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Biochemical and Biophysical Research Communications 299 (2002) 135–141
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β-Adrenergic stimulation controls the expression of a thioesterase specific for very-long-chain fatty acids in perfused hearts

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Received 15 October 2002

Abstract

Arachidonic acid is not freely stored in the cells. A number of different pathways for the mobilization of this compound have been proposed, including a novel mechanism that involves the release of arachidonic acid from arachidonoyl-CoA by a thioesterase with substrate specificity for very-long-chain fatty acids. In rat heart, the acyl-CoA thioesterase activity can be regulated by a mechanism that involves β-adrenoceptors. In this paper we demonstrate that β-adrenergic agonists also regulate the acyl-CoA thioesterase mRNA levels. Isoproterenol (10^{-7} M)—a concentration known to exert physiological responses—increases in a time-dependent manner the acyl-CoA thioesterase mRNA levels, an effect blocked by a specific β-adrenoceptor antagonist. In addition, our results show that cAMP is involved in this process. The acyl-CoA thioesterase mRNA levels are also increased by fasting, but not by di(2-ethylhexyl)phthalate, a peroxisome proliferator. These results may suggest the existence of a β-adrenoceptor-activated regulatory pathway for arachidonic acid release in cardiac tissue.
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Keywords: Acyl-CoA thioesterase; Arachidonic acid; Cardiac tissue; Heart perfusion; Isoproterenol; mRNA levels

Arachidonic acid and its metabolites are critical to a variety of biological processes in cardiac tissue [1]. There are several potential pathways by which arachidonic acid release can be effected by hormonal stimulation. It has been reported that α-adrenergic [2] and muscarinic agonists [3], angiotensin II [4], bradykinin [5], endothelin [6], TNF-α, and IL-1β [7] evoke arachidonic acid release in heart. These agonists are stimulus for phospholipase A_2 and phospholipase C, both of which may provide a source of free arachidonic acid, an essential step in the generation of eicosanoids. It was also reported that β-adrenergic agonists stimulate arachidonic acid release in mammalian heart [8], but the mechanism by which arachidonic acid is released is not well documented.

We have recently proposed a novel mechanism that involves the release of arachidonic acid from acyl-CoAs by hydrolysis of the thioester linkage of the fatty acid to CoA by a thioesterase with substrate specificity for very-long-chain fatty acids including arachidonoyl-CoA [9]. This acyl-CoA thioesterase has first been described in steroidogenic tissues, acting in the regulation of steroid synthesis through the release of arachidonic acid [10]—a well-known intermediary in this pathway—and it was named arachidonic acid-related thioesterase involved in steroidogenesis (ARTISt) [9]. This protein resulted to be 100% and 92% homologous to very-long-chain acyl-CoA thioesterases isolated from liver mitochondria (MTE-I) and cytosol (CTE-I), respectively, that can be induced by peroxisome proliferators [11,12].

The enzymatic activity of the acyl-CoA thioesterase in adrenal cells is controlled by a hormone-regulated protein phosphorylation mechanism, which may then constitute an alternative regulatory pathway for the release of arachidonic acid. In accordance with the postulated role for this enzyme in steroidogenesis, the acyl-CoA thioesterase is under ACTH regulation in adrenal gland, which activates the protein by

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PII: S0006-291X(02)02596-2

phosphorylation [13]. The presence of the mRNA for the acyl-CoA thioesterase was detected in all steroidogenic tissues and it was described to be a very early regulation of the acyl-CoA thioesterase transcript. This regulation is rapid and transient since ACTH affects the amount of transcript as early as 5 min, returning to basal levels in 30 min [9].

