Original Research

Effects of long-term anti-ischemic drug treatment on Na,K-ATPase isoforms in cardiomyopathic hamsters

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Abstract: We investigated the effects of long-term anti-ischemic therapy with trimetazidine on Na,K-ATPase (NKA) activity and protein expression in cardiomyopathy. NKA isoforms in membrane fractions from cardiomyopathic hamsters of the BIO 14.6 strain were studied and compared with those from healthy Syrian golden hamsters (F1B). Trimetazidine was orally administered to a subset of cardiomyopathic hamsters in the early stage of active disease (30 days) until the congestive stage (350 days). In the congestive stage of cardiac failure, the cardiomyopathic hamsters displayed altered NKA activity (-55 % vs. F1B; p<0.01), which was related to a specific decrease in abundance of the membrane NKA α_1 isoform (-27 % vs. F1B). Trimetazidine partially prevented the cardiomyopathy-induced changes in NKA activity (+38 %) and α_1 membrane expression (+ 66 %) without inducing changes in the expression of the α_2 isoform or α_1 isoform of NKA. Cardiac hypertrophy and remodeling were reduced after trimetazidine treatment. Additionally, the abundance of NKA α_1 in membranes was negatively correlated with the ventricular weight/body weight ratio (an index of cardiac hypertrophy) ($r^2 = 0.99$; p<0.0015). These findings suggest that some of the cardioprotective effect of trimetazidine during long-term cardiomyopathy may be achieved via regulation of cardiac remodeling and selective modulation of cardiac NKA isoforms.

Key words: Trimetazidine; Na,K-pump; Ouabain; Ion transport; Hypertrophy; Heart failure.

Introduction

The cardiomyopathic Syrian hamster (CMH; Bio (14.6) strain) is widely used as a model of human hypertrophic cardiomyopathy (1). Abnormal calcium homeostasis (2) and microcirculation spasms producing transient focal ischemia (3) have been postulated to be the causes of cardiac cell death in this animal model. Proteomic analysis of this model has recently been performed (4). CMHs are also characterized by large alterations of Na,K-ATPase (NKA), the receptor of digitalis (5-6). Functionally, NKA is the membrane ion pump that maintains Na$^+$ and K$^+$ homeostasis by hydrolyzing intracellular ATP. In excitable cardiomyocytes, elevation of the intracellular Na$^+$ concentration [Na$^+$]$_{in}$ critically affects the sarcolemmal transmembrane transport of two other ions, Ca^{2+}, through Na$^+$/Ca^{2+} exchange, and H$^+$, through Na$^+$/H$^+$ exchange, as well as mitochondrial Na$^+$/Ca^{2+} exchange (7). During cardiomyopathy, this equilibrium is perturbed, leading to Ca^{2+} overload. Inhibition of the transport function of NKA is well known to be associated with cardiac contracture and arrhythmia. Structurally, the sarcolemmal NKA is an oligomer comprising a catalytic α and a glycosylated β subunit

in 1 to 1 stoichiometry. The β subunit is essential for the normal activity of NKA (6, 8). In rodents, the α_1 and β_1 subunits are the major isoforms in most tissues and serve as housekeeping enzymes, but the α_2 isoform is also present in adult rodent cardiomyocytes (8). Alterations of NKA in this model of cardiomyopathy have been linked to early decreases in α_1 isoform gene expression (5). Indeed, a simultaneous decrease in α_1 protein expression and related NKA activity has been found in CMH cardiac membranes (5,6). In the hamster model of idiopathic congestive heart failure (CHF) caused by cardiomyopathy, long-term treatment with numerous drugs, particularly trimetazidine, increases survival owing to their cardioprotective effects (9). However, the involvement of membrane isoforms of NKA in drug-induced cardioprotection and their relationship with hypertrophy have not been fully explored. Recently, the maintenance of NKA activity and intracellular [Na$^+$]$_i$ by trimetazidine during ischemia was hypothesized by Cross (10) following the work of El Banani et al. (11). We have also shown that NKA isoforms can be sensitive to oxidative stress during ischemia and reperfusion (12-15). In accordance, pretreatment with antioxidants protects NKA isoenzyme activity in animal models of

acute ischemia (16-17). Very recently, it was shown that stimulation of the membrane NKA activity linked to signaling prevented cardiomyopathic hypertrophy (18).

