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Antifibrogenic effects of histone deacetylase inhibitors on pancreatic stellate cells

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ABSTRACT

Pancreatic stellate cells (PSCs) are essentially involved in pancreatic fibrogenesis and considered as a target for antifibrotic therapies. Here, we have analyzed the effects of three histone deacetylase inhibitors (HDACIs), sodium butyrate, sodium valproate (VPA) and trichostatin A (TSA), on profibrogenic activities of PSC and elucidated molecular targets of HDACI action. Therefore, cultured PSCs were exposed to HDACI. Cell proliferation and viability were assessed by 5-bromo-2′-deoxyuridine (BrdU) incorporation and trypan blue staining assays. Exhibition of the myofibroblastic PSC phenotype was monitored by immunofluorescence analysis of α-smooth muscle actin (α-SMA) expression. [^{3}H]-proline incorporation into acetic acid-soluble proteins was measured to quantify collagen synthesis. Levels of mRNA were determined by quantitative reverse transcriptase real-time PCR. Protein expression, phosphorylation and acetylation were analyzed by immunoblotting, and gel shift assays were performed to study DNA binding of nuclear proteins. HDACI enhanced histone H3 acetylation in a dose-dependent manner. In the same dose range, they strongly inhibited cell proliferation, α-SMA expression and collagen synthesis. A significantly increased rate of cell death was observed in response to TSA at 1 μM. While all three HDACI inhibited mRNA expression of endothelin-1, only VPA significantly reduced expression of transforming growth factor-β1. Both mediators exert autocrine profibrogenic effects on PSC. Furthermore, HDACI-treated PSC displayed a diminished DNA binding of AP-1, a key transcription factor in profibrogenic signaling. Together, the results suggest that HDACI exert antifibrogenic effects on PSC. Interruption of AP-1 signaling and autocrine loops enhancing PSC activation might be key mechanisms of HDACI action.

1. Introduction

In chronic pancreatitis, accumulation of extracellular matrix (ECM) proteins frequently leads to the development of pancreatic fibrosis, resulting in an exocrine and endocrine insufficiency of the gland [1,2]. Fibrosis is also a hallmark of pancreatic cancer and likely to play an active role in disease progression (reviewed in [3]). Despite significant efforts in recent years, specific therapies to retard or even reverse pancreatic fibrosis have not been established yet.

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Abbreviations: HDACI, histone deacetylase inhibitor; PSC, pancreatic stellate cell; VPA, sodium valproate; TSA, trichostatin A; BrdU, 5-bromo-2′-deoxyuridine; α-SMA, α-smooth muscle actin; ECM, extracellular matrix; HSC, hepatic stellate cell; PDGF, platelet-derived growth factor; TGF, transforming growth factor; ET, endothelin; CTGF, connective tissue growth factor; IFN, interferon; ECL, enhanced chemoluminescence; IMDM, Iscove's modified Dulbecco's medium; HPRT, hypoxanthine-guanine phosphoribosyl transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; S.E.M., standard error of the mean; AP-1, activator protein-1; ab, antibody.

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Pancreatic stellate cells (PSCs) are considered as the main source of ECM proteins in the diseased organ [3]. In response to profibrogenic mediators, PSC undergo phenotypic changes termed activation: the cells proliferate, synthesize and secrete increased amounts of ECM proteins (e.g., collagen types 1 and 3) and stain strongly positive for the myofibroblastic marker protein α-smooth muscle actin (α-SMA), which is organized in networks of so-called stress fibers [4–9]. Furthermore, in the course of activation PSC loose most of their characteristic vitamin A-containing fat droplets [4]. Together, these phenotypic changes closely resemble the process of hepatic stellate cell (HSC) activation in the fibrotic liver, suggesting common mechanisms in the development of fibrosis in both organs. Typical mediators of PSC activation include, in addition to ethanol metabolites, various cytokines [5,8,9]. Thus, platelet-derived growth factor (PDGF) exerts strong mitogenic effects on PSC, while transforming growth factor (TGF)-β stimulates matrix protein synthesis [10,11]. PSC activation is maintained and further enhanced through autocrine loops that have been suggested for mediators such as endothelin (ET)-1 [12,13], connective tissue growth factor (CTGF) [14,15] and members of the TGF-β family (TGF-β1; Activin A) [16,17]. Extracellular antagonists of PSC activation are less well characterized. In a recent study, we found that interferons (IFNs), particularly IFN-γ, exert antiproliferative effects on PSC and inhibit collagen synthesis in vitro [18], but the in vivo effects of these cytokines remain to be analyzed.

In recent years, epigenetic mechanisms have emerged as major determinants of gene expression and implicated in the regulation of complex differentiation and developmental processes, both under physiological and pathological conditions. In addition to promoter methylation, histone modification, particularly histone acteylation, is considered as a key principle of epigenetic regulation [19,20]. Histone deacetylation, which is associated with a repressed chromatin state, is tightly controlled by two classes of enzymes, histone acetyltransferases and histone deacetylases [19–21]. Inhibitors of histone deacetylases (HDACI) display anti-cancer activities and are, therefore, of growing clinical interest [22]. The role of epigenetic regulatory mechanisms in the control of PSC functions has not been studied so far. Recent studies, however, suggest that HDACI exert antifibrotic effects on hepatic stellate cells [23,24] and lung fibroblasts [25]. The underlying molecular mechanisms are still poorly understood. Here, we have characterized the effects of three structurally diverse HDACI, sodium butyrate, sodium valproate (VPA) and trichostatin A (TSA), on key functions of activated PSC. Furthermore, we have studied target genes of HDACI action in PSC and linked changes in histone acetylation to the modulation of profibrogenic signal transduction pathways. Therefore, an established in vitro model was applied, using PSC that were isolated from the pancreas of healthy rats and activated by sustained culture [26].