The acyl-CoA thioesterase is also expressed in non-steroidogenic tissues such as heart, liver, and kidney [13]. We have demonstrated that in cardiac tissue the activity of the acyl-CoA thioesterase is regulated by β-adrenergic agonists [14]. Using a recombination cell free assay we verified that isoproterenol could regulate the enzymatic activity of the acyl-CoA thioesterase in a mechanism that involves β-adrenoceptors. Moreover, antibodies against the lipase serin-motif and the Cys residue present in the catalytic domain of the acyl-CoA thioesterase blocked the activity present in extracts from isoproterenol-perfused hearts [14]. In cardiac tissue, β-adrenergic stimulation increases cytosolic cAMP, inducing metabolic and trophic effects [15,16]. Apart from these functional actions, it is also involved in the regulation of β-adrenergic function via receptor protein phosphorylation, the modulation of the transcription rate of a variety of cAMP-responsive genes, and the expression of different myocardial regulatory protein syntheses [17].

Taking into account that in steroidogenic tissues, trophic hormones not only regulate the enzymatic activity of the acyl-CoA thioesterase, but also its messenger levels [9], it was of our interest to investigate if β-adrenergic agonists could also regulate the mRNA levels of the acyl-CoA thioesterase in cardiac tissue. This paper describes the regulation by β-adrenergic agonists on the levels of the messenger of the acyl-CoA thioesterase and we demonstrate that the mRNA abundance is controlled by β-adrenoceptor agonists and fasting, but not by di(2-ethylhexyl)phthalate (DEHP), a peroxisome proliferator.

Materials and methods

Materials. Isoproterenol, propranolol, actinomycin D, 8 Br-cAMP, and DEHP were purchased from Sigma (St. Louis, MO, USA). All other reagents were commercial products of the highest grade available.

Production of polyclonal antibodies against recombinant cytosolic acyl-CoA thioesterase. Sequence comparisons of CTE-I and MTE-I showed 92% similarity at the aminoacid level [12]. In view of the high structural similarity, and of the very low yield obtained after purification of recombinant MTE-I, we have used recombinant CTE-I for antibody generation. The obtained rabbit serum recognizes both recombinant CTE-I and MTE-I [32]. Rabbits were injected once with 500 μg recombinant CTE-I [18] as antigen dissolved in 0.5 ml distilled water and mixed with equal volumes of Freund's complete adjuvant, and three times with 500 μg antigen mixed with equal volumes of Freund's incomplete adjuvant. Antibody titer against recombinant CTE-I was determined by ELISA.

Western blot. For Western blot analysis, aliquots (20 μg of protein) of the different subcellular fractions obtained as previously described [14] were loaded and resolved on a 10% SDS–polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membranes using 25 mM Tris, 192 mM glycine, pH 8.3, containing 20% methanol. Membranes were then incubated with the antibody against recombinant CTE-I (1/4000). Antibody binding was visualized by the chemiluminescence detection system (ECL, Amersham Pharmacia Biotech. Inc.) and autoradiography.

Immunohistochemistry. A group of rats were anesthetized with ether and perfused transcardially with saline followed by ice-cold 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS), pH 7.4 [19]. Hearts were removed, post-fixed with paraformaldehyde for 1 day at 10 °C, and then cryoprotected overnight in the same fixative supplemented with 30% sucrose. The tissue was sectioned at 16 μm thickness in a cryostat, thaw-mounted onto gelatinized glass slides, and processed for indirect immunohistochemstry. Briefly, sections were rinsed in PBS and incubated with blocking solution (1.5% goat serum in 0.3% Triton X-100 PBS) for 1 h at room temperature and incubated with rabbit polyclonal antibodies against recombinant acyl-CoA thioesterase (1/100), or vehicle (control) in a humidified chamber for 24 h at 4 °C. Detection of the primary antibody was done by means of a cy3-conjugated goat antirabbit IgG (Molecular Probes, Eugene, OR). After rinsing with PBS, the sections were mounted in FluorSave reagent (Calbiochem, La Jolla, CA) and examined in an Olympus BX-50 epifluorescence microscope. Kodak T-400 C41 film was used for photography.

