Comp Clin Pathol (2014) 23:1639–1644
DOI 10.1007/s00580-013-1838-x

ORIGINAL ARTICLE

Cardiac myosin heavy chains in mice treated with N^G-nitro-L-arginine methyl ester and thyroxine

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Received: 7 June 2013 / Accepted: 30 October 2013 / Published online: 15 November 2013

Abstract The aim of this study was to evaluate the effect of N^G-nitro-L-arginine methyl ester and thyroxine on the distribution of cardiac myosin heavy chains in mice. Myocardial hypertrophy was induced in mice by intraperitoneal injection with L-thyroxine and oral administration of N^G-nitro-L-arginine methyl ester (L-NAME). Mice were randomly allocated into five groups: control, high and low doses of thyroxine and L-NAME. Heart weight divided by body weight was calculated and used as indicator for cardiac hypertrophy. Myosin heavy chains (MHCs) were separated using 4 % polyacrylamide gel electrophoresis. Hypertrophy was induced in mice treated with thyroxine and a high dose of L-NAME and was accompanied by a shift toward α-MHC in thyroxine-treated mice and β-MHC in L-NAME-treated mice. There was no difference with respect to MHC between a low dose of L-NAME and a high dose; however, low doses of L-NAME did not result in cardiac hypertrophy. In conclusion, L-NAME treatment changes the MHC distribution from α- toward β-MHC and this transition in the MHCs occurs before heart hypertrophy. Further investigations are needed to determine whether L-NAME treatment causes this transition via a direct or indirect effect on the heart muscles.

Keywords L-NAME · Myosin heavy chains · Cardiac muscle · Thyroxine

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Introduction

Myosin is a mechanoenzyme molecule which converts chemical energy stored as adenosine triphosphate (ATP) into mechanical energy (muscle contraction). The myosin molecules generate force and motion via cyclic interactions with actin molecules (Nikitina et al. 2008). Myosin present in the heart is the conventional myosin (class II) which is also expressed in skeletal, smooth muscle, and non-muscular tissues (Sellers 2000). According to its electrophoretic mobility and molecular weight, cardiac myosin can be further classified into A1 and A2 (atria); V1, V2, and V3 (ventricular) isozymes. These components have different calcium-activated myosin ATPase activity (Hoh et al. 1978). Cardiac myosin isozymes differ in their heavy chain structures (Hoh et al. 1979) of which there are two types, alpha and beta myosin heavy chains (MHCs) (Mahdavi et al. 1982), whereas the light chains show no difference (Hoh et al. 1978).

The myocardial contractility and the mechanical properties of the heart are dependent upon calcium-activated myosin ATPase activity and the pattern of distribution of specific myosin isozymes within the myocardium. In adult mice and rats, myosin V1 isozyme, which resembles in its ATPase activity that of fast-twitch skeletal muscle myosin, predominates in the mice ventricular myocardium over V3 isoenzyme (Lompre et al. 1981). Myosin isoenzyme V3, which behaves like slow-twitch skeletal muscle, predominates over V1 myosin in adult dogs, cattle, and human ventricular myocardium (Lompre et al. 1981). Myosin V3 isoform is more economical in force development and slower in velocity of contraction than myosin V1 isoform fibers (Van der Velden et al. 1998; Narolska et al. 2005).

Administration of drugs that enhance the myocardial contractility and peripheral vasodilation such as thyroxine and isoproterenol favors the distribution of V1 myosin isoenzyme (Carter et al. 1987; Hoh and Egerton 1979; Rupp et al. 1991;

Rundell et al. 2005). However, the impact of increasing the afterload on the redistribution of myosin isoforms in the heart has not yet been elucidated.

Nitric oxide (NO), an endogenous vasodilator, is synthesized in vivo from the amino acid L-arginine by the effect of an enzyme called nitric oxide synthase (NOS). Inhibition of NO production can be achieved by competitive enzyme inhibitors such as N^G-nitro-L-arginine methyl ester (L-NAME). NO is believed to contribute in the pathogenicity of septic shock related to systemic vasodilation which is insensitive to treatment with vasopressors (Petros et al. 1991). L-NAME is used in clinical practice to reverse hypotension associated with septic shock (Kiehl et al. 1997; Avontuur et al. 1998).

