Growth Inhibitory Properties of Endothelin-1 in Activated Human Hepatic Stellate Cells: A Cyclic Adenosine Monophosphate–mediated Pathway

Inhibition of both Extracellular Signal-regulated Kinase and c-Jun Kinase and Upregulation of Endothelin B Receptors

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Abstract

During chronic liver diseases, hepatic stellate cells (HSC) acquire an activated myofibroblast-like phenotype, proliferate, and synthetize fibrosis components. We have shown that endothelin-1 (ET-1) inhibits the proliferation of activated human HSC via endothelin B (ETB) receptors. We now investigate the transduction pathway involved in the growth inhibitory effect of ET-1 in activated HSC.

Endothelin-1 and the ETB receptor agonist, sarafotoxin-S6C, increased synthesis of PGI2 and PGE2, leading to elevation of cAMP. The cyclooxygenase inhibitor ibuprofen and the adenylyl cyclase inhibitor SQ22536 both blunted the growth inhibitory effect of ET-1. Analysis of early steps associated with growth inhibition indicated that: (*a*) similar to ET-1, forskolin decreased c-jun mRNA induction without affecting c-fos and krox 24 mRNA expression; (*b*) ET-1, sarafotoxin-S6C, as well as forskolin, reduced activation of both c-Jun kinase and extracellular signal-regulated kinase. Finally, forskolin, PGI2, and PGE2 raised by fivefold the number of ET binding sites after 6 h, and increased the proportion of ETB receptors from 50% in control cells to 80% in treated cells.

In conclusion, ET-1 inhibits proliferation of activated HSC via ETB receptors, through a prostaglandin/cAMP pathway that leads to inhibition of both extracellular signal-regulated kinase and c-Jun kinase activities. Upregulation of ETB receptors by prostaglandin/cAMP raises the possibility of a positive feedback loop that would amplify the growth inhibitory response. These results suggest that ET-1 and agents that increase cAMP might be of interest to limit proliferation of activated HSC during chronic liver diseases. (*J. Clin. Invest.* 1996. 98:2771–2778.) Key words: liver • fibrosis • proliferation • prostaglandins

Introduction

Endothelins (ET)[1] are a family of three homologous peptides (ET-1, ET-2, and ET-3) that display a wide variety of biological activities (1) such as vasoconstriction (2), glycogenolysis (3–5), and positive (6) or negative (7) effects on cell growth. The peptides bind at least three G protein–coupled receptors, which have been cloned. The ETA receptor binds ET-1 with a higher affinity than ET-3, the ETB receptor displays a similar high affinity for both peptides, and the ETC receptor exhibits a higher affinity for ET-3 than ET-1 (8, 9). In keeping with the pleiotropic functions of ETs, stimulation of ET receptors is coupled to several transduction pathways, including calcium, nitric oxide, arachidonic acid, and cyclic AMP (8, 10).

Hepatic stellate cells (HSC, also known as lipocytes, fat-storing cells, or perisinusoidal cells) play a crucial role in the development of liver fibrosis (11). After acute or chronic liver injury, HSC transdifferentiate to an activated myofibroblastic phenotype (12), proliferate, accumulate, and secrete components of fibrosis (11). Activated HSC display a high number of ET binding sites (7, 13) that mediate at least two biological effects of ET-1, contraction (14, 15) and growth inhibition (7). In human activated HSC, ETA receptors are responsible for contraction via a calcium-dependent pathway (16), and we have shown that ETB receptors mediate the antiproliferative effect of ET-1 (7). The aim of the present study was to determine the second messenger responsible for the antiproliferative effect of ET-1, and to investigate its mechanism of action. We show that activation of the ETB receptor stimulates the production of prostaglandins, leading to an increase in cAMP, which in turn inhibits cell proliferation. Stimulation of the cAMP pathway by ET-1 reduces activation of both extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal protein kinase (JNK). In addition, both cyclic AMP and prostaglandins markedly upregulate ETB binding sites, suggesting the possibility of a positive feedback regulatory loop.

Methods

Materials. ET-1 and Sarafotoxin-S6C (SRTX-C) were from Neosystem (Strasbourg, France), SQ 22536 from Biomol (Tebu, Le Perray en Yvelines, France). Forskolin (Sigma Chemical Co., L'Isle d'Abeau

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1. *Abbreviations used in this paper:* ERK, extracellular signal-regulated kinase; ET, endothelin, G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HSC, hepatic stellate cell; JNK, c-Jun kinase; SRTX-C, sarafotoxin S6C.

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Chesnes, France) was dissolved in dimethylsulfoxide. Ibuprofen, prostaglandin E2, and carbacyclin, the stable analog of prostaglandin I2, were obtained from Sigma Chemical Co. and dissolved in ethanol. [Methyl-^{3}H]thymidine (25 Ci/mmol), [α^{32}P]ATP and [γ^{32}P]ATP (5,000 Ci/mmol) were from Amersham (Les Ulis, France). Fetal calf serum was from JBio Laboratories (France). Pooled human AB positive serum was supplied by the National Transfusion Center. cDNAs encoding mouse c-fos and c-jun were kindly provided by Dr. Yaniv (Institut Pasteur, Paris, France) and the human krox-24 probe was a generous gift of Dr. Casellas (Sanofi, Montpellier, France).

