Biochemical Pharmacology, Vol. 54, pp. 575–582, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00178-0

Thiamine Deficiency in Cardiac Cells in Culture

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ABSTRACT. Rat heart cells in culture were found to be a unique model for studying biochemical and pharmacological aspects of thiamine deficiency. When thiamine was excluded from the growth medium, the following effects were observed: (1) Morphological examination did not show any difference between control and thiamine-deprived cells during the first 10 days. However, after 10–11 days spontaneous contractions ceased, accompanied by initiation of cell degeneration; (2) Intensive degeneration and cell death were observed after 14–16 days. (3) Thiamine pyrophosphate (TPP) concentration in thiamine-deprived cells was decreased gradually, with an elimination half-life of 4–5 days. (4) [^{3}H]deoxyglucose uptake by the cells was increased, even after 1 day of thiamine deprivation. (5) ATP level decreased after 8 days and reached 50% of control cells after 10 days. (6) In thiamine-deprived cells, thiamine addition caused a 60% rise in contraction amplitude but contraction rate was not altered significantly. (7) All these effects were reversible if thiamine was supplied before the initiation of the degeneration processes. BIOCHEM PHARMACOL 54;5:575–582, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. thiamine; rat cardiomyocytes; ATP; glucose uptake; contraction amplitude

Thiamine pyrophosphate (TPP)† is an important cofactor in several vital enzymatic reactions involved in metabolism and energy production. After its uptake by the cell, thiamine (vitamin B1) undergoes pyrophosphorylation yielding thiamine pyrophosphate, which is the active substance.

Thiamine deficiency causes damage to the nervous system ("dry beriberi") and/or to the cardiovascular system ("wet beriberi"). In the Western world, thiamine deficiency is observed in malnourished and alcoholic patients [1, 2], and may also occur in seemingly healthy elderly [3–5]. Chronic use of diuretic drugs may also cause unsuspected thiamine deficiency [6–8]. Congestive heart failure in thiamine deficiency is related to vasodilatation and to increased salt and water retention, but very little is known about the direct effects of thiamine deficiency on the heart [7]. In rat models, complete deprivation of thiamine from food for 21 days caused a focal necrosis, especially in the left ventricle and also structural alterations in mitochondria [9]. It was not clear whether ATP production was impaired [10] or not [11, 12]. Another study reports a decrease in contractile properties of isolated heart muscle after 35 days without thiamine [13].

In this work we examined the direct effect of thiamine depletion on morphology of cultured rat heart cells, TPP concentrations, ATP levels, glucose uptake and spontaneous contraction of the cells. Our results indicate that thiamine deficiency causes direct biochemical and morphological alterations in cardiac cells.

MATERIALS AND METHODS

Cell Culture

Rat (1–2 days old) hearts were removed under sterile conditions and washed three times in phosphate-buffered saline (PBS) to remove excess blood cells. The hearts were minced to small fragments and then agitated gently in a solution of proteolytic enzyme-RDB (Biological Institute, Ness-Ziona, Israel) prepared from a fig tree extract. The RDB was diluted 1:50 in Ca^{2+}- and Mg^{2+}-free PBS, at 25°C for a few cycles of 10 min each, as described previously [14, 15]. Thiamine-free Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum (Biological Industries, Kibbutz Beit Haemek, Israel) was added to supernatant suspensions containing dissociated cells. The mixture was centrifuged at 500 *g* for 5 min, the supernatant phase was discarded and the cells were suspended again. The suspension of the cells was diluted to 1.0×10^6 cells/mL and 1.5 mL were placed in 35 mm collage–gelatin-coated plastic culture dishes. The cultures were incubated in a humidified atmosphere of 5% CO_2, 95% air at 37°C. Confluent monolayers, which exhibit spontaneous contractions, developed in culture within 2 days. The growth medium was replaced after 24 hr by a special "thiamine-free" chemical defined medium based on DMEM without thiamine, supplemented with bovine serum albumin (100 μg/mL), transferin (10 ng/mL), insulin (0.3 U/mL), sodium selenite (1 nM), and thyroxine (100

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† *Abbreviations:* [^{3}H]-DG, [^{3}H]deoxyglucose; DMEM, Dulbecco's modified Eagle's medium, PBS, phosphate-buffered saline; TCA, trichloroacetic acid; TPP, thiamine pyrophosphate.

