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Posttranscriptional regulation of expression of plasminogen activator inhibitor type-1 by cAMP in HepG2 liver cells

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Altered expression of plasminogen activator inhibitor type-1 (PAI-1), a physiologic fibrinolysis inhibitor, is implicated in atherosclerosis. Cyclic adenosine monophosphate (cAMP) alters PAI-1 expression in several cells. Nevertheless, posttranscriptional regulation of PAI-1 has not been elucidated. To determine whether cAMP affects PAI-1 expression at posttranscriptional level, we determined promoter activity, mRNA levels, 3′-untranslated region (UTR) activity and protein levels of PAI-1 using HepG2 cells. cAMP decreased PAI-1 promoter activity at 24 h and mRNA expression at 4 h while it increased mRNA expression and accumulation of PAI-1 protein into media at 24 h. Human PAI-1 mRNA exists in two subspecies (3.2 and 2.2 kb), and cAMP increased baseline luciferase activity of 3′-UTR of the 3.2 kb PAI-1 mRNA [3′-UTR (+1358–3176)] and 1 kb fragment of 3′-terminus of 3′-UTR of 3.2 kb mRNA [3′-UTR (+2177–3176)]. cAMP increased PAI-1 protein expression despite decrease in promoter activity, presumably by regulating PAI-1 expression at the posttranscriptional level and thereby affecting mRNA stability. The 53-nt fragment in 3′-UTR (+2591 to +2643 nt) was involved in posttranscriptional regulation by cAMP. Thus, cAMP can stabilize 3.2 kb PAI-1 mRNA mediated by specific effects on 3′-UTR, and these effects are associated with increased expression of PAI-1 protein.

Keywords: 3′-untranslated region/cAMP/mRNA stability/PAI-1/posttranscriptional regulation.

Abbreviations: ARE, AU-rich element; cAMP, cyclic adenosine monophosphate; ORF, open reading frame; PAI-1, plasminogen activator inhibitor type-1; RBP, RNA binding protein; UTR, untranslated region.

Plasminogen activator inhibitor type-1 (PAI-1) is a major physiologic inhibitor of fibrinolysis in blood (*1*). Increased expression of PAI-1 has been implicated as a risk factor for myocardial infarction (*2*). Putative mechanisms include shifting the balance between thrombosis and fibrinolysis in blood towards thrombosis, thereby favouring evolution of thrombosis as well as acting in vessel walls and accelerating evolution of atherosclerotic plaques vulnerable to rupture by inhibiting migration of vascular smooth muscle cells in response to atherogenic stimuli such as oxidized LDL (*3*). Augmentation of expression of PAI-1 is a result of several mechanisms already elucidated. One is oxidative stress that can increase production of PAI-1 protein (*4*). Simvastatin, an agent with anti-oxidative properties (*5*), can attenuate expression of PAI-1 protein (*6*). The 3′-untranslated regions (UTRs) of mRNA affects stability of the mRNA and the efficiency of its translation. Pharmacologic strategies targeting modulation of expression of PAI-1 mediated by the 3′-UTR may be effective in attenuating otherwise increased expression of PAI-1.

Cyclic adenosine monophosphate (cAMP) is an intracellular messenger regulating various cell functions and gene expression. Cyclic AMP regulates PAI-1 expression in many cells. In human mast cells, cAMP increases PAI-1 mRNA and protein by increasing PAI-1 promoter activity (*7*). In keratinocytes, cAMP suppresses PAI-1 expression induced by TGF-β through protein kinase A (*8*). An anti-platelet agent, cilostazol, elevates blood cAMP levels by inhibiting phosphodiesterase and decreases PAI-1 in blood vessels (*9–11*). In liver, the effects depend on the growth state of cells. For example, cAMP increases PAI-1 mRNA levels in freshly isolated rat hepatocytes (*12*) but decreases PAI-1 mRNA levels in proliferating rat hepatocytes or hepatoma cells (*13*, *14*). Cyclic AMP also regulates some mRNAs at posttranscriptional levels and rat PAI-1 mRNA 3′-UTR contains a cAMP responsive element (*15*). We performed the present study to characterize the effects of cAMP on expression of PAI-1 in HepG2 cells and to elucidate the underlying molecular mechanisms. The results showed that posttranscriptional modifications appear to be critical in the cAMP-mediated effects on the expression of PAI-1.

Materials and Methods

Cell culture and reagents

Human hepatocarcinoma HepG2 cells (*16*) were grown in Dulbecco's modified Eagle's medium (Wako Pure Chemical, Osaka, Japan) containing 4.5 mg/ml glucose and supplemented with 10% foetal bovine serum (Life Technologies) at 37°C in 5% CO_2. Cells were grown to 80% confluence, and incubated in serum-free medium for 16 h. 8-bromo cAMP (Nacalai Tesque, Kyoto, Japan) was added to the medium to assess its effects on expression

of PAI-1 mRNA and PAI-1 protein. Actinomycin D (Wako Pure Chemical) was used as a transcriptional inhibitor.

Plasmid constructs

To construct the pGEM-PAI-1 plasmid, the PAI-1 coding region was amplified using total cDNA prepared from HepG2 cells and primers 5′-GCGAATTCCACCATGCAGATGTCTCCAGCCCTC AC-3′ and 5′-GGCCCGGGTTCCATCACTTGGCC-3′. The amplified fragment was then cloned into pGEM-T Easy (Promega, Madison, WI, USA) to generate pGEM-PAI-1.

