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# Cholecalciferol affects cardiac proteins regulating malonyl-CoA availability and intracellular calcium level

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**Abstract.** Cholecalciferol improves insulin signaling and glucose metabolism in the heart and reduces circulating non-esterified fatty acids. Cholecalciferol effects on the cardiac fatty acid (FA) metabolism and the consequences on calcium handling were examined. Blood lipid profile was determined. Western blot and qRT-PCR were used to examine protein and mRNA expression. Cholecalciferol-treated rats had increased acetyl CoA carboxylase 2 protein expression and decreased expression of malonyl CoA decarboxylase. In addition, the expression of uncoupling protein 3 was elevated. Also, the level of peroxisome proliferator-activated receptor-gamma coactivator in the nucleus of heart cells was increased along with the level of sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ATPase in the microsomal fraction. In parallel, the L-type calcium channel and ryanodine receptor expression was reduced. In the heart of healthy rats, cholecalciferol affects proteins regulating malonyl CoA availability and intracellular $Ca^{2+}$ handling proteins.

**Key words**: Vitamin D — Heart — Fatty acid metabolism — Calcium handling

## Introduction

Metabolic flexibility is a fundamental feature of the heart's energy metabolism, necessary to fulfill its function. It implies the existence of highly efficient metabolic machinery that allows the use of different substrates, depending on their availability (Bertero and Maack 2018). Under normal conditions, the heart mainly uses energy from fatty acids (FA) oxidation. At the same time, a smaller share is made up of energy obtained by the oxidation of pyruvate formed in glycolysis as well as by the oxidation of lactate (Bertero and Maack 2018).

The heart primarily obtains FA from circulation. Upon entering the cardiomyocytes, most of the FA is esterified into fatty acyl-CoA and transported to the mitochondria (Bayeva et al. 2013). Finally, FA enter β-oxidation in mitochondria to produce acetyl-CoA, which enters the tricarboxylic acid cycle for ATP production (Bayeva et al. 2013).

Continuous contractile activity of the heart requires high energy expenditure and precise regulation of intracellular $Ca^{2+}$ concentration. Rhythmic contractions and relaxations of cardiomyocytes require cyclic changes in intracellular $Ca^{2+}$ concentration and are regulated by ion channels and exchangers (Hamilton and Terentyev 2018). Transient increase in $Ca^{2+}$ concentration in the cytosol activates cross-bridge cycling of myofilaments and muscle contraction (Bertero and Maack 2018; Hamilton and Terentyev 2018). Most of the ATP produced in mitochondria is expended on these processes (Bertero and Maack 2018).

A large part of the total ATP produced in energy metabolism is spent on heart contraction, where sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ATPase (SERCA2) consumes about one-third (Nagoshi et al. 2011). In addition, it has been observed that intracellular calcium level affects the selection of cardiac energy substrate (Schonekess et al. 1995).

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Agents that would act to optimize the metabolism of the substrate in the heart while not causing adverse hemodynamic effects are particularly interesting because they could work with existing therapies (Stanley et al. 2005). Vitamin D is best known for its role in calcium absorption and bone mineralization (Latic and Erben 2020). However, the presence of Vitamin D receptors in cardiomyocytes as well as in the endothelial and vascular smooth muscle cells has indicated a role of Vitamin D in the cardiovascular system (Latic and Erben 2020). Low Vitamin D levels are associated with an increased risk of cardiovascular disease (Rai and Agrawal 2017). In addition, literature data have suggested that Vitamin D participates in the regulation of energy metabolism (Yang et al. 2021). Since maintaining a balance of energy metabolism is necessary to maintain normal heart function, Vitamin D/VDR (Vitamin D receptor) is thought to play a role in protecting cardiomyocytes by regulating the use of FA (Yang et al. 2021). Considering all this, Vitamin D seems to be a good candidate for additional therapy that would affect pathological changes in the diabetic heart. In addition, Vitamin D is an essential regulator of extracellular calcium homeostasis, which affects both intracellular calcium and, consequently, the contractility of the heart (Fanari et al. 2015). Although Vitamin D has been shown to affect calcium currents in the heart (Tamayo et al. 2017), and the interaction of VDR with calcium handling proteins was detected, literature data connecting Vitamin D with intracellular calcium turnover in cardiac cells are inconclusive (Zhao and Simpson 2010).

As we recently reported, Vitamin D treatment improved insulin signaling and stimulated the metabolism of glucose in the heart of healthy rats. Vitamin D also reduced the concentration of non-esterified fatty acids (NEFA) in circulation (Ivkovic et al. 2021). Therefore, we assumed that the observed changes strongly reflect the metabolism of FA in the heart. Meanwhile, our assumption was strongly supported by the finding that Vitamin D3 reduced lipid consumption and stimulated glucose uptake in the heart of hyperlipidemic mice (Yang et al. 2021). We hypothesize that Vitamin D, in addition to the indirect effect through the availability of NEFA, directly affects the regulation of energy metabolism and the contraction-relaxation process in the heart under physiological conditions.