Trimetazidine [1-(2,3,4, trimethoxy-benzyl) piperazine dihydrochloride] also shows interesting effects in the clinical treatment of heart failure (19). Trimetazidine exerts its protective effects by preventing ATP levels from decreasing and limiting free radical, proton, sodium and calcium ion accumulation in cardiomyocytes (20-22). The efficacy of trimetazidine administered to young animals has been previously reported in CMH (9, 23-24); however, it is not known whether trimetazidine can also preserve cardiac NKA activity and isoform expression in the CMH model and in long-term follow-up. Using the experimental design of a trimetazidine treatment with a long-term follow-up, the present study aimed to i) characterize NKA activity in relation to the expression of its α and β subunits in the CMH model, ii) determine whether long-term trimetazidine treatment could prevent NKA alterations and iii) define the relationship between membrane NKA changes and cardiac hypertrophy.

Materials and Methods

Animals and drug treatment

Sixteen male Syrian cardiomyopathic hamsters of the BIO 14.6 strain, purchased in Olivet (France), were used in the membrane study. Sex- and age-matched healthy Syrian golden hamsters (F1B; n=5) served as controls (4). The cardiomyopathic hamsters were randomly divided into two groups, CMH and CMH-T, which received normal drinking water and 18 mg/kg b.w./day trimetazidine in their drinking water, respectively (9). Treatment was given from the age of 30 to 350 days as described previously (23-24). All animals were fed a standard laboratory diet. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health NIH publication No 8523, revised 1985).

Tissues and membrane preparations

The hearts of CMHs were rapidly removed and rinsed with cold (+4°C) buffer as described previously (25). The ventricular cardiac tissues and septa were frozen in liquid nitrogen and stored at -80°C until use. After tissue homogenization, NKA-enriched membrane microsomal fractions were obtained by sequential differential centrifugation according to a previously described procedure (12-13, 25).

NKA (Na,K-ATPase) assay

Analysis was performed on microsomal membrane fractions, with an enzyme yield of more than 50 %. This preparation represents a compromise between crude homogenates, with low NKA-specific activities, and sarcolemmal membrane fractions, which contain higher specific NKA activities but low total enzyme activity. The NKA-specific activity was determined using the coupled assay method with or without ouabain, a specific inhibitor of NKA, as described previously (12-14, 16-17). NKA activity was measured at 37°C in ATP regenerating medium in the absence or presence of ouabain by continuously recording NADH produced via NAD

oxidation using a UV/Vis Unicam spectrophotometer. Enzymatic activity was estimated at 37°C as a function of the amount of membrane protein (from 10 to 20 μg) after a 10 min incubation. The specific activity of NKA expressed as μM of Pi released per hour and per mg of protein was calculated in the presence of ouabain ($4 \cdot 10^{-4}$ M) and digitoxigenin (10^{-3} M) after correcting for the ouabain- or digitoxigenin-insensitive activity measured in the presence of ouabain ($4 \cdot 10^{-4}$ M) and digitoxigenin (10^{-3} M).

The assays were carried out with either native or detergent-permeabilized vesicles. Detergent treatments were performed by incubating the membranes with 0.1, 0.2, and 0.3 mg of sodium dodecyl sulfate (SDS) per mg of protein for 30 min at 20°C (17, 25).

Sodium Dodecyl Sulfate Gel Electrophoresis (SDS-PAGE) and Western Blotting

Expression of the α and β NKA isoforms was assessed by immunodetection with specific antibodies after sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) and Western blotting as previously described (13) using an anti-rat α_1 polyclonal antibody provided by E. Feraille (Hôpital Cantonal, Genève, Switzerland), an anti-rat α_2 (McB2) monoclonal antibody provided by K. Sweadner (Harvard University, Boston, USA), an anti-human β_1 polyclonal antibody provided by P. Martin Vasallo (Universidad de la Laguna, Tenerife, Spain), and secondary commercial anti-mice and anti-rabbit antibodies (Upstate Biotechnology, Lake Placid, NY, USA). Samples of highly purified plasma membrane NKA from the rat brain (α_2) and kidney (α_1) were used as controls according to Feschenko et al. (26). Equal protein loading was verified by protein determination and Ponceau staining. At least three independent blots were analyzed with reproducible results as determined by quantitative densitometry using a scanning densitometer (Arcus; Agfa Gevaert, Morbel, Belgium). The amount of protein was quantitated using imaging software and is expressed as arbitrary units.

