Regulation of \( apoA_1 \) gene expression with acidosis: requirement for a transcriptional repressor

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ABSTRACT

Serum apolipoprotein A\( _1 \) (apoA\( _1 \)) concentration is inversely correlated with the risk of premature atherosclerosis. Serum apoA\( _1 \) concentrations are regulated, in part, at the transcriptional level. ApoA\( _1 \) mRNA is synthesized primarily in the liver and small intestine, under the direction of a number of signaling molecules and tissue-specific regulatory elements. Previously, we demonstrated that extracellular acidosis suppresses apoA\( _1 \) mRNA levels at the level of transcription. Here we demonstrate that intracellular acidosis, in the absence of extracellular pH changes, represses \( apoA_1 \) promoter activity. Repression occurs through a pH responsive element (pH-RE) located within the \( apoA_1 \) gene promoter. Acidosis increases the specific DNA binding activity of a putative repressor protein within the immediate 5'-flanking region of the \( apoA_1 \) gene. The cis-element that binds the putative repressor protein contains a negative thyroid hormone response element (nTRE) located 3' and adjacent to the \( apoA_1 \) TATA box. Mutation of the nTRE/pH-RE abrogates protein binding and alters the activity of reporter genes controlled by this element. Repression by acidosis did not require \textit{de novo} mRNA and protein synthesis. Inhibition of tyrosine kinase activity and diacylglycerol-stimulated protein kinase C (PKC) signaling pathways with tyrphostin A47 and phorbol myristate acetate, respectively, did not affect the repression of \( apoA_1 \) promoter activity with acidosis. These results suggest that transcriptional repression of the \( apoA_1 \) gene by alterations in ambient pH is associated with enhanced DNA binding activity of a repressor protein, through a mechanism which appears to be independent of \textit{de novo} mRNA and protein synthesis, tyrosine kinase activity, or PKC activation.

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INTRODUCTION

Serum apolipoprotein A\( _1 \) (apoA\( _1 \)) concentration is inversely related to the risk of developing atherosclerosis (Miller & Miller 1975, The Lipid Research Clinics Coronary Primary Prevention Trial Results 1984). Synthesis and secretion of apoA\( _1 \) by hepatocytes and epithelial cells of the small intestine are regulated at both the transcriptional and post-transcriptional levels (Sastry \textit{et al}. 1988, Widom \textit{et al}. 1991, Shah \textit{et al}. 1995). Clearance of cholesterol from the circulation is thought to occur through a process called reverse cholesterol transport (Franceschini \textit{et al}. 1991). Augmenting \( apoA_1 \) expression through dietary or pharmacologic means should provide a significant benefit for those with insufficient HDL concentrations. In order to achieve this goal, it is necessary to determine how liver and intestinal cells control \( apoA_1 \) gene expression.

Transcription of the \( apoA_1 \) gene is regulated by several hormones and second messengers, including retinoids (Zolfagari & Ross 1994), insulin (Murao \textit{et al}. 1998, Hargrove \textit{et al}. 1999), thyroid hormone (Mooradian \textit{et al}. 1996, Taylor \textit{et al}. 1996b), sex hormones (Patsch \textit{et al}. 1980, Harnish \textit{et al}. 1998), and others (Taylor \textit{et al}. 1996a, Vu-Dac \textit{et al}. 1998, Murao \textit{et al}. 1997). Several of these pathways function to increase the expression of the \( apoA_1 \) gene, whereas others act to decrease \( apoA_1 \) gene
expression. For instance, hyperglycemia inhibits \(apoA_1\) promoter activity and decreases \(apoA_1\) mRNA levels through an insulin response core element (IRCE) located between nucleotides −425 and −390, relative to the transcriptional start site (Murai et al. 1998). This element is also responsible for induction of the \(apoA_1\) gene by insulin (Murao et al. 1998). Likewise, thyroid hormone can act as either a transcriptional enhancer or, in a more artificial fashion, as a transcriptional repressor (Taylor et al. 1996b). The enhancer effect requires the presence of a thyroid hormone response element (TRE) located between nucleotides −208 and −193, in site A (Taylor et al. 1996b), whereas the repressor effect is observed only when the TRE in site A is removed from reporter constructs, and requires the presence of a negative TRE (nTRE), AGGTCA, located between nucleotides −25 and −20. In this case, the nTRE, located 3′ end adjacent to the \(apoA_1\) TATA box, acts as a transcriptional repressor in the presence of the ligand-activated thyroid hormone receptor (THR) (Taylor et al. 1996b, Carr & Wong 1994). The relevance of this cis-element in regulating expression of the \(apoA_1\) gene in vivo is not known.

Expression of the \(apoA_1\) gene is repressed at the transcriptional level by acidosis (Haas et al. 2000). Acidosis repressed \(apoA_1\) mRNA levels by 25–30%, and \(apoA_1\) promoter activity by more than 50%, relative to alkalosis. Repression due to acidosis is observed in HepG2 liver cells, and in Caco-2 cells (Haas et al. 2000) – a cell line with the characteristics of an epithelial cell from the small intestine and which expresses the \(apoA_1\) gene. In order to understand the mechanism(s) by which acid–base balance may regulate \(apoA_1\) gene expression, we mapped the cis-element responsible for repression by acidosis. The results of our analyses indicate that acidosis controls the DNA-binding activity of a factor that binds to nTRE and has the properties of a transcriptional repressor.

Materials and Methods

Materials

Actinomycin D, acetylcoenzyme A, bafilomycin A1, cycloheximide, nigericin, ouabain, phorbol-myristate acetate (PMA), tyrophostin A47 and valinomycin were purchased from Sigma Chemical Company (St Louis, MO, USA). Lipofectamine was purchased from Life Technologies Inc. (Gaithersburg, MD, USA). New England Nuclear (Boston, MA, USA) was the supplier for [14C]chloramphenicol, and [α-32P]dCTP was purchased from Amersham-Pharmacia Biotech (Piscataway, NJ, USA). Tissue culture media and fetal calf serum were purchased from BioWhittaker Inc. (Walkersville, MD, USA). All other chemicals were of reagent quality and were purchased from either Sigma Chemical Company or Fisher Scientific Company (Pittsburgh, PA, USA).

