc Natl Acad Sci USA* **100**: 15548–15553.

Chen X, Ren S, Ma MG, Dharmalingam S, Lu L, Xue M *et al*. (2003). Hirulog-like peptide reduces restenosis and expression of tissue factor and transforming growth factor-β in carotid artery of atherosclerotic rabbits. *Atherosclerosis* **169**: 31–40.

Clements RT, Minnear FL, Singer HA, Keller RS, Vincent PA (2005). RhoA and Rho-kinase dependent and independent signals mediate TGF-β-induced pulmonary endothelial cytoskeletal reorganization and permeability. *Am J Physiol* **288**: L294–L306.

Davies PF (1995). Flow-mediated endothelial mechanotransduction. *Physiol Rev* **75**: 519–560.

Florian JA, Kosky JR, Ainslie K, Pang Z, Dull RO, Tarbell JM (2003). Heparan sulfate proteoglycan is a mechanosensor on endothelial cells. *Circ Res* **93**: e136–e142.

Frater-Schroder M, Muller G, Birchmeier W, Bohlen P (1986). Transforming growth factor-β inhibits endothelial cell proliferation. *Biochem Biophys Res Commun* **137**: 295–302.

Gingras D, Gauthier F, Lamy S, Desrosiers RR, Beliveau R (1998). Localization of RhoA GTPase to endothelial caveolae-enriched membrane domains. *Biochem Biophys Res Commun* **247**: 888–893.

Grainger DJ (2004). Transforming growth factor β and atherosclerosis: so far, so good for the protective cytokine hypothesis. *Arterioscler Thromb Vasc Biol* **24**: 399–404.

Heimark RL, Twardzik DR, Schwartz SM (1986). Inhibition of endothelial regeneration by type-β transforming growth factor from platelets. *Science* **233**: 1078–1080.

Hirakawa M, Oike M, Karashima Y, Ito Y (2004). Sequential activation of RhoA and FAK/paxillin leads to ATP release and actin

reorganization in human endothelium. *J Physiol (London)* **558**: 479–488.

Kanzaki T, Tamura OK, Takahashi K, Saito Y, Akikusa B, Oohashi H *et al.* (1995). *In vivo* effect of TGF-β1. Enhanced intimal thickening by administration of TGF-β1 in rabbit arteries injured with a balloon catheter. *Arterioscler Thromb Vasc Biol* **15**: 1951–1957.

Kawamura S, Miyamoto S, Brown JH (2003). Initiation and transduction of stretch-induced RhoA and Rac1 activation through caveolae: cytoskeletal regulation of ERK translocation. *J Biol Chem* **278**: 31111–31117.

Kimura C, Cheng W, Hisadome K, Wang YP, Koyama T, Karashima Y *et al.* (2002). Superoxide anion impairs contractility in cultured aortic smooth muscle cells. *Am J Physiol* **283**: H382–H390.

Kimura C, Koyama T, Oike M, Ito Y (2000). Hypotonic stress-induced NO production in endothelium depends on endogenous ATP. *Biochem Biophys Res Commun* **274**: 736–740.

Koyama T, Oike M, Ito Y (2001). Involvement of Rho-kinase and tyrosine kinase in hypotonic stress-induced ATP release in bovine aortic endothelial cells. *J Physiol (London)* **532**: 759–769.

Kranenburg O, Poland M, Gebbink M, Oomen L, Moolenaar WH (1997). Dissociation of LPA-induced cytoskeletal contraction from stress fiber formation by differential localization of RhoA. *J Cell Sci* **110**: 2417–2427.

Malek AM, Izumo S (1996). Mechanism of endothelial cell shape change and cytoskeletal remodeling in response to fluid shear stress. *J Cell Sci* **109**: 713–726.

Mallat Z, Gojova A, Marchiol-Fournigault C, Esposito B, Kamate C, Merval R *et al.* (2001). Inhibition of transforming growth factor-β signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice. *Circ Res* **89**: 930–934.

McCaffrey TA (2000). TGF-βs and TGF-β receptors in atherosclerosis. *Cytokine Growth Factor Rev* **11**: 103–114.

Moncada S, Palmer RM, Higgs EA (1988). The discovery of nitric oxide as the endogenous nitrovasodilator. *Hypertension* **12**: 365–372.

Nikol S, Weir L, Sullivan A, Sharaf B, White CJ, Zemel G *et al.* (1994). Persistently increased expression of the transforming growth factor-β1 gene in human vascular restenosis: analysis of 62 patients with one or more episode of restenosis. *Cardiovasc Pathol* **3**: 57–64.

Oike M, Kimura C, Koyama T, Yoshikawa M, Ito Y (2000). Hypotonic stress-induced dual Ca^{2+} responses in bovine aortic endothelial cells. *Am J Physiol* **279**: H630–H638.

Ota T, Fujii M, Sugizaki T, Ishii M, Miyazawa K, Aburatani H *et al.* (2002). Targets of transcriptional regulation by two distinct type I receptors for transforming growth factor-β in human umbilical vein endothelial cells. *J Cell Physiol* **193**: 299–318.

Saura M, Zaragoza C, Cao W, Bao C, Rodriguez-Puyol M, Rodriguez-Puyol D *et al.* (2002). Smad2 mediates transforming growth factor-β induction of endothelial nitric oxide synthase expression. *Circ Res* **91**: 806–813.

Shyy JY, Chien S (2002). Role of integrins in endothelial mechanosensing of shear stress. *Circ Res* **91**: 769–775.

Takahashi M, Ishida T, Traub O, Corson MA, Berk BC (1997). Mechanotransduction in endothelial cells: temporal signaling events in response to shear stress. *J Vasc Res* **34**: 212–219.

Tzima E, Irani-Tehrani M, Kiosses WB, Dejana E, Schultz DA, Engelhardt B *et al.* (2005). A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. *Nature* **437**: 426–431.