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Effect of trimetazidine and verapamil on the cardiomyopathic hamster myosin phenotype

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1 In this study we investigated whether long-term trimetazidine (anti-ischaemic drug) therapy alters the ventricular myosin heavy chain (MHC) isoform composition in a model of cardiomyopathy.

2 MHC isoforms were analysed in the native state by electrophoresis in a pyrophosphate buffer. Myosin isoform patterns were studied in cardiac muscle from cardiomyopathic hamsters (CMH) of the BIO 14:6 strain during the time course of the disease and compared with those of healthy golden hamsters (F1B). The correlation between myosin profile and Ca^{2+}-activated ATPase activity was determined from 220 days.

3 At the stage of insufficiency (350 days), CMH presented the most abnormal phenotype with 53% V1-24% V3 compared to 79% V1-7% V3 ($P<0.001$), in F1B. Trimetazidine was administered to cardiomyopathic hamsters from the early stage of active disease (30 days) to the congestive stages (220–350 days). Within 65 days, trimetazidine treatment, in CMH and F1B, reduced V1 to a low level (53% and 62%, respectively), which remained constant throughout the treatment. This level was similar to that in 220 and 350 days-old untreated-CMH. In sharp contrast, a standard calcium blocker, verapamil, administered to CMH in the same conditions resulted in a higher V1 (about 70%) and higher global myosin ATPase activity from 220 days.

4 Previous results in terms of hypertrophy and survival, compared to these results, suggest that verapamil and trimetazidine treatments reveal a disssociation between ventricular hypertrophy and isomyosin distribution. In addition, the shift in favour of V3 may not necessarily be an aggravating factor of the disease but an adaptative compensatory event.

Keywords: Cardiomyopathy; cardiac muscle; trimetazidine; verapamil; myosin isozymes; myosin ATPase activity; hypertrophy; survival; ischaemia

Introduction

The BIO 14:6 Syrian cardiomyopathic hamster (CMH) is a widely used model of both cardiac and skeletal abnormalities (Kagiya *et al*., 1991; Davidoff & Gwathmey, 1994). Abnormal cellular calcium homeostasis and spasms of the microcirculation, producing transient focal ischaemia, have been postulated to be at the origin of the cell death in these animals (Natelson *et al*., 1991; Conway *et al*., 1994). In the heart, cell loss is associated with compensatory ventricular hypertrophy and the degree of hypertrophy correlates with the myosin isoform restructuring (see Kitsis & Scheuer, 1988; Lompré *et al*., 1991 for reviews). The distribution of ventricular myosin isozymes (V1, V2, V3) in the CMH changes during development and especially as the pathological process of cardiomyopathy progresses. Isomyosin V1 consists of two myosin heavy chain α (α-MHC) exhibits higher Ca^{2+}-ATPase activity and is associated with higher shortening velocity than V3, which contains two β-MHC. V2, a heterodimer of α- and β-MHC, has intermediate ATPase activity. The change from predominantly V1 to slow migrating V3 isozyme parallels the depressed myosin Ca^{2+}-ATPase activity in the hypertrophic and final stages of cardiomyopathy (Malhotra, 1990). Production of myosin isoforms favouring the V3 form may, like hypertrophy, be a compensatory event in the evolution of the disease process (Schwartz *et al*., 1988). It was therefore of interest to test the effects of an anti-ischaemic agent on the cardiomyopathic isomyosin phenotype. Trimetazidine, 1-(2,3,4 trimethoxybenzyl) piperazine di-hydrochloride (marketed as Vastarel by Biopharma, Neuilly-sur-Seine, France) is used in the clinical treatment of anginal disease (Dalla-Volta *et al*., 1990; Detry *et al*., 1994). It was synthesized in the 1960s and first tested in the clinic in 1964. It protects myocardial cell function during ischaemia by preventing the fall in adenosine 5′-triphosphate (ATP) (Lavanchy *et al*., 1987), limiting the content of free radicals (Maupoil *et al*., 1990; Devynck *et al*., 1993) and prevention of the accumulation of Na^+ and Ca^{2+} in the myocyte (Renaud, 1988). However, it is not known whether anti-ischaemic treatment, begun when young, could maintain the V1 level, thereby preventing cardiomyopathy-related V1 myosin isozyme decrease. Our previous results have shown that trimetazidine possesses anti-Ca^{2+} properties in CMH (D'hahan *et al*., 1997). In addition, some calcium blockers modulate the myosin pattern in the heart (Factor *et al*., 1988; Raizada *et al*., 1994). The aims of the present study were thus (i) to follow the relative proportion of heavy-chain myosin isozymes in healthy golden hamsters (F1B) and CMH, during the progression of the disease; (ii) to determine whether long-term treatment with trimetazidine, in comparison with the standard Ca^{2+} blocker verapamil also used as an anti-anginal agent, normalizes the myosin isozyme profile and (iii) to study its effects on the myosin Ca^{2+}-activated ATPase activity.