For characterization of effects of the PAI-1 3′-UTR, full-length 3′-UTR fragments (+1358 to +3176 nt) [3′-UTR (+1358–3176)], 0.8 kb region of 3′-UTR fragment (+1358 to +2176 nt) [3′-UTR (+1358–2176)], and the 1 kb region of 3′-UTR fragment (+2177 to +3176 nt) [3′-UTR (+2177–3176)] were converted to cDNA with oligo dT primer and PrimeScript RT-PCR kit (TaKaRa Bio, Shiga, Japan) from HepG2 cell total RNA isolated using TriPure Isolation Reagent (Roche Diagnostics, Basel, Swizerland). PCR was performed using PrimeSTAR Max DNA Polymerase (TaKaRa Bio) and primers containing XbaI recognition sequences. Amplified materials were treated with XbaI and ligated to the restriction enzyme site of XbaI-treated (+1934 to +1939 nt) pGL3-control vector (Promega) with In-Fusion Advantage PCR cloning Kits (TaKaRa Bio). The following primers were used; for 3′-UTR (+2177–3176), forward (5′-GCCGTGTAATTCTAGAGAAAGAGAACTACTAA GG-3′) and reverse (5′-CCGCCCCGACTCTAGGTCCTGACATA TTCTTCGTATTTAT-3′); for 3′-UTR (+1358–2176), forward (5′-GCCGTGTAATTCTAGCCCTGGGGAAAGACGCCTTCA TC-3′) and reverse (5′-CCGCCCCGACTCTAGCTCTAACAAGC ACTCAAGGGGAAGG-3′); and for 3′-UTR (+1358–3176), forward (5′-GCCGTGTAATTCTAGCCCTGGGGAAAGACGCCT TCATC-3′) and reverse (5′-CCGCCCCGACTCTAGGTCCTGAC ATATTCTTCGTATTTAT-3′).

3′-UTR fragments with deletions, 3′-UTR (+2177–2486) and 3′-UTR (+2177–2806), were prepared in a similar manner. The following primers were used; for 3′-UTR (+2177–2486) forward (5′-G CCGTGTAATTCTAGAGAAAGAGAACTACTAAGG-3′) and reverse (5′-CCGCCCCGACTCTAGAAATCTATTGAACACATT GGAAACC-3′); for 3′-UTR (+2177–2806) forward (5′-GCCGTGT AATTCTAGAGAAAGAGAACTACTAAGG-3′) and reverse (5′-CCG CCCCGACTCTAGGACGGGGGTCTTGGTATGTTG-3′).

3′-UTR (+2177–3176) fragments containing mutated AU-rich element (ARE) m2, AREm3 and AREm4 were constructed by site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) from pGL3-control vector containing 3′-UTR (+2177–3176). The following primers were used; for AREm2, forward (5′-TACTAAGGAAAAT AATATTAGGGAAACTCGCTCCTAGTGTTTC-3′) and reverse (5′-GAAACACTAGGAGCGAGTTTCCCTAATATTATTTTCC TTAGTA-3′); for AREm3, forward (5′-TCCAATGTGTTCAATA GAGGGAGGAGCAGAAATGCAAGG-3′) and reverse (5′-CCTT GCATTTCTGCTCCTCCCTCTATTGAACACATTGGA-3′); for AREm4, forward (5′-TATATATTTTAAAGACCAAGGGATGG GAGAATTGCACACAG-3′) and reverse (5′-CTGTGTGCAATT CTCCCATCCCTTGGTCTTTAAAATATATA-3′).

Quantitative real-time RT-PCR

Total RNA was converted to cDNA using PrimeScript RT reagent kits for real time (TaKaRa Bio). Quantitative real-time PCR was performed using the SYBR Premix Ex Taq (TaKaRa Bio) on a Thermal Cycler Dice Real Time System (TaKaRa Bio). After the reaction had proceeded and the amplification and melting curves had been confirmed, mRNA was quantified with the ΔΔCt method as described previously (*6*). The following primers were used; for human PAI-1, forward (5′-TGATGGCTCAGACCAACAAG-3′) and reverse (5′-CAGCAATGAACATGCTGAGG-3′); for human 3.2 kb PAI-1, forward (5′-AATCAGCCCACCATGTTCTC-3′), and reverse (5′-CACCGTCCAGTGCAAAATC-3′). For human β-actin primer set (HA067803) were purchased from TaKaRa Bio and used as a control for normalization.

Northern blotting

Northern blotting was performed as we described previously (*17*), with minor modifications. Specifically, total RNA blot containing 12.5 μg RNA from HepG2 cells was performed on Hybond XL membrane (GE Healthcare). The PAI-1 probe was prepared using a 0.6 kb DNA fragment corresponding to the middle region of the PAI-1 ORF, which had been amplified from pGEM-PAI-1 plasmid using primers 5′-GTGAGGGTGTTTCAGCAGGT-3′ and 5′-GGG CGTGGTGAACTCAGTAT-3′. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was prepared using a 0.45 kb DNA fragment corresponding to the C-terminus region of the GAPDH ORF, which had been amplified using total cDNA prepared from HepG2 cells and primers 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TC CACCACCCTGTTGCTGTA-3′. The amplified fragments were then labelled with [α-^{32}P]dCTP (3000 Ci/mmol; MP Biomedicals) using a *Bca*BEST^{TM} Labelling Kit (TaKaRa Bio). After hybridization with ExpressHyb^{TM} Hybridization Solution (TaKaRa Bio) for 2 h at 68°C, the membrane was analysed using a Bio-Imaging Analyzer, BAS-1800II (Fujifilm, Tokyo, Japan).