2. Materials and methods

2.1. Materials

The enhanced chemoluminescence (ECL) Plus kit, horseradish-peroxidase-labelled antibodies and [^{3}H]-proline were purchased from GE Healthcare (Freiburg, Germany), Taqman™ reagents from Applied Biosystems (Foster City, CA, USA) and recombinant rat PDGF-BB from R&D Systems (Minneapolis, MN, USA). Iscove's modified Dulbecco's medium (IMDM) and all supplements for cell culture were delivered by Biochrom (Berlin, Germany). Ascorbat, β-aminoproprionitrile, the α-SMA antibody, TSA, sodium butyrate, VPA, ET-1 and standard laboratory chemicals were from Sigma–Aldrich (St. Louis, MO, USA). Nycodenz was obtained from Nycomed (Oslo, Norway), TRIzol from Invitrogen (Carlsbad, California, USA), Alexa Fluor goat anti-mouse IgG from MoBiTec (Göttingen, Germany) and all further antibodies from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

2.2. Cell culture

Stellate cells were isolated from the pancreas of male LEW.1W inbred rats by collagenase digestion of the organ followed by Nycodenz density gradient centrifugation as previously published [26,27]. PSCs collected from the top of the gradient were washed and resuspended in IMDM supplemented with 10% fetal calf serum, 1% non-essential amino acids (dilution of a 100× stock solution), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. All experiments were performed with cells growing in primary culture, or, depending on the experimental settings, with cells of the first passage. If replating of the cells was required, PSCs were harvested by trypsinization on day 7 after isolation and recultured at equal seeding densities. Trypan blue staining was performed to distinguish live from dead cells and to determine absolute cell counts.

2.3. Quantification of DNA synthesis

To quantify DNA synthesis, incorporation of 5-bromo-2′-deoxyuridine (BrdU) was measured using the BrdU labelling and detection enzyme-linked immunosorbent assay kit (Roche Diagnostics, Mannheim, Germany). Therefore, cells were plated in 96-well plates in complete culture medium supplemented with HDACI as indicated. After 24 h, BrdU labelling was initiated by adding labelling solution at a final concentration of 10 μM. Another 24 h later, labelling was stopped, and BrdU uptake was measured according to the manufacturer's instructions.

2.4. Quantitative reverse transcriptase-PCR using real-time TaqMan™ technology

Total RNA from PSC pretreated with HDACI for 24 h as indicated was isolated with TRIzol reagent according to the manufacturer's instructions. Next, 1 μg of RNA was reverse transcribed into cDNA by means of TaqMan™ Reverse Transcription Reagents and random hexamer priming. Relative quantification of target cDNA levels by real-time PCR was performed in an ABI Prism 7000 sequence detection system (Applied Biosystems) using TaqMan™ Universal PCR Master Mix and the following Assay-on-Demand™ rat gene-specific fluorescently labelled TaqMan™ MGB probes: 00561129_m1 (ET-1), Rn00572010_m1 (TGF-β1), and Rn01527838_g1 (hypoxanthine-guanine phosphoribosyl transferase [HPRT]; house-keeping gene control).

Expression of α-SMA was monitored applying a Custom TaqMan™ Gene Expression Assay specific for rat α-SMA (GenBank accession no. X06801). The primer and probe sequences for α-SMA were as follows—forward primer: 5′-CAGGATGCAGAAGGAGATCACA-3′; reverse primer: 5′-GCCG-ATCCAGACAGAATATTTGC-3′, and FAM-labelled probe 5′-CCA-TGAAGATCAAGATTATTG-3′. The following PCR conditions were used: 95 °C for 10 min, 50 cycles of 15 s at 95 °C/1 min at 60 °C. PCR reactions were performed in triplicate, and repeated five times with independent samples. The relative expression of each mRNA compared with HPRT was calculated according to the equation: $\Delta C_t = C_{t\ target} - C_{t\ HPRT}$. The relative amount of target mRNA in control cells and cells treated with HDACI as indicated was expressed as $2^{-(\Delta\Delta C_t)}$, where $\Delta C_{t\ treatment} = \Delta C_{t\ HDACI} - \Delta C_{t\ control}$.

2.5. Quantification of collagen synthesis

Collagen synthesis was assessed through the quantification of [^{3}H]-proline incorporation into acetic acid-soluble proteins. Therefore, cells were plated in 12-well plates and grown to subconfluency before they were exposed to HDACI for 24 h as indicated. Afterwards, culture medium was supplemented with 50 μg/ml ascorbate, 50 μg/ml β-aminoproprionitrile and 2.5 μCi/ml [^{3}H]-proline (48 Ci/mmol) per well, and incubation in the presence of HDACI continued for another 24 h. All further steps were performed as described before [27]. Raw data of [^{3}H]-proline incorporation were normalized on the basis of absolute cell counts determined by trypan blue staining of PSC cultured in parallel under identical conditions, except of that no [^{3}H]-proline was added.

2.6. Immunoblotting

Protein extracts of PSCs (pretreated as indicated) were prepared as described [26]. After separation by SDS-PAGE, proteins (15 μg per sample) were blotted onto nitrocellulose filters. Next, membranes were blocked with 1% BSA (Sigma–Aldrich) and incubated with the indicated antibodies (diluted according to the manufacturer's instructions) overnight at 4 °C. After a final incubation with a horseradish-peroxidase-labelled anti-rabbit or anti-mouse Ig antibody for 2 h at room temperature, blots were developed using the ECL Plus kit. Prior to reprobing with additional antibodies, membranes were stripped by incubation in stripping buffer (62.5 mM Tris–HCl, pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol) at 50 °C for 30 min. Chemoluminescence signals were quantified using a Kodak image station.