Preliminary accounts of this work were presented at the XXIV and XXV European Muscle Conferences (European Society for Muscle Research), Florence, Italy, September 13-16, 1995 and La Grande Motte, France, September 14-17, 1996.

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Methods

Animals, study design and experimental protocol

This study was conducted with 50 30 days-old cardiomyopathic hamsters of the BIO 14 : 6 strain, CMH (Olivet, France) and with 50 control F1B golden hamsters (Depré, France). To follow the evolution of the myosin phenotype during the disease, we analysed the myosin isozyme distribution at the four pathological stages (cited in Venkatakrishnan & Rourke, 1979) : 30 days, prenecrotic stage (4 CMH and 4 F1B), 65 days, necrotic stage (5 CMH and 9 F1B), 220 days, hypertrophic stage (10 CMH and 6 F1B), and 350 days (5 CMH and 7 F1B), final stage. Animals were treated from age 30 days and treatment efficiency was determined in CMH exhibiting well-established cardiac disease after 220 days and 350 days.

Protocol A Four CMH and 4 F1B received 18 mg kg^{-1} day^{-1} trimetazidine (gift from IRIS (Institut de Recherches Internationales Servier, France)) administered in drinking water from age 30 days to 65 days. Four CMH and 4 F1B similarly received 120 mg kg^{-1} day^{-1} verapamil (Sigma).

Protocol B Five CMH and 4 F1B received 18 mg kg^{-1} day^{-1} trimetazidine administered in drinking water from age 30 days to 220 days. Five CMH and 4 F1B similarly received 120 mg kg^{-1} day^{-1} verapamil. Ca^{2+}-ATPase activity was determined on 3 animals, from each group.

Protocol C Four CMH and 4 F1B received 18 mg kg^{-1} day^{-1} trimetazidine administered in drinking water from age 30 days to 350 days. Four CMH and 4 F1B similarly received 120 mg kg^{-1} day^{-1} verapamil.

The plasma concentration of trimetazidine (about 75 nM) and of verapamil (about 60 nM) were determined in a previous study and correspond to the therapeutic range (0.1 – 1 μM) i.e. to a concentration found *in vivo* and *in vitro* to limit the consequences of ischaemia (see in D'hahan *et al*., 1997). Note that the kinetic half-life of the drug is about 6 – 7 h. All animals were individually housed at CE-Saclay under identical controlled conditions with standard laboratory diet. Animals, at every age studied, were anaesthetized with ether, their hearts (including both ventricles) were removed and, after the atria were dissected, weighed.

Myosin extraction and isoenzyme composition

Myosin isozymes in CMH, F1B and treated animals (trimetazidine and verapamil) were analysed after 30, 65, 220 and 350 days by pyrophosphate (PP_i) polyacrylamide gel electrophoresis, as independently developed by Hoh *et al*. (1978) and d'Albis *et al*. (1979). Crude extraction of myosin without further purification was performed according to d'Albis & Janmot (1993). Electrophoresis was carried out at 2°C for 22 h, with a Pharmacia GE-4 electrophoresis chamber and cylindrical gels (6 × 0.5 cm). The gels were fixed and stained with Coomassie brilliant blue and scanned by a scanning densitometer at 600 nm. The areas under the peaks were measured to determine the relative amounts of V1, V2 and V3 isomyosins.