This study was designed to investigate, by blocking the natural production of nitric oxide using L-NAME the effect of increasing the myocardium afterload, on the redistribution of cardiac myosin isoforms and to compare such effects with the myosin isoform redistribution caused by thyroid hormone treatment.

Methods

Animal grouping and treatment

Thirty seven white adult male Swiss mice balb/C, ranging in weight between 18.4 and 32.1 g, were used in this study. They were kept in a temperature-regulated environment receiving a mice pellet diet and water ad libitum. All animals were treated according to guidelines for animal care and use stipulated by the committee of Jordan University of Science and Technology, Jordan. The mice were distributed randomly into five groups (Table 1): group I received 0.2 ml of normal saline (0.9 % NaCl) intra-peritoneally (IP) daily for 14 days, groups II and III received freshly prepared L-thyroxine (Sigma) IP (500 μg/kg body mass/day and 250 μg/kg body mass/day, respectively) for 14 days (Suarez et al. 2010), and groups IV and V received L-NAME (Sigma) in the drinking water for 5 weeks (250 mg/L and 600 mg/L, respectively). L-NAME is highly water soluble; it was prepared every other day to ensure its effectiveness and freshness. The amounts of L-NAME in the drinking water was equivalent to doses of approximately 4 and 10 mg/kg body mass/·day, respectively. The doses of L-NAME used were extrapolated from referenced literature (Zeidan et al. 2012).

Each animal's body weight was recorded at the beginning and end of the study. At the end of the experiment, animals were sacrificed; the heart of each mouse was removed, flushed with normal saline to remove any clotted blood, blotted with filter paper, and weighed. A small tissue sample was excised from the left ventricular wall and chopped into fine pieces on ice. The chopped heart muscle was mixed with Laemmli sample buffer (Laemmli 1970) which consists of 1 g glycerol, 0.23 g SDS, 0.6 g TRIS base, 0.45 ml mercaptoethanol, and few crystal of bromophenol blue for each 10 ml. The mixture was shaken on a vortex for 1 min and placed in boiled water for 5 min and then cooled. The samples were centrifuged at $6000 \times g$/min and supernatant removed and stored at −20 °C until use.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

A long gel electrophoresis (SCIE-PLAS) with parallel glass slabs and a 0.75-mm spacer was used. The optimum voltage for long gel electrophoresis run was a continuous 100 V/slab. After the staining and destaining procedures, the gel was scanned with computer scanner and densitometer (GS 800 Bio- rad).

Statistical analysis

Paired and unpaired two sample t tests were performed with the help of the Minitab computer program. Values were considered statistically significant if the probability (p) value was less than 0.05. Data were presented as mean ± standard error of mean (SE).

Results

Thirty four out of 37 mice completed the project; three mice died in the thyroxine-treated groups. One was from group II (high dose thyroxine-treated groups) and the other two from group III (low dose thyroxine-treated group).

Table 1 Study groups, number, and treatment

Groups of study	Number	Treatment
Group I (control)	6 mice	IP 0.2 ml of normal saline (0.9 % NaCl) daily for 14 days
Group II	8 mice	IP 500 μg/Kg/day of L-thyroxine for 14 days
Group III	8 mice	IP 250 μg/Kg/day of L-thyroxine for 14 days
Group IV	8 mice	250 mg of L-NAME /L in the drinking water for 5 weeks
Group V	7 mice	600 mg of L-NAME /L in the drinking water for 5 weeks

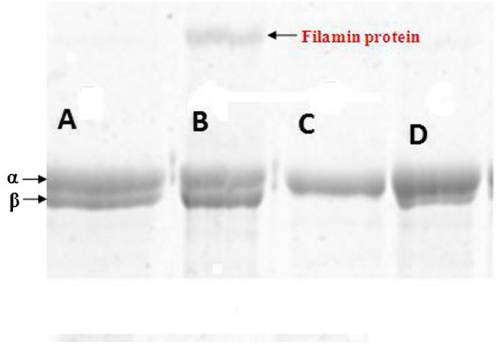

Fig. 1 Comparison between cardiac MHCs bands in different groups; (**a**) L-NAME, (**b**) chicken gizzard (used as a marker protein), (**c**) thyroxine, and (**d**) control