Immunoblotting

Cells were cultured for 24 h, and conditioned media were collected. After quantification of concentrations of protein with BCA protein assay kits (Thermo Fisher Scientific, Waltham, MA, USA), samples were diluted with 4×SDS sample buffer [250 mM Tris–HCl (pH 6.8), 8% SDS, 40% glycerol, 10% 2-mercaptoethanol and a trace amount of bromophenol blue]. Proteins (20 μg) were subjected to SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was incubated with a specific primary antibody (1 μg/ml; rabbit anti-PAI-1 IgG, from Merck) for 1 h and then with a secondary antibody (40 ng/ml; peroxidase-conjugated goat anti-rabbit IgG, from Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h. Labelling was detected with the ECL Plus Western Blotting Detection Reagents (GE Healthcare) and was quantified using a lumino-image analyser, LAS-3000 mini (Fujifilm).

Transfection and luciferase assays

For measuring activities of the promoter of PAI-1 gene and the 3′-UTR of PAI-1 mRNA, DNA transfection and luciferase assays were performed. The plasmid pGL3-basic vector (Promega) containing human PAI-1 promoter region (−825 to +42 nt) was kindly supplied by Dr. S. Imagawa (Hokkaido University, Sapporo, Japan) (*17*). Cells were transfected with 1 μg of vectors containing the firefly luciferase gene-fusioned PAI-1 promoter or 3′-UTR sequence and 5 ng of pRL-TK or 50 ng of phRL-null vector (Promega), a *Renilla* luciferase reporter plasmid to control for the efficiency of transfection, for 5 h using the Lipofectamine LTX reagent (Life Technologies). The cells were serum-starved for 18 h and then stimulated with 8-bromo cAMP in serum-free DMEM for 24 h and harvested. Luciferase activity was detected in cell extracts with the passive lysis buffer and the use of the Dual-Luciferase Reporter Assay Systems (Promega) and a luminometer (GloMax 20/20 n, Promega). Normalized luciferase activity was calculated as the ratio of firefly luciferase activity to control *Renilla* luciferase activity. Results for each reporter construct were expressed in terms of the fold induction compared with results in transfected, unstimulated cells.

Statistical analysis

Data are expressed as means ± standard deviations. After verification that the data were normally distributed differences were assessed using Welch's *t*-tests. Significance was defined as a $P < 0.05$.

Results

Cyclic AMP decreases the activity of the PAI-1 promoter at 24 h and expression of PAI-1 mRNA at 4 h while it increases the expression of PAI-1 mRNA and PAI-1 protein at 24 h

We first examined the effects of cAMP on whole processes of PAI-1 expression. Treatment of cells with cAMP (0.1, 1 mM) decreased the activity of the PAI-1 promoter (−825 to +42 nt) at 24 h (Fig. 1A). Cyclic AMP inhibited the expression of PAI-1 mRNA in a dose-dependent manner at 4 h (Fig. 1B),