## Materials and Methods

### *Chemicals*

Cholecalciferol, a form of Vitamin D, was provided by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies raised against SERCA2 (sc-376235), carnitine palmitoyltransferase 1 (CPT1, sc-98834), Lipin1 (sc-98450), fatty acid transport protein 1 (FATP1, sc-25541), FA translocase CD36 (sc-9154), peroxisome proliferator-activated receptor alpha (PPARα, sc-398394), $Na^+/Ca^{2+}$ exchanger (NCX1, sc-30304-R), L-type calcium channels (LTCC, sc-398433), and β-actin (sc-1616-R), as well as secondary horseradish peroxidase (HRP)-conjugated anti-rabbit, and anti-mouse antibodies, were also obtained from Santa Cruz Biotechnology, Inc. Anti-phospho-acetyl-CoA carboxylase (Ser79) (ACC, #3661S), and anti-ACC (#3676S) antibodies were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA), while antibodies against Peroxisome proliferator-activated receptor-gamma coactivator (PGC-1, ab54481), and calnexin (ab22595) were obtained from Abcam (Cambridge, UK). Reagents for the bicinchoninic acid (BCA) assay were purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany). Electrophoretic reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

### *Animals*

All animal procedures were in compliance with the EU Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes. The experimental protocol was approved by the Serbian Ministry of Agriculture and Environmental Protection after validation by the Ethical Committee of the "Vinca" Institute of Nuclear Sciences, University of Belgrade. Twelve-week-old Wistar rats were divided into the control group ($n$ = 10) and the Vitamin D group ($n$ = 10). They were fed *ad libitum* with standard chow, with *ad libitum* access to drinking water, and maintained at 22 ± 2°C under a 12 h/12 h light/dark cycle. During the six weeks, rats from the Vitamin D group were intraperitoneally injected with 1000 IU/kg of cholecalciferol in corn oil every second day. The rats from the control group were injected with the vehicle at the same volume. According to the literature (Salum et al. 2012) and our previous work (Ivkovic et al. 2021), the applied dose of cholecalciferol does not lead to hypercalcemia. Animals were sacrificed by decapitation 24 h after the last treatment, thus avoiding the acute effect of cholecalciferol. At the time of sacrifice, blood was collected, and the hearts were isolated, measured, and stored at −80°C until the preparation of samples for the Western blot and qRT-PCR analysis.

### *Blood plasma preparation and biochemical measurements*

Before measurement of blood lipid profile, rats fasted overnight. For the determination of triglyceride, total cholesterol, HDL, and LDL concentration, blood was collected in anticoagulant-coated tubes at decapitation, and plasma samples were obtained by centrifugation. Triglyceride, total cholesterol, HDL, and LDL concentrations were measured using the Bio Systems analyzer A25 (Barcelona, Spain).

*Cell lysate*

Heart tissue was homogenized in modified RIPA buffer (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 1% Triton X-100, 0.2% Na-deoxycholate, 0.2% SDS, 1 mmol/l EDTA, protease inhibitors, phosphatase inhibitors) in 1:6 (w/v) ratio, on ice with an Ultra-Turrax homogenizer (3×30 s). The supernatant, representing the cell lysate, was obtained by centrifugation of the homogenate at 15,000 × *g* for 30 min. After protein concentration assessment by the BCA method, samples were prepared for Western blot analysis.

*Plasma membrane isolation*

The plasma membrane isolation protocol was performed according to Luiken et al. (2002). Heart tissue samples were incubated for 30 min at 4°C in preincubation buffer (2 mol/l NaCl, 20 mmol/l HEPES, 5 mmol/l NaN3, pH 7.4) and then centrifuged (5 min, 1000 × *g*). The resulting pellets were homogenized on ice with Ultra-Turrax homogenizer (3×30 s) in TES buffer (20 mmol/l Tris, 1 mmol/l EDTA, 250 mmol/l sucrose) with protease inhibitors. The homogenates were centrifuged (5 min; 1000 × *g*). The pellets were rehomogenized in TES buffer in a glass-glass homogenizer, after which supernatants were added. From the following centrifugation (10 min; 100 × *g*), the supernatants were separated and centrifuged (10 min; 5000 × *g*). The pellets obtained were a fraction of plasma membranes, in which the protein concentration was determined by the BCA method, and samples for the Western blot analysis were prepared.

*Nuclear and microsomal extract isolation*

Heart tissue samples were homogenized on ice with Ultra-Turrax homogenizer (3×30 s) in TEMG buffer (50 mmol/l Tris-HCl, 1 mmol/l EDTA, 12 mmol/l monothioglycerol, 10% glycerol, pH 7.5 with protease and phosphatase inhibitors) and then centrifuged (15 min; 1000 × *g*). Pellets (P1) were further used to obtain the nuclear fraction, while supernatants (S1) were used to obtain the microsomal fractions. P1 was washed once in TMG buffer (20 mmol/l Tris-HCl, 12 mmol/l monothioglycerol, 10% glycerol, pH 7.5) and twice in TMG buffer with 0.2% Triton X-100. Centrifugation (15 min; 1000 × *g*) was performed between each wash. The washed pellets were resuspended in lysis buffer (TEMG + 0.5 mol/l KCl) and left on ice for 60 min stirring with a glass rod every 15 min. After lysis, the suspensions were centrifuged (60 min; 105,000 × *g*). The obtained supernatants were a nuclear fraction in which the protein concentration was determined by the Lowry macro method. S1 was centrifuged (30 min; 12,000 × *g*) at 4°C, and then the supernatants were centrifuged (60 min; 100,000 × *g*). Pellets were resuspended in TEMG buffer and represented microsomal fractions. Protein concentration was determined by the Lowry macro method. All samples were prepared for Western blot analysis.