Received 8 November 1996; accepted 14 March 1997.

nM). To the control cultures, thiamine was added to 10 μM (as in normal DMEM). The growth medium was replaced every 3 days.

Determination of Intracellular TPP

Analysis of TPP was done by high-performance liquid chromatography (HPLC) (Waters M-510; Milford, MA) using the method described by Tallaksen *et al.* [16], with minor modifications adjusting the assay for cardiac cultures (instead of red blood cells). The cardiac cells were washed with PBS, harvested with a rubber policeman under 1 mL of 1% Triton X-100 solution and homogenized for 10 sec at 4°C. Deproteinization was done by addition of 70 μL of 50% trichloroacetic acid (TCA) solution to 0.7 mL of the cell homogenate. After 10 min of centrifugation at 200 × *g*, the supernatant phase was transferred to fresh glass tubes, and TCA was extracted twice with 5 vol of water-saturated diethyl ether. The TPP was derivatized by addition of 60 μL of 0.3 M solution of cyanogen bromide to 0.6 mL of the sample. A 100 μL aliquot of the derivatization sample was injected to the HPLC column. A Licrosorb NH_2 column (15 × 4.6 mm i.d.) from Merck was used. The mobile phase consisted of acetonitrile and phosphate buffer (85 mM, pH 7.5) 50:50 (v/v). The flow rate was 1.5 mL/min and the retention time of TPP was 5.6 min. The detection limit was 4 pg on column and the intraassay coefficient variation, calculated on the basis of 10 analyses of the same sample on one day, was 6.2%.

Determination of ATP Levels

Bioluminescent somatic cell assay KIT (FL-ASC, Sigma) was used for the ATP measurements. Cell's medium was removed and 1 mL of "somatic cell releasing reagent," which immediately causes complete ATP release, was added. After 60 sec, an aliquot was diluted 1:80 in sterile water, and a 200 μL diluted sample was used for ATP determinations. The reaction mix contains luciferin and luciferase which exhibit a photochemical reaction in the presence of ATP. Bioluminescence was measured using a scintillation counter (Kontron, MR 300, Basel, Switzerland), and ATP levels were calculated according to a standard curve measured in each experiment.

Uptake of [^{3}H]Deoxyglucose

[^{3}H]deoxyglucose ([^{3}H]DG) uptake was determined according to the method described by Shainberg and Pearl [17]. [^{3}H]DG (9.57 Ci/mmol) was obtained from the Nuclear Research Center, Negev, Israel. Cultures were incubated in PBS supplemented with glucose (5.5 mM) and 1 μCi/mL [^{3}H]DG, at 37°C for 10 min. The cells were washed five times with cold PBS, and solubilized in 1% Triton X-100. Radioactivity was counted using a liquid scintillation counter (Kontron, MR 300). To obtain background, sister cultures were incubated at 0°C.

Measurement of Rate and Amplitude of Spontaneous Contractions

The video technique for contraction measurement has been described previously by Rich, Langer, and Klassen [18]. A culture dish containing adherent cells was attached to the stage of an inverted phase interference microscope. The image of a selected area was passed through the lens to an interlaced video camera at a final magnification of ×800. The brightness of the end border of the selected cell relative to background was enhanced by video subtraction, that is by mixing the positive (white) and negative (dark) video signals through an amplifier. The movement of the cell border was monitored 400 times/sec. The time variation was then converted to voltage, filtered, and analyzed by a computer program. Each measurement was done during 10 sec of spontaneous contractions, and the dishes were not moved during the 200 min of the experiment. Rate of contractions was calculated according to the number of peaks in each measurement. As the units of amplitude are not absolute, only the % changes in the average amplitude in each measurement during the whole experiment are presented. The dishes were kept under temperature and pH control (37°C, pH = 7.2–7.4) during the experiment.

Determination of Cell Number

The number of cells was determined under the microscope using a cell counter unit. Percentage of dead cells was determined using trypan blue, as described by Walum *et al.* [19].