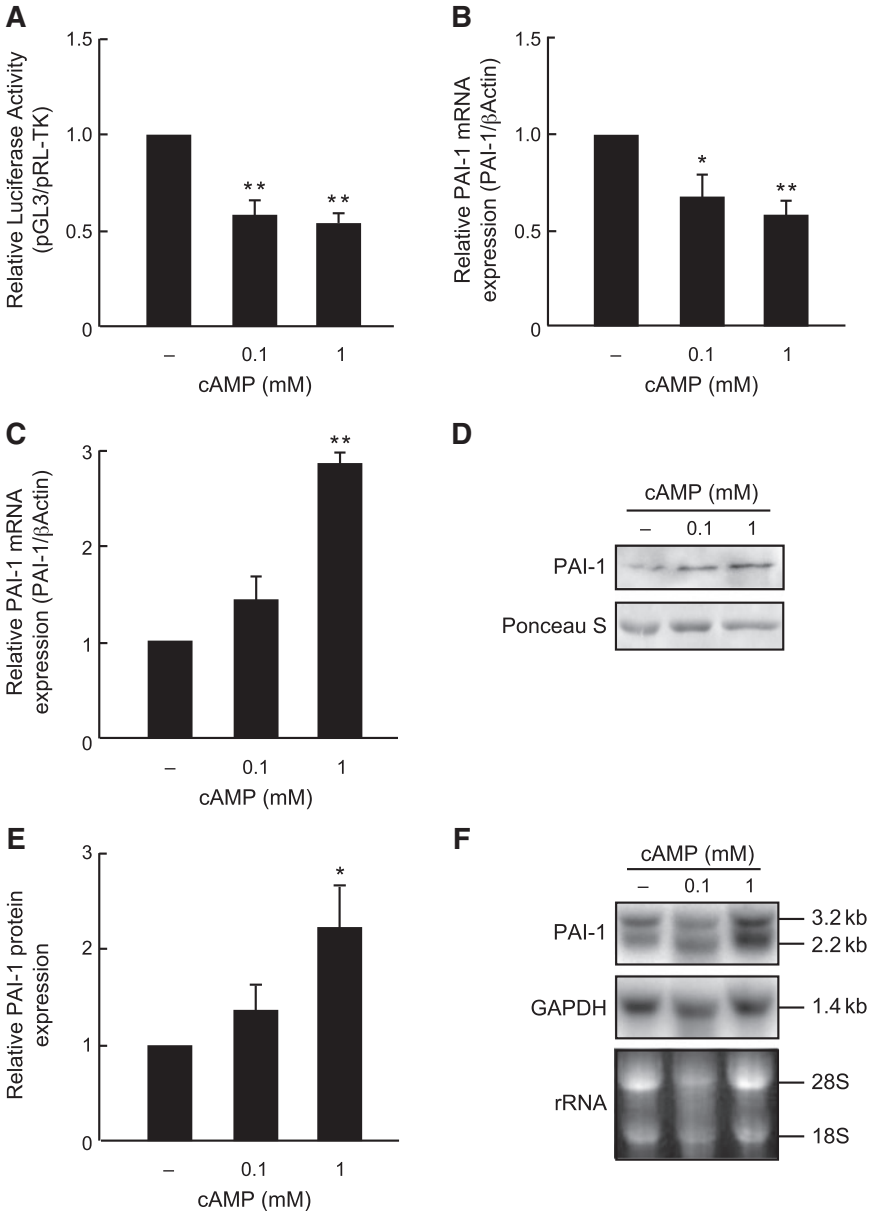

Fig. 1 cAMP decreases the activity of the PAI-1 promoter at 24 h and expression of PAI-1 mRNA at 4 h yet increases the expression of PAI-1 mRNA and PAI-1 protein at 24 h. (A) HepG2 cells at 50% confluency were pre-incubated for 24 h. Transient transfection was performed with the lipofection method with 1 μg of pGL3-basic vector containing PAI-1 gene promoter area (–825 to +42 nt) and 5 ng of pRL-TK vector. After transfection, the cells were stimulated with 8-bromo cAMP (0.1–1 mM). Luciferase assays were performed after 24 h. (B and C) HepG2 cells were stimulated with 8-bromo cAMP for 4 h (B) and for 24 h (C). Total RNA (10 ng) was subjected to quantitative real-time PCR. (D and E) HepG2 cells were stimulated with 8-bromo cAMP for 24 hours. Conditioned media were collected, and PAI-1 protein was subjected to western blot analysis (D). The upper panel shows the representative western blot analysis, and the lower panel shows Ponceau S staining as a loading control (D). Protein bands corresponding to PAI-1 were quantitated using a lumino-image analyser, LAS-3000 mini (E). All values are means ± SD of the fold increase over control without 8-bromo cAMP ($n = 3$, $^{*}P < 0.05$ compared with control, $^{**}P < 0.01$ compared with control). (F) Effects of cAMP on PAI-1 mRNA. HepG2 cells were stimulated with 8-bromo cAMP for 24 h and then total RNA was isolated. ^{32}P-labelled PAI-1 probe (upper panel) was hybridized to total RNA from HepG2 cells immobilized on a nylon membrane. The PAI-1 and GAPDH control (middle panel) were detected using the same membrane. The lower panel shows 18S and 28S rRNA stained with ethidium bromide as a loading control. A representative blot from three separate experiments is shown.

but it increased the expression of PAI-1 mRNA at 24 h (Fig. 1C). Despite the disparate effects of cAMP on expression of PAI-1 mRNA, cAMP increased the accumulation of PAI-1 protein in the conditioned media of the HepG2 cells at 24 h (Fig. 1D and E). The effect of cyclic AMP on the expression of PAI-1 mRNA was confirmed by northern blot (Fig. 1F).

Cyclic AMP increases the activity of the 3.2 kb full length PAI-1 mRNA 3′-UTR (+1358–3176) and the 1 kb fragment of 3′ terminus of PAI-1 mRNA 3′-UTR (+2177–3176), while decreasing the activity of the 2.2 kb full length PAI-1 mRNA 3′-UTR (+1358–2176)

To examine whether cAMP regulates PAI-1 at post-transctiptional level, the effects of cAMP on PAI-1

3′-UTR were evaluated. The two species of PAI-1 mRNA have different 3′-UTR (Fig. 2A). These 3′-UTRs were found to contain some AREs. The 3.2 kb full length PAI-1 mRNA 3′-UTR (+1358–3176), the 2.2 kb full length PAI-1 mRNA 3′-UTR (+1358–2176) and the difference of two PAI-1 mRNA 3′-UTR (+2177–3176) were cloned into pGL3-control vector (Fig. 2B). To determine the location of the 3′-UTR responsible for the cAMP, the effects of cAMP on the full length of 2.2 and 3.2 kb PAI-1 mRNA 3′-UTR were evaluated. Induction of activity from the full length 3.2 kb PAI-1 mRNA 3′-UTR (+1358–3176) was observed by cAMP (1 mM) (Fig. 2C), while decreasing the luciferase activity of the full length 2.2 kb PAI-1 mRNA 3′-UTR (+1358–2176) (Fig. 2D). Additionally, cAMP (1 mM) increased the luciferase activity of 3′-UTR (+2177–3176) (Fig. 2E). These results showed that the distinct region responsive to cAMP resides within the 3′-UTR (+2177–3176) unique to the 3.2 kb form. Accordingly, at least one cAMP responsive region is operating within the 3′-UTR.