*SDS polyacrylamide electrophoresis and Western blot*

Proteins were separated by SDS-polyacrylamide gel electrophoresis using 7.5% or 10% polyacrylamide gel and then transferred from the gel to a PVDF membrane. After Ponceau staining and imaging, membranes were blocked in 5% skim milk in tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) and blotted with an antibody against CD36, FATP1, PPARα, Lipin1, PGC-1, phospho-ACC, CPT1, LTCC, SERCA2, and NCX1. After extensive washing, membranes were incubated with secondary HRP-conjugated antibodies and used for detection with ECL substrate. After blotting with phospho-ACC, the membrane was stripped and reprobed with an antibody against ACC. Protein loading was controlled by probing blots and normalizing protein band density with the β-actin antibody for lysate samples and calnexin antibody for microsomal samples. Ponceau stained membrane was the loading control for the plasma membrane and nuclear samples. Films were scanned, and band density was analyzed using ImageJ software (NIH, USA).

*Quantitative real-time PCR (qRT-PCR) analysis*

Total RNA was extracted from rat heart tissues using TRI Reagent (Ambion Inc., Foster City, CA, USA) following the manufacturer's instructions. The single-stranded cDNA was synthesized from 1 μg of total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK). qRT-PCR analysis was performed on Real-time 7500 system (Applied Biosystems, Foster City, CA, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Primer sequences are listed in Table 1. A standard curve was made for all primers, and PCR efficiency values were similar. All reactions were performed in duplicates, in a total volume of 10 μl of reaction mixtures under the following conditions: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 60 s. The relative mRNA expression was assayed using the $2^{-\Delta Ct}$ formula, where ΔCt = Ct (target gene) − Ct (housekeeping gene) and normalized to that of RPL19 mRNA.

*Statistical analysis*

All results are expressed as mean ± SD. Statistical analysis was determined by Student's t-test (2 groups were compared: Vitamin D-treated group *vs.* control group). $p < 0.05$ was considered significant. The number of animals used in the analysis is 10 *per* group and 6 *per* group for Western blot and qRT-PCR, respectively.

**Table 1.** Primer sequences of genes used in the qRT-PCR method

| Gene | Forward primer (5'–3') | Reverse primer (5'–3') |
|---|---|---|
| *acadl* | GGAATGAAAGCCCAGGACACAG | TCAAACATGAACTCACAGGCAGAA |
| *rpl19* | TCGCCAATGCCAACTCTC | AGCCCGGGAATGGACAGT |
| *ucp3* | GACTCACAGGCAGCAAAGGAA | GAGGAGATCAGCAAAACAGGC |
| *mlycd* | ACTTCTTCTCCCACTGCTCC | GCTTTATGAGGAAGGTGCCG |
| *spt1* | CAGTGCAGCCTGCTTTGCTA | GCCTTTCGAGGATTCTTTTGATC |
| *ryr2* | GGCTTCTTCCGCATTGTCTC | TCTCCTCGCACCTCATCCT |

*acadl,* acyl-CoA dehydrogenase long chain; *rpl19*, ribosomal protein L19; *ucp3*, uncoupling protein 3; *mlycd*, malonyl-CoA decarboxylase; *spt1,* serine palmitoyltransferase 1; *ryr2*, ryanodine receptor 2.

## Results

The plasma lipid profile is presented in Table 2. Vitamin D treatment did not alter plasma triglyceride (TG) or cholesterol levels.

Effects of Vitamin D on FA transport in the heart were assessed by protein expression analysis of primary FA transporters in the plasma membrane, CD36 and FATP1. The expression of neither of these two transporters was altered upon treatment (Fig. 1). Changes in protein expression of CPT1, the key regulatory enzyme that controls the rate of FA uptake by the mitochondria, were not observed (Fig. 2A).

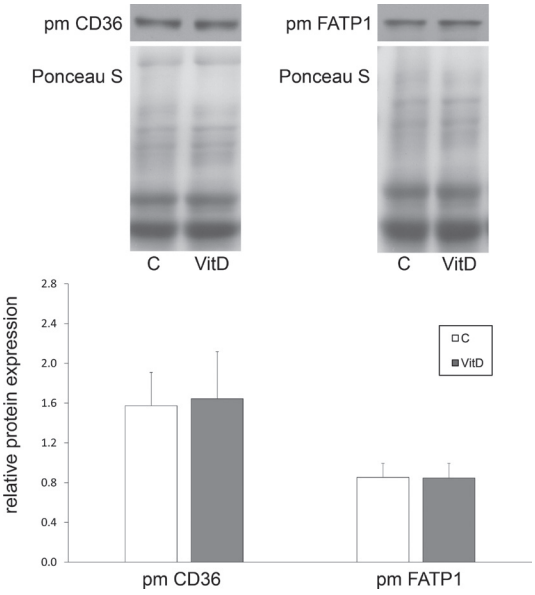


**Figure 1.** Plasma membrane protein content of CD36 and FATP1 in the heart. Protein levels of CD36 and FATP1 were determined by Western blot in the cardiac plasma membrane (pm) of control (C) and Vitamin D-treated (VitD) rats. Ponceau staining was used as a loading control. The representative blots are placed above the histograms. The results are expressed as mean ± SD of 10 animals *per* group. Comparisons between the two groups were made by Student's *t*-test.