Protein Determination

Protein content was determined according to the method of Lowry *et al.* [20], using bovine serum albumin as a standard.

Statistics

The significance of the differences of the means for different experimental conditions was evaluated by using Student's *t*-test.

RESULTS

Morphological Effects of Thiamine Depletion

When cardiac cells after 24 hr in culture were transferred to thiamine-free medium, two phases could be observed. In the first phase, which lasted during the first 10 days, no morphological alteration could be detected. However, between 12–14 days after plating (the second phase), dramatic morphological changes could be observed. First, the spontaneous contractions stopped completely (days 11–12). This was followed by decreased cell volume and initial signs of degeneration of the cells (Fig. 1C). After 1–2 more days, the cells died as determined by trypan blue staining. If thiamine was added to the medium during the second phase

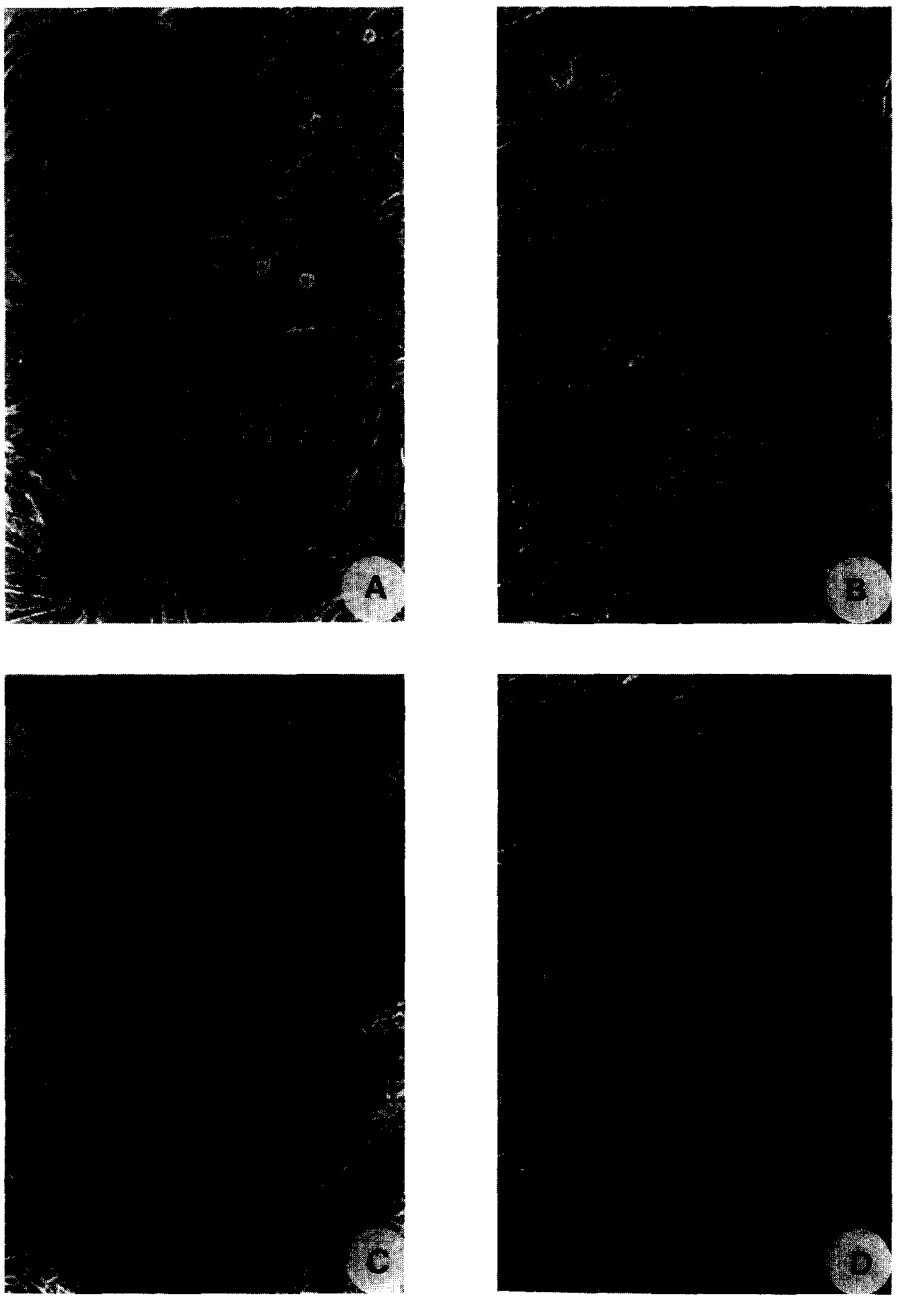

FIG. 1. Photomicrographs of cultured rat cardiac cells in thiamine-free medium (magnification ×336). (A) Seven-day-old cardiac cells grown in chemical-defined thiamine-free medium. (B) Seven-day-old cardiac cells grown in chemical-defined medium supplemented with 10 μM thiamine. (C) Fourteen-day-old cardiac cells grown in chemical-defined thiamine-free medium. (D) Fourteen-day-old cardiac cells grown in chemical-defined medium supplemented with thiamine.

(12–14 days), the cells were saved and returned to normal morphology. In some cases they even resumed their ability to contract spontaneously.

Rate of Cell Death

The number of living cells was determined following thiamine depletion. No differences from control were detected during the first 12 days. After 14 days, a 25% decrease in viable cell number appeared in the thiamine depleted group, and after 3 more days, the number of living cells decreased by 90%. There was no significant change in viable cell number at the control cultures during the experimental period (Fig. 2).