Cell culture

HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS), and penicillin and streptomycin (100 units/ml and 100 µg/ml, respectively). Caco-2 intestinal cells were maintained in Earl’s modified essential medium (EMEM) supplemented with 15% FBS, non-essential amino acids, 0.1 mg/ml sodium pyruvate, and penicillin and streptomycin. Cells were housed in a humidified incubator at 37 °C with 5% CO₂, 95% air.

An acidic environment was established by switching the cells to sodium bicarbonate-deficient DMEM, supplemented with serum and antibiotics as described above. The pH was established with N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid (HEPES), adjusted to either 6.7 or 7.9 with KOH. The pH of the culture medium was maintained at either pH 6.7 or pH 7.9 by the addition of HEPES (at pH 6.7 or pH 7.9 respectively) to a final concentration of 25 mM. No HEPES-related toxicity was noted at either pH extreme.

In some experiments, cells were treated with various ionophores or proton pump inhibitors. Bafilomycin A1, nigericin, ouabain and valinomycin were added to final concentrations of 5 µg/ml, 10 µg/ml, 1 mM, and 2 µM respectively, 24 h after transfection.

Cells were exposed for 16–24 h, then harvested and assayed for reporter enzyme activity.

Plasmids and transient transfection analysis

Plasmids containing various lengths of the rat \(apoA_1\) gene promoter were transfected into both HepG2 and Caco-2 cells for analysis of \(apoA_1\) promoter activity. The plasmid pAI.474.CAT contains 467 bp of the rat \(apoA_1\) gene promoter fused to the chloramphenicol acetyltransferase (CAT) reporter gene (Murao et al. 1998). Reporter constructs with various lengths of the \(apoA_1\) promoter, generated using the polymerase chain reaction (PCR) followed by subsequent cloning of the amplified product in the parent CAT construct, were described previously (Taylor et al. 1996b, Murao et al. 1998). CAT constructs containing deletions within the
apoA1 5'-flanking sequence from −7 to −474 (pA1-474.CAT), −425 (pA1-425.CAT), −375 (pA1-375.CAT), −325 (pA1-325.CAT), −186 (pA1-186.CAT), −170 (pA1-170.CAT), −144 (pA1-144.CAT), and −46 bp (pA1-46.CAT) were used as described previously (Taylor et al. 1996b, Murao et al. 1998). The apoA1 CAT constructs containing a mutant nTRE, pA1.m474.CAT and pA1.m46.CAT, were described previously (Taylor et al. 1996b).

Cells cultured to 80% confluence in six-well plates were transfected with 1 µg of the apoA1 reporter plasmid and 1 µg of the plasmid pCMV.SPORT-β-Gal (Life Technologies, Inc.) using Lipofectamine. The latter plasmid, containing the β-galactosidase gene driven by the cytomegalovirus (CMV) immediate-early promoter, was used to normalize reporter gene activity to transfection efficiency. The latter plasmid containing the β-galactosidase gene driven by the cytomegalovirus (CMV) immediate-early promoter, was used to normalize reporter gene activity to transfection efficiency. After 24 h, the cells were switched to DMEM at a pH of either 6.7 or 7.9. After a further 24 h, the cells were harvested and assayed for CAT activity (Gorman et al. 1982).

A portion of the extract was used for determination of β-galactosidase activity (Herbomel et al. 1984).

RNA isolation and northern blotting

ApoA1 mRNA levels were assessed by northern blotting with a previously characterized apoA1 cDNA probe (Mooradian et al. 1996). Total RNA was prepared from cells cultured at either pH 6.7 or pH 7.9, and treated with either actinomycin D (1 µg/ml) or cycloheximide (CHX) (10 µg/ml) for 24 h, as described previously (Chomczynski & Sacchi 1987). Fifteen micrograms RNA were fractionated on an agarose gel containing 2% formaldehyde, using established procedures (Sambrook et al. 1982). The RNA was transferred to a nylon membrane (Hybond, Amersham-Pharmacia Biotech), and hybridized with a cDNA probe specific to the apoA1 gene. After appropriate exposures were obtained, the nylon membrane was stripped and rehybridized with a second cDNA probe specific for the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene. The amount of signal present on the autoradiographs was quantified with a scanning laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

ApoA1 western blotting

ApoA1 protein concentrations were assessed by western blotting (Towbin et al. 1979) with an antibody prepared and characterized in this laboratory (Mooradian et al. 1997). HepG2 cells in 25 cm² flasks were cultured under either acidic (pH 6.7) or alkaline (pH 7.9) conditions for 24 h. Cells were washed twice with ice-cold phosphate-buffered saline (PBS, 50 mM sodium phosphate (pH 7.4), 150 mM NaCl) and resuspended in Laemmli sample buffer (Laemmli 1970). Protein concentration was determined with the Bradford protein assay (Bradford 1976) with bovine serum albumin as the standard. Twenty five micrograms protein were used in the analysis.

Nuclear protein extract preparation and EMSA analysis

Nuclear protein extracts were prepared from cells at 90% confluence, cultured at either pH 6.7 or pH 7.9 for 24 h. Cells were washed three times with ice-cold PBS, and resuspended in 10 ml of nuclear wash buffer (10 mM HEPES (pH 8.0), 5 mM NaCl, 15% sucrose, 1 mM ethylene diaminetetra-acetic acid (EDTA), 0.5% Triton X-100, 1 mM dithio-reitol (DTT), 5 mM MgCl2, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) and incubated on ice for 10 min. The mixture was underlaid with 5 ml of nuclear wash buffer containing 30% sucrose, but no Triton X-100, and centrifuged at 3000 g for 30 min at 5 °C. The nuclei in the pellet were resuspended in 1 ml of a buffer containing 10 mM HEPES (pH 8.0), 500 mM NaCl, 10 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF and 5 mM spermidine, and placed on ice for 1 h. The supernatant fraction was obtained by centrifugation at 10 000 g for 10 min, and was dialyzed extensively against 20 mM HEPES (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 20% glycerol. Protein concentration was determined as previously described (Bradford 1976), with bovine serum albumin as the standard.