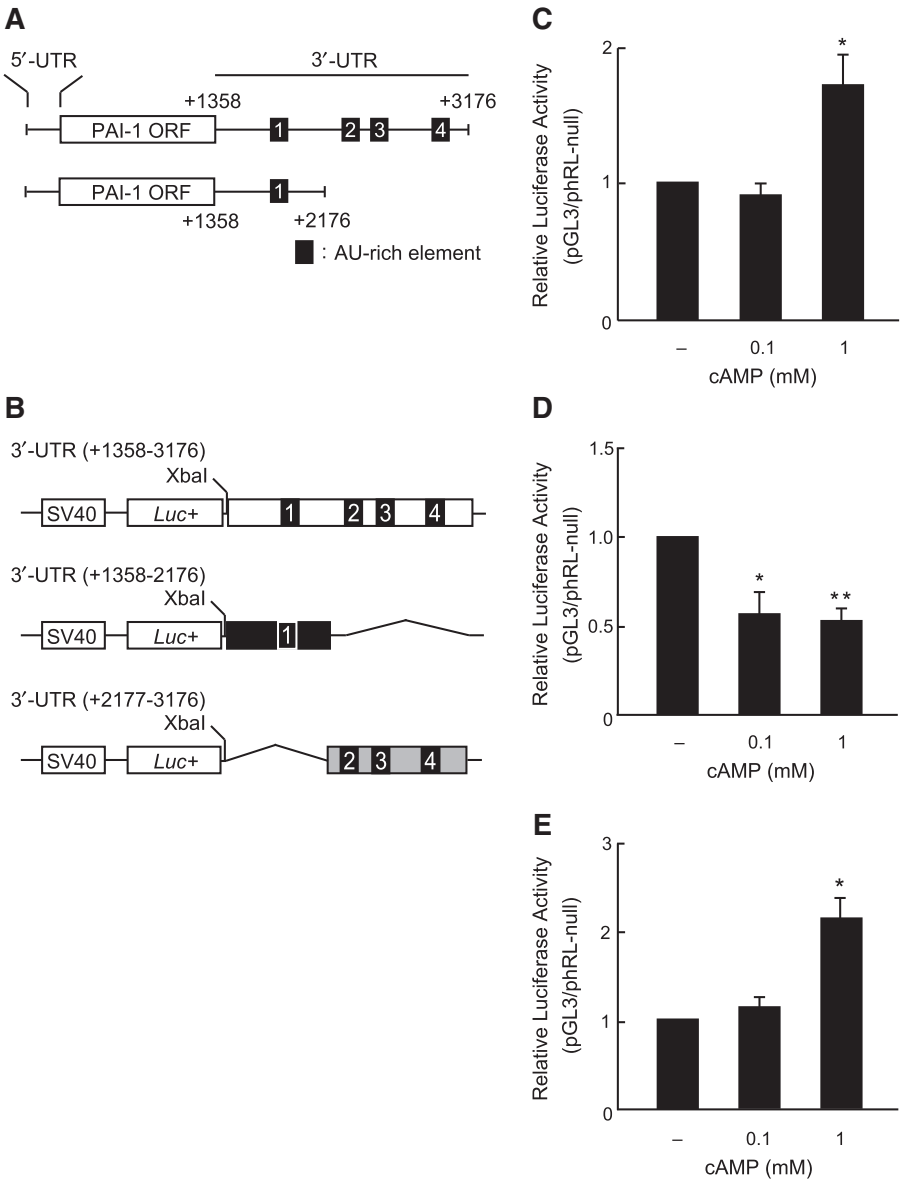

Fig. 2 cAMP increases the activity of 3′-UTR (+2177–3176). (A) Two species of PAI-1 mRNA are shown. ARE is a sequence of AUUUA. These AREs represent ARE1 (+2141–2145), ARE2 (+2207–2211), ARE3 (+2483–2487) and ARE4 (+2889–2893). ORF: open reading frame. (B) PAI-1 3′-UTR cDNA was cloned into XbaI site located downstream of the luciferase gene of pGL3-control vector. Open box indicates the 3.2 kb full length PAI-1 mRNA 3′-UTR (+1358–3176); closed box indicates the 2.2 kb full length PAI-1 mRNA 3′-UTR (+1358–2176); and shaded box indicates the PAI-1 mRNA 3′-UTR (+2177–3176). (C–E) HepG2 cells at 50% confluency were co-transfected with 1 μg of the firefly luciferase reporter constructs containing 3′-UTR (C: +1358–3176; D: +1358–2176; E: +2177–3176) and 50 ng of the phRL-null *Renilla* luciferase reporter vector. After transfection, the cells were stimulated with 8-bromo cAMP (1 mM). Luciferase assay were performed after 24 h. The hPAI-1 3′-UTR-directed firefly luciferase activities were normalized to *Renilla* luciferase activities to compensate for differences in the efficiencies of transfection. Values are means ±SD of the fold increase over control without 8-bromo cAMP and with empty pGL3-control vectors ($n = 3$, $^{*}P < 0.05$ compared with control, $^{**}P < 0.01$ compared with control).