Rate of TPP Elimination From Cardiac Cells

TPP concentration was determined in cardiac cells grown in thiamine-free medium following the depletion period. It was found that the concentration of TPP was reduced to below 100 pg/mg protein after 8 days (Fig. 3). The half-life was calculated to be 4.5 days, when excluding the period of cell division (days 0–2). In the control group, there were no significant changes in TPP concentration during the experimental period.

When thiamine (10 μM) was added to the thiamine-free medium, the level of TPP in the cardiac cells reached control values within 2 hr, even after 1 hr the value of TPP in the cells was almost as in the control cells (data not shown).

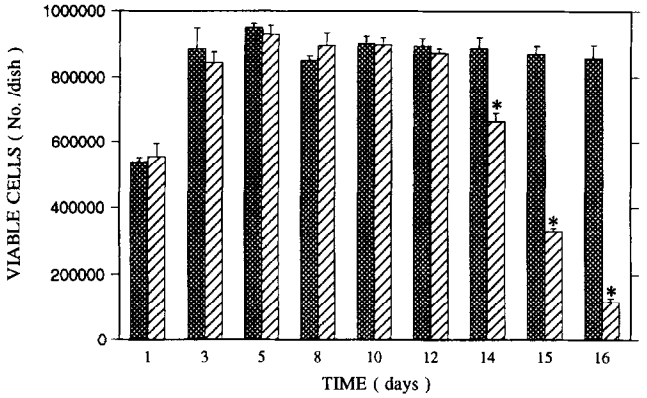

FIG. 2. Effect of thiamine depletion on cell viability. The number of viable cardiac cells was measured at various ages by excluding trypan blue from cells grown in thiamine-free medium (slanted rules) or from control (crosshatched). Each bar represents the mean ± SD (n = 8), *P < 0.01.

Effect of Thiamine Depletion on ATP Levels in Cardiac Cells

ATP levels were determined during the thiamine depletion period. During the first 5 days in culture, intracellular ATP levels remained unchanged and similar to levels in the control group (~25 nmol/mg protein). ATP levels started to decrease at 6–8 days *in vitro*, the fall becoming statistically significant only after 8 days. After 2–3 more days, the decrease became drastic, and ATP levels reached below 10 nmol/mg protein (Fig. 4). In Fig. 4, five experiments are combined for each point, at which the drastic decrease appeared in an interval of 1–2 days, so the decrease seems more moderate.

