Stimulation of tissue-type plasminogen activator gene expression by sodium butyrate and trichostatin A in human endothelial cells involves histone acetylation

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We have previously shown that the pleiotropic agent sodium butyrate strongly stimulates tissue-type plasminogen activator (t-PA) expression in human umbilical vein endothelial cells (HUVEC). Here we provide the following evidence that the butyrate-induced t-PA expression in HUVEC involves histone H4 acetylation. (1) t-PA induction by butyrate occurs at the transcriptional level and does not require new protein synthesis, indicating a direct effect. (2) t-PA induction by butyrate can be fully mimicked by a specific, structurally unrelated, histone deacetylase inhibitor, trichostatin A. (3) At optimally stimulatory

conditions, a combination of butyrate and trichostatin A does not enhance t-PA production more than each of the compounds alone, indicating that both compounds act through a common regulatory mechanism. (4) Induction of t-PA transcription by butyrate and trichostatin A was found to be preceded by histone H4 acetylation; at suboptimal inducing concentrations of butyrate and trichostatin A, the degree of acetylation of histone H4 caused by each agent was similarly reduced. These results are consistent with a role for histone H4 acetylation in t-PA induction by butyrate in HUVEC.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) plays a key role in the onset of the fibrinolytic process by converting the zymogen plasminogen into the active enzyme plasmin. Plasmin can degrade fibrin, the matrix structure of a blood clot. The importance of the role of t-PA *in vivo* has recently been demonstrated once more by Carmeliet et al. [1], who showed that t-PA-deficient mice have a reduced thrombolytic capacity. The activity of t-PA in plasma is regulated by, amongst others, a specific inhibitor, plasminogen activator inhibitor 1 (PAI-1). The vascular endothelium plays an important role in determining plasma t-PA activity by synthesizing both t-PA and PAI-1. Consequently, cultured human endothelial cells are frequently used as a model to gain insight into the regulation of t-PA and PAI-1 synthesis [2].

We have previously shown that sodium butyrate is a strong and rather selective inducer of t-PA production in cultured human umbilical vein endothelial cells (HUVEC), without markedly affecting PAI-1 synthesis [3]. The effects of butyrate with respect to t-PA and PAI-1 synthesis were confirmed at the mRNA level. However, the regulatory mechanism by which butyrate stimulates t-PA expression remained unknown. Although butyrate is a pleiotropic agent, one of the most evident changes brought about by butyrate is the acetylation of histones via inhibition of the enzyme histone deacetylase [4,5]. The introduction of acetyl groups neutralizes positive charges in the histone molecules, which results in diminished interaction of the histones with the DNA. Consequently, there is increased accessibility of transcriptional factors to their DNA recognition motifs, leading to transcriptional activation [6–8]. Besides having a direct effect via histone acetylation, butyrate may also affect t-PA gene expression by the induction of transcriptional factors such as c-jun [9–13]. For example, differentiation of the embryo carcinoma cell line F9 by butyrate or another differentiating agent, retinoic acid, is accompanied by an induction of t-PA gene

expression and preceded by the induction of c-jun. Transfection of F9 cells with a c-jun expression vector was shown to induce t-PA expression, suggesting that c-jun may be involved in the induction of t-PA by butyrate or retinoic acid [11].

In this paper we have sought evidence for a role for histone acetylation in the induction of t-PA gene expression by butyrate in HUVEC by comparing changes in t-PA transcription rate with the degree of histone H4 acetylation. Also, we have compared the effect of butyrate with that of (*R*)-trichostatin A (TSA), a compound structurally unrelated to butyrate, which has been shown to be a potent and specific inhibitor of histone deacetylase at very low concentrations [14]. To examine a possible role for c-jun in butyrate-stimulated t-PA production, we have analysed c-jun mRNA levels in butyrate- and retinoic acid-treated HUVEC.