The electrophoretic mobility shift assay (EMSA) was performed by incubating 10–15 µg nuclear protein extract with 15 000 c.p.m. of a 32P-labeled oligonucleotide containing 46 bp of DNA corresponding to the proximal promoter region of the apoA1 gene. The double-stranded oligonucleotides (5'-*GCCCCTGCCACACACATATAGTTCA GGGAAAGACCTGGAACCCACAGCT-3', 5'-TC AGTCTGGTGTCAGTTCATTCCCTG ACCTATATATGTTGTGTCAGCAG-3') were annealed and the probe labeled by filling in the 5'-overhang with [α-32P]dCTP using DNA polymerase I. The * denotes which base in the template strand was labeled in the experiments described here. The underlined bases indicate the position of the nTRE (Taylor et al. 1996b), and the bold bases indicate the position of the apoA1 TATA box (Widom et al. 1991). Incubation of the
protein extract with the labeled DNA probe was accomplished in a solution containing 12% glycerol, 12 mM HEPES (pH 7.9), 60 mM KCl, 5 mM MgCl₂, 4 mM Tris-(hydroxymethyl)aminomethane hydrochloride (Tris–Cl) (pH 7.9), 0.6 mM EDTA, 0.6 mM DTT and 2 μg of poly(dI-dC)·poly(dI-dC), then placed on ice for 30 min. The mixture was loaded onto a 5% polyacrylamide gel, and subjected to electrophoresis in 0.5 × TBE (1 × TBE is 45 mM Tris base, 32 mM boric acid, and 1.25 mM EDTA (pH 8.3)) at room temperature, 200 V, for 90–120 min. The gel was dried and exposed to Hyperfilm (Amersham-Pharmacia Biotech).

Specificity of binding was assessed by adding a 10- and 100-fold molar excess of either an unlabeled double-stranded competitor oligonucleotide consisting of the wild type (WT) 46 bp DNA fragment or the unrelated DNA sequence, Site A (sense strand: 5′-TGACCCCTTTGATCCCA-3′). Competition with a 10- and 100-fold molar excess of an unlabeled, double-stranded oligonucleotide containing the apoA₁ mutant nTRE (Taylor et al. 1996b) (5′-ACACACATATAGGCTGGAGCTCCAGACCTGG-3′) was performed in a similar manner. The underlined bases in the nTRE were altered to electrophoresis, was identical to the procedure described above.

Statistical analysis

Changes in reporter gene activity, and apoA₁ protein and mRNA levels were evaluated with the two-tailed Student’s t-test. P < 0.05 was considered the limit for statistical significance. The results are expressed as means ± S.E.M.

RESULTS

Effect of acidosis on apoA₁ protein content

We have recently shown that apoA₁ mRNA content and apoA₁ promoter activity are suppressed by acidosis in both Caco-2 intestinal cells and HepG2 hepatocytes (Haas et al. 2000). To confirm and extend our previous findings, apoA₁ protein content in HepG2 cells was measured by western blotting. The results (Fig. 1) demonstrate that apoA₁ protein concentrations are significantly reduced when cells are cultured under acidic conditions. Quantitation of the results with a densitometer showed that acidosis reduced apoA₁ protein concentrations in HepG2 cells nearly 20%, from 100 ± 2.3% to 80.3 ± 5.4% (P < 0.01). In this cell line, apoA₁ mRNA levels are also reduced by acidosis, by 20% (Haas et al. 2000). This suggests that the steady-state apoA₁ protein concentration is likely to be directly related to the decrease in apoA₁ mRNA levels.

Effect of cation pump inhibitors and ionophores on apoA₁ promoter activity

To determine whether the changes in apoA₁ promoter activity with acidosis (Haas et al. 2000) that we had observed could be reproduced with various chemicals known to reduce intracellular pH directly or indirectly, the effect of various cation pump inhibitors and ionophores on apoA₁ promoter activity was evaluated. Changes in intracellular Na⁺ and K⁺ concentration results in changes in intracellular pH as a result of the activity of a Na⁺/H⁺ antiporter (Aronson 1985, Krapf et al. 1991). In order to determine if changes in intracellular Na⁺/K⁺ concentration can affect apoA₁ promoter activity, we assessed what the effects the Na⁺/K⁺ ATPase inhibitor ouabain, and the ionophores nigericin and valinomycin, may have on apoA₁ promoter activity. HepG2 and Caco-2 cells were transfected with the plasmids pA1-474.CAT and pCMV.SPORT.β-gal, and after 24 h, the culture media was switched to DMEM at pH 6.7 or pH 7.9. Ouabain (1 mM), nigericin (10 µg/ml), or valinomycin (2 µM) were added to cells in pH 7.9 culture media. After 24 h, the cells were harvested and assayed for CAT and β-galactosidase activity. The results (Table 1) clearly show that each effector was as potent at inhibiting CAT activity as acidosis itself. In this experiment, acidosis repressed apoA₁ promoter activity to 32.8% in HepG2 cells (P < 0.05). Nigericin, ouabain, and valinomycin repressed apoA₁ promoter activity to 57.7% (P < 0.01), 45.3% (P < 0.01), and 37.9% (P < 0.05) of controls respectively. Similar results were obtained with valinomycin and nigericin in Caco-2 cells. Additional experiments with bafilomycin A₁-treated Caco-2 cells showed a similar reduction in apoA₁ promoter activity (Table 1). Bafilomycin A₁, a potent inhibitor of the vacuolar H⁺/ATPase (Harada et al. 1997, Keeling et al. 1997), prevents endosome acidification and decreases cytosolic pH. Caco-2 cells transfected with the plasmid pA1-474.CAT and treated with bafilomycin A (5 µg/ml) showed decreased apoA₁ promoter activity to 57.8% relative to untreated cells (Table 1). These effects were accomplished without changes in extracellular pH, as assessed by measurements of media pH (data not shown). These results suggest that changes in intracellular pH through pharmacological means, in the absence of changes in extracellular pH, repress apoA₁ promoter activity to an extent similar to that produced by extracellular acidosis.
Localization of the pH responsive element (pH-RE)

