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Steroid-Hormone Regulation of Myosin Subunit Expression in Smooth and Cardiac Muscle

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Abstract We investigated the effects of ovarectomy and the steroid hormones estrogen and testosterone on the in vivo expression of heavy (MHC) and light (MLC) chains of myosin in the heart, uterus, and aorta of rats. In the heart, ovarectomy decreased alpha-MHC expression, while both steroid hormones normalized it. Differential steroid hormone effects could be observed on myosin subunit expression of smooth muscle. Testosterone but not estrogen normalized the ovarectomy-induced decreased expression of SM1 and strongly increased the expression of 5′-inserted MHC in the uterus. Estrogen but not testosterone normalized the ovarectomy-induced diminished MLC17a expression. In contrast to the uterus, no steroid hormone effects on myosin subunit expression could be observed in the aorta.

Key words: steroid-hormone regulation, MHC, MLC, myosin, rats

The myosin molecule of all muscle types is composed of two heavy chains (MHC) and four light chains. Each MHC has about 200 kDa and consists of a globular N-terminal (ATP and actin binding) and a fibrous coiled-coil C-terminal domain. The globular domains are noncovalently associated with two types of light chains (MLC) [Lowey and Risby, 1971]. Two different genes, beta-MHC and alpha-MHC, located in tandem 4 kb apart on chromosome 14 are expressed in the ventricle of rats [Leinwand et al., 1983]. The MHC associate to dimers forming alpha-alpha-homodimers, alpha-beta-heterodimers, and beta-beta-homodimers which can be separated by pyrophosphate polyacrylamide gel electrophoresis, designated as V1, V2, and V3, respectively [Hoh et al., 1978]. The heart switches myosin subunit expression by changing transcriptional activity of different genes during development as well as in response to differing environmental demands [Swynghedauw, 1986]. It could be demonstrated that the myosin isoforms expressed determined both the energetical and mechanical characteristics of the heart [Swynghedauw, 1986].

In smooth muscle cells (SMC) three different genes coding for MHC are expressed, i.e., one smooth-muscle specific (SM-MHC) and two genes coding for nonmuscle (NM) MHC. The single SM-MHC gene transcript is alternatively spliced at both the 3′-carboxyl terminus (myosin tail) and the 5′-amino-terminus. Inclusion of the 39 nucleotide (nt) exon at the most 3′-terminus generates SM2 (200 kDa), exclusion SM1 (204 kDa) [Rovner et al., 1986; Nagai et al., 1989; Hamada et al., 1990]. 5′-Splicing is accomplished by the insertion of 21 nt (7 amino acids) near the ATP-binding 25K/50K-junction [White et al., 1993; Babij, 1993; Kelley et al., 1993]. SM-MHC with the 5′-insert were designated as B-forms (SM1B and SM2B), those without 5′-insertion as A-forms (SM1A and SM2A).

Two genes coding for NM-MHC are expressed in SMC designated as NM-MHCA (196 kDa) and NM-MHCB (198–200 kDa) [Kawamoto and Adelstein, 1991]. NM-MHCB is expressed in the embryonic SMC (designated, therefore, also as SMemb) [Kuro-o et al., 1991; Aikawa et al., 1994], in proliferating SMC [Schwartz et al., 1986], and in culture [Kawamoto and Adelstein, 1991]. In the adult state myometrial cells express NM-MHCA in addition to SM-specific MHC isoforms [Morano et al., 1993].