2.7. Immunofluorescence detection of α-SMA

Cells growing in primary culture were harvested by trypsinization, seeded onto glass coverslips and allowed to attach before they were exposed to HDACI for 24 h as indicated. For immunofluorescence staining, cells were fixed with ice-cold methanol followed by incubation with a mouse monoclonal antibody to α-SMA. Antibody binding was determined by a fluorescein-labelled goat anti-mouse IgG and visualized using

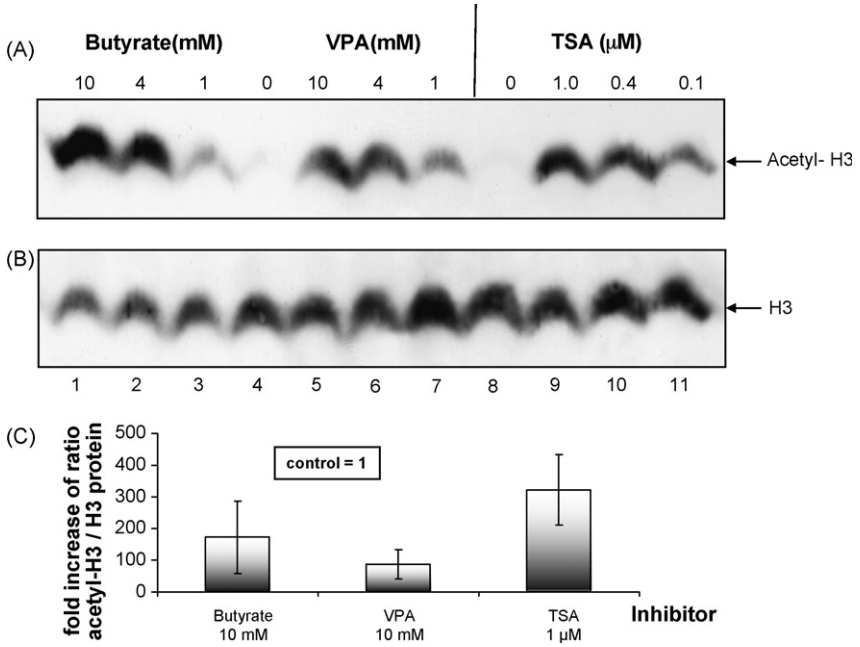

Fig. 1 – Enhancement of histone H3 acetylation by sodium butyrate, VPA and TSA. PSC growing in 6-well plates were treated with sodium butyrate, VPA and TSA at the indicated concentrations for 24 h. (A) Histone H3 acetylation was analyzed by immunoblotting. (B) Reprobing of the blot with an anti-H3 protein-specific antibody revealed no systematic differences of the histone H3 amount among the samples. (C) Acetyl-histone H3 levels were further investigated using image analysis software and related to the histone H3 protein level. Therefore, acetyl-histone H3 and H3 protein signal intensities were determined, and the ratio acetyl-histone H3/H3 protein was calculated. Data are expressed as the fold increase of this ratio in HDACI-treated cells vs. control cultures. Data of three independent experiments were used to calculate mean values and S.E.M.

a fluorescence microscope. Samples were further evaluated using a scoring system to assess α-SMA expression and organization of the protein in stress fibers in a semiquantitative manner. Therefore, samples were blinded for the investigator and scored from 0 (low or undetectable α-SMA expression; absence of stress fibers) to 3 (high expression levels; extensive stress fiber bundles).

2.8. Electrophoretic mobility shift assays (EMSA)

PSC growing in 6-well plates in complete culture medium were exposed for 24 h to HDACI or treated with PDGF (10 ng/ml; 60 min) as indicated. Cell lysis and preparation of nuclear extracts were performed essentially as previously described [26,28]. For EMSA experiments, nuclear proteins of 10^5 cells were incubated with double-stranded oligonucleotides which were end-labelled with [γ^{32}P] ATP by polynucleotide kinase. The sequence of the activator protein (AP)-1 probe was 5′-CGCTTGATGACTCAGCCGATC-3′ (consensus binding motif underlined). Binding reaction and supershift analysis (initiated by adding 1 μg antibody) were performed as described [20,36]. Protein–DNA complexes were analyzed by electrophoretic separation on a 6% non-denaturating polyacrylamide gel. Dried gels were exposed to X-ray film. The results shown are representative of three independent experiments.

2.9. Statistical analysis

Results are expressed as mean ± standard error of the mean (S.E.M.) for the indicated number of separate cultures per experimental protocol. Statistical significance was checked using Wilcoxon's rank sum test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of HDACI on histone acetylation as well as PSC growth and survival

Sodium butyrate, VPA and TSA induced acetylation of histone H3 in a dose-dependent manner, suggesting inhibition of