Cyclic AMP stabilizes 3.2 kb PAI-1 mRNA

As cAMP affected PAI-1 mRNA 3′-UTR, we next examined the effect of cAMP on the stability of PAI-1 mRNA. We inhibited new transcription using actinomycin D (5 μg/ml) and then treated the cells with cAMP. The half-life of total PAI-1 mRNA was extended from 2.8 to 3.8 h by cAMP (Fig. 3A). The half-life of 3.2 kb PAI-1 mRNA was also extended from 1.9 to 3.3 h (Fig. 3B). This suggests that cAMP stabilizes 3.2 kb PAI-1 mRNA. The effect of cyclic AMP on the stabilization of PAI-1 mRNA was confirmed by northern blot and cyclic AMP preferentially stabilized 3.2 kb PAI-1 mRNA (Fig. 3C).

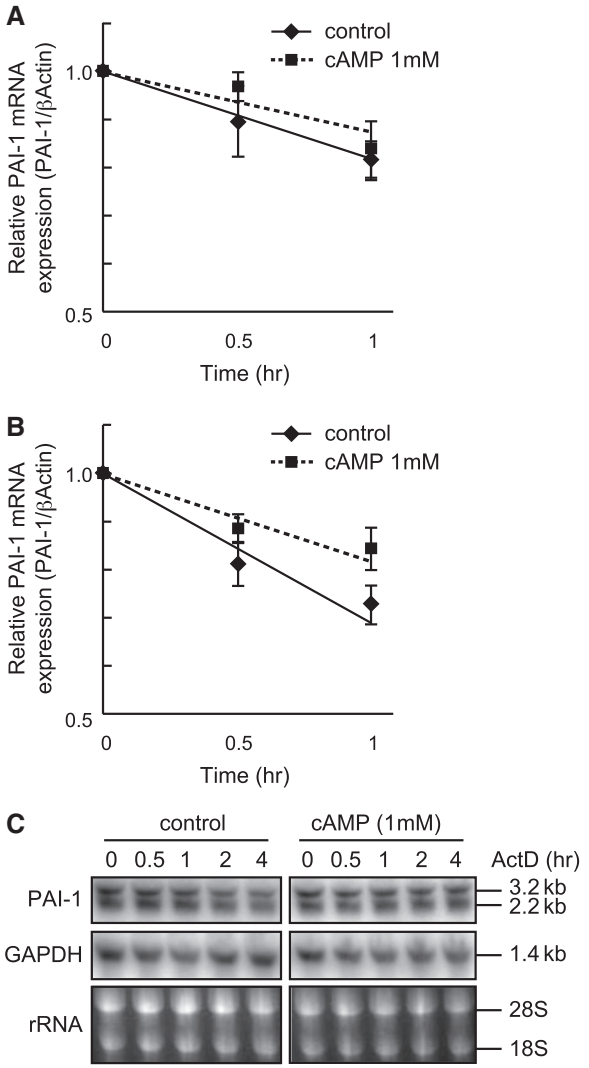

Fig. 3 Cyclic AMP stabilizes 3.2 kb PAI-1 mRNA. HepG2 cells were pre-treated with actinomycin D (5 μg/ml) for 30 min, and then the cells were additionally treated for the indicated times with or without 8-bromo cAMP (1 mM). Total RNA (10 ng) was subjected to quantitative real-time PCR. Data represent total (A) and 3.2 kb (B) PAI-1 mRNA. Solid line with the square and dashed line with the diamonds represent without or with 8-bromo cAMP, respectively. Values are means ± SD of the fold increase over control (0 h) ($n = 3$). (C) Effects of cAMP on PAI-1 mRNA. HepG2 cells were stimulated with 8-bromo cAMP as in (A) and total RNA was isolated. ^{32}P-labelled PAI-1 probe (upper panel) was hybridized to total RNA from HepG2 cells immobilized on a nylon membrane. The PAI-1 and GAPDH control (middle panel) were detected using the same membrane. The lower panel shows 18S and 28S rRNA stained with ethidium bromide as a loading control. A representative blot from three separate experiments is shown.

The increased activity of 3′-UTR (+2177–3176) induced by cAMP was mediated through the region residing in +2591 to +2643 nt of 3′-UTR but not AREs

To determine whether cAMP affects 3′-UTR (+2177–3176) through AREs, to which RNA binding proteins (RBPs) bind, we performed the following studies. Mutation of ARE2, ARE3 and ARE4 did not inhibit the increased activity of 3′-UTR (+2177–3176) induced by cAMP (1 mM). Multiple mutations of ARE2, ARE3 and ARE4 did not inhibit the increased activity of 3′-UTR (+2177–3176) induced by cAMP, either (Fig. 4A). To define the precise region residing in 3′-UTR responsible for cAMP effect, deletion of 3′-UTR (+2177–3176) was performed (Fig. 4B). As a result, cAMP (1 mM) increased the activity of 3′-UTR (+2177–2806) and 3′-UTR (+2177–2643). In contrast, cAMP did not affect the activity of 3′-UTR (+2177–2590), 3′-UTR (+2177–2538) and 3′-UTR (+2177–2486) (Fig. 4C). This suggests that the increased activity of 3′-UTR (+2177–3176) induced by cAMP was mediated through the region residing in +2591 to +2643 nt of 3′-UTR but not AREs. As we found that ARE binding proteins are not involved in the effects of cAMP, we used a computational RNA motif prediction analysis of 3′-UTR (+2177–3176) using a newly developed RNApromo indicating regions that may regulate mRNA decay in 3′-UTR (+2177–3176) (*18*). The program predicted secondary structure of +2589 to +2631 nt of 3′-UTR with motif score 55.5 (Fig. 4D).