Regarding the metabolism of FA in the heart, the effects of Vitamin D were assessed by protein or mRNA content analysis of important molecules involved in β-oxidation. Since malonyl-CoA is a key regulator of FA oxidation, we analyzed the effects of Vitamin D treatment on enzymes responsible for its formation and degradation, ACC and malonyl-CoA decarboxylase (MCD). Vitamin D treatment increased the protein level of ACC ($p < 0.01$), while it did not affect the inhibitory phosphorylation of ACC on Ser79 (Fig. 2A). Also, gene expression analysis by qRT-PCR method revealed that treatment significantly decreased gene expression of MCD ($p < 0.05$, Fig. 2B).

In nuclear fraction, we determined the expression level of transcriptional regulators of FA β-oxidation. Lipin1 and PPARα protein content remained unchanged after Vitamin D treatment, while PGC-1 nuclear protein level was increased ($p < 0.01$, Fig. 3A). Also, gene expression of acyl-CoA dehydrogenase long-chain (ACADL), the enzyme that catalyzes the initial step of mitochondrial β-oxidation, was not changed (Fig. 3B). We also examined uncoupling protein 3 (UCP3) gene expression. Although the role of uncoupling proteins expressed in tissues that do not have the ability of adaptive thermogenesis is mainly unknown, their expression in the heart could be related to FA metabolism. Vitamin D treatment led to an increase in UCP3 gene

**Table 2.** Effect of cholecalciferol on plasma lipid profile in rats

| | Control | Vitamin D |
|---|---|---|
| TG | 0.53 ± 0.15 | 0.47 ± 0.12 |
| Total-Ch | 1.47 ± 0.21 | 1.46 ± 0.34 |
| HDL-Ch | 0.81 ± 0.14 | 0.84 ± 0.16 |
| LDL-Ch | 0.17 ± 0.04 | 0.15 ± 0.06 |

All parameters were determined in the plasma. Data are presented as mean ± SD from the experiment with 10 animals *per* group. TG, triglycerides; Ch, cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

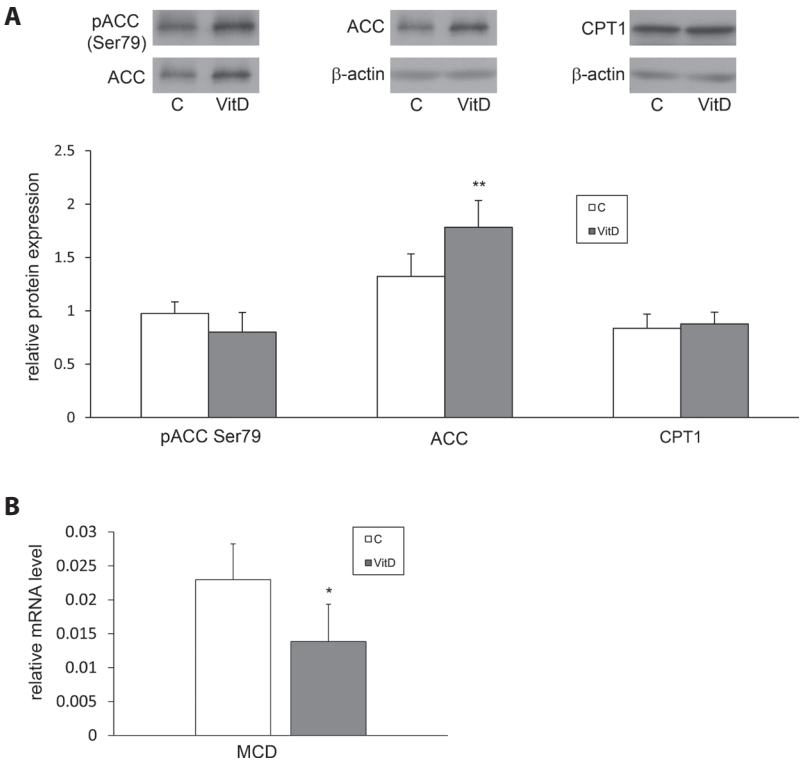


**Figure 2.** Cardiac ACC and CPT1 protein level and MCD mRNA level. The protein levels of phosphorylated or total ACC and CPT1 (**A**) were determined by Western blot in the cardiac cell lysate of Vitamin D-treated (VitD) and control (C) rats. β-actin was used as a loading control for total ACC and CPT1. The phosphorylated-ACC (pACC) level was normalized to the total ACC protein level. MCD mRNA level was determined using the SYBR Green qPCR method (**B**). The results are expressed as mean ± SD of 10 animals *per* group for Western blot and mean ± SD of 6 animals *per* group for qPCR. Comparisons between the two groups were made by Student's *t*-test. * $p < 0.05$, ** $p < 0.01$. ACC, acetyl-CoA carboxylase; CPT1, carnitine palmitoyltransferase 1; MCD, malonyl-CoA decarboxylase.