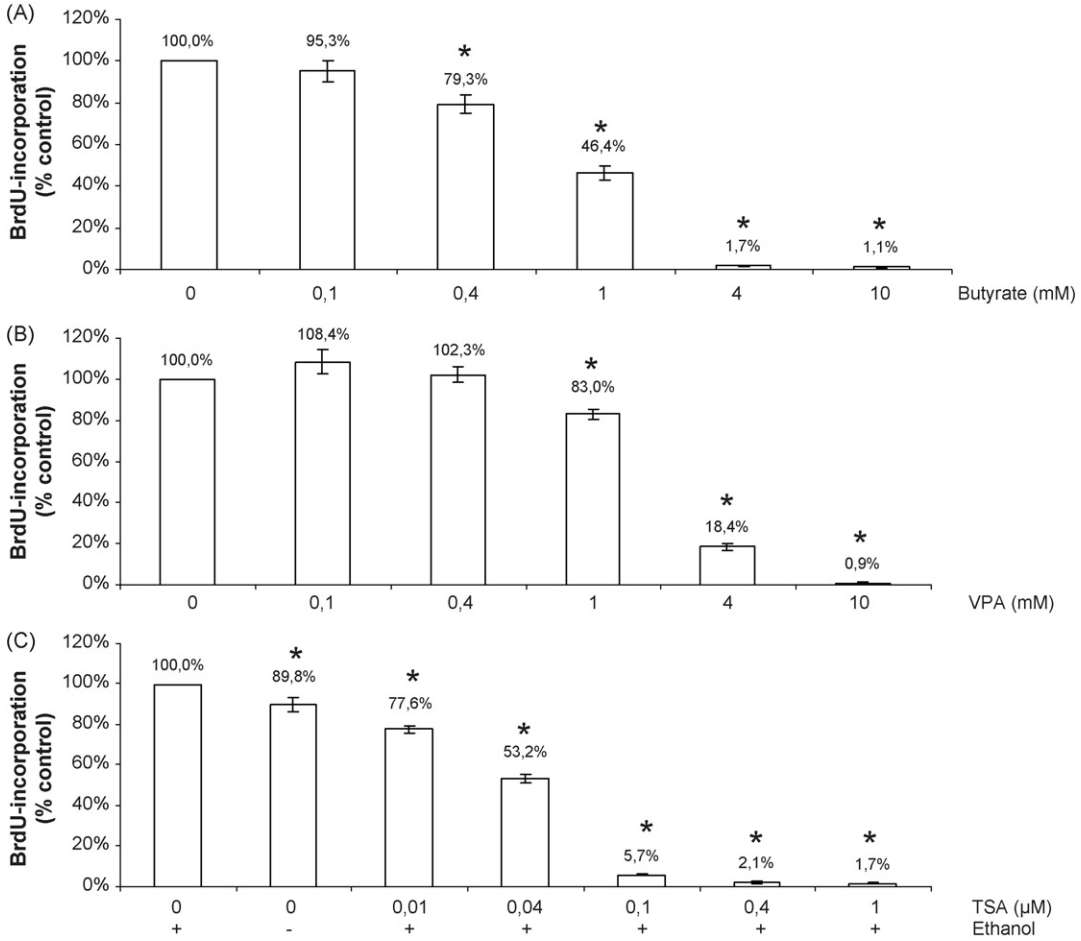

Fig. 2 – Effects of HDACI on DNA synthesis of PSC. PSCs growing in primary culture were harvested, replated at equal seeding densities in 96-well plates and treated with sodium butyrate (A), VPA (B) and TSA (C) at the indicated concentrations for 24 h. Subsequently, cells were labelled with BrdU for 24 h, and proliferation assessed with the BrdU DNA-incorporation assay. Control cultures were exposed to the solvents water (sodium butyrate and VPA) and ethanol (TSA; final concentration 5 mM), only. One hundred percent BrdU incorporation corresponds to solvent-treated PSC. Data from six separate cultures were used to calculate mean values and S.E.M. *$P < 0.05$ vs. control cultures.

histone deacetylase activity (Fig. 1). Average data of three experiments revealed that TSA incubation was associated with the strongest increase of acetyl-histone H3 levels. While TSA was efficient at concentrations of 1 μM and below, millimolar concentrations of the other two drugs were required to enhance histone acetylation. Based on these results, HDACI concentrations for subsequent experiments were selected.

To analyze the effects of HDACI on PSC proliferation, DNA synthesis after 48 h of HDACI treatment was measured (Fig. 2A–C). All three HDACI inhibited DNA synthesis significantly and in a dose-dependent manner. The data also indicate a small but significant stimulatory effect of the TSA solvent ethanol at the low concentration of 5 mM compared to control cultures exposed to water; the solvent of sodium butyrate and VPA (Fig. 2C).

As shown in Fig. 3A, inhibition of DNA synthesis by HDACI was accompanied by a significant decrease of cell counts (number of live cells). Again, comparison of control cultures suggested a growth-promoting effect of ethanol, which was, however, not significant. Cell counting after trypan blue staining (Fig. 3B) also revealed a significant increase of dead cells in cultures exposed to TSA at 1 μM (but not 0.1), whereas a quantitatively similar effect of VPA at 10 mM did not reach statistical significance. Sodium butyrate even at 10 mM did not cause a significant increase of dead cells in the trypan blue assay.

3.2. *Collagen synthesis and α-SMA expression in HDACI-treated PSC*

Incubation of PSC with sodium butyrate at ≥1 mM, VPA at ≥1 mM and TSA at ≥0.1 μM was associated with a significant decrease of [^{3}H]-proline incorporation into acetic acid-soluble proteins, suggesting inhibition of collagen synthesis (Fig. 4).

Phenotypic transition of PSC towards myofibroblast-like cells was monitored by immunofluorescence detection of α-SMA. Culture-activated untreated PSC stained strongly positive for α-SMA, while PSC exposed to either one of the three HDACI expressed less of the protein. Furthermore, organization of α-SMA in typical stress fibers was most pronounced in untreated PSC (Fig. 5A–D). Semiquantitative assessment of α-

