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# Statins, fibrates and retinoic acid upregulate mitochondrial acylcarnitine carrier gene expression

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ABSTRACT

In this study, we investigated the effects of statins, fibrates, 9-cis-retinoic acid and forskolin on the transcription of the mitochondrial carnitine/acylcarnitine carrier (CAC) gene. Statins, fibrates, retinoic acid and forskolin activate luciferase gene reporter activity driven by the −334/+3 bp region of the human CAC promoter containing wild-type (but not mutated) PPRE. These four agents also increase CAC transcript and protein levels. The combinations of statins and fibrates, retinoic acid and fibrates and fibrates and forskolin act synergistically. Mevalonate abolishes the activation of CAC gene expression by statins; the inhibitor of the PKA pathway H89 suppresses the stimulation of CAC gene expression by forskolin. Because CAC is essential for fatty acid β-oxidation, the above results on the regulation of CAC gene expression provide a novel contribution to the understanding of the hypolipidemic action of statins, fibrates and retinoic acid.



## Introduction

The carnitine/acylcarnitine carrier (CAC) is an integral protein of the inner mitochondrial membrane that transports acylcarnitine esters (in exchange for free carnitine) into mitochondria where the acyl groups are oxidized. It therefore plays an essential role in fatty acid β-oxidation, which is the major source of energy for heart and skeletal muscles during fasting and physical exercise. The function of CAC has been extensively investigated in mitochondria and in reconstituted liposomes [1,2 and references therein]. In man, CAC is encoded by the SLC25A20 gene [3,4] that maps to chromosome 3p21.31 [5]. CAC deficiency provokes a disorder of fatty acid β-oxidation (OMIM 212138) that presents two phenotypes: the first and more severe form with early onset in the neonatal period and the second milder form with onset in infancy [6 for a review]. Patients usually display a good correlation between phenotype and reduction of CAC activity.

In this study, we provide evidence that statins, fibrates, 9-cis-retinoic acid and forskolin upregulate the transcription of the human CAC gene via the PPRE site of its promoter. We also show that the combinations of statins and fibrates, retinoic acid and fibrates and forskolin and fibrates act synergistically on CAC gene expression.

## Materials and methods

*Construction of plasmids.* The human CAC gene promoter (chromosome 3p21.31) from −334 to +3 bp, containing wtPPRE (5′-CAAAAGTGGGTGAAAGGTCG-3′) or mutPPRE (5′-CAAAAGGGTGTGACAGGGCG-3′) in the −99/−80 positions, was amplified by PCR and cloned into the pGL3 basic-LUC vector (Promega) upstream of the LUC gene-coding sequence.

*Cell culture and transient transfection.* C9 rat hepatocytes (Sigma) were maintained in Ham's F12 containing 10% (v/v) heat-inactivated fetal calf serum and 2 mM L-glutamine at 37 °C in 5% $CO_2$. HepG2 (Sigma) and L6.C11 rat skeletal muscle myoblast (Sigma) cells were grown in high-glucose DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U penicillin and 100 μg/ml streptomycin at 37 °C in 5% $CO_2$. Transient transfection was performed as

*Abbreviations:* CAC, carnitine/acylcarnitine carrier; GW7647, 2-(4-(2-(1-cyclohexanebutyl)-3-cyclohexylureido)ethyl)phenylthio)-2-methylpropionic acid; LUC, luciferase; mutPPRE, mutated PPRE; PKA, protein kinase A; PPARα, peroxisome proliferator-activated receptor alpha; PPRE, peroxisomal proliferator response element; RXRα, retinoic X receptor alpha; wtPPRE, wild-type PPRE; WY-14643, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid.

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reported [7] using 0.5 μg of pGL3 basic-LUC vector containing the −334/+3 bp region of the CAC gene promoter, and 10 ng of pRL-CMV (Promega) to normalize the extent of transfection [8]. Transfected cells were assayed for LUC activity using the Dual-Luciferase® Reporter Assay System (Promega). Where indicated HepG2, C9 or L6.C11 cells were incubated for 24 h with WY-14643 (Sigma), GW7647 (Sigma), simvastatin (Tocris Bioscence), fluvastatin (Calbiochem), forskolin (Sigma), mevalonate (Sigma) and 9-cis-retinoic acid (Sigma) starting 24 h after having been depleted of serum; H89 (Sigma) was added 1 h before forskolin.