Determination of ATPase activity on gels

Ca^{2+}-activated ATPase activity of myosin was determined according to the method of Hoh *et al*. (1978), modified by d'Albis & Janmot (1993). After 20 h of electrophoresis, the current was stopped, buffer replaced by an ATPase electrophoresis buffer containing 190 mM glycine, 20 mM Tris, 5 mM ATP, 10% glycerol, pH 8.8, and electrophoresis resumed for 2 h. Extruded tube gels were then incubated with shaking in ATPase assay buffer at 37°C. This buffer contained 190 mM glycine, 600 mM KCl, 15 mM $CaCl_2$, 25 mM Tris, 0.05% mercaptoethanol, pH 8.8 and 5 mM ATP was added immediately before use. We followed and scanned the appearance of white bands of calcium phosphate precipitate, which corresponds quantitatively to the hydrolysis of ATP. The slope of the regression line of the function 'area under calcium phosphate peak/area under protein peak, as a function of time' was taken as the specific activity of the myosin (AU; arbitrary units). This analysis was performed on 220 days-old F1B, CMH and CMH-treated animals.

Data analysis

All variables are presented as means ± s.e.mean. The data were analysed by two-way analysis of variance (ANOVA; treatment and strain (F1B/CMH) as factors), followed, if significant, by Newman-Keuls test for pairwise comparisons (SigmaStat Software, Jandel Scientific). The number of animals in each group is indicated as n. Statistical significance was set at $P = 0.05$. Linear regressions were also performed by use of SigmaStat Software, with Pearson's test for correlation assessment (r, correlation coefficient).

Results

Electrophoretic pattern of native myosins

In both control and cardiomyopathic hamsters, there was a tendency for the relative amount of V1 to decrease progressively with ageing, as has already been demonstrated (Lompré *et al*., 1981) (Table 1). However, from 220 days, the decrease was larger for CMH than F1B. This difference was confirmed at 350 days: the V1 myosin isozyme was 34% lower ($P < 0.001$) in untreated CMH than F1B and the relative content of V3 increased at the expense of that of V1. Thus, at the age of insufficiency (350 days), CMH presented the most significant phenotype with 53% V1-24% V3 compared to 79% V1-7% V3 ($P < 0.001$) in F1B (Figure 1).

At 65 days, ANOVA revealed a significant effect of treatments on V3 ($F(2, 25) = 52.2$; $P < 0.001$) and V1 levels ($F(2, 25) = 20.3$; $P < 0.001$). *Post-hoc* multiple pairwise comparisons showed a significant effect of trimetazidine at the beginning of the disease: trimetazidine decreased V1 towards a value similar to that for untreated- and trimetazidine-treated CMH at 220 days and 350 days (Table 1). When trimetazidine was applied to healthy hamsters, it similarly reduced V1 levels and increased V3 levels ($P < 0.05$) (Table 1). By sharp contrast, verapamil had no significant effect at 65 days, on either CMH or the normal profile (Table 1).

At 220 and 350 days, the effects of the treatments were significant, as assessed by ANOVA on V3 level ($F(2, 27) = 15.0$; $P < 0.001$ at 220 days and $F(2, 25) = 5.9$; $P < 0.01$ at 350 days) and on V1 level ($F(2, 27) = 30.7$; $P < 0.001$ and $F(2, 25) = 7.1$; $P < 0.005$). *Post-hoc* multiple pairwise comparisons showed that trimetazidine did not reverse the V1 decline: the V3 level was not significantly different from that in untreated CMH animals (about 54%). However, with verapamil, a higher V1 with a simultaneous reciprocal decrease in V3 was found in CMH ($P < 0.001$) (Figure 1, Table 1). V1 and V3 in verapamil-treated CMH were not significantly different from the values for verapamil-treated F1B.