Statistical analysis

Analysis of variance (ANOVA) of the data was performed, if significant, using the Newman–Keuls test for pairwise comparisons (SigmaStat Software; Systat Software, San Jose, CA, USA), except for the NKA activity results, for which the data were compared using the Student' t-test.

Results

Changes in the ventricular weight of cardiomyopathic hamsters

At 350 days of age, BIO 14.6 cardiomyopathic hamsters were smaller than control animals (Table 1). CMHs had a significantly lower body weight (-14 %; p<0.05) than that of age-matched control hamsters, and the ventricular weight-to-body weight ratio of CMHs was increased significantly by 27 % compared with that of age-matched control hamsters. Trimetazidine treatment over 320 days (18 mg/kg b.w./day) significantly reduced myocardial hypertrophy (ventricular weight/body weight ratio, 4.2 to 3.6 mg/g; p<0.05).

Table 1. Anatomical characteristics of healthy cardiomyopathic and trimetazidine-treated cardiomyopathic hamsters at 350 days of age.

	F1B	CMH	CMH-T
Body weight (g)	162 ± 8	140 ± 3*	161 ± 4.7§
Ventricular weight/body weight (mg/g)	3.3 ± 0.1	4.2 ± 0.1*	3.6 ± 0.1§
Number of animals	5	8	8

Healthy (F1B), cardiomyopathic (CMH) and trimetazidine-treated cardiomyopathic (CMH-T, 18 mg/kg b.w./day) hamsters. Values are means ± SEM. *: $p<0.05$ vs. F1B, §: $p<0.05$ vs. CMH.
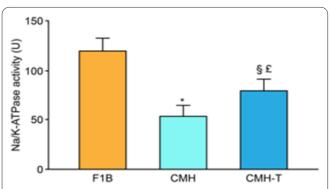
Table 2. General characteristics of cardiac microsomal membrane fractions from healthy, cardiomyopathic and trimetazidine-treated cardiomyopathic hamsters at 350 days of age.

	F1B	CMH	CMH-T
Tissue weight (mg)	195 ± 22	260 ± 31*	193 ± 24§
Membrane proteins (mg)	2.5 ± 0.8	3.7 ± 0.9	2.5 ± 0.8
Yield in protein (%)	12 ± 3	14 ± 3	13 ± 3
Number of animals	5	8	8

Healthy (F1B), cardiomyopathic (CMH) and trimetazidine-treated cardiomyopathic (CMH-T, 18 mg/kg b.w./day) hamsters Values are means ± SEM. *: $p<0.05$ vs. F1B, §: $p<0.05$ vs. CMH.

Na,K-ATPase (NKA) activity in cardiac microsomal fractions

The characteristics of NKA were examined in the three experimental groups. For this analysis, we isolated cardiac membrane fractions from control, cardiomyopathic, and trimetazidine-treated cardiomyopathic hamsters at 350 days of age (Table 2). Despite the increased ventricular weight of the CMHs, the yield of membrane proteins remained unchanged, at 12–14 %. The effect of cardiomyopathy was estimated by comparing CMHs with control animals, and the effect of trimetazidine was estimated by comparing CMHs with and without trimetazidine treatment. These comparisons required the measurement of NKA activity in leaky membranes, to allow access to both ATP and ouabain binding sites. Detergent treatments with different concentrations of SDS were used to permeabilize vesicles. However, these SDS treatments decreased the NKA activity of the three study groups, indicating that all the membrane vesicles were leaky (data not shown). The results are presented in Figure 1 and indicate that sarcolemmal NKA activity was markedly decreased in CMHs compared with that in control animals. In trimetazidine-treated CMHs, sarcolemmal NKA activity was significantly higher than that

in CMHs ($p<0.05$). Additionally, this activity was still 34 % lower than that in control F1B animals ($p<0.05$).