Cell isolation and culture. Human HSC were obtained in their activated phenotype by outgrowth from explants of normal liver obtained after surgery of benign or malignant liver tumors. This procedure is in accordance with ethical regulations imposed by French legislation. Explants were incubated, as described previously, in DMEM containing 10% serum (5% fetal calf serum/5% pooled human serum), thereafter referred to as DMEM 5/5 (17). Exhaustive characterization of these cells has already been published (17). All cell isolates were routinely characterized by a positive staining for smooth muscle α-actin, a marker of HSC in their myofibroblastic phenotype. Experiments were performed between passages 3 and 8, without any noticeable difference in results observed with cells from various passages, or obtained from various livers.

Cyclic AMP assay. Confluent cells in 24-well plates were serum starved in Waymouth medium over 48 h. After preincubation with 1 mM iso-butyl-methyl-xanthine for 15 min, cells were stimulated in PBS with indicated effectors, over 5 min (unless otherwise specified). Assays were performed in duplicate. Cyclic AMP was extracted in 95% ethanol for 2 h at 25°C. Extracts were stored at −80°C, pending assay. Cyclic AMP was assayed by a commercial radioimmunoassay (Amersham). When indicated, cells were pretreated with 1 mM SQ 22536 or vehicle. As reported by Haraguchi et al., SQ 22536 cross-reacted with the cAMP assay; hence, calculations were performed as described (18).

Adenylyl cyclase activity of a particulate fraction of HSC. A particulate fraction of HSC was obtained from confluent, quiescent cells as previously described (7). Briefly, cells were washed twice in PBS, pH 7.4, scraped in 1 mM $NaHCO_3$ at 4°C, homogenized three times with a Polytron homogenizer for 10 s, and subsequently centrifuged for 30 min at 30,000 *g.* The pellet was resuspended in 50 mM Hepes, pH 7.4, and stored in liquid nitrogen. Adenylyl cyclase activity was measured as previously described (19), using 15–20 μg proteins.

Prostaglandin release. Confluent quiescent cell monolayers in 24-well plates were washed twice in PBS and further incubated over various periods of time (0–30 min) with 100 nM SRTX-C or ET-1. Supernatants were collected and stored at −80°C. PGE2 and 6-keto-PGF1α (the stable breakdown product of PGI2) were assayed by a specific enzyme immunoassay, as previously described (20). Release of 6-keto-PGF1α and PGE2 was monitored in the absence or presence of 100 nM SRTX-C and results were expressed as percent increase from control levels. During the incubation period, spontaneous release (control levels) of 6-keto-PGF1α and PGE2 increased progressively, achieving 23 and 46.3 ng/mg proteins after 30 min, respectively.

DNA synthesis assay. DNA synthesis was measured in triplicate wells by incorporation of [^{3}H]thymidine, as previously described (7). Confluent cells in 96-well plates were made quiescent in serum-free Waymouth medium over 3 d. Cells were stimulated for 30h with 2% human serum in the presence of ET-1, forskolin, or prostaglandins. In some experiments, cells were pretreated for 30 min, with either the cyclooxygenase inhibitor ibuprofen (25 μM), the adenylyl cyclase inhibitor SQ 22536 (1 mM), or vehicles. [^{3}H]Thymidine (0.5 μCi/well) was added during the last 6h of incubation. Radioactivity incorporated into trichloroacetic acid–insoluble material was recovered and measured by scintillation counting.

RNA preparation and Northern blot analysis. Confluent quiescent cells were stimulated with 5% human serum, in the absence or presence of SRTX-C, as indicated. Total RNA was extracted in guanidium isothiocyanate, according to Chomczynski and Sacchi, and quantified by A_{260} spectrophotometry (21). RNA samples (20 μg/lane) were denaturated, fractionated by electrophoresis through a 0.8% agarose/formaldehyde gel, and subsequently transferred to a Hybond N membrane (Amersham). Prehybridization was performed at 42°C in 5× SSC (1× SSC = 150 mM NaCl, 15 mM Na citrate), 50% formamide, 1× Denhardt's, 50 mM NaH_2P0_4 and 250 μg/ml salmon sperm DNA. cDNA probes for mouse c-fos (0.5 kb NCO1 fragment), mouse c-jun (1 kb Sma1 fragment), human glyceraldehyde-3-phosphate dehydrogenase (G3PDH, 1.3-kb EcoR1 fragment), and human krox 24 (prepared from a PCR product obtained from amplification with specific primers for krox24 [5′ATTGTGAGGGACATGCTCAC3′ and 5′ACAAAAATCGCCGCCTACTC3′, krox 24, 246 bp]) were labeled with [α^{32}P]dATP by nick translation with a commercially available kit (Promega Corp., Madison, WI). Blots were subsequently hybridized overnight at 42°C with the ^{32}P-labeled probes, washed once at 22°C for 15 min with 1× SSC, 0.1% SDS (fos and jun mouse probes) or 0.1× SSC, 0.1% SDS (G3PDH and krox 24 human probes), and twice in the same medium for 45 min at 52°C (fos and jun) or 68°C (G3PDH or krox 24). Blots were then exposed to x-ray film (Hyperfilm; Amersham) at −70°C. Hybridization signals were quantified at 633 nm by scanning densitometry. Results are relative to G3PDH expression, which was used as an internal standard to correct for variations in loading and transfer. Autoradiograms show representative experiments and densitometric data are the mean±SEM of three independent experiments.