SDS-PAGE procedures

The difference in molecular weight of the two heavy chains is small, making the separation of these proteins difficult for the purpose of scanning and quantification of each. Therefore, a very porous polyacrylamide gel (4 %) was used. Numerous methods were tried to obtain good separation to enable good scanning, the best separation of cardiac MHCs was obtained using the Bio-rad procedure and the separation was further optimized when long gel was used. Therefore, the Bio-rad procedure was chosen for cardiac MHC separation and quantification. Figure 1 shows a typical separation of MHCs in the cardiac muscle in different groups compared with MHCs in control animals.

Body weight changes during study period

All animals except those treated with low-dose-thyroxine group ($p=0.024$) showed no statistical alteration in the body weight at the end of the study.

Degree of the heart hypertrophy

The degree of heart hypertrophy in the treated groups was assessed by calculating the heart weight/body weight (HW/BW) ratio and comparing it to the control group. Both the high-dose- and low dose thyroxine-treated groups (groups II and III, respectively) produced a highly significant degree of heart hypertrophy. The HW/BW ratio was higher in group II (mean of 5.9±0.02) and group III (mean of 5.4±0.02) compared to the control group (mean of 4.2±0.01), $p<0.001$ in both cases. (See Table 2 and Fig. 2)

The degree of hypertrophy caused by L-NAME showed an obvious dose-dependent response. Low dose L-NAME-treated mice had a HW/BW ratio of 4.4±0.01 which was not significantly different from the control group (4.2±0.01) ($p=0.127$). When compared with the control group, the high dose L-NAME treated-mice (4.7±0.01) showed a significant difference $p=0.004$.

When comparing the effect of different doses with the degree of heart hypertrophy, our study revealed no significant difference in the HW/BW ratio between group II and group III (5.5±0.02 and 5.4±0.02, respectively) $p=0.164$ (see Table 3). However, the comparison between the two L-NAME-treated groups (group V 4.7±0.01 vs group IV 4.4±0.01) showed a significant difference $p=0.02$ (see Table 3). Also, group II (high-dose thyroxine) treated mice showed a higher degree (5.9±0.02) of heart hypertrophy than group V (high-dose L-NAME) treated mice (4.7±0.01), $p<0.01$.

Redistribution of myosin heavy chains after treatment with thyroxine and L-NAME

MHCs have been classified according to their electrophoretic migration into slow and fast migrating MHC which correspond to alpha and beta MHCs, respectively. On comparing the effect of different doses of thyroxine and L-NAME on the redistribution of the MHCs our data showed that thyroxine treatment increases the percentage of the slow migrating MHCs when compared with the control group. The percentage of slow migrating bands have a mean of 92.8 %±4.5 in high dose thyroxine-treated group and 94.2 %±3.6 in low dose thyroxine-treated groups compared to 78.3 %±1 in the control group with p values 0.004 and 0.001, respectively (Table 4, Fig. 3).

Interestingly, the comparison of L-NAME treatment with controls showed the reverse effect in the MHCs redistribution to that seen with the thyroxine treatment. The slow MHC was lower in group V (high dose L-

Table 2 HW/BW ratio for each study group

Group	Treatment (no.)	BW (g)	HW (mg)	HW/BW (mg/g) Mean ± SE	p value
I	Control ($n=6$)	26.62±1.1	111.8	4.2±0.01	–
II	High dose of thyroxine 500 μg/kg body mass/day ($n=6$)	24.13±0.94	130.3	5.4±0.02	<0.001***
III	Low dose of thyroxine 250 μg/kg body mass/day ($n=7$)	21.01±1	124	5.9±0.02	<0.001***
IV	Low dose of L-NAME 4 mg/kg body mass/day ($n=8$)	23.06±0.62	101.5	4.4±0.01	0.127
V	High dose of L-NAME 10 mg/kg body mass/day ($n=7$)	22.94±1.3	107.8	4.7±0.01	0.004***