Heart perfusion. Adult Wistar rats (200–300 g) were used throughout. Animals were anesthetized with ether and received heparin. The thorax was opened and the heart was removed and placed in Krebs–Henseleit buffer (KHB) containing 114 mM NaCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4.7 mM KCl, 25 mM $NaHCO_3$, and 5.5 mM glucose, equilibrated with 95% oxygen–5% CO_2. Hearts were perfused via the aorta according to the method of Langendorff, as previously described [14], with isoproterenol (10^{-7} M), 8 Br-cAMP (10^{-4} M), or with propranolol (10^{-5} M) prior to isoproterenol perfusion. Control hearts were perfused only with KHB.

Northern blot. Total RNA from cardiac and hepatic tissue was extracted by the method of Chomczynski [20]. RNA samples (24 μg) were denatured at 65 °C for 15 min in 45% formamide/5.4% formaldehyde and electrophoresed at room temperature on a 1.2% agarose/2.2 M formaldehyde gel. RNA was transferred by capillarity to nylon membranes in 20× SSC (1× SSC: 150 mM NaCl, 15 mM sodium citrate) and fixed by baking of the membranes for 2 h at 80 °C. Blots were prehybridized for 4–5 h at 42 °C in 10 ml of 50% formamide, 1% SDS, 1× Denhardt's reagent, 5× SSC, and 100 μg/ml denatured salmon sperm DNA. Hybridization was performed overnight at 42 °C as previously described [9] and detected by autoradiography using intensifying screens for 18–72 h at −80 °C.

In vitro recombination heterologous recombination assay. The activity of cardiac acyl-CoA thioesterase was analyzed using an in vitro recombination assay as described before [14].

Statistical analysis. Results are shown as means ± SEM. Statistical significance was evaluated using Student's t test or ANOVA followed by Tukey's test; $P < 0.05$ was considered significant.

Results

In a previous work [14], we showed that a specific acyl-CoA thioesterase for very-long-chain fatty acids and its mRNA are expressed in cardiac tissue. Here, we studied the subcellular localization of the enzyme by Western blot analysis of the different fractions obtained by centrifugation. The 43 kDa band corresponding to

