Myosin Light Chain Kinase in Vascular Smooth Muscle. An Immunohistochemical Study in Normotensive and Spontaneously Hypertensive Rats

Giorgio Vescovo, Gianluigi Scannapieco, Elisabetta Spanò, Irene Calliari, Cristiana Leprotti, Francesco Serafini, Giovanni Battista Ambrosio and Luciano Dalla Libera[1]

Complesso Convenzionato Università di Padova, Ospedale Civile Venezia, Divisione Medica I, Ospedale Civile, Venezia, Italy and (1) CNR Unit for Muscle Biology and Physiopathology, Department of Biomedical Sciences, University of Padova, Padova, Italy

Abstract

Purpose of this study was to see whether during development of vascular hypertrophy and post-natal growth, quantitative changes of myosin light chain kinase (MLCK) occurred in the kidney arteriolar media. MLCK is a Calcium-Calmodulin dependent enzyme able to trigger smooth muscle cell contraction and regulating vascular tone. We studied newborn normotensive Wistar Kyoto (WKY) and age-matched spontaneously hypertensive (SHR) rats together with adult normotensive and age-matched hypertensive rats. On kidney cryosections we assessed, by means of a computerized interactive morphometric method, the area of the arteriolar media stained with a polyclonal antibody anti chicken gizzard myosin light chain kinase, highly specific for smooth muscle cells. We showed that both normotensive and spontaneously hypertensive adult rats have a significantly lower content of myosin light chain kinase in their media when compared with newborns (24.2 ± 7.1 vs. 49.2 ± 4.2 $p < 0.001$ WKY) (11.3 ± 4.5 vs. 52.3 ± 5.9 $p < 0.001$ SHR). Control newborns showed myosin light chain kinase levels similar to those of spontaneously hypertensive rats. However, adult hypertensive rats showed an even lower expression of the enzyme than the age-matched normotensive animals (11.3 ± 4.5 vs. 24.2 ± 7.1 $p < 0.05$). The degree of vascular hypertrophy was found to be significantly lower in this latter group. Furthermore there was a negative linear correlation in adult rats between myosin light chain kinase content and degree of hypertrophy of the vascular media.

We conclude that both during growth and occurrence of vascular smooth muscle hypertrophy due to hypertension, cells tend to adapt by down-regulating the expression of myosin light chain kinase and therefore by modulating their vascular reactivity.

Key words: Myosin light chain kinase, vascular smooth muscle, development, vascular hypertrophy, hypertension.

BAM 6 (3): 183-187, 1996

Polymorphism of contractile proteins is well documented in vascular smooth muscle [11]. During development contractile protein pattern of smooth muscle cells undergoes dramatic changes with expression of different isoforms. Actin in mature smooth muscle cells is represented by the α and γ isoforms, however during embryonic development the proportion of each isoform changes markedly [15]. α-SMC actin has been used by Romano et al. as a marker for studying the ontogeny in the intrarenal vasculature [15]. Similarly myosin isoforms are expressed to a different degree during postnatal development. Pauletto et al. [14] showed the existence of two myosin heavy chains isoforms namely the adult type, which represents the majority in the adult rabbit aorta, and the post-natal type, which is the minority. It has been shown by Frid et al. [9] that these differences in contractile proteins pattern correlate with the existence of at least four different smooth muscle cells phenotypes which are differently expressed during vessel maturation. The extracellular matrix protein fibronectin seems to be a marker of smooth muscle cell

differentiation in normal vessels and in vascular diseases [12]. The same proteins in certain pathophysiological conditions show changes of their phenotypic expression [4, 14, 17]. In experimental models of hypertension and atherogenesis there are in fact adaptive changes of myosin heavy chains, fibronectin and other proteins of intracellular matrix, which accompanies smooth muscle cells growth and hypertrophy with expression of immature cells phenotypes characteristic of the early stages of development [3, 13, 17].

Myosin Light Chain Kinase (MLCK) is a a calcium calmodulin-dependent enzyme which triggers actin-myosin interaction by phosphorylating the 20 KD myosin subunit which regulates smooth muscle cells contraction and tone [16, 18].