To determine whether the ATP decrease is reversible, some of the cultures in thiamine depleted medium were supplemented by 10 μM thiamine (as in normal medium), 2 hr before ATP determination. As described in Fig. 4, the addition of thiamine resulted in a return of ATP levels to near control values, showing that the ATP decrease is reversible. After 12 days without thiamine, more time was

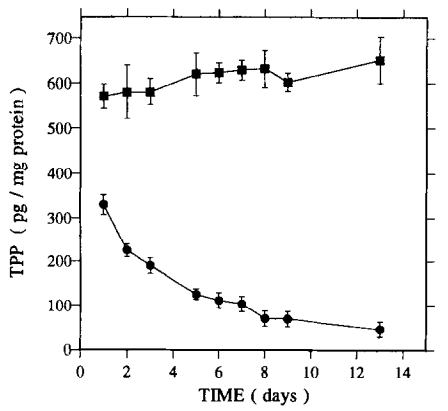

FIG. 3. TPP elimination from cardiac cells. TPP concentration was determined in cells grown in thiamine-free medium (●) or in the control cells (■) (n = 5).

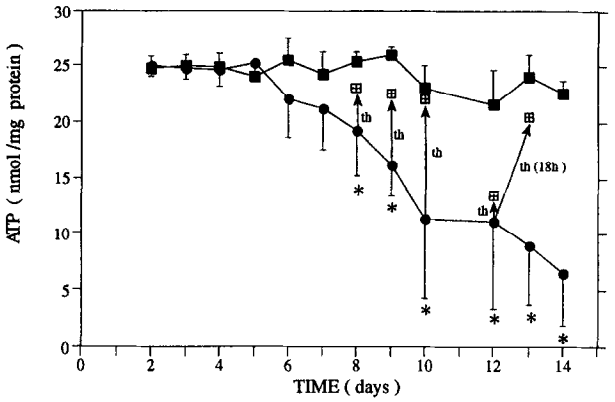

FIG. 4. Effect of thiamine depletion on ATP level. The level of ATP was determined in cardiac cells grown in thiamine-free medium (●), control medium (■), and thiamine-free medium supplemented with thiamine (10 μM) 2 hr before ATP measurement (square with plus sign) (n = 5) *P < 0.05 (control medium vs. thiamine-free medium).

required for elevation of ATP level after thiamine addition (Fig. 4).

Effect of Thiamine Depletion on [^{3}H]Deoxyglucose Uptake

Thiamine depletion caused an increase in [^{3}H]DG uptake. The increase was observed already after 1 day of thiamine depletion (Fig. 5). The percent increase was not constant throughout the thiamine deprivation period, but was usually significantly above control.

To determine whether the increased [^{3}H]DG uptake is reversible, cultures treated in thiamine-depleted medium were supplemented with 10 μM thiamine, 2 hr before the [^{3}H]DG uptake measurement. As described in Fig. 5, the addition of thiamine resulted in the return of [^{3}H]DG uptake to control levels, indicating the reversibility of this effect.

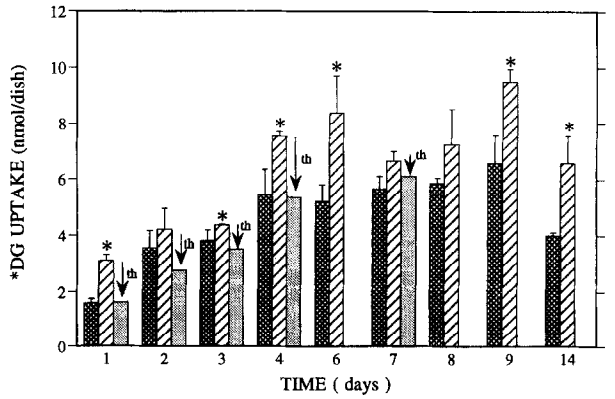

FIG. 5. Effect of thiamine depletion on [^{3}H]deoxyglucose uptake. The uptake of ^{3}H-DG was determined in cardiac cells grown in thiamine-free medium (slanted rules), control medium (crosshatched), and thiamine-free medium supplemented with thiamine (10 μM) 2 hr before ^{3}H-DG uptake measurements (dotted). (n = 3–4) *P < 0.05 (control medium vs. thiamine-free medium).