As the effect of acidosis on expression of the apoA1 mRNA and protein is due to inhibition of apoA1 promoter activity (Haas et al. 2000), we wished to determine what cis-element is necessary for mediating this response. CAT reporter constructs containing 474, 425, 375, 325, 186, 170, 144, and 46 bp of 5'-flanking sequence of the apoA1 promoter region (Fig. 2) were transfected into both HepG2 and Caco-2 cells. After 24 h, the cells were switched to bicarbonate-deficient DMEM adjusted to either pH 6.7 or 7.9 by the addition of HEPES. CAT activity
Table 1. Effect of ion perturbation on apoA1 promoter activity. HepG2 and Caco-2 cells were transfected with the plasmids pA1-474.CAT and pCMV.SPORT-β-gal. After 24 h, the media were switched to DMEM at either pH 6-7 or 7-9, at which time baflomycin (Bafl.), 5 μg/ml), nigericin (10 μg/ml), ouabain (1 mM), and valinomycin (Valinol., 2 μM) were added to cells containing pH 7-9 DMEM. After 24 h, cells were harvested and assayed for CAT and β-galactosidase activity. In all cases, baflomycin, nigericin, ouabain, and valinomycin treatment repressed apoA1 promoter activity significantly.

<table>
<thead>
<tr>
<th>Treatment/pH</th>
<th>HepG2 CAT activity</th>
<th>Percent change</th>
<th>Caco-2 CAT activity</th>
<th>Percent change</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/6-7</td>
<td>39.8 ± 1.9</td>
<td>-32.8†</td>
<td>5.6 ± 1.1</td>
<td>-72.8†</td>
</tr>
<tr>
<td>C/7-9</td>
<td>59.3 ± 2.5</td>
<td>—</td>
<td>20.6 ± 2.3</td>
<td>—</td>
</tr>
<tr>
<td>Bafl./7-9</td>
<td>N.D.</td>
<td>—</td>
<td>8.7 ± 2.4</td>
<td>-57.8†</td>
</tr>
<tr>
<td>Nigericin/7-9</td>
<td>25.1 ± 1.7</td>
<td>-57.7†</td>
<td>0.7 ± 0.2</td>
<td>-96.9†</td>
</tr>
<tr>
<td>Ouabain/7-9</td>
<td>32.4 ± 2.2</td>
<td>-45.3†</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Valino./7-9</td>
<td>36.8 ± 2.0</td>
<td>-37.9†</td>
<td>2.5 ± 0.8</td>
<td>-87.9†</td>
</tr>
</tbody>
</table>

C, untreated cells; N.D., not determined; †P<0.01 relative to C/pH 7-9, n=4.

in HepG2 and Caco-2 cells cultured at pH 6-7 for 24 h decreased 45–46% relative to the activity observed in cells at pH 7-9 (Fig. 2) for the pA1-474.CAT plasmid. All of the other apoA1 deletion constructs were repressed to a similar degree by acidosis (Fig. 2). In all cases, the decrease was statistically significant. These results suggest that a pH-RE is located in the 46 bp DNA fragment immediately adjacent to the transcriptional start site.

Effect of acidosis on protein–DNA binding activity

In order to determine if the effect of acidosis on the apoA1 promoter is due to modulation of the activity of a DNA binding protein, we performed EMSAs with nuclear protein extracts prepared from HepG2 and Caco-2 cells cultured at either pH 6-7 or pH 7-9 for 24 h. The probe used in the gel shift assay consisted of a double-stranded oligonucleotide containing the region of the apoA1 promoter from -48 to +5bp, relative to the transcriptional start site (+1). This fragment includes the region present in the smallest CAT construct (Fig. 2) used in the pH-RE localization experiments described above. One major protein–DNA complex was observed in HepG2 cells at both pH 6-7 and pH 7-9 (Fig. 3). However, the amount of complex formation in the extracts from the cells maintained at pH 6-7 was increased relative to the amount of complex formation with extracts prepared from cells at pH 7-9 (compare lanes 2 and 3 with lanes 4 and 5). The amount of complex formation was equivalent in identically treated cells (compare lanes 2 and 3 with lanes 4 and 5), suggesting that the degree of variability in complex formation is small. Quantitation of the results by densitometry showed that complex formation was increased by 30.1±3.9% with acidosis. This finding suggests that acidosis may increase the DNA-binding activity of a transcriptional repressor protein. Similar results were obtained with nuclear protein extracts prepared from Caco-2 cells (Fig. 3) maintained under either acidic (lane 6) or alkaline conditions (lane 7). These results suggest that acidosis affects apoA1 gene expression in a similar manner in both cell types.

Gel shift assays with probes containing the apoA1 IRCE (Murao et al. 1998) and site A suggest that protein–pH-RE binding is real, because the amount of complex formation with these probes did not show change with acidosis (data not shown).