Table 1 Effects of treatments on myosin pattern at various ages, in cardiomyopathic and control hamsters

Myosin isoform	*Animal group*	*Prenecrotic stage 30 days*	*Necrotic stage 65 days*	*Hypertrophic stage 220 days*	*Terminal stage 350 days*
	F1B	86.3±1.2 (4)	79.3±4.5 (9)	80.5±1.6 (6)	79.4±3.9 (7)
	vs F1B-T; F1B-V		$P<0.05$; NS	$P<0.001$; NS	$P<0.05$; NS
	CMH	83.3±2.8 (4)	80.4±2.7 (5)	56.0±1.2 (10)	52.2±2.9 (5)
	vs F1B	NS	NS	$P<0.001$	$P<0.001$
	CMH-T	*oooooooooo*	53.5±1.3 (4)	54.3±0.9 (5)	54.3±2.9 (4)
V1%	vs CMH		$P<0.001$	NS	NS
	CMH-V	*oooooooooo*	71.7±1.3 (4)	70.6±1.8 (5)	70.3±1.3 (4)
	vs CMH		NS	$P<0.001$	$P<0.05$
	F1B-T	*oooooooooo*	61.7±1.1 (4)	62.0±1.1 (4)	62.7±0.7 (4)
	vs CMH-T		$P<0.005$	$P<0.01$	$P<0.05$
	F1B-V	*oooooooooo*	74.0±0.8 (4)	74.3±0.9 (4)	74.0±0.9 (4)
	vs CMH-V		NS	NS	NS
	F1B	3.0±0.6 (4)	7.3±1.7 (9)	7.3±0.6 (6)	6.7±1.7 (7)
	vs F1B-T; F1B-V		$P<0.01$; NS	$P<0.001$; NS	$P<0.05$; NS
	CMH	6.6±0.8 (4)	6.6±0.9 (5)	19.0±1.1 (10)	23.6±0.7 (5)
	vs F1B	NS	NS	$P<0.001$	$P<0.001$
	CMH-T	*oooooooooo*	23.6±0.8 (4)	20.6±0.6 (5)	22.0±2.8 (4)
V3%	vs CMH		$P<0.001$	NS	NS
	CMH-V	*oooooooooo*	9.5±0.4 (4)	12.3±1.3 (5)	13.3±0.8 (4)
	vs CMH		NS	$P<0.001$	$P<0.001$
	F1B-T	*oooooooooo*	18.2±0.8 (4)	17.0±1.0 (4)	15.5±0.9 (4)
	vs CMH-T		$P<0.01$	$P<0.05$	$P<0.05$
	F1B-V	*oooooooooo*	9.7±0.8 (4)	10.3±0.6 (4)	11.5±1.8 (4)
	vs CMH-V		NS	NS	NS

Number of animals studied is indicated in parentheses. F1B: controls; CMH: cardiomyopathic hamsters; CMH-T/F1B-T: trimetazidine treated CMH/F1B; CMH-V/F1B-V: verapamil treated CMH/F1B. *oooooooooo*: no isomyosin measurement was performed at 30 days, which was the beginning of the treatment. NS, not significant.

Specific Ca^{2+}-ATPases activities

The whole myosin Ca^{2+}-ATPase activity was lower in CMH than in F1B, at the late stage of the disease (10.3 AU in F1B ($r=0994$; $P=0.0055$) compared to 5.6 AU in CMH ($r=0.998$; $P=0.0012$) (Figure 2). These values were close to the values for other small mammals, including rabbit, guinea-pig and pig, containing myosins with low activities (2.6, 3, 3.5, respectively) (Lompré *et al*., 1981). In 220 days-old animals, V1 exhibited higher Ca^{2+}-ATPase activity than V3 in F1B and in CMH (not shown). The time courses of the changes in Ca^{2+}-ATPase activities fell into two distinct groups: that of the CMH and trimetazidine-treated CMH with low activities (5.6 AU ($r=0.998$; $P=0.0012$) and 6.0 AU ($r=0.992$; $P=0.0078$), respectively) and that of the F1B and verapamil-treated CMH with higher activities (10.3 AU ($r=0.994$; $P=0.0055$) and 8.3 ($r=0.997$; $P=0.0031$), respectively) (Figure 2). Note that the two treatments did not modify the Ca^{2+}-ATPase activity of the two major components, V1 and V3 (not shown).