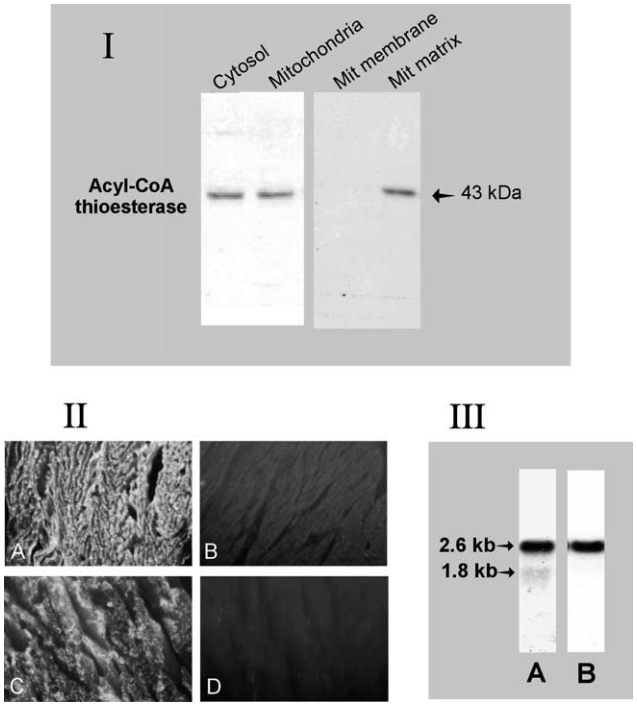

Fig. 1. Detection of the acyl-CoA thioesterase in rat heart. Panel I: Subcellular localization of the enzyme. Aliquots of subcellular fractions of rat heart were analyzed by immunoblotting with a polyclonal antibody against rat acyl-CoA thioesterase (1/4000). Panel II: Immunohistochemical localization of the acyl-CoA thioesterase in cardiac cells. Sections of rat heart tissue were incubated with an antibody against recombinant acyl-CoA thioesterase (1/100) (A,C) or vehicle (B,D) and detected as described in Materials and methods. Magnification: (A) and (B) 200×; (C) and (D) 600×. Panel III: Detection of the acyl-CoA thioesterase mRNA. Total RNA from cardiac tissue (24 μg) was analyzed by Northern blot using a 795 bp fragment of the acyl-CoA thioesterase cDNA as probe (A). The membrane was then stripped and analyzed with a cDNA probe corresponding to the 5′ end of the open reading frame encoding the putative mitochondrial leader sequence of the acyl-CoA thioesterase. Exposure time: 48 h.

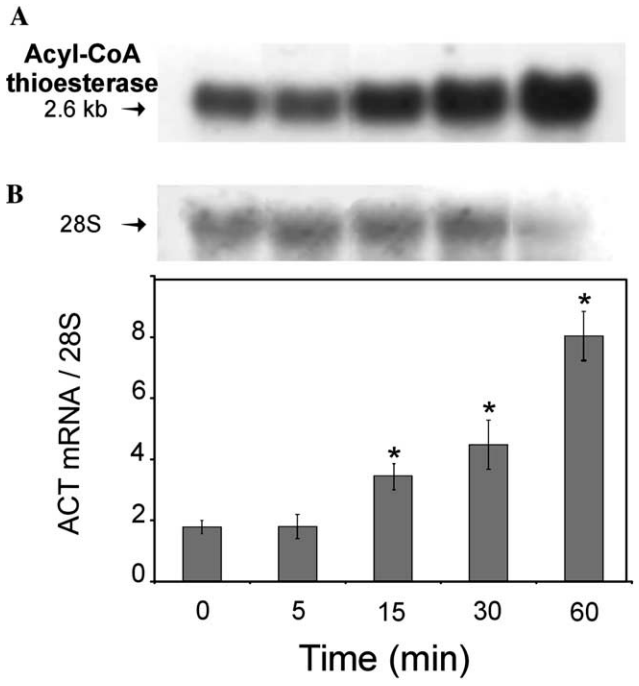

Fig. 2. Time-course effect of isoproterenol. Rat hearts were perfused for the times indicated with isoproterenol (10^{-7} M). Total RNA (24 μg) was analyzed by Northern blot with a 795 bp fragment of the acyl-CoA thioesterase cDNA (A)—after a 48 h period of exposure—or 28S rRNA (B) as probes. The histogram shows data representing the signal integration quantitated by densitometric scanning of the acyl-CoA thioesterase (ACT) mRNA signal normalized to 28S rRNA. Each value represents the mean (±SEM) of five independent experiments. *Significantly different from control values ($P < 0.05$).

the acyl-CoA thioesterase was detected in the cytosol and in the mitochondrial matrix (Fig. 1, panel I). To study if the enzyme is present in cardiac muscular cells, immunohistochemical staining of sections prepared from rat heart tissue was performed. As shown in Fig. 1 (panel II), immunoreactivity was detected using specific antibodies against the acyl-CoA thioesterase.

The presence of the acyl-CoA thioesterase mRNA was detected in heart as two transcripts of 2.6 and 1.8 kb by Northern blotting using a 795 bp PCR amplification product as a probe [14]. To detect the specific MTE-I mRNA, a cDNA probe corresponding to the 5′ end of the open reading frame encoding the putative mitochondrial leader sequence was used. Using that probe, we have detected the mRNA encoding the mitochondrial isoform in cardiac tissue as a single transcript of 2.6 kb (Fig. 1, panel III).

The following experiments were conducted to study the effect of the β-adrenergic agonist isoproterenol on the levels of the acyl-CoA thioesterase mRNA. The abundance of the mRNA was analyzed by Northern blotting when rat hearts were perfused with isoproterenol 10^{-7} M for 0, 5, 15, 30, and 60 min. Isoproterenol increased the abundance of the 2.6 transcript as early as 15 min (Fig. 2). The intensity of the 1.8 kb transcript was very low and no difference could be detected.