SMC express two essential MLC isoforms with 17 kDa by alternative splicing, namely, MLC_{17a} and MLC_{17b}. In smooth muscle cells of human [Lenz et al., 1989] and chicken [Nabeshima et al., 1987] MLC_{17b} differs from MLC_{17a} by inclu-

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sion of 44nt (human) or 39nt (chicken) inserts at the C-terminus. MLC_{17a} is also expressed in non-muscle cells whereas MLC_{17b}, which contains the insertion, seems to be SM specific [Hasegawa and Morita, 1992]. Two isoforms of the 20 kDa phosphorylatable MLC, designated as the MLC_{20A} and MLC_{20B} with increasing acidity [Erdödi et al., 1987; Inoue et al., 1989] have been identified in SMC. Phosphorylation of the MLC_{20} by Ca^{2+}-calmodulin dependent myosin light chain kinase is a key event in the initiation of smooth muscle contraction and the extent of MLC_{20} phosphorylatation determines tension development and shortening velocity [Kamm and Stull, 1985, for review].

Little is known about the regulation of alternative splicing as well as the functional consequences of alternatively spliced MHC and MLC isoforms in the SMC. MHC containing the 25K/50K-insertion revealed a higher ATPase activity and moved actin filaments faster in in vitro motility assays than MHC without the 5'-insert [Kelley et al., 1993]. MLC_{17} isoforms have been correlated with SM energetics and contractility: there is a positive correlation between MLC_{17a} expression and both shortening velocity [Malmqvist and Arner, 1991; Morano et al., 1993] and ATPase activity [Helper et al., 1988]. These results suggest that similar to the striated muscle types isoforms of myosin subunits modulate SMC contractility.

In this study we investigated the regulation of myosin subunit expression in the heart and two types of smooth muscle cells of the rat by steroid hormones in vivo. We found that steroid hormones selectively change the expression of myosin subunits in the ventricle and uterus but not in the aorta. These results suggest that steroid hormones can regulate the contractile and energetic behaviour of smooth and cardiac muscle cells.

MATERIALS AND METHODS

Experimental Groups

Eight-week-old Sprague-Dawley rats were ovarectomized or sham-operated by the Versuchstieranstalt (Hannover, Germany) using a technique previously described [Waynforth, 1980]. The animals were shipped to our laboratory one week after surgery. All animals were fed commercially available food (Altromin) and water ad libitum. After 1 week in our laboratory (10 weeks old) sham-operated animals were treated daily with appropriate volumes of benzylbenzoate-castor oil (N) for 2 weeks. Gonadectomized animals were treated with estrogen (E) (estradiol-valerat, 1mg/d, Schering, Berlin, Germany), testosterone (T) (testosterone propionat testosterone enanthat, Schering, Berlin, Germany, 1 mg/d), or appropriate volumes of benzylbenzoate-castor oil (S) for 2 weeks (subcutaneous application each). Adequacy of gonadectomy and hormone treatments were evaluated by analysis of uterus weight and serum levels of testosterone and estrogen. At the age of 12 weeks animals were killed by cervical dislocation, tissues (left ventricle of the heart, descending aorta, uterus) excised, blotted, weighed, frozen in liquid nitrogen, and stored for a maximum of 2 weeks at −80°C. 2 ml blood samples were collected for determination of testosterone and estrogen serum concentrations using commercial radioimmunoassay kits: 17β-estradiol using the Delfia-test (Pharmacia, Freiburg, Germany), testosterone using Testosteron-RIA COAT-A-COUNT (Biermann Diagnostica, Bad Nauheim, Germany).

Analysis of In Vivo Myosin Heavy Chain (MHC) Isoform Expression

Cardiac (left ventricle) MHC isoenzymes were analyzed by pyrophosphate PAGE (PP-PAGE) as described [Hoh et al., 1978]. In short, the ventricles were crushed in liquid nitrogen, extracted for 20 min at +4°C with 3.4 µl/mg muscle wet weight with a modified Schneider-Hasselbach solution (mM): KCl (300), $NaPO_4$ (100), $MgCl_2$ (1), $Na_4P_2O_7$ (10), EDTA (10), 0.1% beta-mercaptoethanol, 1% NaN_3, pH 6.5. The extracts were centrifuged (20,000 rpm) for 20 min. Supernatants were mixed with glycerol 1:1 and stored at −20°C. Electrophoresis was performed in 4% polyacrylamide gels using pharmacia GE-4 apparatus which allows circulation of the upper and lower buffer chamber. The buffer was composed of 20 mM $Na_4P_2O_7$, 1 mM EDTA, 10% glycerol, 0.1% beta-mercaptoethanol, pH 8.5. The temperature was maintained at +2°C. Electrophoresis was run for 20 h at 84 V (constant). Gels were stained for 30 min (0.025% Coomassie-blue R-250, 10% methanol, 7% acetic acid), scanned densitometrically and evaluated geometrically. The relative amount of the individual isoenzyme was expressed as percentage of the total sum of areas under the entire trace. Percentage alpha-MHC was determined by the