Thus, as early as 65 days, trimetazidine reduced V1 and increased V3 percentages in CMH and F1B, a profile which remained constant throughout the treatment. In sharp contrast, verapamil induced from 220 days, higher V1 and lower V3 levels, parallel to an increased myosin Ca^{2+}-ATPase activity.

Discussion

The present results demonstrate qualitative and quantitative changes in myosin isoforms during the progression of cardiomyopathy and treatment with trimetazidine, in comparison with a classical calcium blocker, verapamil.

At 220 days, both the myosin Ca^{2+}-ATPase activity and the percentage of V1 isoenzyme were lower in CMH than in F1B. The isomyocin pattern was not different in CMH and F1B before 220 days. Thus the shift from V1 to V3 seems to occur later than in other cardiomyopathic hamster strains (Wiegand *et al*., 1983; Malhotra *et al*., 1985; Lambert *et al*., 1995). Moreover, no difference in myosin isoform Ca^{2+}-activted ATPase activity was observed between F1B and CMH at this stage. The effects of verapamil on myosin isoforms were observed by 220 days. Verapamil prevented the myosin isoform shift in CMH, suggesting that it inhibits the stimuli responsible for increased V3 or decreased V1 expression. Such a correction of the myosin pattern has previously been observed with verapamil in the BIO 53 : 58 strain (Factor *et al*., 1988) and in young rats treated with another calcium blocker, nifedipine (Raizada *et al*., 1994). How exactly verapamil prevents the shift by maintaining V1 myosin content in CMH is unclear. Possibly verapamil, through its peripheral vasodilator action (Godfraind *et al*., 1991), reduces the peripheral vascular resistance and myocyte stress (Raizada *et al*., 1994). As a result, the V3 synthesis would be repressed. Thus, the effects of verapamil on myosin phenotype would be specific to the disease state and possibly mediated through effects upon the systemic circulation. Nevertheless, it is obvious that, as Lubic *et al*. (1995) underlined in a recent study, it remains difficult to differentiate, *in vivo*, whether the effects of the calcium channel blockers are a result of an indirect effect via their action in lowering blood pressure or a direct negative inotropic effect on the myocardium. A decreased contractile activity has been shown to alter contractile protein synthesis (Samarel & Engelmann, 1991; Sharp *et al*., 1993). We cannot exclude a possible direct effect of verapamil on the myocardium. In sharp contrast to drugs

such as verapamil, trimetazidine, another anti-anginal agent, improves exercise capacity without altering blood pressure, heart rate or contractility (Timour *et al*., 1991; Detry *et al*., 1994). Trimetazidine, like other piperazine derivatives (Williams *et al*., 1992), is considered to belong to a new class of anti-anginal agents, the mechanism of which involves direct myocardial cytoprotection (Chiercha & Fragasso, 1993; Veitch *et al*., 1995). However, the precise mechanism(s) of the beneficial effect of trimetazidine is still unknown (Fantini *et al*., 1994). At 220 and 350 days, no effect on myosin isoform and myosin ATPase activity could be detected; the level in V3 in trimetazidine-treated CMH was the same as that in untreated CMH. The effects of trimetazidine treatment were different at earlier stages of the disease. At 65 days, while the myosin isoform profile was the same in CMH and F1B (about 80% V1 and 6% V3), in trimetazidine-treated CMH and F1B, the proportion of V1 was only 53% (about 20% V3) and remained constant thereafter. Trimetazidine may therefore not act specifically on the CMH myosin profile. Thus, trimetazidine effects resulted in the same level of V3 being maintained in CMH and F1B, and this from the beginning to the end of the treatment.