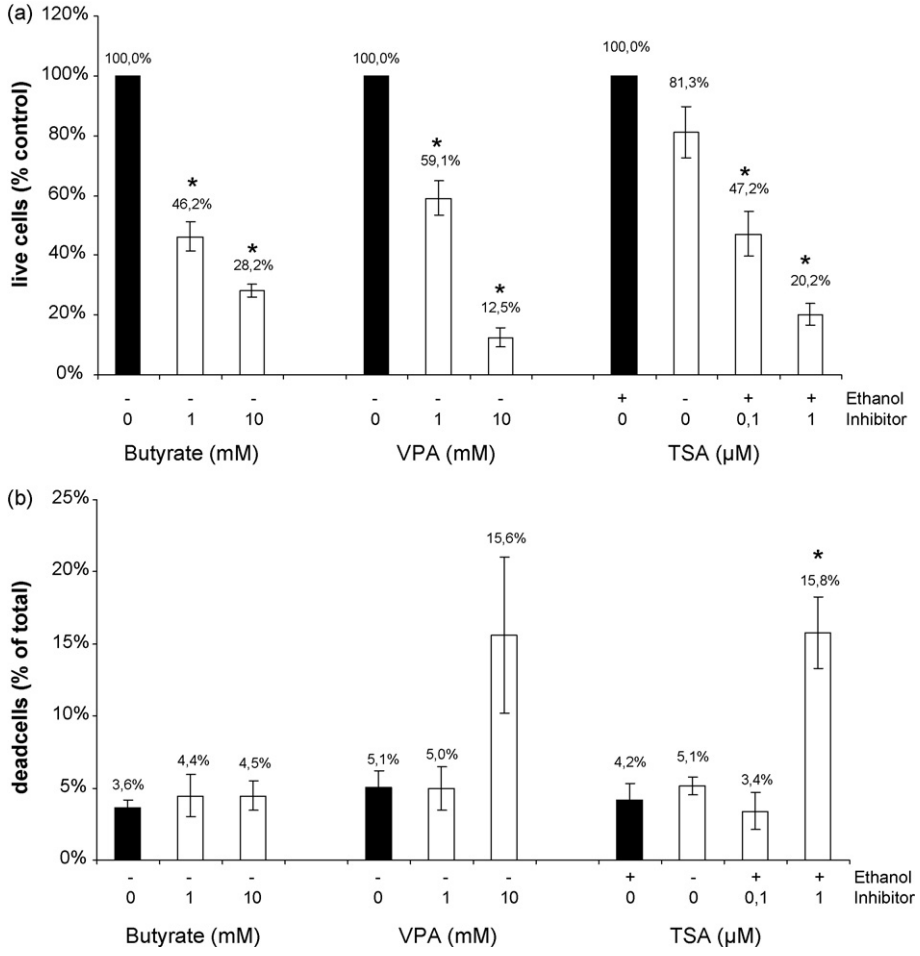

Fig. 3 – Cell counts of HDACI-treated PSC. PSCs growing in primary culture were harvested, replated at equal seeding densities in 6-well plates and treated with sodium butyrate, VPA and TSA at the indicated concentrations for 24 h. Control cultures were exposed to HDACI solvents (see legend to Fig. 1) only. Cell counts of live cells (A) and dead cells (B) were determined by trypan blue staining. In (A), 100% of live cells corresponds to solvent-treated cultures. In (B), 100% are built by the sum of live and dead cells. Data from six separate cultures were used to calculate mean values and S.E.M. *$P < 0.05$ vs. control cultures.

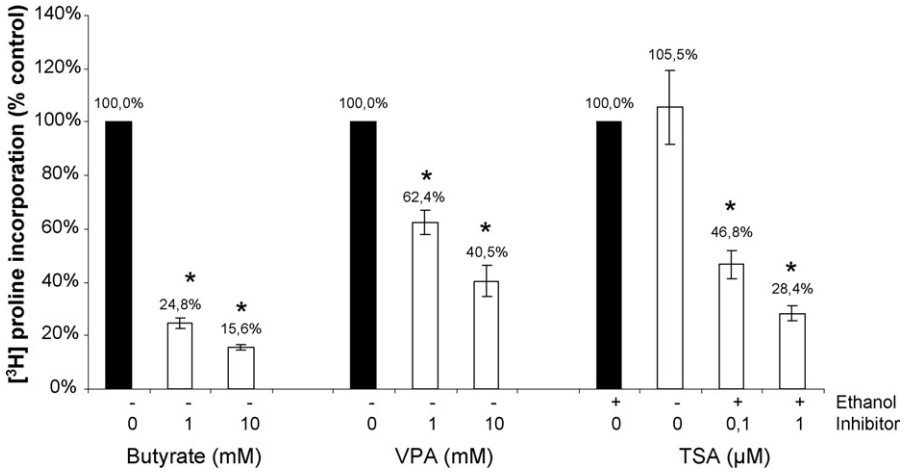

Fig. 4 – HDACI inhibit collagen synthesis in PSC. PSCs growing in 12-well plates (one passage) were exposed for 48 h to HDACI or their solvents (see legend to Fig. 1) as indicated. Collagen synthesis was analyzed through the quantification of [^{3}H]-proline incorporation into acetic acid-soluble proteins, and raw data were normalized for differences in cell growth rates as described under Section 2. One hundred percent [^{3}H]-proline incorporation corresponds to solvent-exposed PSC. Data are expressed as mean (±S.E.M.) of eight independent experiments. *P < 0.05 vs. solvent-treated control cultures.

SMA expression and stress fiber formation, applying a scoring system (see Section 2) to blinded samples, revealed significant inhibitory effects of sodium butyrate, VPA and TSA (Fig. 5E). Furthermore, all three HDACI strongly and in a dose-dependent manner suppressed α-SMA mRNA expression (Fig. 6A). Exposure of PSC to the TSA solvent ethanol alone neither significantly affected [^{3}H]-proline incorporation (Fig. 4), nor α-SMA expression (Figs. 5E and 6A).

3.3. *Effects of HDACI on TGF-β1 and ET-1 gene expression as well as on AP-1 activation*

We next addressed the question if HDACI modulate the mRNA expression of ET-1 and TGF-β1 which act on PSC through autocrine loops. At two concentrations tested, the three HDACI uniformly exerted significant inhibitory effects on the expression of ET-1 (Fig. 6B). In contrast, only VPA at 10 mM induced a significant reduction of TGF-β1 mRNA expression (Fig. 6C).