*Other methods.* Electrophoretic mobility shift assays (EMSA) were performed as described [9]. The double-stranded oligonucleotide probe was 5′-end labeled using T4 polynucleotide kinase and [$\gamma^{32}$P]-ATP at 37 °C for 30 min. The gels were dried and images acquired by phosphoimager (Bio-Rad). Total RNA was extracted from $1 \times 10^6$ cells, and reverse transcription was performed as reported [10]. Real-time PCR was carried out as described previously [11]. Assay-on-demand for human or rat CAC (Catalog Nos. Hs01088810_g1 or Rn01403582_m1, respectively) and for human or rat actin (Catalog Nos. Hs00357333_g1 or Rn00667869_m1, respectively) were purchased from Applied Biosystems. All transcript levels were normalized against the β-actin expression levels. For Western blot analysis, proteins were electroblotted onto nitrocellulose membranes (Bio-Rad) and subsequently treated with anti-CAC (specific for total rat mitochondrial CAC) [12], the β-subunit of $F_1$-ATPase (sc-58619, Santa Cruz) antibodies. The immunoreaction was detected by the ECL plus system (Amersham).

## Results

### *Statins and fibrates increase CAC mRNA levels*

To investigate the effects of statins and fibrates on CAC gene expression, HepG2 cells were incubated for 24 h with various concentrations of two statins, simvastatin and fluvastatin, and two fibrates, WY-14643 and GW7647. After incubation, total mRNA was extracted and used to determine CAC transcript levels. As shown in Fig. 1A, the CAC mRNA level in HepG2 cells was increased by both statins and fibrates in a dose-dependent manner. Fluvastatin and GW7647, which were the most effective in increasing CAC mRNA in HepG2 cells, were also tested on rat hepatocytes (C9) and myocytes (L6.C11) at a concentration of 100 μM and 1 μM, respectively (Fig. 1B). 1 μM GW7647 caused a CAC mRNA increase of 3.5-, 1.6- and 2.3-fold in hepatocytes, myocytes and HepG2 cells, respectively; and 100 μM fluvastatin an increase of 3.9-, 3.3- and 3.2-fold in hepatocytes, myocytes and HepG2 cells, respectively. Finally, when GW7647 and fluvastatin were used in combination a synergistic effect was observed (Fig. 1B). These results clearly indicate that both statins and fibrates increase the CAC transcript level in primary and secondary cell lines.

### *The human CAC promoter contains an active PPRE*

Because statins are known to act on gene transcription by activating PPARα or SREBP-2 [13,14], we checked for the presence of PPRE and SRE in the CAC gene promoter. A computer search of the vertebrate transfactor database (http://www.cbrc.jp/research/db/TFSEARCH.html) with the −5100/+3 bp region of the CAC gene revealed the presence of a PPRE sequence from −99 to −80 bp, which shares 80.9% identity with the canonical PPRE [15]. No SRE binding site was found. The protein-binding activity of the CAC promoter PPRE was investigated by EMSA experiments using HepG2 cell nuclear extracts and a labeled DNA probe from −105 to −75 bp encompassing the PPRE region. A band shift was observed which disappeared by competition with unlabeled probe containing wtPPRE, but not mutPPRE (results not shown), indicating that the CAC gene promoter contains a functional PPRE site.