Despite the observed increase in messenger abundance, no increase in protein expression could be detected (results not shown). In view of the high amount of protein present in the control, one possible explanation is that the sensitivity of the method employed is not sufficiently high to detect small increases in protein abundance.

To analyze the possibility that cardiac acyl-CoA thioesterase mRNA is regulated via a β-adrenoceptor-associated mechanism, rat hearts were perfused with the β-adrenergic receptor antagonist propranolol (10^{-5} M) for 15 min or KHB, and then for another 15 min with isoproterenol 10^{-7} M or KHB. As shown in Fig. 3, the effect of isoproterenol was blocked by the addition of propranolol.

We also investigated if the second messenger cAMP is involved in the regulation of the levels of the 2.6 kb

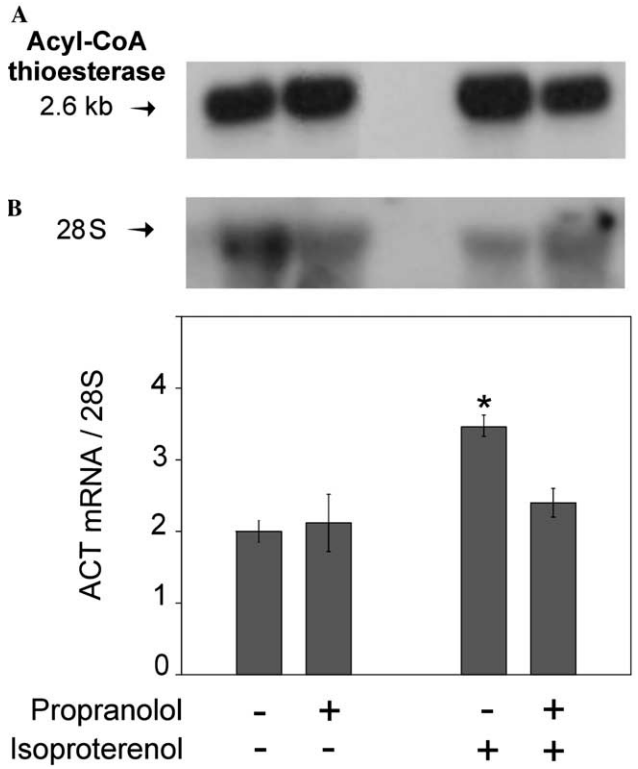

Fig. 3. Effect of isoproterenol in propranolol perfused hearts. Rat hearts were perfused with propranolol 10^{-5} M for 15 min or KHB and then for another 15 min with isoproterenol 10^{-7} M or KHB. Total RNA (24 μg) was analyzed by Northern blot with a 795 bp fragment (A)—after a 72 hours period of exposure—or 28S (B) as probes. The histogram shows data representing the signal integration quantitated by densitometric scanning of the acyl-CoA thioesterase (ACT) mRNA signal normalized to 28S rRNA. Each value represents the mean (±SEM) of four independent experiments. *Significantly different from control values ($P < 0.05$).

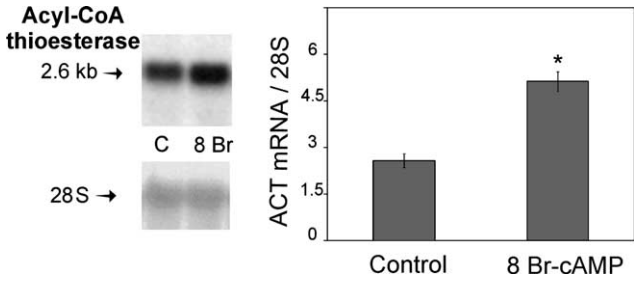

Fig. 4. Effect of 8 Br-cAMP. Rat hearts were perfused with 8 Br-cAMP 10^{-4} M or KHB for 15 min. Total RNA was isolated from cardiac tissue and Northern blot was carried out as already described. The histogram shows data representing the signal integration quantitated by densitometric scanning of the acyl-CoA thioesterase (ACT) mRNA signal normalized to 28S rRNA. Each value represents the mean (±SEM) of four independent experiments. *Significantly different from control values ($P < 0.05$). Exposure time: 48 h.