AP-1 transcription factor complexes have previously been implicated in the transcriptional induction of PSC activation [26,28] as well as ET-1 and TGF-β1 gene expression [29–31]. All three HDACI inhibited DNA binding of AP-1 in a dose-dependent manner (Fig. 7). Control samples indicated activation of AP-1 by PDGF (as previously established [26,28]; lane 13), and presence of the c-Fos protein in the DNA-binding complex (supershift analysis; lane 14). In contrast, HDACI did not inhibit protein binding to probes with nuclear factor (NF)-κB and Smad consensus motifs (data not shown).

4. Discussion

Stellate cells in liver and pancreas are essentially involved in the development of liver and pancreas fibrosis, respectively [6,32,33]. Profibrogenic mediators induce a phenotypic transition of stellate cells from a quiescent to an activated, myofibroblast-like phenotype that is characterized by a high capacity of ECM synthesis [4,5,8–11,32,33]. The physiological role of stellate cells in their quiescent stage is less clear. Interestingly, recent studies with HSC and stellate-like cells from the pancreas suggest that these cells display features of progenitor cells and are capable of differentiating into different cell lineages [34–36]. In this case formation of fibrotic tissue by activated stellate cells might reflect a process of dysregulated regeneration under the pathological circumstances of chronic pancreatitis and pancreatic cancer. With respect to the questions addressed in this study, it is important to note that under the experimental conditions used here PSC after a few days in culture uniformly express the myofibroblastic marker protein α-SMA, and that this phenotype is maintained for at least one passage. Therefore, all experiments were performed with primary or only one time recultured PSC.

The results of this study indicate that three structurally diverse inhibitors of histone deacetylase, sodium butyrate, VPA and TSA, inhibit proliferation of cultured PSC, diminish collagen synthesis, and strongly suppress exhibition of the myofibroblastic marker α-SMA both at the mRNA and protein level. Incubation with TSA at 1 μM, the highest concentration tested, was also associated with a significant (although relatively small) decrease of cell viability. This finding might be explained by the results of pilot experiments that showed a pro-apoptotic effect of HDACI at high concentrations after prolonged exposure of PSC (Jaster, unpublished data). Together, our data suggest an interference of HDACI with profibrogenic activities of culture-activated PSC in vitro, and a capability of these inhibitors to promote exhibition of a quiescent PSC phenotype. The results are in accordance with previous studies with hepatic stellate cells [23,24] and lung fibroblasts [25], which also suggested an antifibrotic efficiency of HDACI. Given that VPA is in clinical use for decades as an

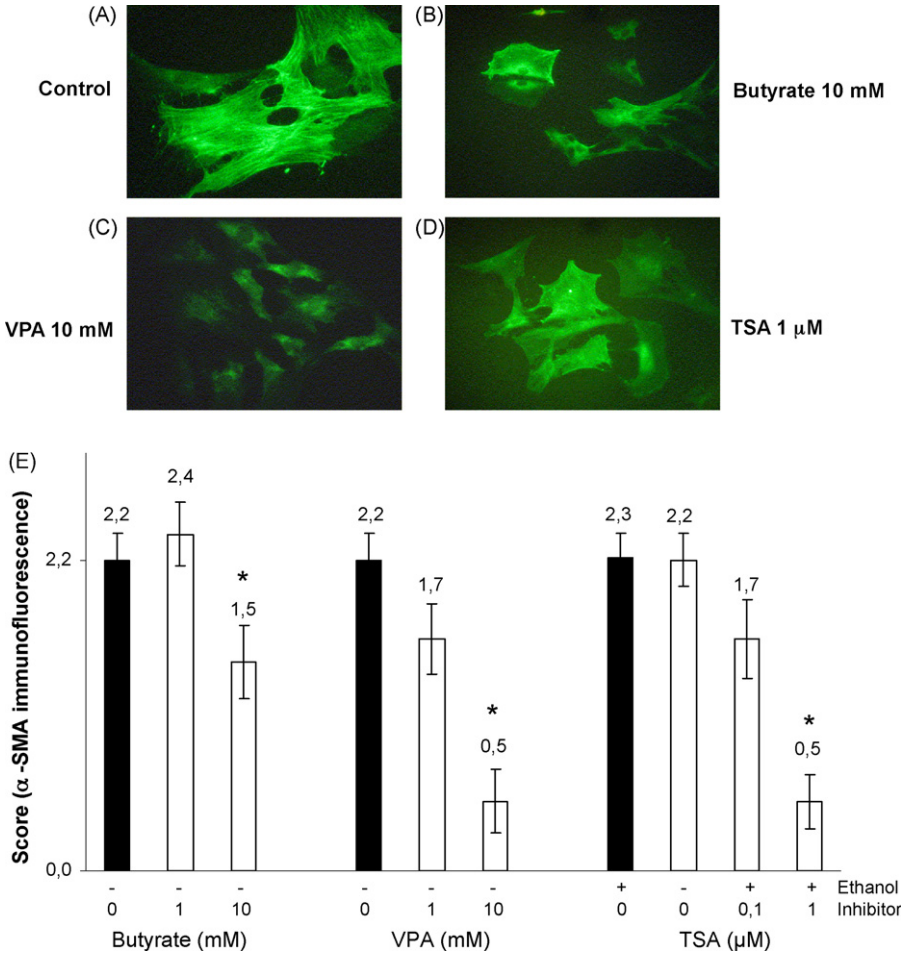

Fig. 5 – Effects of HDACI on α-SMA expression and stress fiber formation in PSC. PSC growing on glass coverslips were treated for 24 h with the sodium butyrate/VPA solvent water (control; A) sodium butyrate (10 mM; B), VPA (10 mM; C), TSA (1 μM; D), or HDACI and their solvents (see legend to Fig. 1) as indicated (E). (A–D) Expression and structural organization of α-SMA were analyzed by immunofluorescence staining of the protein and documented by fluorescence microscopy (original magnification, 630×). (E) Semiquantitative assessment of samples was performed as described under Section 2. Data are expressed as mean (±S.E.M.) of 12 independent samples. *P < 0.05 vs. solvent-treated control cultures.

anti-epileptic drug [37], the observations are also of potential practical value. They encourage further studies aimed at elucidating the efficiency of VPA in animal models of pancreatic fibrosis.