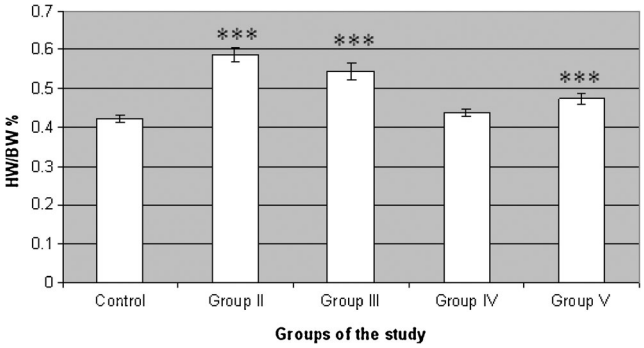

Fig. 2 Difference in the HW/BW ratio for each study group, ***$p<0.01$

NAME) treated mice (67.3 %±2), and group IV (low-dose L-NAME) treated mice (67.2 %±2.7) compared to controls (78.3 %±1), $p=0.005$, and 0.002, respectively (Table 4, Fig. 3).

Discussion

The mechanical properties of the cardiac muscle depend on the types of MHCs. Cardiac myocytes containing predominantly α-MHC have different contractile properties than cardiac myocytes containing predominantly β-MHC. The heart muscle containing a large amount of α MHC has higher ATPase activity and greater velocity of contraction (Hoh et al. 1978; Locher et al. 2011). Hence, it is important to understand the effect of the physiological, pathological, and pharmacological factors that affect the mechanical properties of the myocardium on the percentile distribution of α- and β-MHC within the myocytes.

L-NAME (N^G-nitro-L-arginine methyl ester) is a nitric oxide (NO) inhibitor. This drug has clinical effects on body system pathologies; it has a protective effect against peptic ulcer disease (Oztürk et al 2002; Takeuchi et al 1995) and pancreatitis (Sugiyama et al 2005) as well as reducing increased bleeding time in uraemia (Brunini et al. 2003). On the cardiovascular system, L-NAME causes a decrease in the heart rate, cardiac output, stroke volume, peak thoracic aortic blood flow and the total peripheral conductance but the mean arterial pressure increases while there is no change in central venous pressure (Gardiner et al. 1990). In leukocytopenic patients with severe septic shock, L-NAME causes an increase in the mean arterial pressure, systemic vascular resistance, and left ventricular stroke work index compared to baseline values (Avontuur et al. 1998). This study was designed to explore the effect of L-NAME on cardiac MHC redistribution and to compare it with MHCs redistribution caused by thyroid hormone treatment.

Table 3 HW/BW ratio in different study groups

Experimental group	HW/BW (mg/g) Mean ± SE	p value
Group II	5.9±0.02	0.164
Group III	5.4±0.02	
Group V	4.7±0.01	0.02
Group IV	4.4±0.01	
Group II	5.9±0.01	0.001
Group V	4.7±0.01	

Table 4 Slow migrating MHCs % in different groups

Experimental group	Slow migrating MHC % (Mean ± SE)	p value
Control	78.3±1	–
Group II	92.8±4.5	0.004***
Group III	94.2±3.6	0.001***
Group IV	67.2±2.7	0.002***
Group V	67.3±2	0.00***

***$p<0.01$

Cardiac hypertrophy was evident in group II (high-dose thyroxine) and group V (high-dose L-NAME) (Fig. 2 and Table 2), as both groups showed an increase in the heart weight/body weight (HW/BW) ratio with no body weight reduction during the course of the study. These findings are in agreement with the finding of Carter et al. (1987) and Hropot et al. (1994). Our data indicated that the low-dose L-NAME treatment (group IV) was insufficient in inducing cardiac hypertrophy.