NKA isoform expression in cardiac microsomal fractions

To evaluate the changes in the NKA isoforms that might explain the differences in NKA activity, immunodetection by western blot analysis was performed on cardiac microsomal membranes. In the presence of 10 μg of protein, the α_1, α_2 and β_1 isoforms of NKA were identified in all three groups. As shown in Figure 2, the intensity of the 114-kDa band corresponding to the α_2 isoform was not significantly different among the
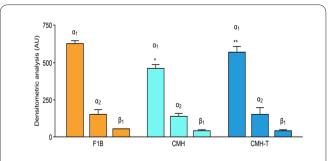

Figure 2. Immunoblot and densitometric analyses of Na,K-ATPase α_1-, α_2-, and β_1-subunit expression. Samples from microsomal membrane fractions purified from healthy (F1B), cardiomyopathic (CMH) and trimetazidine-treated cardiomyopathic (CMH-T) hamsters, corresponding to the membranes purified for the NKA activity measurements, were used. Samples were electrophoresed on a 4–15 % polyacrylamide gradient gel; blotted onto nitrocellulose; probed with isoform-specific anti-α_1 (A), β_1- (B) and α_2-(C) antibodies; and detected by the enhanced chemiluminescence method. Isoform detection was performed using 10 μg of membrane proteins with 1/250 antiserum for α_1, 1/20 antiserum for α_2 and 1/1000 antiserum for β_1. The apparent molecular weights of the α and β subunits were deduced by linear regression from those of prestained commercial SDS-PAGE standards for each gel (data not shown). The bar graphs (D) (means ± SEM) correspond to immunoblot densitometric analysis of the NKA α_1, α_2, and $\beta_?$-subunits in at least 6 lanes from 3 independent immunoblots.

Figure 1. Sarcolemmal NKA activity from healthy (F1B), cardiomyopathic (CMH) and trimetazidine-treated cardiomyopathic (CMH-T) hamsters. (U): μmoles Pi/mg Prot./h. Values are presented as means ± SEM (n=5 to 8 per group), *: $p<0.05$ vs. F1B; £: $p<0.05$ vs. CMH; §: $p<0.05$ vs. F1B.

samples, suggesting that the expression of this isoform was not modified by cardiomyopathy or the trimetazidine treatment. Similarly, no significant difference was found in the immunoreactivity of the β_1 isoform (42 kDa) among the three groups in this study. In contrast, cardiomyopathy induced a decrease in the intensity of the 113-kDa band, corresponding to the α_1 isoform, and the trimetazidine treatment partially prevented these changes (+66 %; $p<0.05$). The intensity of the 114-kDa band corresponding to the α_2 isoform was not significantly different among the samples, suggesting that the expression of these isoforms was not modified by cardiomyopathy or the trimetazidine treatment. Similarly, there was no significant difference in the immunoreactivity of the β_1 immunoreactivity (42 kDa) among the three groups in this study (Figure 2).
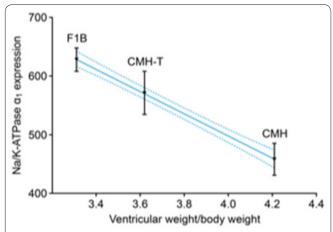
Relationship between the membrane expression of the α_1 isoform to myocardial ventricular hypertrophy

Given the observed changes in cardiac NKA activity and abundance, we next evaluated the relationship between the expression of the α_1 isoform and cardiac hypertrophy. The changes in the expression of the α_1 isoform were significantly negatively correlated with the changes in ventricular hypertrophy (Figure 3). These data suggest that ventricular hypertrophy is influenced by cardiac membrane expression of the α_1 isoform of NKA.

Discussion

This study shows the beneficial effect of long-term oral therapy with an anti-ischemic drug, such as trimetazidine, in a cardiomyopathic Syrian hamster model, as evidenced by significant improvements in ventricular hypertrophy and the preservation of sarcolemmal NKA activity and its isoforms.