The expression of MLCK maintains the same primary sequence during post-natal development, although post-translational modifications and quantitative changes may occur [6, 7, 10]. This protein is of paramount importance in regulating smooth muscle cell contraction, presumably not only in physiological but also in pathological conditions. There are however very few studies on its expression during development [8] and in pathophysiological conditions [2].

In this paper we wanted to investigate whether in the rat during physiological and pathological post-natal development there were quantitative changes of MLCK in smooth muscle cells. We did that by immunostaining the kidney arteriolar media of normotensive and spontaneously hypertensive rats with a highly specific antibody raised against smooth muscle MLCK [5, 6, 13].

Materials and Methods

Animals

Five newborn 3-day-old normotensive Wistar Kyoto (WKY) and six Spontaneously Hypertensive Rats (SHR) were studied together with five adult, 9-week-old WKY and six age matched SHR.

Animals were killed by cervical dislocation and the right kidney was immediately frozen in liquid nitrogen.

Immunohistochemistry

On 5 µm thick transverse cryosections of the right kidney an immunohistochemical analysis was performed by using a polyclonal antibody against chicken gizzard MLCK raised in the rabbit [5] as primary antibody and anti rabbit IgG conjugated with peroxidase as a second antibody.

Quantitation of MLCK

The percent area of the arteriolar media occupied by positive staining for MLCK was measured by means of a computerized interactive morphometric method (Kontron IBAS 2000). The TV camera signal is visualized in black and white (768 x 512 pixels) and the image is stored and analyzed. The area measurements performed by our computer system are based on a gray scale that analyzes 256 gray levels (level 0 corresponds to black and 255 to white).

Two intervals are interactively fixed within these 256 levels and correspond to the area positive (dark intervals) and to that negative (bright intervals) for MLCK staining.

These intervals were defined by two trained observers [1].

A 300 times magnification photograph of each section (Figure 1) was put under a camera connected to the instrument. The cross section of each artery was divided into four 90 degree segments and on each segment a field entirely occupied by the media was randomly chosen so that 4 measurements were done on a single vessel. For each animal 4 to 5 arteriolae were chosen at random and analyzed. The mean values are reported.

Degree of the hypertrophy of the vascular media

The degree of hypertrophy of the vascular media was calculated on cross sections of the arteriolae by using the following equation:

$$\frac{(R1 + R2)/2}{(M1 + M2)/2}\ \%$$

where R1, R2 and M1, M2 are respectively the two vessels radii and the two media thicknesses taken perpendicularly.

Statistical analysis

Student t test for unpaired data and linear regression were used.

Results

Percent area of the media occupied by positive staining for MLCK

Typical immunohistochemistry with antibody against MLCK of cross sections of kidney arteriolae from newborn and adult SHR and WKY rats are presented in Figure 1.

The percent area of kidney arteriolar media occupied by positive staining for MLCK, determined as specified in Materials and Methods section, is illustrated in Fig. 2. Newborn WKY and SHR showed a significantly higher percent area occupied by MLCK when compared to the adult animal of the same species (49.2 ± 4.2 vs 24.2 ± 7.1 $p < 0.001$ for WKY; 52.3 ± 5.9 vs 11.3 ± 4.5 $p < 0.001$ for SHR). Adult SHR animals showed a lower percentage of area occupied by MLCK when compared to the age-matched adult WKY (11.3 ± 4.5 vs 24.2 ± 7.1 $p < 0.05$). There were no significant differences between newborn SHR and WKY.

Hypertrophy of the vascular media

Newborn SHR showed a degree of hypertrophy of vascular media of $47.7 \pm 8.65\%$ whilst in newborn WKY it was $48.1 \pm 6.77\%$ (p = ns). In the adult animals the degree of hypertrophy of SHR was significantly higher ($p < 0.003$) than that of WKY ($24.2 \pm 3.2\%$ vs. $18.2 \pm 2.6\%$).

In the adult animals there was a negative linear correlation between percent area of MLCK and degree of hypertrophy ($r^2 = -0.30$; $p = 0.052$).