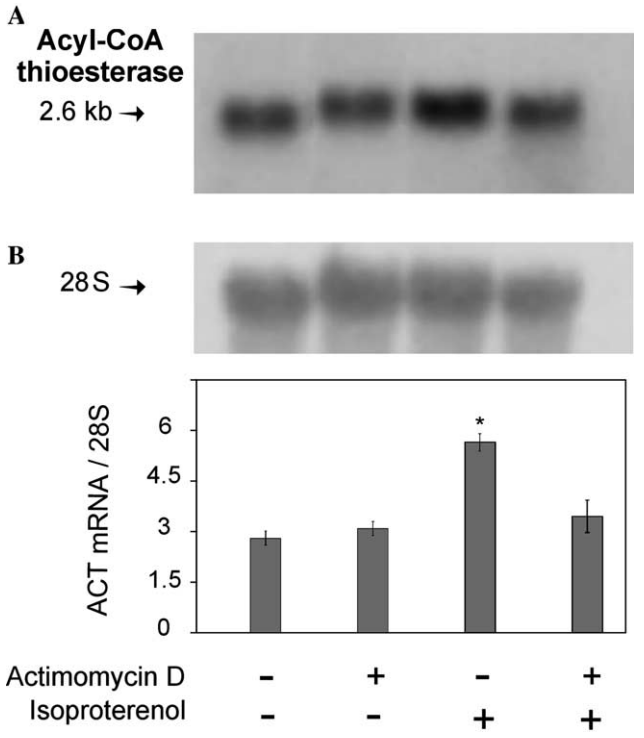

Fig. 5. Effect of actinomycin D. Rats were injected with actinomycin D (500 μg/kg) or its vehicle 45 min before heart perfusion. Cardiac perfusion was carried out with isoproterenol 10^{-7} M or KHB. After perfusion, total RNA (24 μg) was analyzed by Northern blot with a 795 bp fragment (A)—after a 36 h period of exposure—or 28S (B) as probes. The histogram shows data representing the signal integration quantitated by densitometric scanning of the acyl-CoA thioesterase (ACT) mRNA signal normalized to 28S rRNA. Each value represents the mean (±SEM) of four independent experiments. *Significantly different from control values ($P < 0.05$).

transcript. For this purpose, rat hearts were perfused with KHB for 15 min or 8 Br-cAMP, a permeable analog of the messenger. We observed that the levels of the mRNA were significantly increased by 8 Br-cAMP perfusion (Fig. 4).

It seems likely that the effects of isoproterenol on the expression of cardiac mitochondrial acyl-CoA thioesterase in vivo are due to enhanced transcription. Thus, rats were injected with actinomycin D (500 μg/kg) or its vehicle 45 min before heart perfusion. Cardiac perfusion was then carried out with isoproterenol or KHB. The levels of mRNA were reduced in actinomycin D treated rats (Fig. 5), suggesting that the transcript levels are controlled, at least in part, by an increase in transcription.

To study the influence of actinomycin D on the regulation of enzyme activity, an in vitro heterologous recombination assay was performed. We observed an inhibition of progesterone production when actinomycin D was administered prior to isoproterenol stimulation (Fig. 6). This demonstrates a correlation between the expression of the messenger and the activity of the enzyme.