Discussion

Human PAI-1 mRNA exists in two subspecies (3.2 and 2.2 kb). The difference of 1 kb is attributable to polyadenylation at the 3′-UTR. PAI-1 genomic DNA exon 9 has two splicing variations (*19*). The two transcripts encode an identical PAI-1 protein, but the stability is not equal. The half-life of 2.2 kb PAI-1 mRNA is reported to be 168 min while that of 3.2 kb PAI-1 mRNA is reported to be 56 min (*20*). Insulin-like growth factor type 1 stabilizes both 3.2 and 2.2 kb mRNA while insulin and TGF-β stabilizes only the 3.2 kb mRNA (*20*, *21*). Many processes affecting posttranscriptional regulatory mechanisms occur within elements present in the 3′-UTR of diverse mRNAs (*22*). RBPs are involved in these processes and act on mRNA at the posttranscriptional level by binding to mRNA thereby affecting its stability (*23*). AREs are known as AUUU A or UUAUUUAUU motifs and are targets of RBP binding (*24*). The 3′-UTR of the human 3.2 kb PAI-1 mRNA contains four AREs. 2.2 kb PAI-1 mRNA 3′-UTR contains 1 ARE and 1 kb fragment contains three AREs. Some RBPs are known to bind PAI-1 AREs. For example, human antigen R binds to ARE of rat PAI-1 3′-UTR (*25*) and p53 binds to ARE of human PAI-1 3′-UTR (*26*). Both of them enhance the stability of PAI-1 mRNA. 6-phospho-D-gluconate-NADP oxidoreductase binds to non-ARE 33-nt fragment in the human PAI-1 3′-UTR (*27*). In rat