MATERIALS AND METHODS

Materials

All-*trans*-retinoic acid, n-butyric acid (sodium salt) and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). TSA was generously provided by Dr. M. Yoshida, Department of Agricultural Chemistry, University of Tokyo, Japan. Stock solutions of retinoic acid (10 mM) and TSA (10 mM) were prepared in DMSO. A stock solution of butyrate (0.5 M) was prepared in 0.9% (w/v) NaCl. All stock solutions were stored at –20 °C. The compounds were diluted with incubation medium immediately before use to the concentrations indicated. All experiments involving retinoic acid or TSA were performed in subdued light, and the tubes containing the solutions were covered with aluminium foil. Culture reagents were obtained from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.), and plastics were supplied by Costar (Cambridge, MA, U.S.A.). Enzyme immunoassay kits for determination of human t-PA antigen ('Fibrinostika t-PA') were from Organon Teknika (Boxtel, The Netherlands). Bradford

Abbreviations used: CM, conditioned media; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cells; PAI-1, plasminogen activator inhibitor 1; t-PA, tissue-type plasminogen activator; TSA, (*R*)-trichostatin A; AUT gel electrophoresis, acid/urea/Triton gel electrophoresis.

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protein reagent was from Bio-Rad (Veenendaal, The Netherlands). Deoxycytidine 5-[α - 32 P]triphosphate ($3 \mu\text{Ci}/\mu\text{mol}$), uridine 5-[α - 32 P]triphosphate ($> 400 \mu\text{Ci}/\mu\text{mol}$) and Megaprime kit were obtained from Amersham International. Other materials used in the methods described below have been specified in detail in related references.

Cell culture experiments

Endothelial cells were isolated from human umbilical cord veins by the method of Jaffe et al. [15], and cultured as previously described [16]. Cells were grown on fibronectin-coated dishes in Dulbecco's modified Eagle's medium supplemented with 20 mM Hepes, 10% (v/v) newborn calf serum (heat-inactivated), 10% (v/v) human serum, 5 i.u. of heparin/ml, 150 μg of endothelial cell growth supplement/ml [17], 2 mM glutamine, 100 i.u. of penicillin/ml and 100 μg of streptomycin/ml at 37 °C under a 5% CO_2 atmosphere. The medium was replaced every 2–3 days. Subcultures were obtained by trypsin/EDTA treatment at a split ratio of 1:3. For experiments, confluent cultures were used at second or third passage, and the cells were always re-fed the day before the experiment with incubation medium, i.e. Dulbecco's modified Eagle's medium supplemented with 10% (v/v) human serum, glutamine, penicillin and streptomycin. Conditioned media (CM) were obtained by incubating cells at 37 °C for various lengths of time with incubation medium containing the appropriate concentration of the test compound or stock solvent [DMSO; final concentration maximally 0.1% (v/v)]. CM were centrifuged for 2 min in a Beckman Microfuge to remove cells and cellular debris, and the samples were frozen at –20 °C until use. The cells were washed twice with ice-cold PBS (0.14 M NaCl, 0.01 M Na_2HPO_4 , 0.015 M KH_2PO_4 , pH 7.4), and were used for isolation of RNA or extraction of histones.

Extraction of histones

Histones were extracted from HUVEC according to the procedure described by Cousens et al. [5]. About 8×10^6 cells were incubated for various lengths of time with or without various concentrations of butyrate, TSA or retinoic acid, and nuclei were isolated. All buffers contained 10 mM butyrate to minimize residual histone deacetylase activity. The nuclear pellet was suspended in 0.1 ml of ice-cold water, and concentrated H_2SO_4 was added to a final concentration of 0.2 M. In order to allow precipitation of nuclear membranes, the suspension was kept at 4 °C for at least 1 h. Next, the sample was centrifuged for 5 min at 5000 g , and the supernatant was mixed with 1 ml of acetone. After keeping the preparation overnight at –20 °C, the coagulated material was collected by centrifugation (15 min at 5000 g). This acid-soluble histone fraction was dissolved in 50 μl of water. Protein yields (quantified using Bradford protein reagent) were about 30 μg , 15 μg of which was used for an analysis by acid/urea/Triton (AUT) gel electrophoresis.