DNA–protein binding was not suppressed by the addition of an unlabeled oligonucleotide containing the apoA1 site A region (Fig. 4, lanes 7 and 8), but was completely suppressed when the homologous oligonucleotide containing the pH-RE was added (Fig. 4, lanes 3 and 4). These results support the conclusion that binding of the pH-responsive factor in HepG2 cells to the pH-RE is specific. A similar specificity profile was obtained using nuclear protein extracts prepared from Caco-2 cells (data not shown).

Effect of pH-RE mutation on pH-RE activity

The region of the apoA1 promoter which contains the pH-RE activity and is able to bind the putative repressor protein contains a consensus nTRE motif located immediately adjacent to the apoA1 TATA element (Carr & Wong 1994, Taylor et al. 1996a,b). Therefore, we investigated whether or not mutation of the nTRE would affect the ability of acidosis or alkalosis to affect reporter gene expression. Two different constructs were used, one of which contained a mutant nTRE in the context of the full-length apoA1 promoter (pA1.m474.CAT), the other of which contains the mutant nTRE within only 39 bp of the apoA1 5’-flanking region (Fig. 5) (pA1.m46.CAT). In both cases, the central nucleotides within the WT nTRE, TCA, were replaced with CTG. When Hep G2 cells were transfected with the plasmid pA1-474.CAT and subjected to acidic culture conditions for 24 h, CAT activity decreased to 81-6% relative to CAT activity at
pH 7·9 (Fig. 5). However, CAT activity HepG2 from cells transfected with the plasmid pA1.m474.CAT, containing the TCA→CTG mutation, was suppressed by acidosis to 77·2% relative to CAT activity at pH 7·9. Although this difference was statistically significant (P<0·01), the lack of complete abrogation of acidosis-related suppression indicates that the nTRE is not the sole pH-RE in the apoA₁ promoter.

Different results were obtained with the apoA₁-CAT reporter construct containing only 39 bp of 5′-flanking DNA (Fig. 5). When HepG2 cells were transfected with pA1.m46.CAT and cultured at either pH 6·7 or 7·9, no statistically significant differences in CAT activity were observed in HepG2 cells (5·1% acetylation at pH 7·9 compared with 4·9% acetylation at pH 6·7, P>0·05) or in Caco-2 cells (3·9% at pH 7·9 compared with 3·5% at
pH 6·7, \( P > 0.05 \)). Taken together, these experiments indicate that the previously described nTRE may also act as a pH-RE. However, there must be additional cis-acting elements within the promoter that can also function as pH-REs when the nTRE/pH-RE is mutated.

Effect of pH-RE mutation on protein–pH-RE binding

Because mutation of the pH-RE appeared to be at least partly involved in the repressive effect of acidosis on the \( \text{apo}A_1 \) promoter, we next determined

**FIGURE 3.** Effect of acidosis on factor binding to the 46 bp DNA fragment. EMSAs were used to examine the effect of acidosis on protein–DNA binding. The probe, consisting of the 46 bp DNA fragment to which the pH-RE was localized, was incubated with either 15 or 10 \( \mu \)g of nuclear extract protein from HepG2 or Caco-2 cells respectively, cultured at either pH 6·7 or pH 7·9 for 24 h. Increased protein–DNA binding can be observed in extracts prepared from cells cultured at pH 6·7 (lanes 2 and 3), relative to extracts prepared from cells cultured at pH 7·9 (lanes 4 and 5). Increased protein–DNA binding was also observed in cell extracts prepared from Caco-2 cells at pH 6·7 (lane 6) relative to pH 7·9 (lane 7). Migration of the bound (B) and free (F) \( ^{32} \)P-labeled DNA probe is indicated. The experiment was repeated three times.
whether or not the same nucleotide changes also effect pH-RE–protein binding. Double-stranded oligonucleotides containing the TCA→CTG substitution were synthesized, annealed, and used as competitors in the gel shift assay shown in Fig. 4. Inclusion of this oligonucleotide in the binding reaction displaced protein from the \(^{32}\)P-labeled probe less effectively than the WT sequence. These results indicate that the mutated pH-RE binds to nuclear protein(s) with less affinity than the WT sequence, suggesting that a transcriptional repressor may be involved in the acidosis-related downregulation of the apoA\(_1\) promoter.

Effect of RNA and protein synthesis inhibitors on repression of the apoA\(_1\) promoter by acidosis

In order to determine if either de novo RNA or protein synthesis are required for repression of the apoA\(_1\) gene by acidosis, HepG2 cells were placed in culture medium at pH 7-9 or at pH 6-7 and treated with either 1\(\mu\)g/ml actinomycin D or 10\(\mu\)g/ml cycloheximide (CHX). At these doses, cell survival was 94-3% (actinomycin D) and 95-6% (CHX) relative to cells at pH 6-7 (98-5%), as assayed by trypan blue exclusion. After 24 h, RNA was harvested from the cells and northern blotting was performed with the apoA\(_1\) cDNA probe. Acidosis repressed apoA\(_1\) mRNA levels by 50% (Fig. 6) (P<0.05). Addition of either actinomycin D or CHX when the medium was switched to pH 6-7 failed to inhibit the repression normally observed with acidosis; even in the presence of actinomycin D and CHX, acidosis repressed apoA\(_1\) mRNA levels to 42% and 51% of control respectively. These results suggest that repression of the apoA\(_1\) gene by acidosis does not require de novo mRNA and protein synthesis.