We found that trimetazidine pretreatment minimized the decrease in sarcolemmal NKA activity that accompanies cardiomyopathy. Moreover, administration of trimetazidine partially prevented the decrease in membrane expression of the α_1 isoform. These findings strongly suggest that the cardioprotective effect of the anti-ischemic drug trimetazidine could be achieved, at least in part, by preserving sarcolemmal NKA activity. The decrease in NKA reported in this study is in agreement with previous findings showing decreased NKA activity in a CMH inbred strain (5, 6) as well as in other strains (6,13). Furthermore, our study demonstrated reduced protein expression of the α1 isoform of NKA without detectable changes in protein expression of the α_2 isoform. The magnitude of the decrease in NKA activity (approximately half of that observed in the control) is very similar to that found by Panagia et al. (25) in older hamsters with terminal heart failure. Furthermore, these changes were not an artifact of membrane isolation because the decrease in NKA activity in homogenates was similar to that found in crude membrane fractions (as used in the present study) and in enriched-membrane sarcolemmal fractions (5). These authors reported a similar decrease of 33 % in crude homogenate fractions. We detected NKA alterations without using SDS. Changes in NKA activity and gene expression

Figure 3. Inverse association between α_1 membrane expression and the ventricular weight to body weight ratio. Correlations were evaluated by simple linear regression analysis with average values from each group. A significant association was found for the values from the three groups ($r^2 = 0.99$; $p<0.0015$). The dashed lines represent 95 % confidence intervals.

appear to be among the earliest biochemical signs of cardiomyopathy in the Syrian hamster because they are observed at an age of only 25 days (5). Thus, alterations of NKA by a change in gene expression and by the possible decreased transcription of the α_1 subunit could be a common feature of cardiomyopathy at the molecular level, independent of age, strain and severity of heart failure. The α_1 subunit of Na, K-ATPase is the major isoform contributing to membrane enzyme activity and is at a proportion of 4 or 5 to 1 with the other isoforms of NKA (8,15). In the present study, the decrease in protein abundance fully explains the decrease of enzyme activity. Indeed, only half of the decrease in enzyme activity could be attributed to a lack of α_1 membrane protein (-27 % in protein level vs. -55 % in enzyme activity). Oxidant-induced inactivation of NKA activity without an effect on protein abundance, as observed *in vitro* (12), may also contribute to injury in the present model over the long term. The molecular basis of these alterations in NKA activity in hamsters remains speculative and requires further investigation. One indication of decreased transcription of the α_1 subunit has previously been shown in hamsters (5) and humans (27).

An elevated cytosolic Na^+ concentration is the most direct consequence of reduced NKA activity and density in the sarcolemmal membrane. Sodium fluxes are coupled to hydrogen and Ca^{2+} fluxes through sarcolemmal Na/H- and Na/Ca-exchangers, respectively, particularly during ischemic insults (7, 10-11,28). Thus, a decrease in NKA activity may potentiate intracellular acidosis induced by ischemia in this genetic model of cardiomyopathy. Such decreases are important for the maintenance of the cytosolic calcium concentration, as previously evidenced in this model after trimetazidine treatment (24). Inhibition of NKA activity by digitalis increases cardiac contractility during heart failure. Therefore, one consequence of the reduced NKA activity may be increased cardiac contractility in the presences of progressive cardiomyopathy. Finally, calcification and necrosis in CMHs (28) could, in part, result from excessive NKA alterations.

In this long-term study, drug treatment began before

the onset of cardiac hypertrophy and appeared to minimize disease progression. We found a close relationship between NKA expression and cardiac hypertrophy (Figure 3). This relationship could result from molecular mechanisms discovered recently in rat cardiomyocytes. Rat and hamster hearts have the same expression pattern of NKA isoforms, as shown in the present study (29). In contractile cardiac cells, partial inhibition induced by ouabain has also been demonstrated to stimulate hypertrophic growth (30). Markers of cardiac hypertrophy have been characterized and correspond to the activation of early- and late-response genes. Furthermore, given the action of trimetazidine as an antioxidant, it may be relevant that oxygen reactive species are important mediators in the ouabain signaling pathway, leading to hypertrophy following partial NKA inhibition in rat cardiomyocytes (31). Thus, the trimetazidine-induced preservation of NKA activity over the long term could be responsible for the reduced hypertrophy observed in our treated CMHs. Reactive oxygen signals from oxidant stress have also been described to be second messengers within several signal pathways involved in the control of gene transcription (31-33). The protective effect of trimetazidine obtained by treating animals from 30 to 320 days of age is similar to that obtained with vitamin C or E treatment (33-34). Trimetazidine, via its antioxidant properties, could prevent the possible growth-related effects of NKA inhibition in CMH, as hypothesized by Cross in an editorial (10).