AUT gel electrophoresis

Acetylation of histones was analysed by gel electrophoresis using an AUT gel, basically as described by Cousens et al. [5], with some minor modifications as suggested by Hoshikawa et al. [18]. The original procedure was modified by layering an upper gel [1 M acetic acid, 6.3 M urea, 4.4% (w/v) acrylamide] on to the separating gel [1 M acetic acid, 8 M urea, 0.5% (v/v) Triton X-100, 45 mM NH_3 , 16% (w/v) acrylamide]. Before being applied to the gel, the histone preparations were incubated with an equal volume of loading buffer (7.4 M urea, 1.4 M NH_3 , 0.01 M dithiothreitol) for 5 min at room temperature, and were then

mixed with 1/8 vol. of 0.1% (w/v) pyronine G in acetic acid. The histone preparations were electrophoresed in 0.2 M glycine/1 M acetic acid for 11 h at 13 mA. Gels were stained with Coomassie Brilliant Blue and subjected to densitometric analysis. The individual histones were designated by comparing the stained gel with published migration patterns of histones [5]. In two experiments, control and butyrate-treated HUVEC were labelled for 6 h with [^3H]acetate. Histone preparations were obtained as described above and subjected to AUT gel electrophoresis. The gel was first stained and then fluorographed to determine the distribution of [^3H]acetyl groups among the various histones [5]. The identity of the unacetylated H4 species was confirmed: the fastest running H4 species visible on the stained gel was not visible on the fluorogram, since it is not labelled by [^3H]acetate.

Preparation of RNA, Northern blot hybridization and run-on assay

Total RNA was isolated from about 5×10^6 HUVEC by the method of Chomczynski and Sacchi [19]. The cells were lysed directly in the plates. RNA samples were dissolved in water, and RNA concentrations were determined spectrophotometrically. RNA was fractionated by electrophoresis in a 1% (w/v) agarose gel under denaturing conditions using 1 M formaldehyde [20], and blotted to a Hybond-N filter according to the manufacturer's instructions. The filters were hybridized overnight at 63 °C in NaPI hybridization mix [7% (w/v) SDS, 0.5 M NaPI ($\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.2), 1 mM EDTA] containing 3 ng of [α - 32 P]CTP-labelled probe/ml. The probes were labelled with a Megaprime kit, yielding an average activity of 0.2 $\mu\text{Ci}/\text{ng}$. After hybridization, the filters were washed twice with 0.3 M NaCl, 0.03 M trisodium citrate and 1% (w/v) SDS, and twice with 0.15 M NaCl, 0.015 M trisodium citrate and 1% (w/v) SDS for 20 min periods at 63 °C. The filters were then exposed to Kodak Hyperfilm with an intensifying screen at –80 °C. The relative intensity of the bands present on the autoradiogram was read on a scanning densitometer.

Nuclear run-on assays were essentially performed according to Groudine et al. [21], with the modifications suggested by Twisk et al. [22].

cDNA probes

The following cDNA probes were used: a 2.5 kb *Eco*RI fragment of human PAI-1 cDNA [23]; a 1.9 kb *Bgl*II fragment of human t-PA cDNA [24]; a 1.2 kb *Pst*I fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (kindly provided by Dr. R. Offringa, University of Leiden) [25]; a 1.0 kb *Pst*I fragment of mouse c-jun cDNA (kindly provided by Dr. B. Hagmeyer, University of Leiden) [26]; and a 1.5 kb *Eco*RI fragment of mouse c-fos cDNA (kindly provided by Dr. B. Hagmeyer) [27].