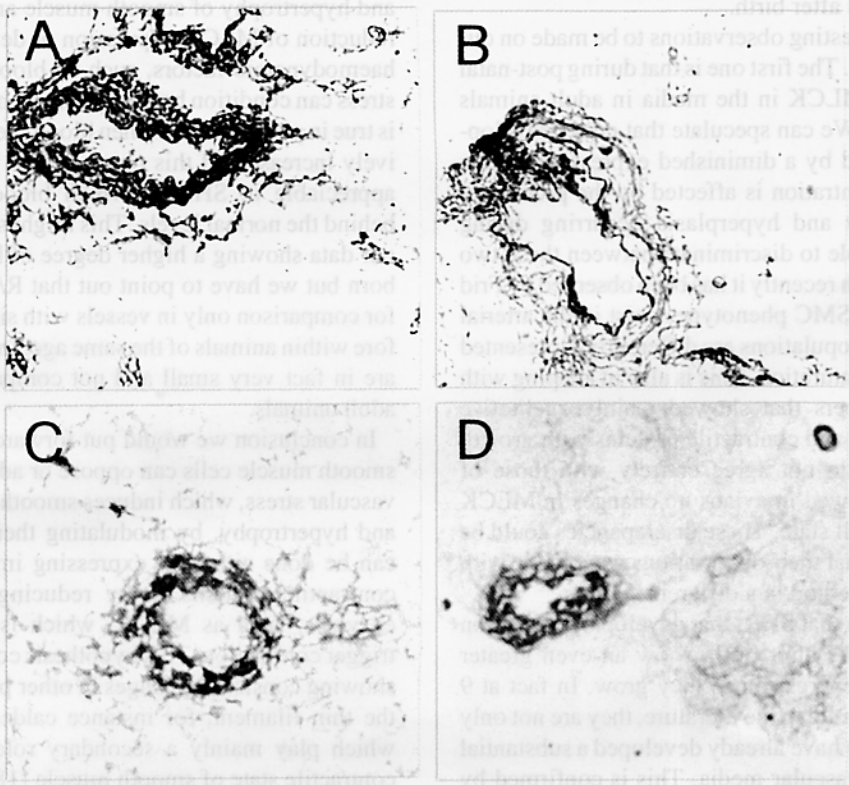

Figure 1. Cross sections of kidney arteriolae. Immunohistochemistry with antibody against MLCK. A) Adult WKY; B) Adult SHR; C) Newborn WKY; D) Newborn SHR. Magnification times 300.

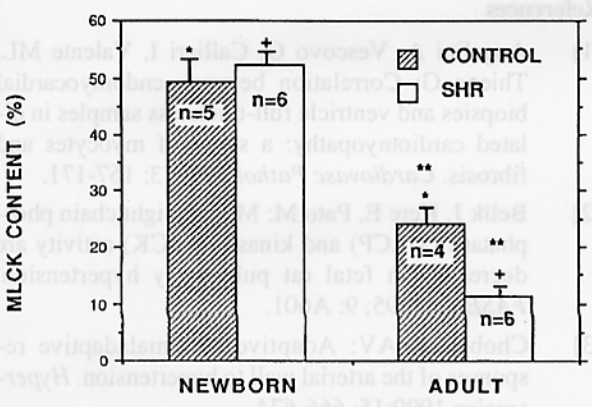

*Figure 2. Quantitative measurement of MLCK content in kidney arteriolar media of newborn and adult rats as expressed as the percent area of arteriolar media occupied by positive staining for MLCK. The standard deviation of the mean is reported. n: number of animals per group; *: $p < 0.001$; **: $p < 0.05$; +: $p < 0.001$*

Discussion

In this study we used a polyclonal antibody against chicken gizzard MLCK which we have already demonstrated to be highly specific for smooth muscle MLCK [5, 13]. This was true especially for vascular smooth muscle MLCK. In fact it has been shown to stain MLCK in several vessels of rat, man and rabbit. No reactivity was found for other non-muscle MLCK such as platelets. It has also been shown that the expression of MLCK maintains the same primary sequence during post-natal development, although post-translational modifications and quantitative changes may occur [6].