Extracellular signal-regulated kinase and JNK assays. Confluent quiescent cells in 60 mm wells were stimulated for 15 min (unless otherwise specified) with the indicated effectors. The reaction was terminated by washing the cell cultures twice with 6 ml ice-chilled PBS. Cells were lysed for 30 min at 4°C in 0.3 ml Hepes 50 mM, pH 7.4, containing 1% Triton-X100, 10% glycerol, 137 mM NaCl, 1.5 mM $MgCl_2$, 10 mM NaF, 0.1 mM DTT, 1 mM EGTA, 5 mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μg/ml pepstatin, 40 mM β-glycerophosphate and 10 mM paranitrophenylphosphate. Lysates were centrifuged at 4°C for 15 min at 12,000 *g.* The supernatants were either used directly, or frozen at −70°C.

Extracellular signal-regulated kinase activity was assayed in situ after electrophoresis of cell lysates (50 μg proteins) on a 10% SDS-PAGE copolymerized with 0.5 mg/ml myelin basic protein, as previously described (7). Phosphorylated gels were exposed to x-ray films and ERK activity was quantified by densitometry.

A c-Jun kinase assay was carried out essentially as previously described, using 25-μg cell lysates (22). JNK activity was assayed in vitro, by the phosphorylation by [γ^{32}P]ATP of its substrate, GST-c-Jun (1-79) fusion protein, followed by SDS-PAGE and phosphorimager analysis. Coomassie staining was used to demonstrate equal protein loading.

Binding of [^{125}I]ET-1 to a particulate fraction of HSC. Confluent quiescent HSC were incubated over various periods of time in Waymouth medium containing either 10 μM forskolin, 50 μM PGE2, or 50 μM carbacyclin, the stable analog of PGI2. The particulate fraction was obtained as described above. Binding of [^{125}I]ET-1 to HSC particulate fraction was performed as previously described (5). Briefly, the HSC particulate fraction (10–20 μg proteins/ml) was incubated for 90 min at 22°C in 200 μl Krebs-Ringer medium containing 20 mM Hepes, pH 7.4, 1% (wt/vol) bovine serum albumin, 300 μg/ml bacitracin, with either 40 pM [^{125}I]ET-1 and varying concentrations of peptide (competition experiments), or varying concentrations of [^{125}I]ET-1 (4.5–750 pM, saturation experiments). Nonspecific binding was determined by incubating with 0.1 μM unlabeled ET-1. Data from saturation and competition experiments were analyzed using the nonlinear regression program LIGAND.

Assay of protein concentration. Protein concentration was measured by the method of Bradford (23), with the exception of cell lysate proteins used for ERK and JNK assays, which were assayed according to Peterson (24).

Results

Stimulation of the ETB receptor raises cyclic AMP levels through the release of prostaglandins in activated HSC. Cyclic AMP has been reported to cause growth inhibition in certain mesenchymal cells (25, 26). Since we have shown that ETB receptors mediate the growth inhibitory effect of ET-1 in activated HSC, we investigated the effect of ETB receptor stimulation on cAMP levels. To that aim, HSC were stimulated with the selective ETB receptor agonist SRTX-C (27). Sarafotoxin-S6C transiently raised intracellular cAMP, a five- to sevenfold increase being observed after 2 to 5 min (Fig. 1 *A*). The natural agonist ET-1 (100 nM) also increased cAMP concentration fivefold (Fig. 1 *C*). We next assayed adenylyl cyclase activity in a particulate fraction prepared from cultured activated HSC. Surprisingly, addition of 100 nM ET-1 or SRTX-C to the particulate fraction did not affect adenylyl cyclase activity, although the enzyme was functional, responding to the pharmacological agent forskolin and to the physiological agonist PGE2 (Fig. 1 *B*). These results indicate that ET-1 and SRTX-C increase cAMP by an indirect mechanism in activated HSC.

Since ET-1 stimulates the cyclooxygenase pathway in several cell types (8), we assessed whether ET-1 elevates cAMP through the production of prostaglandins. Pretreatment of HSC with the cyclooxygenase inhibitor ibuprofen blunted ET-1–induced accumulation of cAMP (Fig. 1 *C*). Among the major cyclooxygenase products, both PGE2 and the stable PGI2 analog, carbacyclin, increased intracellular cAMP by 3.5- and 12.0-fold, respectively, while PGD2 and PGF2α had no effect (Fig. 1 *C*). Moreover, production of both PGE2 and 6-keto-PGF1α (the stable metabolite of PGI2) was stimulated by SRTX-C (Fig. 1 *D*). After a 2–5-min treatment with SRTX-C, secretion of 6-keto-PGF1α and PGE2 was maximally increased by 260 and 350% over basal release, respectively. Stimulation of 6-keto-PGF1α and PGE2 synthesis by ET-1 followed a similar time course (not shown). Ibuprofen totally blunted the increase in the release of 6-keto-PGF1α and PGE2 elicited by SRTX-C (Fig. 1 *D*). It should be noted that 6-keto-PGF1α and PGE2 production remained elevated up to 30 min (Fig. 1 *D*), while cAMP levels returned to basal within 20 min (Fig. 1 *A*). It may be suggested that transient elevation of cAMP in spite of a prolonged enhancement of prostaglandin secretion may be related to desensitization of PG receptors (28).

Taken together, these results indicate that, in activated HSC, stimulation of the ETB receptor increases cAMP indirectly, through the release of prostaglandins.