RESULTS

Effects of butyrate, TSA, retinoic acid and combinations thereof on t-PA synthesis

Figure 1 shows the effects of increasing concentrations of butyrate (0.1–3.0 mM), TSA (0.1–3.0 μM), retinoic acid (0.1–3.0 μM) and combinations thereof on t-PA production in cultured HUVEC over a 24 h period. In three separate experiments butyrate, TSA and retinoic acid maximally stimulated t-PA synthesis 7.7 ± 0.9 -fold (mean $\pm$ S.D.), 5.0 ± 0.5 -fold and 2.4 ± 0.6 -fold respectively. At higher test concentrations cells became partly detached from the matrix, which was accompanied by a fall in t-PA production. Whereas at suboptimal concentrations a combination of butyrate and TSA enhanced t-PA production more than did each com-

Figure 1 Effects of combinations of butyrate, TSA and retinoic acid on t-PA synthesis in HUVEC

HUVEC were incubated for 24 h with various concentrations of butyrate (0.1–3.0 mM), TSA (0.1–3.0 μ M) and retinoic acid (0.1–3.0 μ M), either alone or in combination. CM were collected and analysed for t-PA antigen as described in the Materials and methods section. Results are expressed as percentages of controls and are means $\pm$ S.D. for three independent experiments, with incubations in duplicate. (a) Combination of butyrate (NaB) and TSA; (b) combination of butyrate (NaB) and retinoic acid (RA).

Figure 2 Time course of the effects of butyrate and TSA on t-PA mRNA levels in HUVEC

HUVEC were incubated for various times up to 24 h with butyrate (3 mM), TSA (1 μ M) or control medium, and RNA was isolated as described in the Materials and methods section. Northern hybridizations were performed using t-PA and GAPDH cDNA probes. Equal amounts of RNA, as checked by the GAPDH signal, were applied in each lane. The blot was exposed for 1 week at -80°C to Hyperfilm using an intensifying screen. A representative experiment (out of four) is shown.

pound alone, the synthesis of t-PA in the presence of both compounds was not any higher than with butyrate alone when butyrate and TSA approached maximally stimulatory concentrations (Figure 1a). These results suggest that butyrate and the specific histone deacetylase inhibitor TSA induce t-PA through a common mechanism. In contrast, retinoic acid more than additively enhanced butyrate-induced t-PA stimulation even at optimally stimulatory concentrations (Figure 1b). Similarly,

retinoic acid had an additive effect on TSA-induced t-PA stimulation (results not shown). These data point to distinct mechanisms for t-PA induction by butyrate and TSA on the one hand and by retinoic acid on the other.

Induction of t-PA mRNA and transcription by butyrate and TSA in HUVEC

Figure 2 shows a representative time course of the stimulatory effects of butyrate and TSA on t-PA mRNA in HUVEC. Induction of t-PA mRNA by butyrate (3 mM) or TSA (1 μ M) became visible after 4 h, and t-PA mRNA levels continued to increase up to 16 h for both compounds. Upon further incubation, t-PA mRNA levels declined markedly in the TSA-treated cells but remained high in the butyrate-treated cells. To examine whether the observed t-PA mRNA induction profiles are dependent on the continuous presence of butyrate or TSA in the medium, HUVEC were treated with butyrate (3 mM) or TSA (1 μ M) for 4 h to induce t-PA mRNA, then washed three times to remove the inducing compounds and subsequently incubated with standard incubation medium. Total RNA was harvested at the indicated times after the wash-out and analysed by Northern blotting. Autoradiograms were scanned and values were calculated relative to the corresponding GAPDH control. As shown in Figure 3 in a representative experiment, removal of butyrate or TSA from the incubation medium after 4 h resulted in a rapid decline in t-PA mRNA levels, pointing to a reversible action of the two compounds (compare the t-PA mRNA induction profiles in Figure 2). The slower decrease in the t-PA mRNA concentration in the butyrate-treated cells as compared with that in TSA-treated cells was consistently found in three separate experiments.

Figure 3 t-PA mRNA levels in butyrate- or TSA-pretreated HUVEC, following wash-out of inducers

HUVEC were incubated for 4 h with butyrate (NaB; 3 mM), TSA (1 μ M) or control medium, then washed three times to remove the inducing compounds, and subsequently incubated with control incubation medium. RNA was harvested at the indicated times after the wash-out, and analysed for t-PA mRNA as described in Figure 2. Autoradiograms were scanned and t-PA mRNA levels were expressed relative to the t-PA mRNA level present in control cells immediately after the wash-out. A representative experiment (out of three) is shown.