Effect of PMA and tyrphostin A47 on repression of the apoA\(_1\) promoter by acidosis

As inhibition of mRNA and protein synthesis did not prevent the repressive effect of acidosis on apoA\(_1\) mRNA levels, we examined two potential post-transcriptional mechanisms by which acidosis may affect apoA\(_1\) promoter activity: protein kinase C and tyrosine kinase activation. Protein kinase C is a possible mediator for intracellular signaling by acidosis (Moolenaar et al. 1984, Nishizuka 1984, Williams & Howard 1994). In order to determine if changes in diacylglycerol-stimulated protein kinase C (PKC) isozymes mediate the repression of the apoA\(_1\) promoter by acidosis, two approaches were taken. In one set of experiments, PMA was added immediately after transfection with pA1-474.CAT, to a final concentration of 400 nM. After 24 h, the medium was replaced with bicarbonate-deficient DMEM adjusted to either pH 6-7 or 7-9 with HEPES. PMA was again added to the appropriate wells to a final concentration of 400 nM. After a further 24 h, the cells were harvested and assayed for both CAT and β-galactosidase activity. Under these conditions, PMA has been reported to downregulate PKC activity (Nishizuka 1986, Yamaji et al. 1994). The results (Table 2) indicate that changes in PMA-stimulated PKC isoforms using a high-dose, chronic treatment with PMA failed to alter CAT activity significantly (54-5% decrease in CAT activity in the solvent-treated controls compared with a 50-6% decrease in CAT activity in the PMA-treated HepG2 cells). Similar results were obtained in Caco-2 cells (Table 2). However, basal CAT activity was repressed by PMA-treatment alone in HepG2 cells (from 26-6% to 8-1% in cells at pH 7-9 and from 12-1% to 4-0% in cells at pH 6-7), but not in Caco-2 cells. These results suggest that basal apoA\(_1\) promoter activity is controlled in HepG2 cells, at least in part, by...
protein kinase C. However, downregulation of protein kinase C by chronic exposure to high concentrations of PMA did not alter the ability of acidosis to repress the apoA1 promoter.

In the second set of experiments, PMA was added to the cells 24 h after transfection with reporter constructs containing either the WT pH-RE, or a mutant pH-RE. After another 24 h, CAT activity was assessed. The mutant pH-RE is present in two contexts, either within the full-length 474 bp apoA1 promoter or within the plasmid containing the minimal, 39 bp apoA1 promoter DNA fragment. Acidosis repressed CAT activity from the plasmid pA1.m474.CAT. However, acidosis did not significantly repress CAT activity from the plasmid pA1.m46.CAT. CAT activity in mock transfected cells is less than 0·5%. The percent [14C]chloramphenical conversion without lysate is 0·09%. †P<0.01 relative to pH 7·9; n=5.

**Figure 5.** Constructs used in elucidating the effect of mutating the pH-RE on repression of the apoA1 promoter by acidosis. HepG2 and Caco-2 cells were transfected with reporter constructs containing either the WT pH-RE, or a mutant pH-RE. After 24 h, the media pH was adjusted to either pH 6·7 or pH 7·9. After another 24 h, CAT activity was assessed. The mutant pH-RE is present in two contexts, either within the full-length 474 bp apoA1 promoter or within the plasmid containing the minimal, 39 bp apoA1 promoter DNA fragment. Acidosis repressed CAT activity from the plasmid pA1.m474.CAT. However, acidosis did not significantly repress CAT activity from the plasmid pA1.m46.CAT. CAT activity in mock transfected cells is less than 0·5%. The percent [14C]chloramphenical conversion without lysate is 0·09%. †P<0.01 relative to pH 7·9; n=5.

<table>
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<tr>
<th>Cell Line</th>
<th>HepG2 CAT Activity</th>
<th>Percent Change</th>
<th>HepG2 CAT Activity</th>
<th>Percent Change</th>
<th>Caco-2 CAT Activity</th>
<th>Percent Change</th>
<th>Caco-2 CAT Activity</th>
<th>Percent Change</th>
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<tbody>
<tr>
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<td>pA1.m474.CAT / 7.9</td>
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<td>-</td>
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<td>pA1.46.CAT / 6.7</td>
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<td>pA1.m46.CAT / 6.7</td>
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difference did not achieve statistical significance. Similar results were also obtained with Caco-2 cells (Table 2).

In order to determine if tyrosine kinase activity is important in repression of the apoA1 promoter by acidosis, HepG2 and Caco-2 cells were transfected with the apoA1 reporter plasmid pA1-474.CAT and, after 24 h, treated with either 10 or 100 μM tyrophostin A47 for another 24 h. Tyrophostin A47 is a potent inhibitor of tyrosine kinase activity (Levitzki et al. 1991). Tyrophostin A47 did not repress basal apoA1 promoter activity at either pH 6.7 or 7.9, nor did it significantly affect repression of the apoA1 promoter by acidosis (Table 3). CAT activity was repressed to 44–2% in the control HepG2 cells, compared with 32–5% in cells treated with 0.5 μM tyrophostin A47. This difference was not statistically significant. CAT activity was repressed to 41–4% of control when HepG2 cells were treated with a higher concentration of tyrophostin A47 (5–0 μM). Similar results were obtained with Caco-2 cells (Table 3). These results indicate that repression of apoA1 promoter activity by acidosis is independent of tyrosine kinase activity.

![Figure 6. Effect of RNA and protein synthesis inhibitors on apoA1 mRNA expression with acidosis. Total RNA prepared from HepG2 cells maintained at either pH 7.9 or pH 6.7 with or without 1 μg/ml actinomycin D (Act) or 10 μg/ml cycloheximide (CHX). Northern blots were hybridized with the apoA1 cDNA probe, stripped and hybridized with a probe specific for G3 PDH to normalize apoA1 mRNA expression levels. The 0.9 kb apoA1 mRNA band and the 1.2 kb G3 PDH band were quantified by densitometry. Percent changes in apoA1 mRNA expression are shown, relative to the level of G3 PDH expression. Addition of actinomycin D and CHX had no effect on the ability of acidosis to repress apoA1 mRNA levels. *P<0.01 compared with pH 7.9. Other comparisons did not achieve statistical significance. n = 3.](www.endocrinology.org)

<table>
<thead>
<tr>
<th>Treatment/pH</th>
<th>HepG2 CAT activity</th>
<th>Percent change</th>
<th>Caco-2 CAT activity</th>
<th>Percent change</th>
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<td>P100/6-7</td>
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<td>P100/7-9</td>
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<td>P400/7-9</td>
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†P<0.05; ‡P<0.01, relative to pH 7.9, n=3.