The growth inhibitory effect of ET-1 is mediated by a prostaglandin/cAMP pathway. The growth inhibitory effect of ET-1 was reproduced by forskolin, PGE2, and PGI2, each of which inhibited DNA synthesis of serum-stimulated HSC by 50% at 10 μM (Fig. 2 *A*). Moreover, pretreatment of cells with the adenylyl cyclase inhibitor SQ 22536 suppressed the inhibition of DNA synthesis elicited by ET-1 (Fig. 2 *B*). We verified that SQ 22536 prevented the increase in cAMP induced by 100 nM ET-1 and 10 μM forskolin (1.8- and 2.8-fold, respectively, as compared with 5.1-fold in the presence of ET-1 alone and 57.4-fold in the presence of forskolin alone). Blockade of the cyclooxygenase pathway by ibuprofen also blunted the growth inhibitory effect of ET-1 (Fig. 2 *B*). Taken together, these results show that prostaglandin-induced accumulation of cAMP is responsible for the antiproliferative effect of ET-1 in activated HSC.

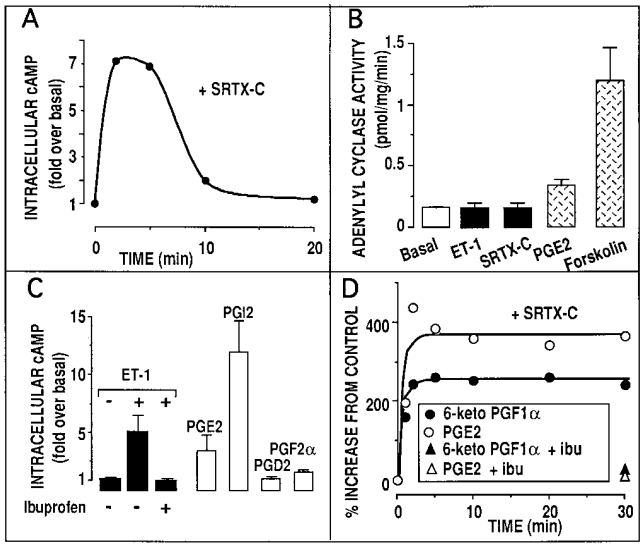

Figure 1. Stimulation of the ETB receptor raises cAMP indirectly by increasing the production of prostaglandins. (*A*) Effect of SRTX-C on cAMP levels (time course). Confluent quiescent activated HSC were incubated with 100 nM SRTX-C for various periods of time. Cyclic AMP was assayed as described in Methods. Results of a typical experiment repeated twice are expressed as fold over basal levels (17 fmol cAMP/μg protein). (*B*) Adenylyl cyclase activity in a particulate fraction of activated HSC. Adenylyl cyclase activity was assayed in a particulate fraction prepared as described in Methods. The assay medium contained 10 μM GTP, 10–20 μg protein and either 100 nM ET-1, 100 nM SRTX-C, 30 μM PGE2, or 10 μM forskolin. Results are the mean±SEM from triplicate determinations of a typical experiment repeated twice. (*C*) Indirect effect of ET-1 on cAMP levels. (*Black bars*) Confluent quiescent cells were exposed for 5 min to 100 nM ET-1, after a 30 min pretreatment with 25 μM ibuprofen or vehicle. In the presence of ibuprofen alone, cAMP levels were 15.4±1.5 fmol/μg protein. (*White bars*) Cells were treated for 5 min with either 30 μM PGE2, 30 μM carbacyclin (the stable analog of PGI2), 30 μM PGD2, or 30 μM PGF2α. Data are the mean±SEM of three to six experiments and are expressed as fold over basal levels obtained in the absence of added agents (22±8.5 fmol/μg protein). (*D*) Sarafotoxin-S6C stimulates production of PGI2 and PGE2 by activated HSC (time-course). Confluent quiescent cells were exposed to 100 nM SRTX-C over various periods of time, after pretreatment with ibuprofen (25 μM) or vehicle. 6-keto PGF1α (the stable metabolite of PGI2) and PGE2 were assayed by enzyme immunoassay, in the presence or absence of ibuprofen (25 μM), as described in Methods. Results are expressed as percent increase from control and show a typical experiment repeated three times. Cyclic AMP and prostaglandin production were also assayed in the conditions used for the DNA synthesis assays; that is, using serum-stimulated cells (see Fig. 2). Serum alone caused a fourfold increase in cAMP levels (basal level, 22 fmol cAMP/μg protein) and a twofold elevation in prostaglandin production (basal level, 12 ng PGE2/mg protein.) Nevertheless, in the presence of serum, SRTX-C and ET-1 increased cAMP by 2.1- and 2.6-fold as compared with levels observed in serum alone (88 fmol/μg protein); in the presence of serum, SRTX-C increased prostaglandin production by 1.9-fold (serum alone, 23 ng/mg protein).