To investigate whether the induction of t-PA mRNA is regulated at the level of transcription, nuclear run-on experiments were performed. Under basal, non-stimulated conditions, t-PA transcription in HUVEC was found to be very low, and its signal was close to a prokaryotic control, pUC, which hampered an accurate assessment of the increases in t-PA transcription rate after treatment of the cells with butyrate or TSA. In two separate experiments we measured an increase in t-PA transcription rate as early as 4 h after the addition of butyrate (3 mM) or TSA (1 μ M) to HUVEC (Figure 4). With both compounds a maximal 4-fold elevated transcription rate was observed after 6–8 h of incubation. In the following 16 h, t-PA transcription rates declined to control levels (with butyrate) or even below (with TSA). None of the experimental conditions resulted in a marked change in the PAI-1 transcription rate (Figure 4).

The induction profile of t-PA seen with butyrate at the transcriptional level deviates from that at the mRNA level beyond 8 h: whereas t-PA transcription declines to control levels, t-PA mRNA levels increase further (compare Figures 2 and 4), suggesting a post-transcriptional effect of butyrate, for example an increase in t-PA mRNA stability. An inhibition of t-PA mRNA degradation in butyrate-incubated HUVEC would be in line with the data presented in Figure 3, where the decline in t-PA mRNA was found to be slower in butyrate-pretreated cells than that in TSA-pretreated cells. Experiments aimed at direct measurement of t-PA mRNA stability by adding actinomycin D to the cells proved to be unsuccessful, since actinomycin D was

found to significantly inhibit t-PA mRNA degradation (results not shown).

Effects of butyrate and TSA on histone acetylation

To assess whether the pattern of butyrate- and TSA-induced changes in t-PA transcription rate corresponds to parallel changes in the acetylation of histone proteins, histone fractions were prepared from butyrate-, TSA- and (for comparison) retinoic acid-treated HUVEC, and subjected to AUT gel electrophoresis. AUT gel electrophoresis allows separation of each individual cellular histone (H1, H2A, H2B, H3 and H4) and their variously acetylated species due to slower migration rates of the acetylated forms. Upon incubation of HUVEC with either butyrate (3 mM) or TSA (1 μ M), the predominantly non-acetylated form of histone H4 present in control cells was converted into mono-, di- and tri-acetylated species of H4, whereas no changes in the gel mobility of histones H1, H2A, H2B and H3 were observed, as illustrated in Figure 5(a) for a 6 h incubation period. No effect of retinoic acid (1 μ M) on histone acetylation was seen. Figures 5(b) and 6 show the time course and the dose-dependence of the changes in the acetylation of histone H4 upon incubation of HUVEC with various concentrations of butyrate and TSA. With both butyrate (3 mM) and TSA (1 μ M), the disappearance of the non-acetylated H4 form was detectable as early as 2 h after the onset of the experiment (Figure 5b). However, whereas with butyrate the disappearance of the non-acetylated H4 form continued, to give almost negligible levels at 16 h, with TSA the maximal disappearance of the non-acetylated H4 form was reached at 2 h, with about 40% of H4 remaining in the non-acetylated form (see also Figure 6). Also, with butyrate the amount of non-acetylated H4 remained very low beyond 16 h, whereas with TSA the amount of non-acetylated H4 had returned to control levels at 16 h. The increases in the amounts of mono-, di- and tri-acetylated species of H4, along with the concomitant decreases in the non-acetylated form, in HUVEC exposed to 0.3, 1.0 or 3.0 mM butyrate or 0.1, 0.3 or 1.0 μ M TSA are illustrated in Figure 6 at various time points. The degree of acetylation showed a similar butyrate- and TSA-dose-dependence as the induction of t-PA. In the case of 0.1 μ M TSA, which barely stimulated t-PA synthesis, the H4 acetylation profile did not differ markedly from that of non-treated cells.