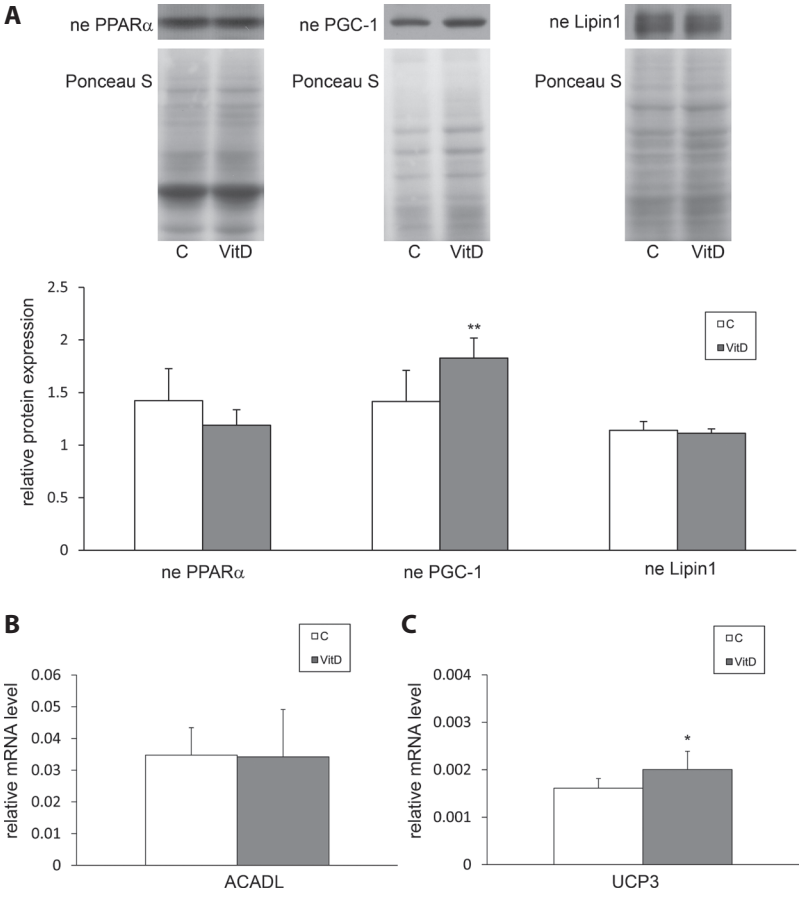


**Figure 3.** Nuclear protein level of PPARα, PGC-1, Lipin1, and mRNA level of ACADL and UCP3 in the heart. The protein levels of PPARα, PGC-1, and Lipin1 (**A**) were determined by Western blot in the cardiac nuclear extract (ne) of Vitamin D-treated (VitD) and control (C) rats. Protein content was normalized to total protein from Ponceau staining. The part of the membrane that was used for quantification contains different sections depending on molecular weight of protein of interest. ACADL (**B**) and UCP3 (**C**) mRNA levels were determined using the SYBR Green qPCR method. The results are expressed as mean ± SD of 10 animals *per* group for Western blot and mean ± SD of 6 animals *per* group for qPCR. Comparisons between the two groups were made by Student's *t*-test. * $p < 0.05$, ** $p < 0.01$. PPAR α, peroxisome proliferator-activated receptor α; PGC, peroxisome proliferator-activated receptor-gamma coactivator; ACADL, acyl-CoA dehydrogenase long-chain; UCP3, uncoupling protein 3.

expression ($p < 0.05$), which was detected by the qRT-PCR method (Fig. 3C).

The protein content of Lipin1 was measured in the microsomal fraction to investigate whether Vitamin D treatment affects TG synthesis in the heart. The protein expression level of Lipin1 in the microsomal fraction was similar between the two experimental groups (Fig. 4A). Another biosynthesis pathway in the heart that could be driven by FA entry is ceramide synthesis. Measuring gene expression of serine palmitoyltransferase 1 (SPT1), an enzyme involved in this pathway, it was demonstrated that Vitamin D treatment decreased its mRNA level ($p < 0.01$, Fig. 4B).

As the metabolism of FA and the availability of nutrients can affect the functioning of $Ca^{2+}$ handling proteins in the heart, their expression was examined. Protein expression of LTCC in the lysate was decreased ($p < 0.001$, Fig. 5A), as well as ryanodine receptor (RyR2) channel gene expression ($p < 0.05$, Fig. 5B). Regarding proteins involved in the relaxation phase, an increase in SERCA2 protein expression was observed in the microsomal fraction ($p < 0.05$), while there was no change in the protein content of NCX1 in the whole-cell lysate (Fig. 5A).