<table>
<thead>
<tr>
<th>Treatment/pH</th>
<th>HepG2 CAT activity</th>
<th>Percent change</th>
<th>Caco-2 CAT activity</th>
<th>Percent change</th>
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<tbody>
<tr>
<td>C/6-7</td>
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<td>8.6 ± 0.2</td>
<td>39.4†</td>
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<td>C/7-9</td>
<td>52.0 ± 0.5</td>
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<td>14.2 ± 4.0</td>
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<td>10T/6-7</td>
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<td>4.8 ± 0.7</td>
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<td>10T/7-9</td>
<td>44.8 ± 0.4</td>
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<td>11.8 ± 3.1</td>
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<td>100T/6-7</td>
<td>30.1 ± 0.5</td>
<td>41.4†</td>
<td>6.2 ± 2.3</td>
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<td>100T/7-9</td>
<td>51.4 ± 1.7</td>
<td>—</td>
<td>14.4 ± 0.5</td>
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</tr>
</tbody>
</table>

†P<0.05; ‡P<0.01, relative to pH 7.9, n=3.
DISCUSSION

ApoA1 mRNA levels are repressed 25–30% in HepG2 and Caco-2 cells maintained in acidic culture media (pH 6.7), relative to cells maintained in alkaline medium (pH 7.9) (Haas et al. 2000). Repression occurred at the level of transcription, as assessed by transient transfection analysis with reporter gene constructs containing the rat apoA1 promoter (Haas et al. 2000). The highest level of CAT activity observed in cells maintained at pH 7.9 gradually decreased by 50–60% when the pH of the culture medium was decreased sequentially to pH 6.7 (Haas et al. 2000). Furthermore, these effects of acidosis were not observed with CAT activity driven by the SV40 virus early region promoter, suggesting that protein translation is not affected by changes in pH (Haas et al. 2000).

Acidosis also suppressed steady-state apoA1 protein levels in HepG2 cells (Fig. 1). This observation suggests that the reduction in apoA1 promoter activity and mRNA concentration is reflected at the protein level. This level of reduction is comparable to the 25–30% decrease in apoA1 mRNA levels reported previously (Haas et al. 2000). Importantly, this result also indicates that the protein synthesis capacity of the HepG2 cell line we used is not drastically affected by the change in pH. This finding is important, because Yamaji et al. (1994) reported that protein synthesis was suppressed by acidosis in some, but not all cell lines they examined.

Changes in Na+ and K+ concentration and distribution by pharmacological means can alter the activity of the membrane-bound Na+/H+ antiporter, resulting in a reduction in intracellular pH. Activity of this ion pump is thought to be necessary for the alkalinization observed in cells observed with growth factor stimulation (Boron 1986, Moolenaar 1986). Nucleus–cytosol pH gradients may also be determined in part by a K+/H+ exchanger (Masuda et al. 1998). The addition of ouabain, a Na+/K+-ATPase inhibitor, and the monovalent cation ionophores nigericin and valinomycin repressed apoA1 promoter activity (Table 1). Thus alterations in intracellular pH, in the absence of changes in extracellular pH, cause repression of apoA1 promoter activity. This finding is important, because the release of carrier-bound molecules and growth factor activation may be increased when culture media containing FBS are acidified. Thus the observation that changes in intracellular pH independent of media pH repress apoA1 promoter activity suggests that there may be an intracellular pH sensor that is capable of regulating apoA1 promoter activity. However, it is also possible that use of ouabain, nigericin and valinomycin may also have unrelated effects on apoA1 promoter activity.

Bafilomycin A1 inhibits endosomal acidification through its ability specifically to inhibit the vacuolar H+/ATPase pump (Forgac 1989, Chinni & Shisheva 1999). Our results show that bafilomycin A1 represses apoA1 promoter activity (Table 1). Whether or not this effect is due to inhibition of endosomal acidification or to a fluctuation in cytosolic pH remains to be determined. Bafilomycin A1 can mimic the effect of insulin on the translocation of glucose transporter 4 to the plasma membrane in 3T3-L1 adipocytes (Chinni & Shisheva 1999). In HepG2 cells, however, insulin, has a positive effect on apoA1 promoter activity (Murao et al. 1998). These results suggest that bafilomycin A1 and insulin function through unrelated mechanisms to regulate apoA1 promoter activity in HepG2 cells.

Transient transfection analysis with reporter genes containing deleted segments of the apoA1 promoter were used to determine if a cis-element responsive to changes in pH could be identified. Deletion of the apoA1 insulin-responsive core element (IRCE) had no effect on the ability of pH to repress reporter gene activity (Fig. 2). Subsequent deletions of sites A, B, and sites C and D together, also had no effect on repression of apoA1 promoter activity (Fig. 2). This finding was unexpected, as two binding sites for the transcription factor Egr-1 exist in site A of the apoA1 promoter (Kilbourne et al. 1995). Egr-1 is an immediate-early, zinc-binding transcription factor (Madden & Rauscher 1993). Egr-1 expression and activity can be induced by mitogenic signals (Sukhatme et al. 1988, Lim et al. 1987), atherosclerosis (McCaffrey et al. 2000) and cellular stress (Santiago et al. 1999, Yan et al. 1999). Egr-1 acts as a transcriptional repressor of the murine deaminase promoter (Ackerman et al. 1991) and the rat malic enzyme promoter (Barroso & Santisteban 1999). Of interest, Yamaji et al. (1994) have previously shown that Egr-1 expression is induced by acidosis in the MCT kidney epithelial cell line as early as 15 min after the change in media pH. Therefore, our results indicate that EGR-1 is probably not the major transcriptional repressor responsible for the effect of acidosis on the apoA1 promoter. However, it may have some role in apoA1 repression with acidosis, because more than one element may be present in the apoA1 promoter.