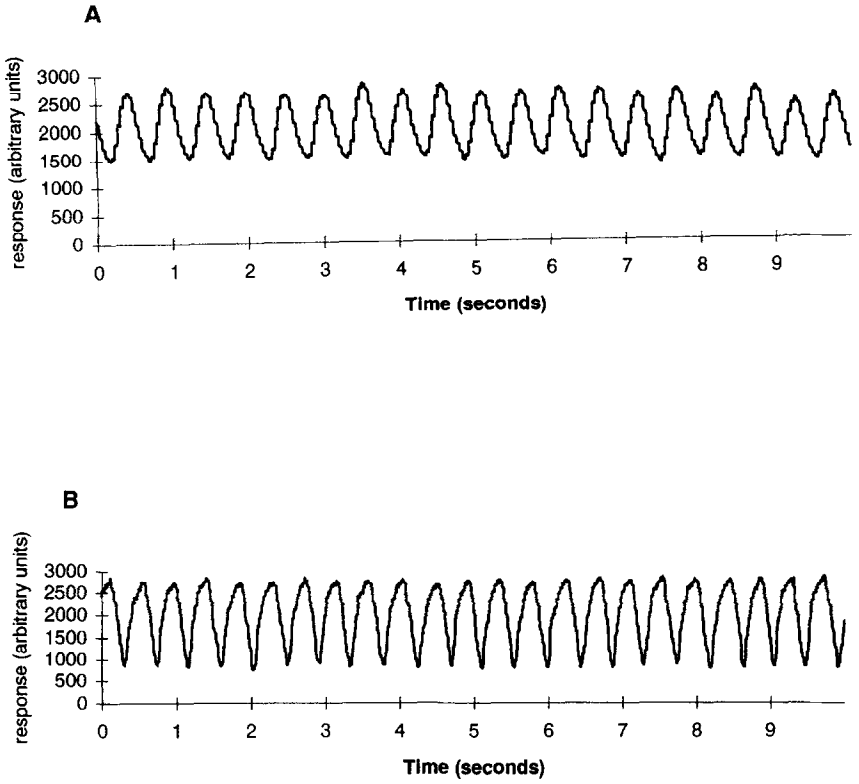

FIG. 6. Effect of thiamine addition on spontaneous contraction of thiamine-depleted cardiac cells. Seven-day-old cardiac cells grown in thiamine-free medium were monitored for contractions from a representative single cell (A). The same cell was monitored 120 min after thiamine (10 μM) addition (B).

Effect of Thiamine Depletion on the Rate and Amplitude of Cell Contraction

Thiamine-depleted cells contracted spontaneously for 10 days *in vitro*, while normally grown cells contracted for 16 days at least. Contraction rate was measured daily, with no significant difference between the control and the thiamine-depleted group (except for the cessation of contraction after 10 days in the depleted group).

When thiamine was added to thiamine-depleted cultures under the microscope, measuring local changes of contraction rate and amplitude in a single cell, no significant change in contraction rate was observed, as shown in the example of Fig. 6 (10% variations in both directions were observed, and were not statistically significant). However, contraction amplitude was significantly increased when thiamine was added after 7–9 days of thiamine depletion. The addition of thiamine caused after 60–120 min an increase of 20–60% in amplitude. This increase was stable for 200 min. A typical experiment is described in Fig. 6. After 5 days of thiamine depletion, thiamine addition did not change the contraction amplitude during the experiment (200 min), while after 7–9 days, the maximal increase (60%) was developed after 120 min of thiamine supplementation (Fig. 7).

In the control cultures, addition of thiamine did not cause any change in the rate or amplitude of contraction.

DISCUSSION

The use of cultured cardiomyocytes as a model for thiamine deficiency enables direct and simple measurements of biochemical and physiological parameters affected by thiamine deprivation. It may also be used for studying pharmacological aspects of thiamine deficiency.