Other authors [21] have reported that in mice liver and heart, fasting increases the mitochondrial and cy-

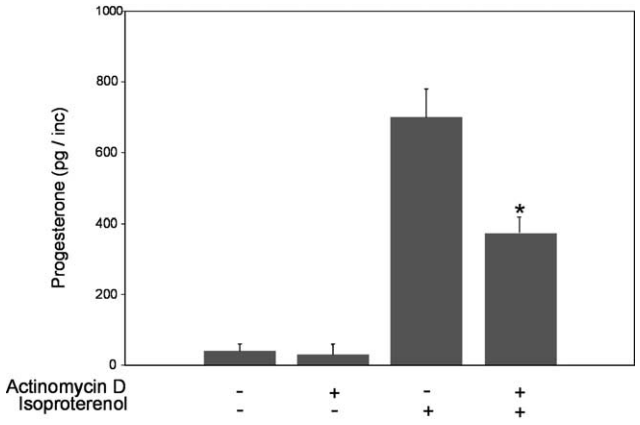

Fig. 6. Effect of actinomycin D on acyl-CoA thioesterase activity. Rats were injected with actinomycin D (500 μg/kg) or its vehicle 45 min before heart perfusion. Cardiac perfusion was carried out with isoproterenol 10^{-7} M or KHB. After perfusion, cardiac acyl-CoA thioesterase was incubated with adrenal mitochondria and microsomes and the capacity to stimulate progesterone synthesis in an in vitro heterologous recombination assay was evaluated. Each value represents the mean (±SEM) of three independent experiments. Expressed as pg of progesterone per incubation. *Significantly different from isoproterenol values ($P < 0.05$).

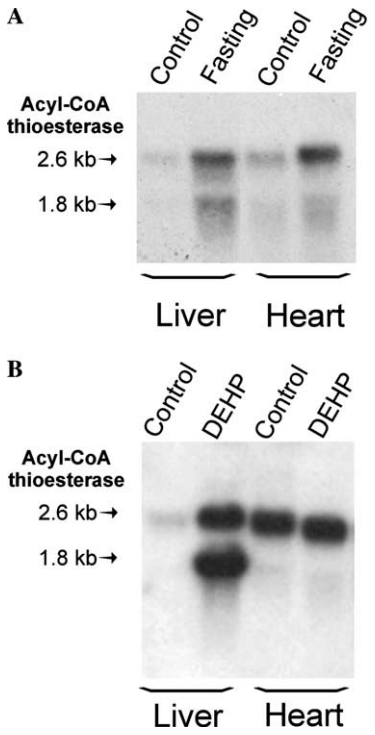

Fig. 7. Regulation of the acyl-CoA thioesterase mRNA expression in heart and liver by fasting and DEHP treatment. (A) A group of rats were fasted for 48 h (fasted). Other group was fed with a normal chow diet ad libitum (control). (B) Rats were fed with 2% (w/w) DEHP for 1 week or with normal diet (control). In both cases, livers and hearts were excised and total RNA was isolated from these tissues and analyzed by Northern blot with the 795 bp fragment. Exposure time: (A) 18 h, (B) 72 h.

tosolic isoforms. They also report an increase of both isoforms in liver with peroxisome proliferator treatment [11]. We investigated the effect of both treatments on the mRNA expression in rat hepatic and cardiac tissue. A group of rats were fasted for 48 h or fed with a normal chow diet ad libitum. The heart and the livers from fasted and fed animals were excised and total RNA was obtained and analyzed by Northern blot. As shown in Fig. 7A, the abundance of both messengers is increased in liver and heart by fasting. Otherwise, rats were fed with 2% (w/w) DEHP for 1 week or with normal diet. Livers and hearts were excised and total RNA was also analyzed by Northern blot. While the 2.6 and 1.8 kb transcripts are strongly induced in liver by peroxisome proliferator treatment, no change was observed in the cardiac messenger (Fig. 7B).

Discussion

Despite a wide role of arachidonic acid and its metabolites in heart physiology, little is known about the mechanism of intracellular arachidonic acid regulation in heart tissue by β-adrenergic agonists.