following formula: %alpha-MHC = %V1 + 0.5%V2.

To obtain in vivo MHC expression, frozen uteri were powdered in liquid nitrogen, mixed with 15% TCA, and denatured for 20 min on ice. The homogenate was centrifuged and TCA was extracted from the protein pellet with ether/ethanol (80/20 v/v). The muscle powder was stored at −20°C under N_2. For analysis the muscle powder was boiled for 1 min in SDS-sample buffer (5% SDS, 50 mM Tris/HCl, pH 6.8), and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [Morano et al., 1990]. In short, we used a 4% stacking gel, 5% separation gel both containing 25% glycerol. Gels were run for 6 h (40 mA per gel) at 15°C under continuous circulation between upper and lower electrode buffers. Gels were stained for protein with Coomassie blue R250, destained overnight, and the MHC were evaluated densitometrically.

Antibody Production and Affinity Purification

A peptide antibody specific for the 25K/50K-insert of rat smooth muscle MHC (5′-insert) was generated based on the amino acid sequence "QGPSFAY" [c.f. White et al., 1993]. At the amino terminus of the peptide N-chloroacetylglycine was added to facilitate cross-linkage to carriers. The peptide was synthesized by the solid-phase method, purified, coupled to KLH, and used for immunization of rabbits. The resulting antibody fractions were purified on a peptide-affinity column. For this, a packed gel of *w*-aminohexyl-agarose (0.5 ml) was activated with a 10-fold molar excess of 2-iminothiolane (Traut reagent) for 20 min at room temperature. The activated gel was poured into a column and extensively washed with 0.1 M $NaHCO_3$, pH 8.0. A solution of 1 ml of N-chloroacetylglycine-QGPSFAY (8 mg) was mixed with the activated gel and immobilized by incubation on a rotating wheel for 3 h at room temperature. The peptide-resin was then incubated for 1 h with 5 ml of 40 mM iodoacetamide to derivatize any remaining sulfhydryl groups. Following extensive washing, the peptide-resin was gently mixed with 5 ml of antiserum diluted 2-fold in 10 mM Tris/HCl-buffer (pH 7.4) containing 200 mg of bovine serum albumin and 0.1 mM phenylmethylsulfonyl fluoride by rotation at 4°C overnight. To elute bound antibodies, 50 mM glycine/HCl-buffer (pH 2.5) was used and the pH was immediately adjusted to pH 7.4. The fractions exhibiting absorption at 280 nm were pooled and stored at −80°C. a25K/50K cross-reacted with MHC derived from rat urinary bladder which contained the 5′-insertion but not or only weakly with rat uterus having MHC mainly without the 5′-insertion [White et al., 1993] (not shown).

Western-Blot Analysis

To analyze expression of 5′-inserted MHC isoenzymes, the polyclonal antibody directed against the 25K/50K insert (a25K/50K) was used. Proteins were transferred from SDS-gels to nitrocellulose (hybond-C 45 μm, Amersham) (90 min, 0.8 mA/cm²). The nitrocellulose was blocked with 3% ovalbumin and subsequently incubated with the primary antibody (2 h at room temperature) and the secondary peroxydase-conjugated antibody (anti-rabbit IgG, Sigma) for 1 h at room temperature. Proteins were visualized by the "enhanced chemiluminescence reaction" kit (ECL, Amersham) using an X-ray film (X-Omat, Kodak).