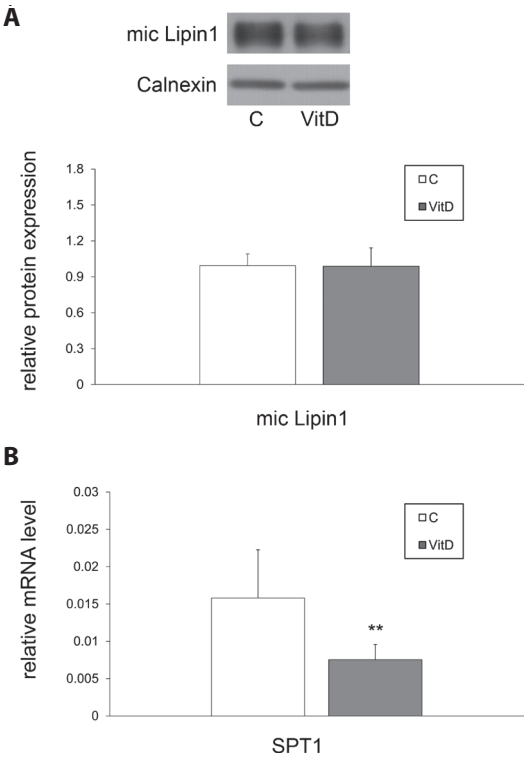


**Figure 4.** Protein level of Lipin1 and mRNA level of SPT1 in the heart. The protein level of Lipin1 was determined by Western blot in cardiac microsomal fraction (mic) (**A**) of Vitamin D-treated (VitD) and control (C) rats. Calnexin was used as a loading control. SPT1 mRNA level was determined using the SYBR Green qPCR method (**B**). The results are expressed as mean ± SD of 10 animals *per* group for Western blot and mean ± SD of 6 animals *per* group for qPCR. Comparisons between the two groups were made by Student's *t*-test. ** $p < 0.01$. SPT1, serine palmitoyltransferase 1.

## Discussion

Regulation of the use of FA as an energy source for cardiac function is performed at various levels starting from the levels of circulating FA, through their transport across the cardiac cell membrane and esterification, to the mitochondrial entry and degradation in the oxidation process (Dyck and Lopaschuk 2002).

In this study, we did not observe changes in the expression of the most important cardiac transporters of FA, CD36 and FATP1. This finding is in line with the study of Yang et al. (2021), which also showed that the level of CD36 transporters in the heart does not change under the action of Vitamin D in hyperlipidemic mice, confirming that it does not affect the transport of FA in the heart. CPT1, a marker of mitochondrial FA uptake, was also unchanged by Vitamin D administration. In contrast to our study, Yang and coworkers (Yang et al. 2021) observed that Vitamin D reduced CPT1 level in the heart of hyperlipidemic mice.

Cholecalciferol did not alter the nuclear content of the most important regulators of β-oxidation in the heart or the expression of the enzymes involved in this process. However, Vitamin D affected the expression of ACC/MCD that participate in the malonyl-CoA-mediated regulation of β-oxidation. Deletion of MCD is associated with increased levels of malonyl-CoA and a shift in the balance of oxidation of energy substrates from FA to glucose in the heart after ischemia/reperfusion injury (Wang et al. 2019). Although an increase in TG levels is expected in such an experimental model, the opposite has been observed. The deletion of MCD was also accompanied by a reduction in SPT1 (Wang et al. 2019), which we also observed. This reduction suggests reduced biosynthesis of harmful ceramides in the heart in parallel with a decrease in MCD level (Samokhvalov et al. 2012). Furthermore, the downregulation of SPT1 level contributes to the improved myocardial insulin sensitivity and glucose utilization in the heart of obese mice (Ussher et al. 2012), which we also recognized in healthy rats (Ivkovic et al. 2021).

Increased UCP3 gene expression in cholecalciferol-treated rats may also be interpreted as a cardiac benefit. Namely, it is known that UCP proteins reduce ROS production, oxidative stress, and lipotoxicity but also increase the survival of heart cells (Ruiz-Ramírez et al. 2016). We did not directly correlate the changes in UCP3 expression with the observed changes in FA metabolism. There is a possibility that UCP3 level correlates with pro-survival Akt/GSK3 pathway activity (Chen et al. 2015), which we have