Selective inhibition of c-jun mRNA expression by cAMP. A peculiar aspect of ET-1 signaling in activated HSC is that stimulation of the ETB receptor selectively reduces c-jun mRNA induction by serum, without affecting that of c-fos and krox 24 (egr-1) mRNAs (7). The addition of forskolin to serum-stimulated cells also reduced by 50% maximal induction

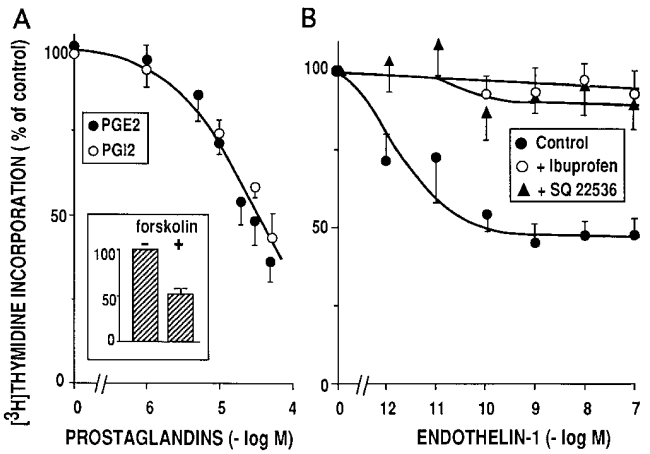

Figure 2. Growth-inhibitory effect of ET-1 is mediated by a prostaglandin-induced accumulation of cAMP. (*A*) Effect of PGE2, PGI2, and forskolin (*inset*) on serum-stimulated DNA synthesis. Quiescent cells were stimulated over 30 h with 5% human serum in the presence of varying concentrations of PGE2 (●) and PGI2 (added in the form of its stable analog carbacyclin) (○) or with 10 μM forskolin (*inset*). Results are the mean±SEM of four to eight experiments. (*B*) The cyclooxygenase inhibitor ibuprofen and the adenylyl cyclase inhibitor SQ 22536 blunt ET-induced inhibition of DNA synthesis. Confluent quiescent cells were pretreated with either 25 μM ibuprofen (○), 1 mM SQ 22536 (▲), or vehicle (●). Cells were subsequently stimulated over 30 h with 5 % human serum, in the presence of varying concentrations of ET-1. Results are the mean±SEM of three experiments and are expressed as percentage of control with serum but without added agent. Ibuprofen and SQ 22536 alone had no effect on the stimulation of [^{3}H]thymidine incorporation by serum. [^{3}H]Thymidine incorporation was determined as described in Methods.

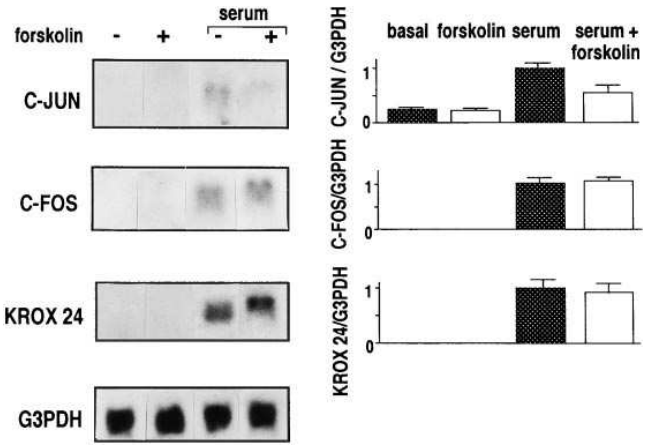

Figure 3. Effect of forskolin on the induction of c-jun, c-fos, or krox 24 mRNAs by serum. Quiescent cells were preincubated for 15 min with or without 10 μM forskolin, and then further incubated for 60 min in medium alone or in medium containing 5% human serum, in the absence or presence of 10 μM forskolin. Total RNA (20 μg/lane) was separated on a 0.8% agarose gel, transferred to a nitrocellulose membrane, and hybridized with ^{32}P-labeled cDNA probes encoding c-jun, c-fos, krox 24, and G3PDH, as described in Methods. (*Left*) Typical autoradiogram. (*Right*) Quantification of c-jun, c-fos, and krox 24 signals relative to G3PDH. Results are the mean±SEM of three experiments.

of c-jun mRNA expression and did not modify induction of c-fos and krox 24 mRNAs (Fig. 3). Forskolin alone did not affect basal expression of c-jun, c fos, or krox 24 mRNAs in quiescent serum-starved cells. Hence, like ET-1, cAMP elicits differential regulation of immediate-early gene expression in HSC.

Endothelin-1 and cAMP inhibit two members of the mitogen-activated protein kinase superfamily, JNK and ERK. ERK and JNK belong to two parallel cascades that lead to phosphorylation of diverse transcription factors that regulate expression of immediate-early genes important for cell proliferation (26, 29–32). We have recently shown that the antiproliferative effect of ET-1 is associated with a reduction of ERK activation by serum (7). Recent reports suggest that JNK may also be involved in cell proliferation (33–35), and we show here that serum increases JNK activity by 10.6±2.0-fold after 15 min (Fig. 4 *A*). Sarafotoxin-S6C reduces activation of JNK by serum (Fig. 4 *A*), resulting in an 80±3.7% inhibition of JNK activity after 15 min. The natural agonist ET-1 also decreases by 60±2.7% JNK activity in serum-stimulated cells (Fig. 4 *B*). In serum-starved cells, JNK activity was either unchanged (Fig. 4, *A* and *B*, *top*, typical) or slightly increased (Fig. 4, *A* and *B*, *bottom*, mean, $n = 3$) by ET-1 and SRTX-C.