We have previously identified a 43 kDa phosphoprotein from adrenal gland which plays an obligatory role in the activation of steroidogenesis through the release of arachidonic acid [10]. This protein has been characterized as a thioesterase and it is member of a highly conserved multigene family involved in lipid metabolism. The four genes described show a very conserved structural organization and encode for a mitochondrial (MTE-I), a cytosolic (CTE-I), and two peroxisomal (PTE-Ia and PTE-Ib) thioesterases [21]. In this paper we show that the mitochondrial and cytosolic isoforms of the acyl-CoA thioesterase family are expressed in cardiac tissue and immunohistochemical staining of sections prepared from rat heart tissue indicates that they are present in cardiac cells.

We have previously demonstrated that in cardiac tissue the acyl-CoA thioesterase activity can be regulated by a mechanism that involves β-adrenoceptors [14]. In addition to the stimulation of the activity, here we show that β-adrenergic agonists regulate the acyl-CoA thioesterase mRNA levels. In the present report we demonstrate that the β-adrenergic agonist isoproterenol increases the abundance of the mRNA of the mitochondrial thioesterase. We are confident on the specificity of the effect of isoproterenol in this study, since the effect was time-dependent, in a concentration known to exert physiological responses and blocked by a specific β-adrenoceptor antagonist. In addition, the results show that cAMP is involved in this process.

It has been reported that β-adrenergic stimulation increases the expression of a variety of genes. Among others, it has been described about the induction of

c-fos, NGF1-A [22], cAMP response element binding protein (CREB-P) [23], and β_1-adrenergic receptor [24] mRNAs by short-term exposure to β-adrenergic agonists. This study provides the first evidence for the regulation of the expression of the acyl-CoA thioesterase gene by a β-adrenoceptor-associated mechanism.

The question then arises as to why β-adrenoceptor stimulation elicits the expression and the activity of the acyl-CoA thioesterase in cardiac tissue. Interestingly, the presence of several types of acyl-CoA synthetases with long-chain fatty acid specificity in cardiac tissue of different species has recently been described [25–27]. An acyl-CoA binding protein, which binds medium- and long-chain acyl-CoA esters with high affinity, has also been detected [28]. It is then possible that in cardiac tissue, the acyl-CoA thioesterase is involved in a new mechanism that controls the intracellular levels of arachidonic acid. Further studies have to be performed to elucidate this question.

The mitochondrial and cytosolic acyl-CoA thioesterase mRNAs from heart are regulated by fasting as it occurs in liver. Recent reports have identified the PPARα as the mediator in the adaptative response to fasting [29]. Hunt et al. have identified a significant involvement of the PPARα in modulating the expression of the acyl-CoA thioesterase genes at the transcriptional level in liver and heart. Using PPARα-null mice, they show that the mitochondrial and cytosolic acyl-CoA thioesterases are dependent on the PPARα signalling pathway. However, CTE-I is also induced in a PPARα-independent manner during fasting, suggesting that other signalling pathways that control the expression of some genes involved in lipid metabolism may be activated in the fasting state [29].

Although conflicting results have been published in relation to noradrenaline turnover during fasting [30,31] our work shows that a β-adrenoceptor mediated mechanism could also be involved in the increase of the transcript during a 48 h fasting.

The effect of the peroxisome proliferator DEHP on the expression of the acyl-CoA thioesterase is likely to be liver-specific, since no differences were found on mRNA levels in cardiac tissue.

Taken together, our current data indicate the presence of a new pathway to regulate intracellular levels of arachidonic acid in cardiac tissue. This study further suggests a new concept in the regulation of intracellular levels of arachidonic acid, in which trophic hormones can release arachidonic acid by a mechanism different than the classical PLA_2-mediated pathway.

Acknowledgments

This work was supported by grants from Universidad de Buenos Aires, Agencia Nacional de Promoción Científica y Tecnológica and Fundación Roemmers. The critical reading of the manuscript by Dr. Carlos Mendez is greatly appreciated. Our thanks are also due to Dr. S.E.H. Alexson, Karolinska Institute, Sweden, for the cDNA probe corresponding to the 5′ end of the open reading frame encoding the putative mitochondrial leader sequence

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