Effects of butyrate, TSA and retinoic acid on c-jun and c-fos expression

Although the above results are consistent with a role for histone acetylation in the induction of t-PA gene expression by butyrate, they do not exclude the possibility that this induction occurs via the expression of newly synthesized transcription factors. Indeed, it has been reported that, upon differentiation of the embryo carcinoma cell line F9 by butyrate or retinoic acid, t-PA gene expression is induced, and that newly synthesized c-jun may participate in this activation of t-PA expression [11–13]. To determine whether c-jun and/or c-fos contribute to the induction of t-PA gene expression by butyrate, TSA or retinoic acid in HUVEC, we analysed RNA extracted from HUVEC for the expression of c-jun and c-fos using Northern blot hybridization at different time points after addition of the compounds. Only retinoic acid was found to induce c-jun mRNA, and none of the three compounds induced c-fos expression (results not shown). These results exclude a role for c-jun and c-fos in the induction of t-PA by butyrate or TSA. Also, we found that the induction of t-PA mRNA by butyrate or TSA, in contrast to the induction by retinoic acid, does not require ongoing protein

Figure 4 Effects of butyrate and TSA on t-PA gene transcription in HUVEC

HUVEC were incubated for various times up to 24 h with butyrate (NaB; 3 mM), TSA (1 μ M) or control medium; nuclei were isolated and t-PA and PAI-1 transcription was determined by nuclear run-on assays as described in the Materials and methods section. Blots were exposed for 4 weeks at -80°C to Hyperfilm using an intensifying screen, and autoradiograms were scanned. Results are expressed as percentages of controls and are presented as means $\pm$ range for two independent experiments.

synthesis, as deduced from experiments in the presence of the protein synthesis inhibitor cycloheximide (results not shown). These results point to a direct involvement of histone acetylation in the induced expression of t-PA by butyrate and TSA, rather than an indirect pathway involving the synthesis of new

Figure 6 Time course and dose-dependence of the effects of butyrate and TSA on histone H4 acetylation in HUVEC

HUVEC were incubated for various times up to 16 h with various concentrations of butyrate (NaB), TSA or control medium, as indicated. Histones were extracted from the cells and analysed by AUT gel electrophoresis as described in the Materials and methods section. Scans are shown of the histone H4 regions of stained AUT gels. The numbers beneath the peaks of the gel scans indicate the numbers of acetyl groups present in H4.

transcription factors. The results also confirm the difference in regulatory mechanism between butyrate/TSA and retinoic acid in inducing t-PA expression.

DISCUSSION

In a previous paper we reported that the synthesis of t-PA in HUVEC is greatly increased by the pleiotropic agent butyrate [3]. The following results in the present paper suggest that histone H4 acetylation is involved in the induction of t-PA in HUVEC by butyrate. (1) Butyrate-induced t-PA stimulation is a fast process, being visible at the transcriptional level within 4 h, as shown by a run-on assay (Figure 4). (2) In contrast to F9 cells, in which butyrate and retinoic acid have been reported to induce c-jun, and c-jun expression has been associated with t-PA induction [11–13], no c-jun (or c-fos) induction occurred in

Figure 5 Time course of the effects of butyrate, TSA and retinoic acid on histone acetylation in HUVEC

HUVEC were incubated for various times up to 24 h with butyrate (3 mM), TSA (1 μ M), retinoic acid (1 μ M) or control medium. Histones were extracted from the cells and analysed by AUT gel electrophoresis as described in the Materials and methods section. (a) A stained gel is shown after a 6 h incubation of HUVEC with control medium (C), butyrate (NaB), TSA or retinoic acid (RA). The numbers 0–3 indicate the numbers of acetyl groups present in histone H4. A representative experiment (out of three) is shown. (b) The concentration of non-acetylated histone H4 (H4-0) versus incubation time is shown for control cells, butyrate-treated cells and TSA-treated cells. Concentrations were determined by scanning of stained acid/urea gels, and are expressed relative to the amount of non-acetylated H4 present at the start of the experiment. The data shown are means $\pm$ range for two independent experiments.