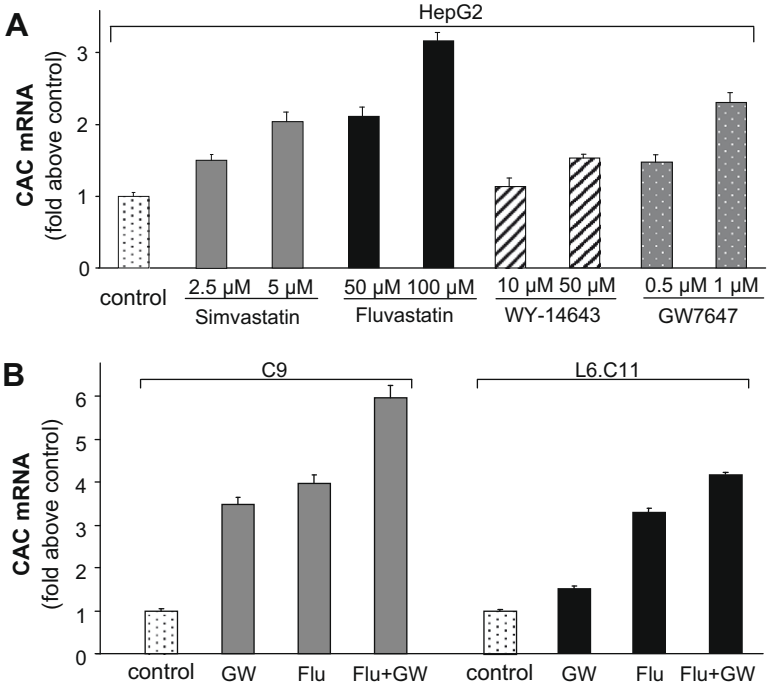


**Fig. 1.** Effects of statins and fibrates on CAC gene expression. (A) Total RNA extracted from HepG2 cells incubated with and without 2.5 or 5 μM simvastatin, 50 or 100 μM fluvastatin, 10 or 50 μM WY-14643 and 0.5 or 1 μM GW7647 for 24 h was used to quantify CAC mRNA by real-time PCR. (B) Total RNA extracted from rat hepatocytes (C9) and myocytes (L6.C11) incubated with or without 1 μM GW7647 (GW), 100 μM fluvastatin (Flu) or both for 24 h was used to quantify CAC mRNA by real-time PCR. In (A) and (B), means ± SD of three duplicate independent experiments are shown; except for 10 μM WY-14643 in (A), differences between samples and control (without any addition) were significant ($P < 0.05$, one-way ANOVA); in (B), differences between CAC mRNA levels in the presence of the combination fluvastatin + GW7647 and in the presence of either fluvastatin or GW7647 alone were also significant ($P < 0.05$, one-way ANOVA).

*The response of the human CAC promoter to fluvastatin and GW7647 is mediated by PPRE*

The promoter activity of the human CAC PPRE was tested by transfecting HepG2 cells with the pGL3 basic-LUC vector containing the −334/+3 bp region of the CAC gene with and without mutations in the PPRE site and by measuring LUC transgene expression activity. As shown in the control experiment of Fig. 2A, gene reporter activity was 5-fold higher in cells transfected with the pGL3 basic-LUC vector harboring wtPPRE (black bar) than with the vector harboring mutPPRE (gray bar).

To assess the involvement of PPRE in the statin/fibrate-mediated activation of CAC gene expression, HepG2 cells transfected with the wtPPRE-containing pGL3 basic construct were incubated with 1 μM GW7647 and/or 100 μM fluvastatin (Fig. 2A). GW7647 and fluvastatin alone increased LUC activity by 119% and 198%, respectively. Co-treatment of cells with fluvastatin + GW7647 triggered a synergistic rise of LUC activity by 304%. Moreover, to verify that the effect of statins on CAC gene expression was due to its pharmacological activity on 3-hydroxy-3-methylglutaryl-CoA reductase [16,17], we tested whether the addition of mevalonate, the product of 3-hydroxy-3-methylglutaryl-CoA reductase, could inhibit the statin-induced increase in CAC gene expression. Mevalonate suppressed both the fluvastatin-induced increase in LUC activity and the synergistic effect of fluvastatin + GW7647 (Fig. 2A). In the latter case, LUC activity was reduced to the level observed with GW7647-treated cells. In addition, the mevalonate + GW7647 combination did not affect the GW7647-induced increase in gene reporter activity (data not shown). Importantly, all the effects indicated by the black bars of Fig. 2A were not observed in cells transfected with the construct containing mutPPRE (gray bars) instead of wtPPRE. Consistently, CAC transcript and protein levels (Fig. 2B and C) were markedly increased in cells treated with GW7647 and fluvastatin alone or in combination as compared to untreated cells. The addition of mevalonate also abolished the effect of fluvastatin on CAC mRNA and protein levels (Fig. 2B and C). Taken together these data show that fluvastatin and GW7647 upregulate CAC gene expression via PPRE.