Because no previous research concerning thiamine deficiency was done on this model, it was first necessary to determine whether TPP is eliminated from the cells during the thiamine depletion period, and what is the elimination rate in this model. The results show a fast decrease in TPP concentration in the cells, with an average half-life of 4.5 days. In a study done on a model of thiamine deficiency in rats, where thiamine was removed from their food, the

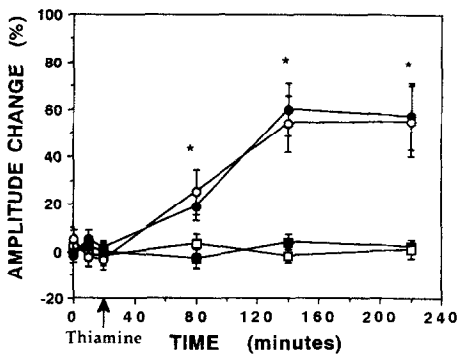

FIG. 7. Amplitude of spontaneous contractions in cells grown in thiamine-free medium, following thiamine supplement. Cardiac cells received thiamine while the rate and amplitude of contractions were monitored from single cells. The results are presented as percentage changes of the amplitude after 60, 120, and 200 min of thiamine addition. (■) cells grown in control medium, (□), (●), and (○) cells grown in thiamine-depleted medium for 5, 7, and 9 days, respectively ($n = 5$). *$P < 0.05$ vs. amplitude before thiamine addition.

elimination rate of TPP was slower: after 1 week, a 54% decrease was measured in red blood cells, 64% decrease in liver tissue, 37% in skeletal muscle tissue, and only 7% in brain tissue. Cardiac tissue was not studied [21]. Another study, using mouse neuroblastoma cells showed a 16-fold decrease in TPP content 8 days after thiamine deprivation [22]. The differences observed between *in vivo* and *in vitro* elimination rates of TPP may be due to release of thiamine from tissue stores [23], even after thiamine is excluded from food. Moreover, the intestinal natural bacterial flora can synthesize thiamine [24], while the cultures are sterile.

As shown in Fig. 3, the TPP levels in the control cells were stable and twice as great as in the thiamine-depleted cultures after 1 day. The reason for that is probably the dilution of TPP in the thiamine-deprived culture caused by cell division occurring mostly in the first day after plating the cells. The finding that 1 hr was enough for most of the thiamine to be absorbed into the cells and phosphorylated was important for the following experimental procedures. Previous works in rat hepatocytes [25] and brush-border membrane vesicles from rat small intestine [26], demonstrated near complete absorption of thiamine by 30 min. However, because TPP levels were not determined, it could be speculated that the phosphorylation process would take some more time. In mouse neuroblastoma cells, TPP levels were determined after thiamine addition to thiamine-depleted cultures, and were found to reach saturation levels only after 8 hr [27].

Thiamine depletion did not cause morphological changes in the cardiac cells over the first 12 days (although TPP levels decreased by 85%). After 12–15 days, acute morphological changes occurred in the thiamine-depleted cultures followed by a decrease in the number of living cells. Cell death started after 14 days, with only 12% of living cells remaining after 16 days. In rats, thiamine depletion from food causes death usually after sudden fatal arrhythmia occurring after 5 weeks in young rats and 8 weeks in adult rats [9, 13, 28]. Histological examination of heart muscle in these rats showed focal necrosis, especially in the left ventricle, occurring after 21 days without thiamine, with structural alterations in the mitochondria [9]. The degradation process *in vivo* is slower, probably because of the slower rate of TPP elimination as mentioned above. Moreover, it was shown that in younger rats death occurs faster, and the source of the cultures is neonatal (in which sensitivity would be even greater).

A statistically significant decrease in ATP levels first appeared after 8 days without thiamine, 5 days before morphological changes were evident, when TPP concentration was less than 100 pg/mg protein (1/6 of the control concentration). TPP is known to be a vital cofactor for enzymes participating in the citric acid cycle and the pentose shunt, which are important for ATP production. Therefore, it was expected that a decrease in ATP levels would start earlier, but these remained equal to the control values over 7 days without thiamine. Previous reports on thiamine deficiency effect on ATP levels are contradictory: McCandless *et al.* [10] showed a 34% decrease in ATP level in rat cardiac muscles after 5 weeks of thiamine deprivation. In contrast, Gubler *et al.* [11] and Sutherland *et al.* [12] reported no decrease in ATP level until death of the rats (after 5 weeks). In neuroblastoma cells, grown for 20 days in low (6 nM) thiamine concentration medium, ATP levels and O_2 consumption only slightly decreased. However, when amprolium (a thiamine uptake inhibitor) was added to the medium, both ATP level and O_2 consumption were decreased, and returned to normal values 1 hr after thiamine (10 μM) addition [22, 29]. In our heart cells ATP levels decreased faster even without addition of a thiamine uptake inhibitor, maybe because the utilization of thiamine and ATP is higher in contracting heart cells, than in neuroblastoma cells. Moreover, the medium of the neuroblastoma cells included 6 nM of thiamine, while our heart cells were grown in a chemical defined medium without thiamine.