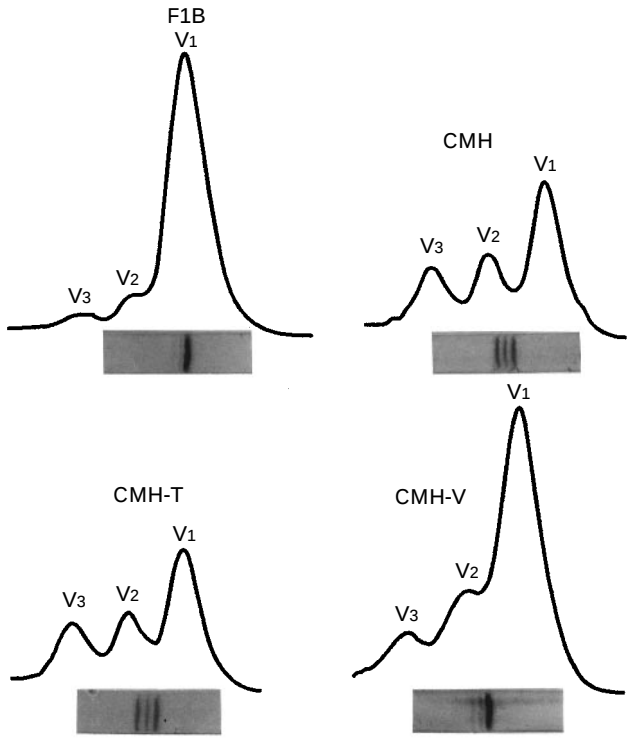

Figure 1 Pyrophosphate gel patterns and scans of gel patterns in representative samples from hearts of control (F1B), untreated myopathic (CMH), and treated animals at 350 days: trimetazidine-treated CMH (CMH-T), verapamil-treated CMH (CMH-V).

These results seem to be in apparent contradiction to previous results, in terms of ventricular hypertrophy. First, as previously described in the literature (Mercadier *et al*., 1981), we observed a correlation in CMH ($r=0.998$; Figure 3) between the V3 isomyosin percentage and the degree of ventricular hypertrophy previously obtained (D'hahan *et al*., 1997). This suggests that the shift is associated with the severity of the haemodynamic overload (Lompré *et al*., 1991). Secondly, we demonstrated a positive and specific effect of trimetazidine and verapamil, abolishing CMH ventricular hypertrophy, from 220 and 350 days, respectively (D'hahan *et al*., 1997). Thus we attempted to prevent the redistribution from V1 to V3, by treating the animals with these drugs. But, as suggested by the absence of correlation between the ventricular to body weight ratio and the percentage of V3 in CMH under treatments (Figure 3), our present results indicate a dissociation between the effects of drugs on hypertrophy and

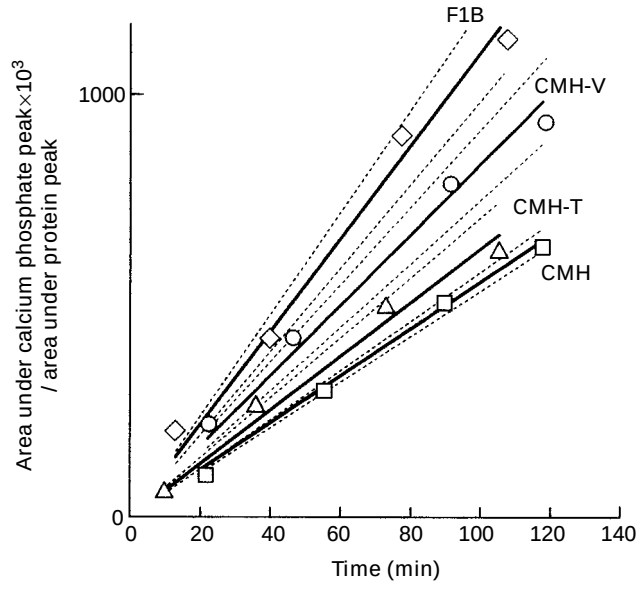

Figure 2 Time-course of Ca^{2+}-activated ATPase activity of whole cardiac myosins extracted from 220 days-old adult hamsters and measured on gels: Controls (F1B; $n=3$), untreated cardiomyopathic Syrian hamsters (CMH; $n=3$) and treated CMH from 30 to 220 days: trimetazidine-treated CMH (CMH-T; $n=3$), verapamil-treated CMH (CMH-V; $n=3$). Each point represents the mean value of two measurements. The slope of the regression line was taken as the specific activity of the myosin (arbitrary units). Dotted lines outline the confidence interval (at 95%) of curves obtained by SigmaPlot regression programme, analysed by ANOVA-Pearson regression test (SigmaStat).