The method we used to measure the percent area occupied by positive staining for MLCK was previously used by our group [1] and demonstrated to be precise and reproducible. The differences in MLCK content detected in the present study seem therefore to be consistent with quantitative changes in MLCK expression occurring during post-natal development in the kidney arteriolae of normotensive and spontaneously hypertensive rats. Our data show that in newborn rats the content of MLCK is substantially higher, without significant differences between WKY and SHR, than in adults. It has to be noticed

that SHR develop hypertension only few weeks after birth and we can therefore conceivably argue that, though not measured, blood pressure values are identical in the two animal strains 3 days after birth.

There are two interesting observations to be made on our series of experiments. The first one is that during post-natal life the content of MLCK in the media in adult animals decreases with age. We can speculate that either development is characterized by a diminished expression of this enzyme or its concentration is affected by the process of cellular hypertrophy and hyperplasia occurring during growth. We are unable to discriminate between these two possibilities, although recently it has been observed by Frid [9] that at least four SMC phenotypes exist in the arterial wall. These cell subpopulations are differently represented during vessel wall maturation. This is also in keeping with data from other papers that showed mainly qualitative changes in structural and contractile proteins with growth [11, 14]. Our data do not agree entirely with those of Duband [8] who showed in avians no changes in MLCK from hatching to adult state. These discrepancies could be ascribed to the fact that their observations were made with a semiquantitative method in a different species.

The second point is that SHR, that develop hypertension in the first five weeks after birth, show an even greater decrease in MLCK expression as they grow. In fact at 9 weeks of age, as reported in the literature, they are not only hypertensive but they have already developed a substantial hypertrophy of the vascular media. This is confirmed by our data. In fact the R/M ratio, which is considered a good index of hypertrophy of the media, is significantly higher in adult SHR as compared to age matched WKY. We can argue that hypertension and vascular hypertrophy are able to further modify the expression of MLCK in vascular smooth muscle by down-regulating it. Our results seem to be confirmed by Belik et al. [2] who showed a decreased MLCK activity in lungs of rats with pulmonary hypertension. Furthermore MLCK level in aorta of SHR animals was found to be lower than in controls (M. Pato, personal communication).

Whether this process is due to hypertension by itself or to hypertrophy cannot be said with certainty. Since a significant inverse correlation is found between the amount of MLCK present in the smooth muscle cells of the media, and the degree of vascular hypertrophy we can speculate on the role played by this latter process in the regulation of MLCK expression. It may be argued that neuro-hormonal factors, which are known to sustain hypertension in SHR such as catecholamines, or blood pressure levels by themselves, could in some way have influenced the expression of MLCK. Unfortunately the limitation of this study is given by the lack of these measurements.

Hypertension has been shown to induce changes in the expression of myosin heavy chains and fibronectin in smooth muscle cells with the appearance of immature isoforms characteristic of the fetal life and these processes have been considered as adaptive [4, 11, 12, 14]. We think that the observed diminished expression of MLCK found with growth and development of vascular hypertrophy could be interpreted in the same way. It looks as if growth and hypertrophy of smooth muscle are accompanied by a reduction of MLCK expression or density. We know that haemodynamic factors, such as blood pressure and wall stress can condition both growth and hypertrophy [3]. This is true in post-natal life when blood pressure does progressively increase and this phenomenon becomes even more appreciable in SHR in which blood pressure rises far behind the normal levels. This might seem in contrast with our data showing a higher degree of hypertrophy in newborn but we have to point out that R/M ratio can be used for comparison only in vessels with similar size and therefore within animals of the same age. In newborns arteriolae are in fact very small and not comparable with those of adult animals.

In conclusion we would put forward the hypothesis that smooth muscle cells can oppose or adapt to the increasing vascular stress, which induces smooth muscle cells growth and hypertrophy, by modulating their contractility. This can be done either by expressing immature isoforms of contractile proteins or by reducing the expression of enzymes, such as MLCK, which is known to actively trigger contraction. This hypothesis could be confirmed by showing consistent changes in other proteins associated to the thin filament, for instance caldesmon and calponin, which play mainly a secondary role in modulating the contractile state of smooth muscle [18].

Address correspondence to:

Dr. Giorgio Vescovo, Divisione Medica I, Ospedale Civile SS Giovanni e Paolo, Castello, 30100 Venice, Italy. Fax +39 (49)-5294651.