Because repression was noted with the smallest promoter fragment analyzed (−46 bp), we examined this region for the presence of protein–DNA interactions. Taylor et al. (1996b) identified an
nTRE 3’ end adjacent to the apoA1 TATA box. This element consists of an nTRE capable of binding only a THRα monomer (Taylor et al. 1996b). Alone, the nTRE is capable of suppressing reporter gene activity, while in the context of the full apoA1 promoter it loses this ability. In fact, site A contains a fully functional TRE that is capable of responding positively to thyroid hormone (Taylor et al. 1996b).

A gel shift probe containing the apoA1, TATA box and nTRE formed a protein–DNA complex with nuclear extract proteins prepared from both HepG2 and Caco-2 cells cultured at pH 7 for 24 h (Fig. 3). The amount of complex formed increased in cells maintained at pH 6·7 for 24 h (Fig. 3). These results indicate that acidosis enhances binding of a putative repressor protein to a pH-RE within this DNA fragment. Furthermore, competition experiments with WT and mutant nTRE oligonucleotides indicated that protein–DNA binding requires the intact nTRE (Fig. 4). Addition of the WT nTRE oligonucleotide at 25-fold molar excess did not compete for factor binding (Fig. 4), although addition of the competitor at a 100-fold molar excess did compete to a small extent (Fig. 4). These results suggest that factor binding may be influenced, stabilized, or both, by factors adjacent to it. A likely candidate for this interaction may be the factors capable of binding the apoA1 TATA box located immediately adjacent to the nTRE. Mutational analysis of this element, however, would probably yield results difficult to interpret, because this element is probably important in basal promoter activity. This finding was confirmed by analyzing the effect of mutations within the nTRE/pH-RE on reporter gene activity. Changing the core TCA sequence within the pH-RE/nTRE to CTG reduced pH-RE protein binding (Fig. 4) and alleviated the repressive effect of acidosis on the apoA1 promoter construct, pAl-474.CAT (Fig. 5). However, mutation of the nTRE in this context did not abolish the effect of acidosis on promoter activity (Fig. 5). This observation suggests that, although nTRE is a pH-RE, it is not the sole pH-responsive element within the apoA1 promoter. Additional pH-REs within the promoter are functional when the nTRE is mutated.

Acidosis has been shown to modulate several intracellular signaling modalities, including PKC activation (Moolenaar et al. 1984, Moolenaar 1986). Therefore, it was somewhat surprising that PMA did not affect the ability of acidosis to repress apoA1 promoter activity (Table 2). It is possible that repression by acidosis requires more than one signaling pathway activated by acidosis, one of which may involve PKC. However, our results with PMA indicate that perturbation of PKC activity affects basal apoA1 expression (Table 2). Similar results were recently demonstrated by Zheng et al. (2000), who showed that PKC modulates apoA1 promoter through the IRCE and the transcription factor SP1.

Inhibition of tyrosine kinase activity with tyrophostin A47 had no effect on the ability of acidosis to repress apoA1 promoter activity (Table 3). Repression of the apoA1 promoter with acidosis occurred to the same extent in the presence of both 10 and 100 µg/ml tyrophostin A47 in both HepG2 and Caco-2 cells (Table 3). These results are somewhat in contrast to those reported by Yamaji et al. (1994) who found that tyrosine kinase activity was necessary for acidosis to stimulate immediately early gene expression in renal epithelial cells.

The precise identity of this transcription factor is not known. However, within the nTRE/pH-RE there are two DNA–protein binding motifs (Quandt et al. 1995). The sequence TGAC is part of the core sequence recognized by AP1. AP1 activity is acutely regulated by oxidative stress (Arrigo 1999, Diamond et al. 1999, Rao et al. 1999), where it may be necessary for upregulating the expression of genes necessary for DNA repair and protection against the carcinogenic effects of oxidative by-products of metabolism (Xanthoudakis & Curran 1996, Primiano et al. 1997).

When combined with the glucocorticoid receptor on a composite glucocorticoid response element, AP1 can act as a repressor (Diamond et al. 1990, Miner & Yamamoto 1992). By itself on such an element, AP1 activity is stimulated by phorbol esters (Mordaq & Linzer 1989). However, in our studies PMA did not significantly influence the ability of acidosis to repress apoA1 promoter activity (Table 2). Furthermore, glucocorticoids have a stimulatory effect on the apoA1 promoter (Saladin et al. 1996, Hargrove et al. 1999), mediated through a glucocorticoid response element located in site A (Taylor et al. 1996a). These findings, although inconclusive, taken together suggest that AP1 is probably not the transcriptional repressor involved in mediating the effect of acidosis on the apoA1 promoter.

The core sequence ACCT is also a binding site for the δ-crystallin enhancer binding protein, δ-EF1 (Sekido et al. 1994). δ-EF1 inhibits gene expression by true transcriptional repression, and by displacing positive-acting DNA-binding transcription factors (Sekido et al. 1997). δ-EF1 binds both to the CACCTG site within the δ-crystallin minimal enhancer, where it prevents binding of the overlapping transcriptional activator δ-EF3 (Sekido et al. 1994), and to the E2-box sequence CACCTG (Sekido et al. 1994), where it may inhibit the
activity of basic region helix-loop-helix-containing transcription factors. δ-EF1 was expressed in most tissues examined (Sekido et al. 1996) and appears to be involved in regulating T-cell development (Higashi et al. 1997). Future experiments should determine whether or not δ-EF1 expression or activity, or both, is influenced by acidosis, and whether it is capable of binding the apoA1 pH-RE.

The present studies show that the acidosis-related reduction in apoA1 promoter activity is also reproduced in the presence of various agents known to alter intracellular pH. The changes observed in promoter activity are mediated by a transcriptional repressor. Future experiments should examine the precise identity of this repressor.

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