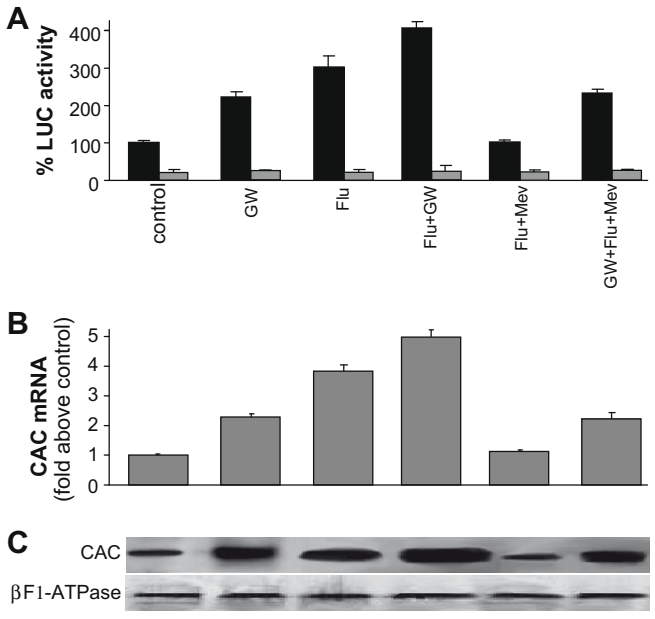


**Fig. 2.** Statin and fibrate stimulation of CAC gene expression via PPRE and effect of mevalonate on statin-induced CAC expression. (A) HepG2 cells, transfected with pGL3 basic-LUC vector containing the −334/+3 bp region of the CAC gene with (gray bars) or without (black bars) mutations in the PPRE, were incubated with or without 1 μM GW7647 (GW), 100 μM fluvastatin (Flu), both, the combination fluvastatin + 6 mM mevalonate (Mev) or the combination GW7647 + fluvastatin + mevalonate for 24 h, and assayed for LUC gene reporter activity. (B) Total RNA extracted from HepG2 cells, incubated as above, was used to quantify CAC mRNA by real-time PCR. (C) CAC and the β-subunit of ATPase of HepG2 cells, incubated as above, were immunodecorated with specific antibodies. In (A) and (B), means ± SD of three duplicate independent experiments are shown. In (A, black bars) and (B), differences between samples in the presence of GW7647, fluvastatin or fluvastatin + GW7647 and controls (without any addition) were significant ($P < 0.05$, one-way ANOVA). Differences between sample of the fluvastatin + GW7647 combination and the samples of fluvastatin or GW7647 alone were significant ($P < 0.05$, one-way ANOVA). However, differences between sample of the fluvastatin + mevalonate combination and control (without any addition) and between sample of the GW7647 + fluvastatin + mevalonate combination and sample of GW7647 alone were not significant.

*PPARα phosphorylation enhances human CAC gene expression*

Because phosphorylation influences PPARα activity [18], we assessed whether the PKA pathway activator forskolin, alone or in combination with GW7647, affects CAC gene expression. When cells were transfected with the wtPPRE-containing pGL3 basic-LUC vector (Fig. 3A, black bars), forskolin and GW7647 stimulated gene reporter activity by 49% and 138%, respectively, compared to control. Combined forskolin + GW7647 treatment further enhanced LUC activity by 1.9-fold as compared to cells treated with GW7647 alone. PKA pathway involvement in CAC gene expression was further demonstrated by adding H89, a specific inhibitor of PKA [19]. H89 repressed LUC activity which was increased by forskolin and the GW7647 + forskolin combination (Fig. 3A, black bars). In the latter case, LUC activity was diminished to the level found with GW7647