References

[1] Angelini A, Vescovo G, Calliari I, Valente ML, Thiene G: Correlation between endomyocardial biopsies and ventricle full-thickness samples in dilated cardiomyopathy: a study of myocytes and fibrosis. *Cardiovasc Pathol* 1994; 3: 167-171.

[2] Belik J, Kerc E, Pato M: Myosin light chain phosphatase (MLCP) and kinase (MLCK) activity are decreased in fetal rat pulmonary hypertension. *FASEB J* 1995; 9: A601.

[3] Chobanian AV: Adaptive and maladaptive responses of the arterial wall to hypertension. *Hypertension* 1990;15: 666-674

[4] Contard F, Sabri A, Glukhova M, Sartore S, Marotte F, Pomies JP, Schiavi P, Guez D, Samuel JL, Rapaport L: Arterial smooth muscle cell phenotype in stroke-prone spontaneously hypertensive rats. *Hypertension* 1993; 22: 665-676.

[5] Dalla Libera L: Avian smooth muscle myosin light chain kinases. A comparison by peptide mapping

study. *Comp Biochem Physiol* 1993; 105B: 147-150.

[6] Dalla Libera L, Collins JH: Myosin light chain kinase expression during smooth muscle development. *Biosc Rep* 1993; 13: 289-295.

[7] Dalla Libera L, Mastroianni M, Sobieszek A: Heterogeneity of smooth muscle myosin light chain kinase. Characterization of isoelectric variants. *Febs Lett* 1994; 346: 213-216.

[8] Duband JL, Gimona M, Scatena M, Sartore S, Small JV: Calponin and SM22as differentiation markers of smooth muscle: spatiotemporal distribution during avian embryonic development. *Differentiation* 1993; 55: 1-11.

[9] Frid M G, Moiseeva EP, Stenmark KR: Multiple phenotypically distinct smooth muscle cell populations exist in the adult and developing bovine pulmonary arterial media in vivo. *Circ Res* 1994; 75: 669-681.

[10] Fasolo M, Cavallini P, Dalla Libera L: Heterogeneity of smooth muscle myosin light chain kinase is revealed by anion exchange high performance liquid chromatography. *Biochem Biophys Res Commun* 1991; 175: 277-284.

[11] Giuriato L, Scatena M, Chiavegato A, Tonello M, Scannapieco G, Pauletto P, Sartore S: Non-muscle myosin isoforms and cell heterogeneity in developing rabbit vascular smooth muscle. *J Cell Sci* 1992; 101: 233-246.

[12] Glukhova MA, Frid MG, Shekhonin BV, Vasilevskaya TD, Grunwald J, Sagginati M, Kotelianski VE: Expression of extra domain A fibronectin sequence in vascular smooth muscle cells is phenotype dependent. *J Cell Biol* 1989; 109: 357-366.

[13] Mastroianni M, Ambrosio GB, Scannapieco G, Vescovo G, Dalla Libera L: Myosin light chain kinase as a marker for smooth muscle: a polyclonal antibody study. *Basic Appl Myol* 1993; 3: 195-200.

[14] Pauletto P, Chiavegato A, Giuriato L, Scatena M, Faggin E, Grisenti A, Sarzani R, Paci MV, Fulgeri D, Rappelli A, Pessina AC, Sartore S: Hyperplastic growth of aortic smooth muscle cells in renovascular hypertensive rabbits is characterized by the expansion of an immature cell phenotype. *Circ Res* 1994; 74: 774-788.

[15] Romano LA, Ferder L, Inserra F, Ercole L, Gomez RA: Intraglomerular expression of α-smooth muscle actin in aging mice. *Hypertension* 1994; 23: 889-893.

[16] Somlyo AP, Somlyo AV: Signal transduction and regulation in smooth muscle. *Nature* 1994; 372: 231-236.

[17] Upadhya A, Samuel M, Cox RH, Bagshaw RJ, Chacko S: Characteristics of arterial myosin in experimental renal hypertension in the dog. *Hypertension* 1993; 21: 624-631.

[18] Walsh MP: Regulation of vascular smooth muscle tone. *Can J Physiol Pharmacol* 1994; 72: 919-936.