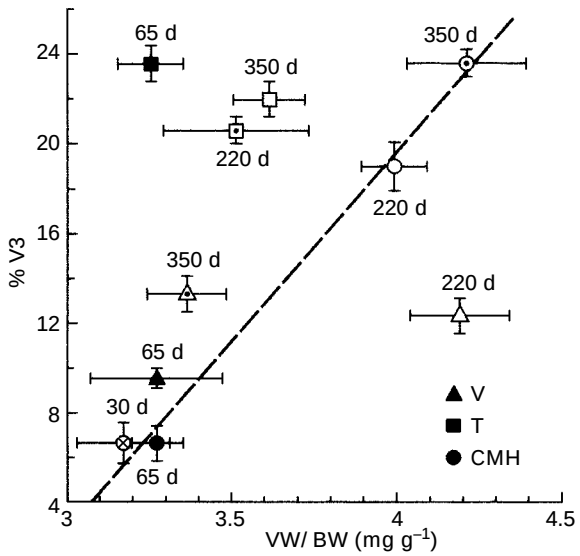

Figure 3 Age-dependent relationship between ventricular weight (VW) to body weight (BW) ratio and the associated changes in the amount of ventricular myosin isoenzyme V3 for cardiomyopathic hamsters (CMH). The linear regression curve was determined for the untreated CMH group and was analysed by ANOVA Pearson regression test, SigmaStat ($r=0.998$, $P<0.001$). Note the shifted correlation after trimetazidine (T) and verapamil (V) treatments, at the different ages: 30 days (cross in symbol); 65 days (solid symbols); 220 days (open symbols) and 350 days (point in symbol).

those on myosin isoforms: hypertrophy and MHC transitions may be completely or partially independent, as has already been observed (Scheuer *et al*., 1982; Wisenbaugh *et al*., 1984).

Trimetazidine unlike verapamil improves the survival of cardiomyopathic hamsters with a substantial gain in longevity (150–200 days) (D'hahan *et al*., 1997). These beneficial effects of trimetazidine do not appear to be the consequence of a normalization of the cardiac myosin isoenzyme composition, but suggest that MHC transitions toward the V3 form are not necessarily an aggravating factor of the disease and in fact may be an adaptative compensatory event, as has often been suggested in overloaded hearts (see Schwartz *et al*., 1988 for review) and in CMH (Momomura *et al*., 1992; Yamashita *et al*., 1993). Furthermore, an increase in V3 has been proposed as an explanation of the increased tolerance of CMH to hypoxia (Momomura *et al*., 1993). How trimetazidine maintains a constant V3 level is not known. It may interfere with metabolism (Guarnieri & Muscari, 1993; Kay *et al*., 1995) and preserve cell energy (Lavanchy *et al*., 1987). Favouring the expression of the myosin V3 form, which has lower ATPase activity and utilizes less energy, trimetazidine would bring the cells either to a reduced activity state or to a 'normoxic' metabolic switch (Demaison *et al*., 1995). In short, trimetazidine may cause slower but more enconomical functioning of the myocardial cell and thus the enhancement of the myocardial energy efficiency (Paulson *et al*., 1992), which could explain the prolonged survival.

In conclusion, trimetazidine and a standard Ca^{2+} blocker agent such as verapamil had different effects on the myosin isoenzyme pattern, revealing a dissociation between hypertrophy and isoform redistribution. We suggest that the positive effects obtained with trimetazidine long-term therapy, in terms of survival and ventricular hypertrophy, might be correlated with the maintenance of an adaptative and economical myosin phenotype.

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