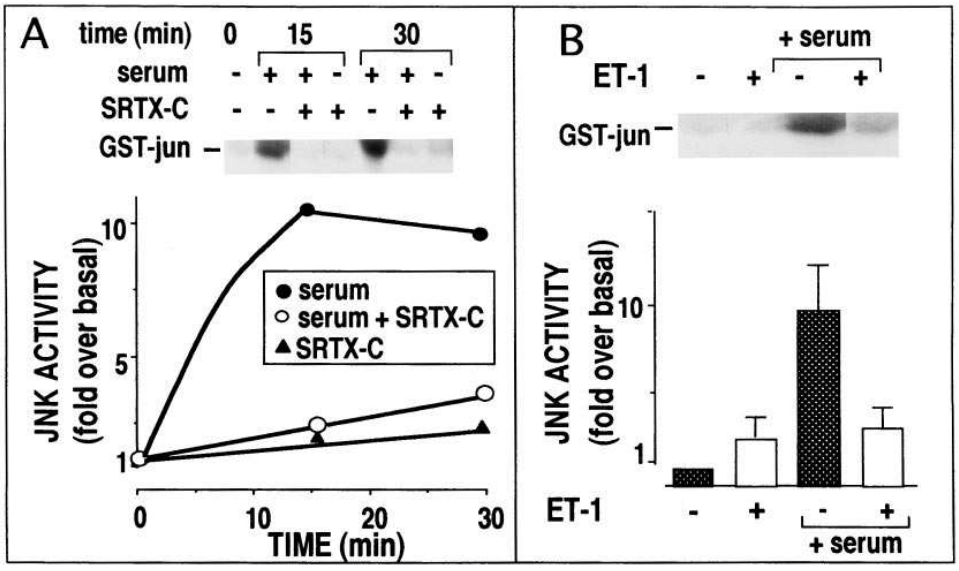

Figure 4. Endothelin-1 and SRTX-C inhibit activation of JNK by serum. (*A*) Time course of the effect of SRTX-C. Confluent quiescent activated HSC were stimulated for the indicated times with 5% serum alone, 5% serum and 100 nM SRTX-C, or 100 nM SRTX-C alone. JNK activity was determined by phosphorylation of the c-jun–GST substrate, as described in Methods. (*B*) Effect of ET-1 on JNK activity. Cells were stimulated for 15 min with the indicated compounds. Results show a typical experiment that was repeated three times. (*Top*) typical autoradiograms. (*Bottom*) Quantification of the JNK signal (mean±SEM of three experiments).

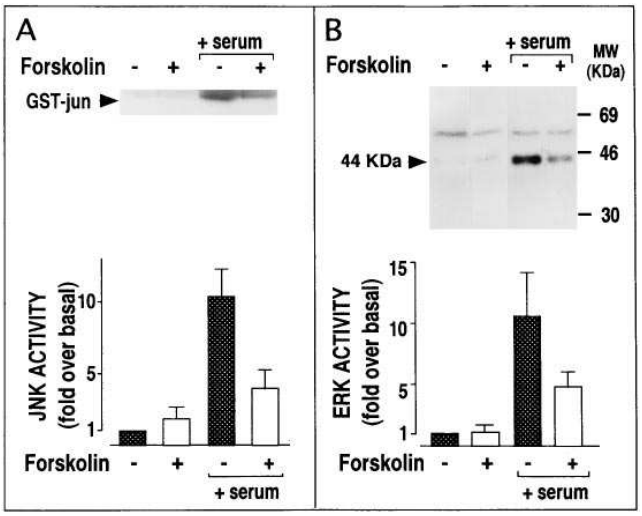

Figure 5. Effect of forskolin on the activation of JNK (*A*) and ERK (*B*) activities. Quiescent cells were preincubated with or without 10 μM forskolin, and then further stimulated for 15 min with 5% human serum alone, 5% human serum and 10 μM forskolin, or 10 μM forskolin alone. JNK and ERK activity were determined as described in Methods. (*Top*) Typical autoradiograms. (*Bottom*) Quantification of the JNK and of the $p44^{erk}$ signals (mean±SEM of three experiments).

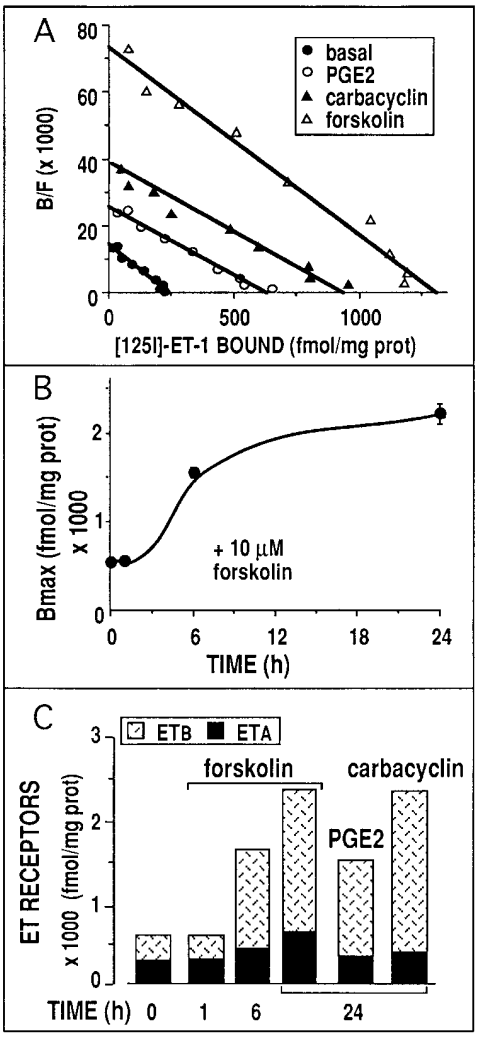

Figure 6. Upregulation of ETB receptors by cAMP and prostaglandins. (*A*) Forskolin and prostaglandins increase the total number of ET binding sites. Confluent quiescent cells were exposed either to medium alone, or to 10 μM forskolin or 50 μM PGI2 (added in the form of carbacyclin), or 50 μM PGE2 for 24 h. Binding of [^{125}I-ET-1] was measured on a cell particulate fraction, as described in Methods. The total number of binding sites (B_{max}) was determined at each time point by Scatchard analysis of saturation experiments performed with increasing concentrations of [^{125}I-ET-1]. (*B*) Time-dependent effect of forskolin on ET binding sites. Experiments were performed as in (*A*). (*C*) Forskolin and prostaglandins selectively upregulate ETB receptors. ETA and ETB receptors were determined in competition experiments, using the ETB receptor agonist SRTX-C and the ETA receptor antagonist, BQ 123. Results show a typical experiment repeated twice.