It is well established that low ATP levels cause activation of phosphofructokinase (PFK-1), which is the rate-limiting enzyme in glycolysis, resulting in faster ATP production [30]. Our results show an increase in [^{3}H]DG uptake by the cardiac cells, even after 1 day of thiamine depletion. This increase can explain the preservation of normal and stable ATP levels over the first 7 days. Beyond this time, elevated glucose uptake probably no longer compensates for the altered ATP production, resulting in lower levels of ATP (when TPP concentrations fall to 1/6 of control). At first (days 7–9), the decrease in ATP level was gradual, but by day 9–12 a dramatic fall was evident, with ATP levels under 10 nmol/mg protein, compared to 25 nmol/mg protein in the control group (this dramatic decrease looks quite gradual in Fig. 4 because the figure takes the average ATP level of five different experiments in which the ATP decrease started during an interval of 1–2 days). This decrease in ATP coincided with the beginning of the morphological changes, just before cell death, suggesting that loss of ATP has a major impact on cell degeneration.

Because our results showed that 2 hr after thiamine addition TPP level reached the control level, we decided on 2 hr of thiamine treatment for testing whether the changes in ATP levels and [^{3}H]DG uptake are reversible. Indeed, reversibility was obtained, however, after 12 days of thiamine deprivation, 18 hr were required for increasing the ATP level to normal. This exception can be explained by the general degradation starting after 12 days, so that TPP rise to the normal levels is not enough for immediate recovery.

Increased glucose uptake by heart cells as a result of thiamine deficiency has not been reported before, but has been shown in thiamine-deficient rat brain [31, 32]. Increased peripheral glucose utilization has been demonstrated in rats after 6 weeks of thiamine deprivation [21]. Our results show increased [^{3}H]DG uptake by the cardiac cells even after 1 day of thiamine depletion. This discrepancy can be explained by the different tissue source (as-

suming that contracting heart cells are more sensitive to partial thiamine deficiency), the faster TPP elimination and the much younger rat source.

The changes in the spontaneous contraction amplitude of the thiamine-depleted cardiac cells indicate that thiamine deficiency can directly cause heart insufficiency (and not only by an indirect mechanism, after vasodilatation, and increased blood volume). This effect was observed only after 7 days of thiamine depletion, and did not change after 2 more days (afterwards, spontaneous contractions are ceased). The decrease in ATP levels also started after 7 days, suggesting a connection between the ATP decrease and the contraction amplitude changes. The complete stop of spontaneous contractions in the culture occurs at the same period of the dramatic ATP decrease (just before the morphological changes and cell death).

It should be mentioned that our experimental procedure can only measure local changes in the amplitude of a single cell under different treatments. The fact that 120 min were required for maximal thiamine effect suits our observation that 120 min were needed for TPP and ATP rise to the control levels. To our knowledge, only two reports [13, 33] have described the effect of thiamine deficiency on contraction amplitude, but only on rat isolated cardiac specimens in response to external stimulation. Those results showed reduction in shortening velocity after 35 days of thiamine deprivation. It could be argued that this reduction does not represent an amplitude reduction of spontaneous contractions. Our results show a significant reduction of spontaneous contraction amplitude.

The present work shows direct mechanisms by which thiamine deficiency could lead to heart failure. The use of cultured cardiomyocytes can be helpful for further studying chronic and partial thiamine deficiency induced by direct thiamine deprivation or by pharmacological treatment.

We are indebted to Mrs. T. Zinman and A. Isaac for their valuable technical assistance, to S. Victor for typing the manuscript, to E. Pearl for designing and building the electronic device for contraction measurements, and to Prof. H. Halkin for his goodwill. This research is supported by Grant No. 93-222 from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel, and by the Otto Meyerhoff Drug Receptor Center at Bar-Ilan University.

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