In ischemic cardiomyopathy resulting from focal ischemia, the relationship between Na,K-ATPase inhibition and ischemic preconditioning should be discussed considering recent results. The binding of ouabain to NKA has recently been shown to reduce the infarct size-limiting effect of preconditioning (15). We hypothesize that ischemic preconditioning may be low in CMHs. It has also been shown that trimetazidine interacts with mitochondria by improving cardiac mitochondrial energy metabolism during ischemia (21-22). In summary, these mechanisms may all contribute to trimetazidine's partial preservation of NKA activity and the selective expression of the α_1 isoform in CMH.

Cardiac remodeling is a rather complex issue (34-36), particularly in hamsters with cardiomyopathy (18). The prevention of myocardial remodeling by trimetazidine in the same animal model and using the same experimental protocol was not found to be associated with the prevention of the isoform shift of myosin from V1 to V3, as previously described in rat cardiac hypertrophy and remodeling [8,23-24]. Furthermore, in these previous studies [23-24], trimetazidine maintained the same level of V3 in CMH and F1B with terminal heart failure from the beginning (30 days) to the end of the study (350 days). Similarly, the effect of remodeling on NKA has been poorly investigated, except in a model of pressure overload in rats in which we described an isoform shift between the α_2 and α_3 isoforms of neonatal NKA (37-39). These differences from the current study may be explained by the severity of hypertrophy and remodeling occurring above the left ventricular mass in more than 60 % (vs. 27 % in the present study) of sham-operated rats and to the specific characteristics of hamsters (36). Irrespective of the mechanism underlying the changes observed in the present study, minimizing left ventricular hypertrophy is a desirable therapeutic goal for patient care because the regression of remodeling improves survival.

Intracellular Na+ (40) and signal transduction (31) during cardiac remodeling and hypertrophy via the activity of membrane NKA as causal links (40) should be further investigated in experimental models and patients during ventricular cardiac dysfunction.

Trimetazidine treatment appears to be responsible for the amelioration of survival and hemodynamics in hamsters with heart failure after 320 days of drug treatment (23). This finding is in accordance with the results of two recent studies by Lin et al 2020 (41), who found increased survival with trimetazidine treatment in an experimental heart failure model, and by Li H et al. 2020 (42), who showed that trimetazidine could preserve metabolic remodeling and improve hemodynamics and survival in heart failure. Whether membrane NKA is a prognostic biomarker during heart failure requires further study in addition to those recently reviewed by Liu et al. (43). This study is the first to monitor the activity and expression of isoforms of NKA during anti-ischemic drug treatment throughout the life of genetic CMH animals.

This study is an experimental study of a unique animal model of cardiomyopathy and a single concentration of trimetazidine. Pressing questions of both clinical and fundamental interest that remain to be answered include the generalization of the protection of trimetazidine to other models, the dose-ranging effect of trimetazidine, the evolution of cardioprotection and NKA at key time points before and during the development of cardiac hypertrophy and at the end of animal life since survival is a key clinical endpoint, the association of trimetazidine with standard-of-care treatments (drug association consisting of vasodilators, diuretics and digitalis), and the control of the therapeutic scheme during the development of cardiac failure.

In conclusion, we demonstrate for the first time the cardioprotective effect of trimetazidine on cardiac ventricular remodeling during long-term treatment in CMH associated with both changes in membrane NKA activity and α_1 isoform expression. The protection of plasma membrane NKA at the molecular level by trimetazidine may contribute to improved myocardial function. Indeed, the maintenance of cardiomyocyte Na$^+$ homeostasis and structural integrity are regulated by fundamental biological functions of NKA, which are, in turn, essential for cardiac preservation. This study suggests that trimetazidine can interfere either directly or indirectly with membrane NKA and the modulation of its molecular functions. Exploitation of this new understanding may allow for the modulation of cardiomyopathies and lead to the development of new clinical therapies linking integrative metabolism from mitochondria to NKA in cardiomyocytes**.**

Author Contributions

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Effect of trimetazidine on Na,K-ATPase ISOFORMS during hypertrophic cardiomyopathy..

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Conflicts of Interest
The authors declare no conflicts of interest.

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Jean Michel Maixent *et al.*

Effect of trimetazidine on Na,K-ATPase ISOFORMS during hypertrophic cardiomyopathy..

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