The fact that stimulation of the ETB receptor decreases activation of both ERK and JNK by serum led us to investigate the effects of cAMP on these two kinases. Like ET-1, forskolin inhibited serum-stimulated JNK and ERK activities by 60 and 50%, respectively (Fig. 5). In quiescent cells, ERK activity was unaffected by forskolin alone (Fig. 5 *B*), while JNK activity was either unchanged (Fig. 5 *A*, *top*, typical) or slightly increased (Fig. 5 *A*, *bottom*, mean, $n = 3$).

Collectively, these data demonstrate that, in activated HSC, a promitogenic stimulus activates both ERK and JNK, and that the cAMP-mediated antiproliferative effect of ET-1 is associated with an inhibition of both pathways.

Cyclic AMP and prostaglandins upregulate ETB receptors in human HSC. Treatment of activated HSC with 10 μM forskolin for 24 h increased ET receptors by four- to fivefold (Fig. 6). This upregulation was not apparent after a 1-h treatment and reached threefold after a 6-h treatment (Fig. 6 *B*). PGI2 (50 μM, in the form of carbacyclin) or PGE2 (50 μM) also raised the number of ET binding sites by four and threefold, respectively, after 24 h (Fig. 6, *A* and *C*), with a similar time course (not shown). There was no significant change in the molecular weight over the time course with either compound (Fig. 6 *A*); after 24 h, the molecular weight for ET-1 was 30.3±4.8 pM (control cells), 27.4±2.2 pM (forskolin), 24.0±1.0 pM (PGE2), and 27.3±1.7 pM (PGI2). Competition experiments using the selective ETB agonist SRTX-C, and the selective ETA antagonist BQ123, indicated that in control cells, ETA and ETB receptors were present in a proportion of 60:40 (mean, $n = 4$). This proportion varied from 50:50 to 70:30 between experiments. Forskolin markedly increased by 6.3-fold the number of ETB receptors after 24 h; there was also a 1.9-fold augmentation of ETA (Fig. 6 *C*). Similarly, treatment of cells with PGE2 or carbacyclin, the stable analog of PGI2, resulted in marked upregulation of ETB receptors (4.1- and 6.7-fold, respectively, after 24 h), and in a modest increment of ETA receptors (1.1- and 1.3-fold, respectively, at 24 h) (Fig. 6 *C*).

Discussion

In the present study, we have investigated the transduction mechanism responsible for the ETB-mediated antiproliferative effect of ET-1 in activated human HSC. We show that cAMP is the messenger involved, based on the following lines

of evidence: (*a*) ETB receptor stimulation increases cAMP; (*b*) cAMP inhibits growth and the adenylyl cyclase inhibitor SQ 22536 blunts the antiproliferative effect of ET-1; and (*c*) like ET-1 (7), cAMP selectively inhibits c-jun mRNA induction. These results are in keeping with the known growth inhibitory effects of cAMP in other cells such as fibroblasts, adipocytes, mesangial and vascular smooth muscle cells (25, 26). Moreover, they also agree with the selective down regulation of c-jun mRNA expression associated with growth inhibitory effects of cAMP in NIH3T3 fibroblasts (36). We investigated whether, in activated HSC, ET receptors are directly coupled to adenylyl cyclase via G proteins. We did not observe any effect of ET-1 on adenylyl cyclase activity in a particulate fraction of cells, in contrast to previous results in iris sphincter cells (37) and vascular smooth muscle cells (38). Instead, our data suggest that increases in PGE2 and PGI2 synthesis can account for ET-1–stimulated cAMP synthesis, as previously shown in anterior pituitary gland (39) and alveolar epithelium (40). The role of prostaglandins in the antiproliferative effect of ET-1 was directly demonstrated by the growth inhibitory properties of PGI2 and PGE2, and by the fact that ibuprofen blocks ET-induced growth inhibition (Fig. 2). These data agree with the growth inhibitory properties of PGE2 and PGI2 in several cell types, including rat activated HSC (41). Overall, our findings demonstrate that in activated HSC, stimulation of the ETB receptor increases the release of PGI2 and PGE2 with autocrine effects on adenylyl cyclase; the resulting elevation of cAMP induces growth inhibition (Fig. 7). Interestingly, it has been shown that ETB receptor stimulation also causes the release of PGE2 from Kupffer cells (42). Hence, it is tempting to speculate that, in vivo, ET-1 could inhibit growth of HSC by two pathways: on the one hand, by binding to ETB receptors of activated HSC and by promoting the secretion of prostaglandins with autocrine effects; and on the other hand, by stimulating ETB receptors of Kupffer cells, thereby releasing prostaglandins with paracrine effects on neighboring activated HSC.