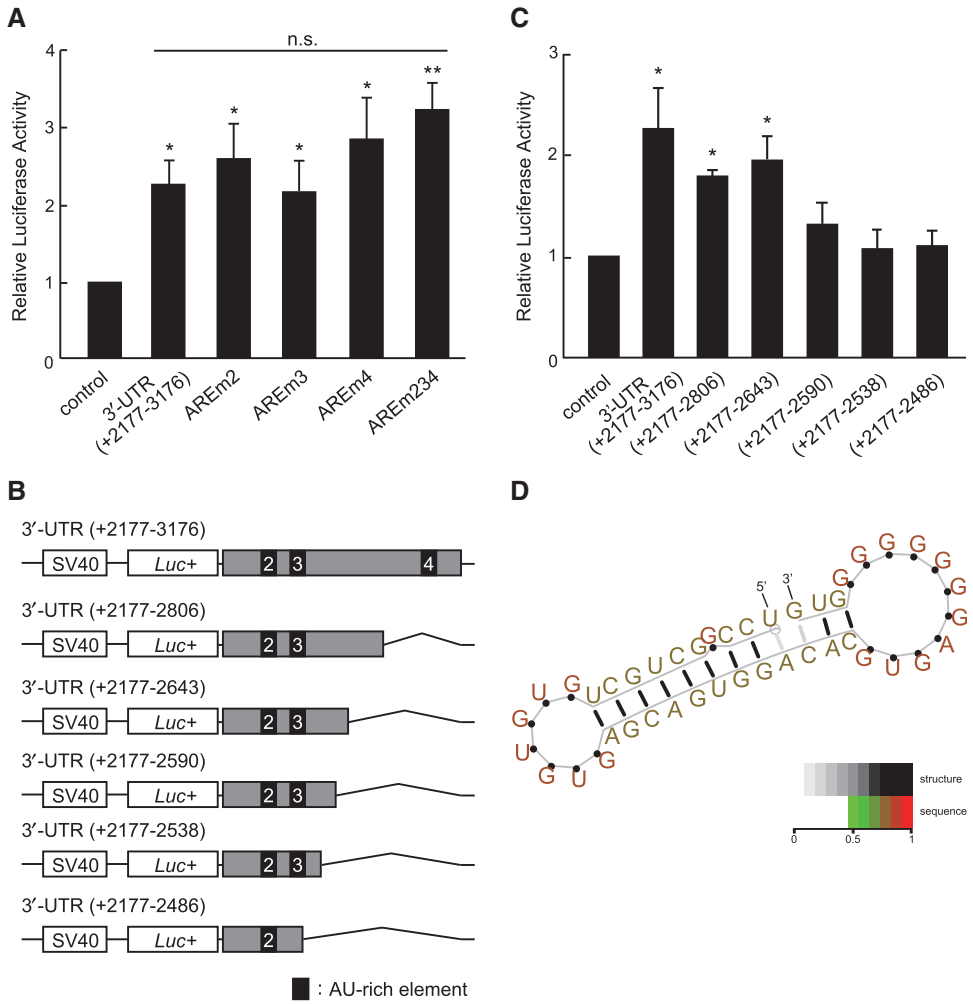

Fig. 4 The increased activity of 3′-UTR (+2177–3176) induced by cAMP are mediated through the region residing in +2591 to +2643 nt of 3′-UTR but not through AREs. (A) HepG2 cells were transfected with 1 μg of AREm2, AREm3, AREm4 or AREm234, respectively (Fig. 2A and B). After transfection, the cells were serum-starved for 16 h, and then stimulated with 8-bromo cAMP (1 mM). Luciferase activities were measured after 24 h. Normalized luciferase activity was calculated as the ratio of the total amounts of protein. Values are means ±SD of the fold increase over the control without 8-bromo cAMP and with empty pGL3-control vectors ($n=4$, $^{*}P<0.05$ compared with control, $^{**}P<0.01$ compared with control, n.s. : not significant). (B) Deletion map in the 3′-UTR (+2177-3176). 3′-UTR (+2177–3176) was deleted by approximately 300 nt at a time, and constructs of 3′-UTR (+2177–2806) and 3′-UTR (+2177–2486) were prepared. Furthermore, the region of +2487 to +2806 nt of 3′-UTR was divided in half at +2643 nt and a construct of 3′-UTR (+2177–2643) was prepared. The region of +2487 to +2643 nt of 3′-UTR was divided into three and constructs of 3′-UTR (+2177–2590) and 3′-UTR (+2177–2538) were prepared. (C) HepG2 cells were transfected with constructs containing 3′-UTR, as shown in Fig. 3B, and then luciferase assays were performed ($n=4$–5, $^{*}P<0.05$ compared with control). (D) The predicted RNA secondary structure in PAI-1 mRNA 3′-UTR is shown. The motif is predicted by RNApromo program.

hepatocytes, a PAI-RBP1 interacts with a cAMP responsive sequence (+2926 to +3054 nt) and modifies stability of PAI-1 mRNA and translational control (*15*). All of them regulate expression of PAI-1. In present study, ARE binding proteins are unlikely to be associated with the responses to cAMP because the effects of cAMP on 3′-UTR were not mediated by AREs (Fig. 4A).

To evaluate the activities of various fragments in the posttranscriptional regulation, we constructed unique reporter plasmids in which the mRNA fragment was inserted downstream of a luciferase cDNA transcribed by the SV40 early enhancer/promoter (*28*). The increase of PAI-1 protein expressions induced by cAMP at 24 h despite the decreases in PAI-1 promoter activity is consistent with increased activity of the PAI-1 mRNA 3′-UTR affecting translation of PAI-1 protein. In fact, PAI-1 mRNA levels induced by cAMP is decreased at 4 h but is increased thereafter. This suggests that cAMP decreases PAI-1 mRNA levels at 4 h, but at 24 h it stabilizes PAI-1 mRNA by the effects of cAMP on PAI-1 mRNA 3′-UTR and increases PAI-1 protein levels. As shown in Figs. 2 and 3, cAMP can stabilize only the 3.2 kb PAI-1 mRNA by affecting the 1 kb terminal of 3′ side of two subspecies of mRNA and that this region may play a major role in the stabilization of PAI-1 mRNA. Because cAMP-response element binding protein (CREB) induced by cAMP binds to HRE2 on PAI-1 promoter (*29*) and insulin stimulates CREB activity (*30*), at least part of the effect of insulin on PAI-1 expression may be mediated by cAMP acting on the 3′-UTR of PAI-1 mRNA.

Thus, the effects of cAMP on expression of PAI-1 may reflect their actions on multiple targets.

In this study, we predicted secondary structure of PAI-1 mRNA 3′-UTR (Fig. 4D). It is reported that there are common structural elements in fast- and slow-decaying mRNAs and linked with binding preferences of several RBPs (*18*). Stem–loop structures on UTRs are targets for RBPs. Iron-responsive element is known to a secondary structure motif in 3′-UTR of transferrin receptor mRNA (*31*). Some regions are identified in PAI-1 mRNA 3′-UTR (+2177–3176). One is the region of +2589 to +2631 nt (Fig. 4D). This region corresponds with that in Fig. 4C (+2591 to +2643 nt). The others are +2693 to +2732 nt with score of 58.2 and +3008 to +3047 nt with score of 59.2. One can expect that RBPs can easily bind to these two regions because they have higher score than +2589 to +2631 nt. This suggests the possibility that yet unknown RBPs may bind these regions.

The present study was undertaken, in part, to identify potentially attractive pharmacologic targets for favourable modification of otherwise adverse effects of increased expression of PAI-1 on fibrinolysis and atherogenesis. Interactions mediated by such targets may exert favourable effects not only on prevention of thrombosis but also on progression of vascular disease by diminishing overexpression of PAI-1 in vessel walls (*32*). Overexpression of PAI-1 has been implicated in both thrombosis and acceleration of atherosclerosis. The present results identify a novel mechanism potentially responsible for these effects, namely, increased expression of PAI-1 mediating the 3′-UTR with activity upregulated by cAMP. The results obtained in the present study imply that pharmacologic amelioration or retardation of accelerated vascular disease associated with PAI-1 may be achievable by sustained down regulation of cAMP-dependent effects on the 3′-UTR of the PAI-1 message.

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Conflict of Interest

None declared.

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