The molecular principles of antifibrotic HDACI action are largely unknown. We have previously shown that the transcription factor AP-1 plays an essential role in culture-induced PSC activation and is involved in the transduction of mitogenic PDGF signals into the nucleus [26,27]. The results of this study now implicate AP-1 in the mediation of HDACI effects in PSC, since all three inhibitors reduced its DNA binding (but not DNA binding of NF-κB and Smad proteins) in a dose-dependent manner. Interestingly, reduction of cyclooxygenase-2 expression by TSA in tumour cells has recently been linked to the inhibition of AP-1-mediated gene transcription through a suppression of the expression of c-Jun [38]. How precisely HDACI target AP-1 in stellate cells remains to be elucidated.

In the perpetuation of PSC activation (after an initial profibrogenic stimulus), autocrine loops of secreted mediators are likely to be important. Thus, PSC express TGF-β1, which is also considered the main stimulator of ECM synthesis in the cells [10,11,16]. Another autocrine factor, ET-1, has been implicated in PSC contraction and migration [12,13]. The results of this study indicate that all HDACI suppress ET-1 expression, whereas only VPA (at 10 mM) significantly inhibited expression of TGF-β1 and sodium butyrate at the same concentration displayed a quantitatively similar but statistically not significant inhibitory effect. Although further autocrine mediators remain to be studied and the results are not completely consistent, it is nevertheless tempting to speculate that inhibition of autocrine loops in PSC represents an important principle of HDACI action in PSC. In pilot experiments, we observed that application of ET-1 polypeptide at 100 nM enhanced BrdU uptake of PSC incubated with sodium butyrate at 1 mM by 21% ($n = 6$ separate cultures; S.E.M. 3.5%; $P < 0.05$). However, there was no significant attenuation of VPA and TSA-mediated growth inhibition by ET-1 (data not shown). Therefore, further studies are required to elucidate the role of ET-1 as a target of HDACI action.