Our study indicated that the redistribution of MHCs caused by thyroxine was opposite to that induced by L-NAME. Thyroxine shifted α-/β-MHC ratio toward alpha (slow migration myosin heavy chain) (Table 4). The α-MHC percentage was significantly increased from 78.3 %±1 in the control group to 92.8 %±4.5 and 94 %±3.6 in the thyroxine-treated groups (high and low doses, respectively). This is in agreement with Carter et al. (1987). Interestingly, it was reported that in the case of a hypothyroid animal, β-MHC was most abundant (Rundell et al. 2005). The effect of L-NAME causes α-/β-MHC ratio to shift toward β-MHC (fast migration).

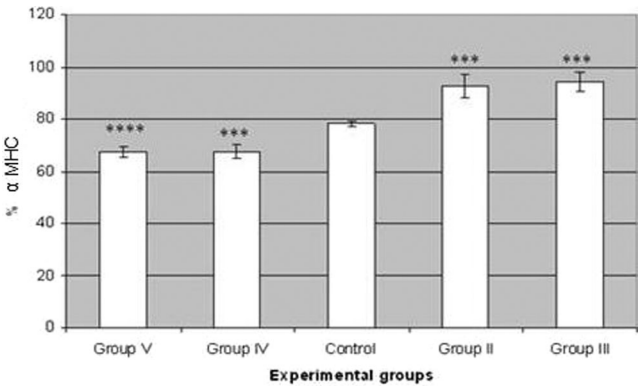

Fig. 3 Slow migrating MHC % in different study groups, ***$p<0.01$

Shift toward α-MHC in thyroxine-treated groups is due to the direct effect of thyroxine on the cardiac muscle (Hoh and Egerton 1979). Rolling out whether the effect of L-NAME on the α-/β-MHC ratio is a direct or an indirect effect on the myocardium is beyond the scope of this study.

By referring to Tables 2 and 4, it is clear that low dose L-NAME-treated mice (group IV) show changes in MHC distribution without significant change in the HW/BW ratio while high-dose L-NAME (group V) changes the MHC distribution but also increases in the HW/BW ratio. Therefore, we would propose that the changes in the MHC distribution caused by L-NAME precede cardiac hypertrophy, similar observation has been reported by Zhang et al. (2003). Since NO is a vasodilating agent, inhibition of NO synthase by L-NAME leads to hypertension (Baylis et al. 1992) and ventricular hypertrophy would be expected to develop. So why was there no hypertrophy in the low dose L-NAME-treated animals? Perhaps a low dose of L-NAME is not enough to produce hypertension, and subsequently, cardiac hypertrophy and the changes in MHC profile toward β-MHC isoform is due to a direct effect of a low dose of L-NAME on the myocytes. Another explanation for this finding is that the left ventricle adapted to hypertension developed without hypertrophy. Ventricular adaptation without hypertrophy to chronic pressure overload caused by L-NAME has been described by Matsubara et al. (1998) and Bartunek et al. (2000). Changes in MHC distribution without hypertrophy seen in low dose L-NAME-treated mice could be due to stress imposed on the cardiac muscle by increased blood pressure caused by L-NAME treatment. Earlier studies indicated that myocardium under strain exhibits redistribution of MHCs toward the beta isoform (Rupp 1981; Yazaki et al. 1989). In fact, the heart muscle containing a large proportion of β-MHC can become more economical in energy consumption (Carter et al. 1987). L-NAME enhances the total peripheral resistance (afterload) and increases the mean arterial pressure (Hropot et al. 1994; Avontuur et al. 1998; Gokcimen et al. 2007), augments the effect of α adrenoceptor agonist (Guc et al. 1992), and increases the activity of the angiotensin-converting enzyme (Korystova et al. 2012).

In light of the above findings, we suggest that the effect of L-NAME on the redistribution of MHCs toward the beta isoform within the ventricular myocardium is an indirect one and could involve different mechanisms.

Conclusion

The L-NAME treatment changes the MHC distribution from α- toward β-MHC and this transition in the MHCs occurs before heart hypertrophy. Further investigations are needed to determine whether the L-NAME treatment causes this transition via a direct or indirect effect on the heart muscles.

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