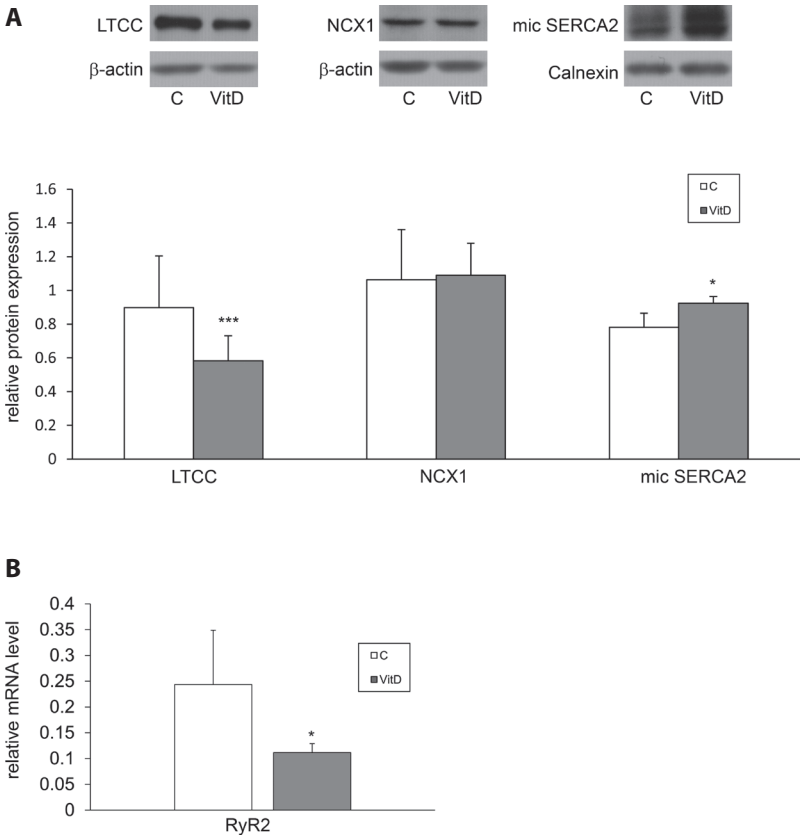


**Figure 5.** LTCC, NCX1 and SERCA2 protein level, and RyR2 mRNA level in the heart**.** The protein levels of LTCC and NCX1 in lysate, and SERCA2 (**A**) in microsomal fraction (mic), were determined by Western blot. β-actin was used as a loading control for lysate and Calnexin was used as a loading control for the microsomal fraction. RyR2 mRNA level was determined using the SYBR Green qPCR method (**B**). The results are expressed as mean ± SD of 10 animals *per* group for Western blot and mean ± SD of 6 animals *per* group for qPCR. Comparisons between the two groups were made by Student's *t*-test. * $p < 0.05$, *** $p < 0.001$. LTCC, voltage-dependent L-type calcium channel; NCX1, $Na^+/Ca^{2+}$ exchanger 1; SERCA2, sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ATPase 2; RyR2, ryanodine receptor 2.

shown in a previous study to be stimulated in the heart of cholecalciferol-treated rats (Ivkovic et al. 2021). There are also data that the expression of UCP3 in the heart is reduced in hyperinsulinemia (Harmancey et al. 2015). As treatment with cholecalciferol tended to reduce insulin concentration in our study (Ivkovic et al. 2021), this can be related to increased expression of the UCP3 gene.

Assuming that an increase in PGC-1 level in the nuclear extract is not associated with the direct regulation of β-oxidation because the content of PGC-1 partners, PPARα and Lipin1, was not altered, this alteration may be associated with other cardiac processes. For example, this result coincided with an increase in the nuclear content of VDR, which we observed in a previous study (Ivkovic et al. 2021). PGC-1 has been shown to enter a complex with VDR and participate as a coactivator in gene regulation (Savkur et al. 2005).

In addition, an increase in nuclear PGC-1 may be associated with increased SERCA2 expression in the heart cells of cholecalciferol-treated rats in this study, as suggested in the study of Lv et al. (2019). It could be one of the mechanisms coupling cardiac energy metabolism with $Ca^{2+}$ handling and myocardial contractility. The probable physiological outcome is increased calcium uptake into the SR during myocardial relaxation. In other words, these findings strongly indicate that Vitamin D *via* PGC-1 could improve the capacity of ventricular diastole, accelerating $Ca^{2+}$ clearance through SERCA2. As indicated by Hamstra et al. (2020), increased expression and activity of SERCA2 in the heart leads to the improvement of diastole, but indirectly also of systole, by increasing the pool of $Ca^{2+}$ in the sarcoplasmic reticulum. Tishkoff et al. (2008) go one step further by suggesting that Vitamin D is important for the maintenance of diastole and that by improving diastole parameters, successful filling of the ventricles of the heart is ensured. Cardiac overexpression of SERCA2 improves myocardial contractility in diabetic rats and protects against the development of arrhythmias (Bayeva et al. 2013; Hamilton and Terentyev 2018). Increased expression of SERCA2 protein also coincides with increased glucose metabolism (Belke et al. 2007), as we also reported in a previous paper (Ivkovic et al. 2021). A possible mechanism responsible for the increased presence of PGC-1 in the nucleus and SERCA2 level could be increased sirtuin 1 (SIRT1) activity (Anderson et al. 2008; Sulaiman et al. 2010). In addition, in the heart of rats treated with Vitamin D, the expression of molecules involved in increasing the content of cytosolic $Ca^{2+}$ during myocardial systole (LTCC and RyR2) was reduced. This result is in complete agreement with the results of Tishkoff et al. (2008), showing that Vitamin D reduces the contraction