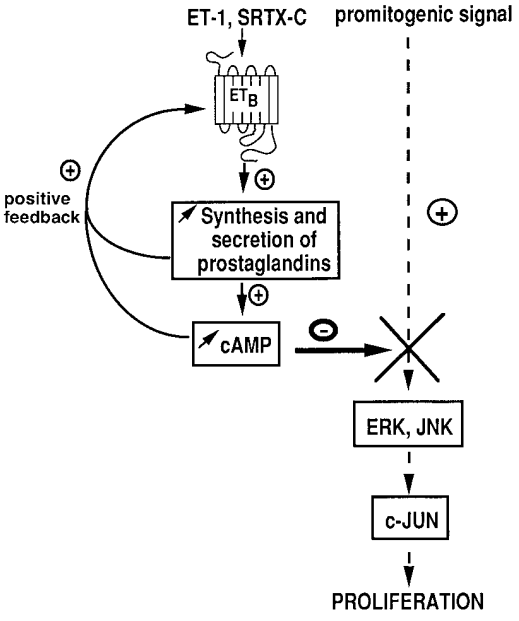

Figure 7. Antiproliferative effect of ET-1 in activated HSC: a hypothetical scheme.

ERK and JNK belong to the mitogen-activated protein kinase family and define parallel cascades that lead to the phosphorylation of transcription factors involved in cell proliferation (26, 29–31). The ERK pathway is predominantly activated by mitogens acting on receptor-protein tyrosine kinases or on G protein–linked receptors (26, 31). The JNKs are activated by stimuli that generally do not stimulate the ERK pathway (29–31), such as cellular stress or inflammatory cytokines (35, 43, 44). However, JNK may also be activated by signals that bind to tyrosine kinase receptors (22, 45) or to G protein–coupled receptors (46, 47). While ERK activation is an early and crucial event leading to mitogenesis (32), the function of JNK is less clear and has been linked to cell proliferation (33–35), cell transformation (48, 49), and apoptosis (50, 51). We previously reported that the growth inhibitory effect of ET-1 in HSC is associated with a decrease of ERK stimulation by mitogenic agents (7). We now show that a promitogenic stimulus also rapidly and markedly activates JNK, and that this effect is strongly reduced in the presence of ET-1 (Fig. 4, *A* and *B*). Moreover, its second messenger cAMP decreases activation of both ERK and JNK (Fig. 5). The ability of cAMP to antagonize activation of ERK by growth factors generally correlates with its growth inhibitory properties (52). The effect of cAMP on JNK has been investigated in few reports that showed dissociated regulation of ERK and JNK by the nucleotide (53, 54). Thus, in T lymphocytes, cAMP inhibited stimulation of JNK and did not affect ERK activation (54). In contrast, in gingival fibroblasts or HepG2, cAMP had no effect on activation of JNK, but inhibited activation of ERK (53). The fact that ET-1 and cAMP reduce activation of both ERK and JNK in activated HSC (Fig. 7), suggests that the blockade of both pathways might be important for growth inhibition. Our results also emphasize the fact that a single factor may regulate both ERK and JNK in selected cell types, either positively, as shown with angiotensin II (47), carbachol (46), or EGF (22, 44), or negatively as observed with ET-1 in human HSC.

In contrast to the antiproliferative effects of ET-1 in activated human HSC, promitogenic effects of the peptide have been described in several cells (6). While growth inhibition is mediated by the ETB receptor (7), ET-1 stimulates cell proliferation via ETA receptors (6). Therefore, the ratio between ETA and ETB receptors might constitute an additional factor that could affect the sensitivity of cells to the growth regulatory effects of ET-1. We found that prolonged treatment with prostaglandins or forskolin increases the total number of ET binding sites by fivefold. This upregulation affects predominantly the expression of ETB receptors, which increase from 50% in control cells to 80% in treated cells (Fig. 6 *C*). The selective upregulation of ETB receptors by prostaglandins/cAMP suggests the possibility of a positive feedback loop that could amplify the growth inhibitory effect of ET-1 (Fig. 7). In keeping with this hypothesis, it has been shown that upregulation of ET receptors by cAMP is correlated with an enhanced responsiveness to ET-1 in vascular smooth cells (55).

Proliferation of activated HSC is well documented in experimental chronic liver injury (56, 57) and constitutes one of the elements that may contribute to the increased deposition of extracellular matrix during chronic liver disease. Therefore, there is growing interest in factors that may limit proliferation of activated HSC (58, 59). It was recently shown by combined

immunocytochemistry and in situ hybridization that, while ET-1 is weakly expressed in sinusoidal endothelial cells of the normal human liver, expression of the peptide is markedly enhanced in cirrhotic liver, and colocalizes with endothelial cells, activated HSC, and bile duct epithelial cells (16). There are currently no data regarding the in vivo pattern of expression of ETA and ETB receptors in HSC, either in normal liver or during chronic liver disease. However, in human HSC in culture, there is a shift from a preponderance of the ETA receptor subtype to a predominant ETB subtype after cell passaging (16). Therefore, increased expression of ET-1 during fibrogenesis and selective upregulation of ETB receptors in activated HSC suggest that the peptide could limit the proliferation of activated HSC during chronic liver disease. Moreover, the fact that cAMP inhibits growth of activated human HSC suggests that pharmacological activators of adenylyl cyclase or inhibitors of phosphodiesterases might be of interest to counteract the development of fibrosis in vivo.

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