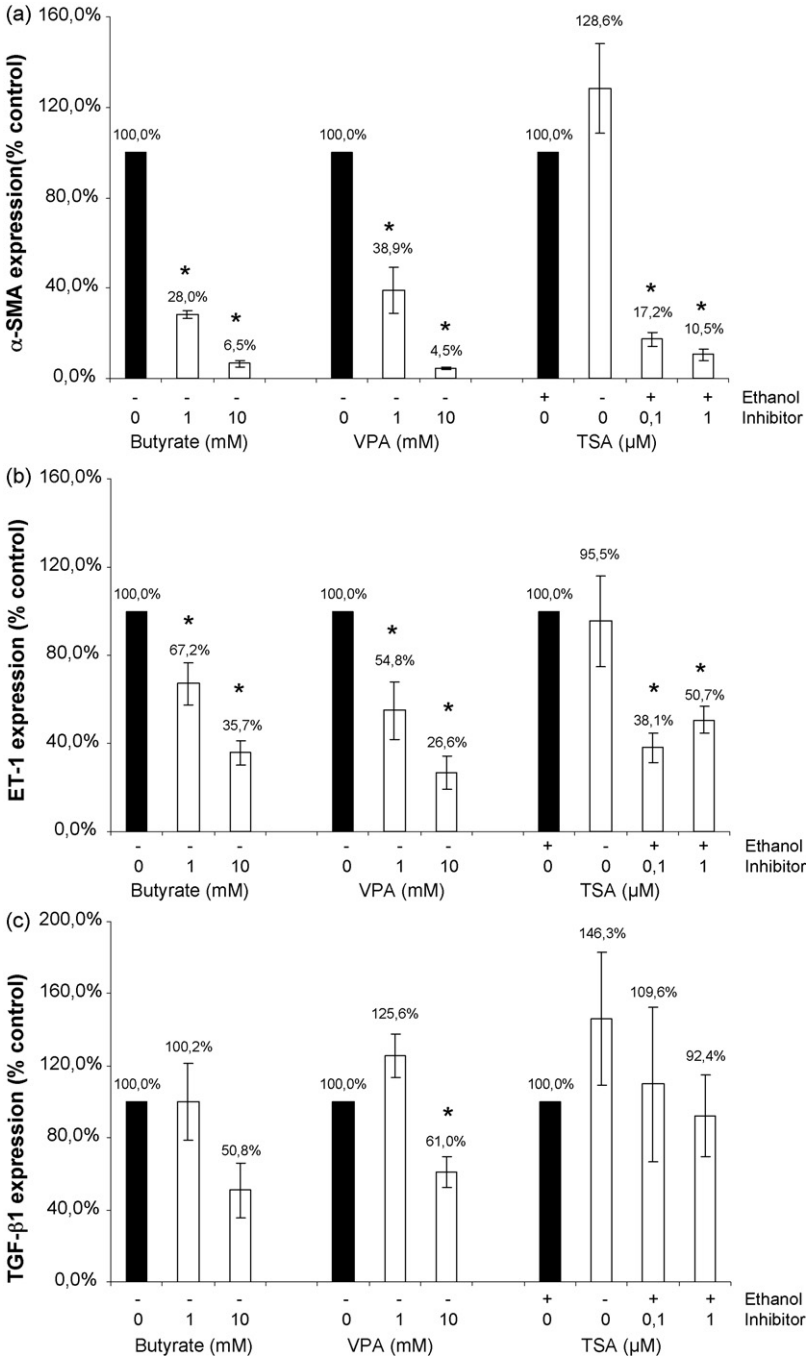

Fig. 6 – Real-time PCR analysis of α-SMA, ET-1 and TGF-β1 gene expression in HDACI-treated PSC. PSC growing in 6-well plates (one passage) were exposed for 24 h to HDACI or their solvents (see legend to Fig. 1) as indicated. The mRNA expression of α-SMA (A), ET-1 (B), TGF-β1 (C) and the house-keeping gene HPRT was analyzed by real-time PCR, and relative amounts of target mRNA were calculated as described in Section 2. One hundred percent mRNA expression of each gene corresponds to cells treated with HDACI solvent only. Data of five independent experiments (with triplicate samples) were used to calculate mean values and S.E.M. *P < 0.05 vs. control cultures (Wilcoxon's rank sum test).

A link between our observations at the levels of transcription factor activation and gene expression is provided by the fact that AP-1 is involved in the regulation of both ET-1 and TGF-β1 gene transcription through the binding to specific promoter elements [29–31]. In this respect, the lack of a significant effect of TSA and sodium butyrate on TGF-β1 expression despite diminished AP-1 activation is somewhat surprising. It is therefore possible that the remaining AP-1 activity was sufficient to maintain TGF-β1 expression in HDACI-treated PSC, and that significant inhibition by VPA reflects an additional specific effect of the drug on an activator of TGF-β1 transcription.

A peculiarity of pancreatic cancer is the extended desmoplastic reaction, which is mainly caused by activated

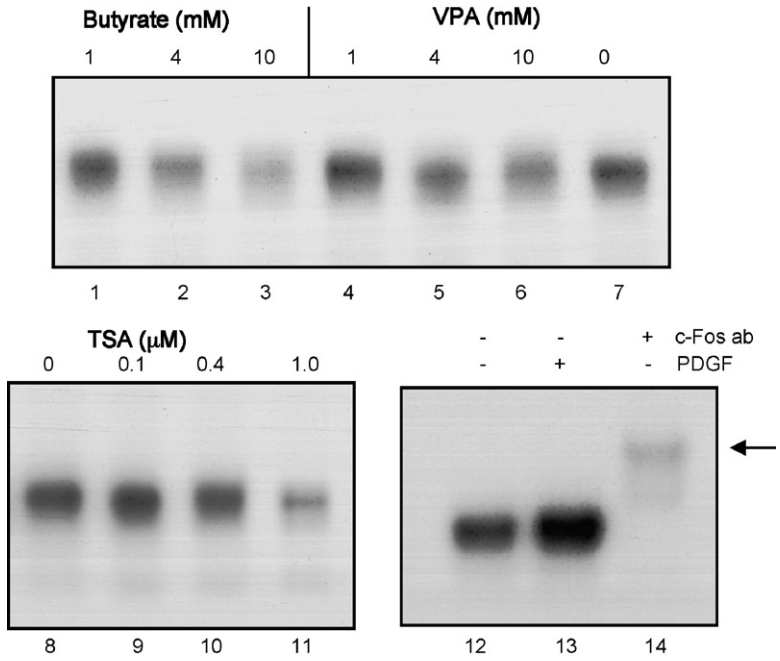

Fig. 7 – Inhibition of AP-1 DNA binding by HDACI. PSC (one passage) were cultured for 24 h with HDACI or their solvents (see legend to Fig. 1) as indicated. Stimulation with PDGF (10 ng/ml; lane 13) was for 60 min. Nuclear extracts were subjected to EMSA analysis using a [^{32}P]-labelled oligonucleotide probe with an AP-1 motif. Supershift analysis was performed by incubating the binding reaction with a c-Fos antibody (ab). The supershifted complex is pointed out by an arrow. Please note that the intensities of bands on different blots cannot be compared because of different times of X-ray exposure. A comparison of untreated PSC with cultures exposed to HDACI solvents (water and ethanol, respectively) revealed no differences in the DNA binding activity of AP-1 (data not shown). Results are representative of three independent experiments.

PSC [39]. Clinical data indicate that presence of a fibrotic focus, suggesting intratumoural fibroblast proliferation, is significantly correlated with shorter survival [40]. Furthermore, experimental studies have provided direct evidence that desmoplasia not only accompanies the tumour disease, but actively stimulates pancreatic cancer progression. Thus, tumour growth in nude mice is significantly accelerated by co-injected PSC [41]. In addition to PSC-derived growth factors and mitogens, ECM proteins are likely to play a key role in this process. Specifically, type I collagen has been shown to induce mitogenesis and to suppress tumour cell apoptosis [42], while laminin and the proteoglycan decorin were found to induce chemoresistance of PC cells [43,44]. PSC not only secrete ECM proteins, but also matrix-degrading enzymes, thereby promoting turnover of ECM. Recently, release of matrix metalloproteinase-2 by activated PSC has been implicated in PC cell invasion and tumour progression [45].

Given the extremely poor prognosis of pancreatic cancer, there is a strong need for novel therapeutic approaches. HDACI, such as TSA, have previously been shown to exert growth-inhibitory and pro-apoptotic effects on pancreatic cancer cells [46,47]. According to the results of this study, they might represent a novel type of drug combining direct antitumour action with inhibitory effects on pancreatic fibrogenesis. Importantly, serum levels of 1 mM valproate, a concentration displaying significant effects on PSC in many of our assays, are in a range that is clinically achievable in cancer patients [48,49]. To further study the efficiency of HDACI (alone and together with classical cytostatic drugs), coculture models of PSC and tumour cells as well as suitable animal models of pancreatic cancer should be employed.

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