HUVEC upon butyrate treatment. Furthermore, t-PA induction by butyrate did not depend on ongoing protein synthesis, indicating a direct effect. (3) The induction of t-PA by butyrate can be mimicked by a specific, structurally unrelated, histone deacetylase inhibitor, TSA, but not by another differentiating agent, retinoic acid (Figures 1 and 4). In accordance with the much lower K_i value of TSA for histone deacetylase [14], much lower concentrations of TSA (optimal concentration 1 μ M) than of butyrate (optimal concentration 3 mM) were required for maximal induction of t-PA expression. (4) Simultaneous addition of both butyrate and TSA gave no additive effect on t-PA stimulation, indicative of a common regulatory mechanism (Figure 1). (5) Both butyrate and TSA induced a time- and dose-dependent increase in the amounts of mono-, di- and tri-acetylated species of histone H4, along with a concomitant decrease in the non-acetylated form; the onset of H4 acetylation preceded the increase in t-PA transcription (Figures 4 and 5), and the changes in the degree of H4 acetylation at differing concentrations of butyrate and TSA paralleled changes in t-PA expression (Figures 1 and 6).

Although the results obtained with the pleiotropic agent, butyrate, resembled largely those obtained with the more potent and specific histone deacetylase inhibitor, TSA, they differed in their effects on t-PA mRNA stability. After 16–24 h of butyrate treatment we found markedly elevated t-PA mRNA levels, despite the fact that t-PA transcription had already returned to basal levels. Similar effects of butyrate, namely modulation of both transcription and mRNA stability, have been reported for c-fos mRNA in F98 rat glioma cells and Caco-2 colon adenocarcinoma cells [28,29]. The mechanism involved remains elusive.

Various lines of experimental evidence have shown a correlation between transcriptional activity and increased histone acetylation, including histone H4 acetylation. A direct link between histone acetylation and active genes was established in the studies of Crane-Robinson and colleagues in which they used an antibody against the epitope *N*- ϵ -acetyl-lysine for the isolation of acetylated nucleosomes from embryonic chicken chromatin [30,31]. On probing with gene sequences, antibody-bound chromatin was found to be enriched in the actively transcribed α -globin gene and not enriched in the inactive ovalbumin gene. The importance of histone H4 acetylation in the control of transcription can be inferred from genetic experiments with *Saccharomyces cerevisiae* in which deletion of the histone H4 N-terminal region or substitution of the specific modifiable lysine residues resulted in a decrease in the activation of the GAL1 and PHO5 promoters [32]. Similarly, the human female inactive X chromosome did not stain with antibodies to acetylated H4, in contrast to all the other chromosomes [33]. Histone acetylation is believed to have a positive role in facilitating transcription factor access to promoter sequences, thus facilitating transcription. For example, *in vitro* reconstitution experiments by Lee et al. [8] showed that the presence of acetylated H4 facilitates the binding of the transcription factor TFIID to its target sequences without nucleosome displacement.

One might expect that such global modifications of chromatin structure induced by butyrate or TSA would lead to a general increase in the expression of many genes that were formerly silent. However, when SDS/PAGE was used to analyse the proteins synthesized after a 24 h incubation with 5 mM butyrate, very few differences were found from the normal proteins made [3]. Also, Northern blot analysis showed no significant increases in mRNA levels of several genes tested, including urokinase-type plasminogen activator, PAI-1 and GAPDH (J. Arts and T. Kooistra, unpublished work). Similar results have been reported

by others for different cell types [5]. Apparently, high levels of histone acetylation are not sufficient for active transcription, but relate rather to the transcriptional competence of the gene [31]. In this respect it should be noted that t-PA transcription in the presence of butyrate had returned to control levels even when histone H4 was still in a highly acetylated form (Figures 4 and 5). It remains to be determined which factors other than histone H4 acetylation itself are responsible for the selective switching of the t-PA gene in HUVEC in the presence of butyrate or TSA.

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