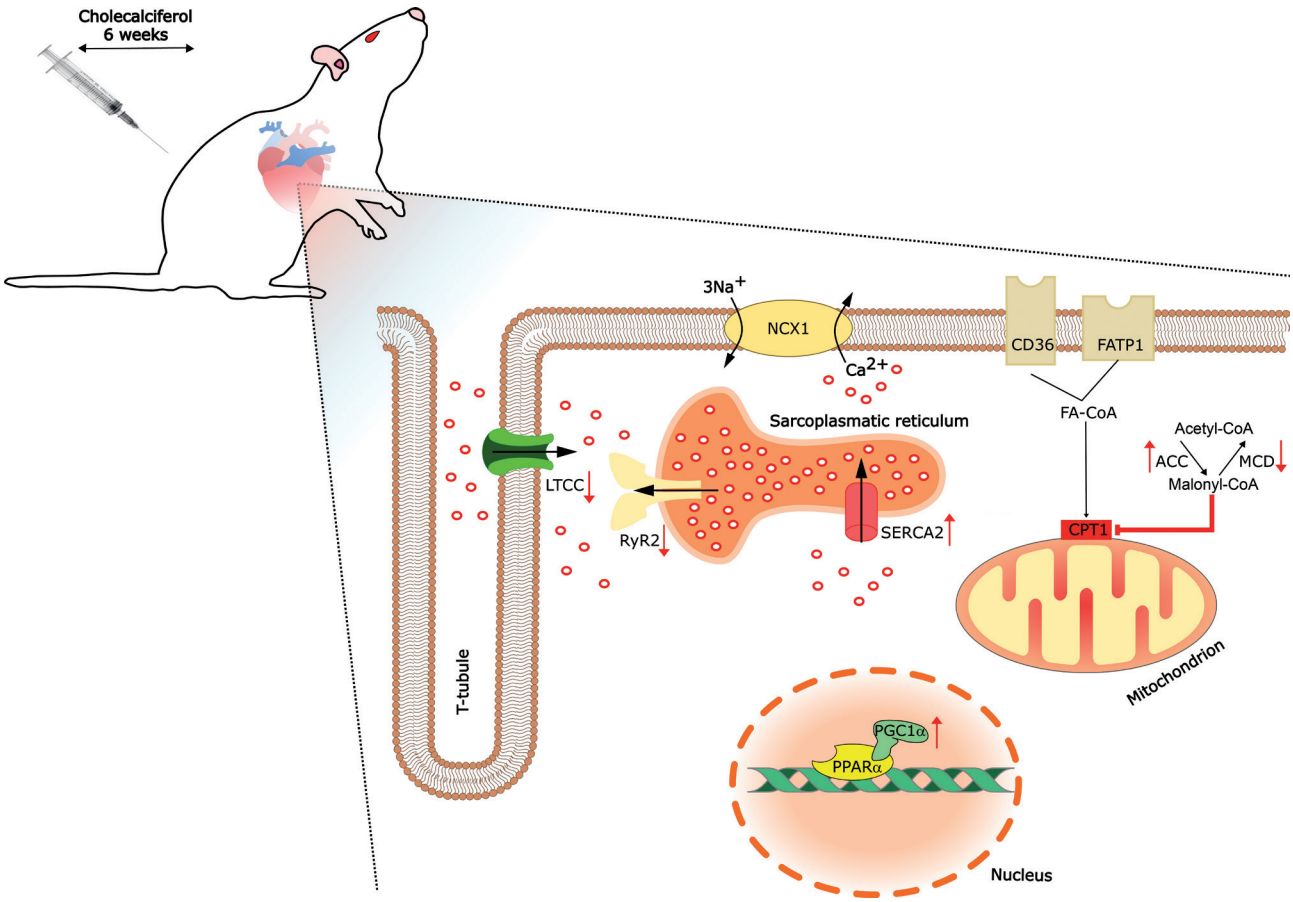


**Figure 6.** Effects of cholecalciferol on cardiac fatty acid metabolism and $Ca^{2+}$ handling. LTCC, voltage-dependent L-type calcium channel; RyR2, ryanodine receptor 2; NCX1, $Na^{+}/Ca^{2+}$ exchanger 1; SERCA2, sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ATPase 2; CD36, cluster of differentiation 36; FATP1, fatty acid transport protein 1; CPT1, carnitine palmitoyltransferase 1; PPAR, peroxisome proliferator-activated receptor; PGC1, peroxisome proliferator-activated receptor-gamma coactivator 1; ACC, acetyl CoA carboxylase; MCD, malonyl-CoA decarboxylase.

rate and increases the heart relaxation rate. Although the abnormal function of LTCC and RyR2 are involved in cardiac pathologies, particularly arrhythmogenesis (Hamilton and Terentyev 2018), we believe that Vitamin D-induced alterations of these molecules' expression observed in this study are delicate modulations of $Ca^{2+}$ handling inside a physiological range.

Angin and coworkers detected an association between high cytosol calcium and increased levels of GLUT4 and CD36 in sarcolemma (Angin et al. 2014). In addition, Balu et al. (2016) observed that a high level of calcium in the cytosol stimulated the oxidation of FA. Considering the status of proteins involved in calcium handling in our study, a decrease in cytosol calcium was indicated, which coincided with unchanged levels of GLUT4 (Ivkovic et al. 2021) and CD36 in the plasma membrane and unchanged β-oxidation parameters.

A possible mechanism that connects the observed changes in FA metabolism with the changes in the expression of proteins involved in maintaining calcium homeostasis is proposed by Aitken-Buck et al. (2020). Namely, increased production of long-chain acylcarnitine by the CPT1 leads to increased cytosol $Ca^{2+}$ in cardiomyocytes. Decreases in the expression of LTCC and RyR2, as well as increases in the levels of SERCA2 detected in the hearts of cholecalciferol-treated rats, may indicate decreased CPT1 activity, despite unchanged expression. Furthermore, increased ACC and decreased MCD expression can reduce CPT1 activity through increased malonyl-CoA.

To conclude, the obtained results indicate that Vitamin D attained subtle attenuation of FA metabolism in the heart, which is associated with a fine-tuning effect on the heart contraction-relaxation cycle in the direction of suppressing systole and stimulating diastole (Fig. 6). Consider-

ing literature data showing that numerous cardiometabolic diseases include altered energy metabolism in the heart, results obtained in this study nominate cholecalciferol as potentially beneficial.

**Conflict of interest.** The authors declare that they have no conflict of interest.

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**Data availability statement.** All materials and data are available from the corresponding author upon a reasonable request. The preprint of this Manuscript is available at: https://doi.org/10.21203/rs.3.rs-2226189/v1.

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