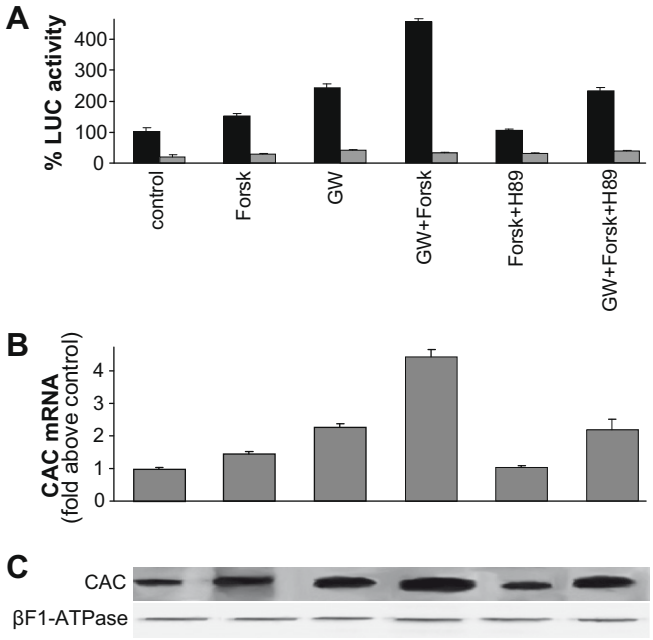


**Fig. 3.** Effect of forskolin on basic and fibrate-induced CAC gene expression and inhibition by H89. (A) HepG2 cells, transfected as in Fig. 2A and incubated with or without 200 μM forskolin (Forsk), 1 μM GW7647 (GW), both, the combination forskolin + 100 μM H89 or the combination GW7647 + forskolin + H89, were assayed for LUC gene reporter activity. (B) Total RNA extracted from HepG2 cells, incubated as above, was used to quantify CAC mRNA by real-time PCR. (C) CAC and the β-subunit of ATPase of HepG2 cells, incubated as above, were immunodecorated with specific antibodies. In (A) and (B), means ± SD of three duplicate independent experiments are shown. In (A, black bars) and (B), differences between samples in the presence of forskolin, GW7647 or GW7647 + forskolin and controls (without any addition) were significant ($P < 0.05$, one-way ANOVA). Differences between sample of the GW7647 + forskolin combination and samples of GW7647 or forskolin alone were significant ($P < 0.05$, one-way ANOVA). However, differences between the forskolin + H89 combination sample and control (without any addition) and between the GW7647 + forskolin + H89 combination sample and GW7647 alone sample were not significant.

alone. Of note, forskolin and GW7647 treatment, alone or in combination, did not affect the gene reporter activity in cells transfected with the mutPPRE-containing pGL3 basic vector (Fig. 3A, gray bars). In agreement with these results, Fig. 3B and C show that forskolin and GW7647 also enhanced CAC transcript and protein levels, whereas H89 abolished the forskolin-dependent increase in CAC transcript and protein levels. These results demonstrate that the PKA pathway plays a role in the activation of CAC gene expression.

*Effect of 9-cis-retinoic acid on human CAC gene expression*

As retinoic X receptor alpha (RXRα) and PPARα form a heterodimer that is essential for binding to PPRE and its activation [20], we tested whether 9-cis-retinoic acid affects CAC gene expression. 9-Cis-retinoic acid increased LUC gene reporter activity 1.9-fold in cells transfected with the pGL3 basic vector containing wtPPRE as compared to control (Fig. 4A, black bars). No effect was observed in cells transfected with mutPPRE-containing pGL3 basic vector (Fig. 4A, gray bars). Combination treatment with GW7647 + 9-cis-retinoic acid resulted in a still greater stimulation of gene reporter activity. These results were confirmed at CAC transcript and protein levels (Fig. 4B and C), demonstrating that 9-cis-retinoic acid, the ligand of RXRα, enhances CAC gene expression.

## Discussion

Statins and fibrates are widely used in the treatment of hypercholesterolemias and hyperlipidemias [21–23]. CAC is key to fatty acid β-oxidation because it catalyzes the entry of fatty acid acyl groups into the mitochondrial matrix where the enzymes of fatty acid β-oxidation are located. The experimental data presented in this study demonstrate that the transcription of the human CAC gene is enhanced by statins and fibrates. The upregulation of CAC gene expression by both drugs is demonstrated by the observation that they stimulate LUC gene reporter activity in HepG2 cells transfected with the LUC vector harboring the −334/+3 bp region of the human CAC promoter. Furthermore, CAC gene transcriptional activation by these two agents is documented by the increase in levels of both CAC transcript and protein. The fact that the effects of both statins and fibrates on gene reporter activity are abolished by mutations in the PPRE site of the CAC gene clearly indicates that CAC transcriptional activation by these drugs is mediated by the PPRE regulatory element present in the human CAC promoter from −99 to −80 bp. This conclusion is also supported by the effect of 9-cis-retinoic acid, because it is known that PPRE is activated when bound by the PPARα–RXRα heterodimer. Both statins and fibrates activate the PPARα transcription factor. However, fibrates exert their effect by binding to PPARα, whereas statins by inhibiting the Rho-signaling pathway as a result of their inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase and, hence, of the decrease of farnesyl and geranyl levels which control the Rho-signaling pathway. Proof of this statin mechanism of action is given by the observation that the effect of statin on CAC gene expression is abolished by mevalonate (Fig. 2), which is able to generate farnesyl and geranyl in the presence of statins. That statins and fibrates activate PPARα through different mechanisms is shown by the fact that their combination results in an additional increase in CAC gene expression, i.e., they act in a synergistic fashion. Moreover, the activation of CAC gene transcription by forskolin and its inhibition by H89 provide evidence that the PKA pathway regulates CAC gene expression by a different mechanism – phosphorylation of PPARα – from those exerted by statins and fibrates.

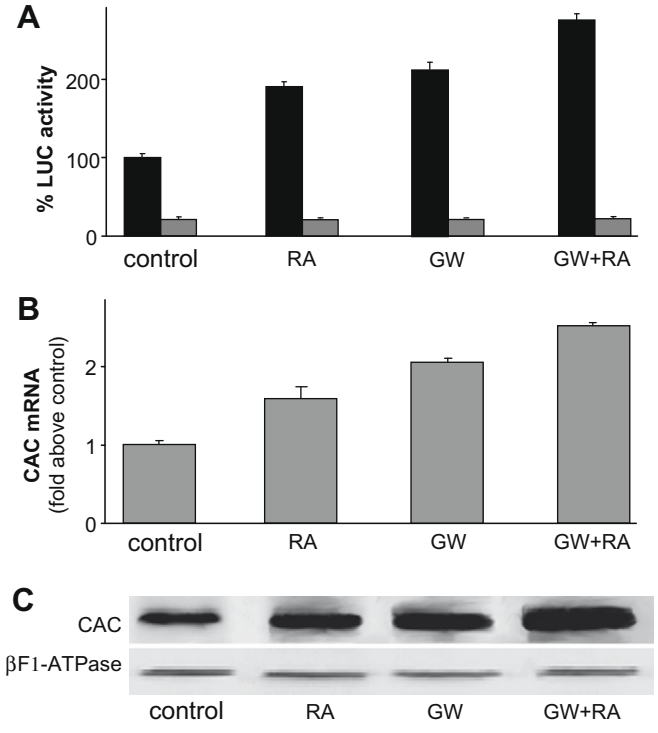


**Fig. 4.** Effect of 9-cis-retinoic acid on CAC gene expression. (A) HepG2 cells, transfected as in Fig. 2A and incubated with or without 1 μM 9-cis-retinoic acid (RA), 1 μM GW7647 (GW), or the combination of 9-cis-retinoic acid and GW7647, were assayed for LUC gene reporter activity. (B) Total RNA extracted from HepG2 cells, incubated as above, was used to quantify CAC mRNA by real-time PCR. (C) CAC and the β-subunit of ATPase of HepG2 cells, incubated as above, were immunodecorated with specific antibodies. In (A) and (B), means ± SD of three duplicate independent experiments are shown. In (A, black bars) and (B), differences between samples and controls (without any addition) were significant ($P < 0.05$, one-way ANOVA); differences between sample of the GW7647 + 9-cis-retinoic acid combination and samples of GW7647 or 9-cis-retinoic acid alone were significant ($P < 0.05$, one-way ANOVA).

The results of the present investigation on the regulation of CAC gene expression extend our knowledge of the molecular mechanisms by which statins, fibrates and retinoic acid exert hypolipidemic action. Furthermore, we propose that patients affected by CAC deficiency, who present a mild phenotype with some residual activity [24–28] and for whom pharmacological strategies are at present very limited [6], might benefit from treatment with statins and fibrates acting via stimulation of CAC gene expression. Indeed, this approach has been used successfully for other disorders of fatty acid β-oxidation [29,30]. While writing this work, Gutgesell et al. [31] reported that fibrates upregulate the expression of mouse CAC gene, a finding confirmed by the present study. Our data further demonstrate for the first time that (i) statins activate CAC gene transcription, (ii) the effects of statins and fibrates on CAC transcription are synergistic, (iii) retinoic acid and the activator of the PKA pathway forskolin stimulate CAC gene expression, and (iv) the combinations of fibrates and retinoic acid or forskolin on CAC transcription are synergistic.

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