Analysis of Myosin Light Chains (MLC)

Phosphorylation of the 20 kDa MLC and the distribution of the 17 kDa MLC isoforms (MLC_{17a} and MLC_{17b}, with decreasing order of acidity) of skinned uteri and TCA-extracts (in vivo expression) was analyzed by 2-dimensional gel electrophoresis (2D-PAGE). Isoelectric focusing (first dimension) was performed in glass capillaries (12.5 cm length, 1 mm inner diameter) using the pH gradient 4.5–5.4 (Pharmalytes, Pharmacia, Sweden). The gels were run overnight at 600 V constant for the first dimensional separation. The second dimension was a SDS-disc electrophoresis [Laemmli and Favre, 1973]. Slab gels were 10.5 × 9.5 cm, 1 mm thick.

Statistics

Statistical analysis were performed using the commerically available statistic program "epistat" on an IBM compatible PC (means, standard deviations (SD), Student's *t*-test for unpaired values). All values are expressed as means ± SD with number of animals investigated in parenthesis.

RESULTS

Tissue and Body Weights

Table I shows tissue and body weights in the female groups. Ovarectomized-sham treated ani-

mals (S) revealed an increased body weight which was prevented by estrogen (E) but not by testosterone (T). Heart weights were similar in all groups, except in testosterone-treated ovarectomized animals where the heart weight increased significantly ($P < 0.05$). The significant decrease of the uterus weight caused by gonadectomy ($P < 0.01$) was prevented by both estrogen (E) and testosterone (T) treatment.

Steroid Hormone Levels

Serum testosterone levels could not be detected in N, S, or E groups (dectection limit 40 pg/ml). In the T group testosterone rose to levels above 10 ng/mg. Estrogen was 135 pg/ml in N and around the detection limit in the S and T groups and increased about 100-fold in the E group.

Myosin Heavy Chain (MHC) Expression

Figure 1 shows the analysis of MHC expressed in the left ventricle of rats by PP-PAGE. Both steroid hormones increased the expression of the alpha-MHC isoenzyme. N animals expressed mainly the V1 isoforms (89% alpha-MHC). Decreasing steroid hormone levels by ovarectomy (S) decreased V1 expression in favor of V3 and V2 (65% alpha-MHC; $P < 0.01$) which normalized upon estrogen (86% alpha-MHC) and testosterone (81% alpha-MHC) treatment.

MHC expression pattern in smooth muscle cells was tissue-specific. In the N group, uterus cells expressed SM1 (204 kDa) and NM (196 kDa) in a ratio of 66/34 but no SM2 could be detected (Fig. 2). However, aorta cells expressed SM1, SM2, and NM-MHC in a ratio of 52/33/15 (Fig. 3).

In the uterus of ovarectomized-sham treated (S) rats SM1 decreased significantly from 66.4% (N) to 53.3% ($P < 0.05$) in favor of NM-MHC. Hormonal substitution with testosterone increased SM1 significantly to 85.9% ($P < 0.01$), while estrogen was without statistically significant effect (57.5% SM1) (Fig. 2). In the aorta, neither ovarectomy nor any hormonal substitution changed the MHC expression (Fig. 3).

To analyze whether steroid hormones affected alternative splicing of SM-MHC at the 5′ terminus, identical amounts of uterus MHC of N, S, E, and T were applied to SDS-gels for Western-blot analysis with a25K/50K (Fig. 4). No cross-reactivity of a25K/50K with NM-MHC could be observed proving the specificity of the peptide antibody. However, SM1 of rat uterus showed some immunoreactivity with a25K/50K. This immunoreactivity remained upon ovarectomy but became hardly detectable upon estrogen substitution. SM1 of testosterone treated rats, however, strongly reacted with a25K/50K.

Myosin Light Chain (MLC) Expression

Both aorta and uterus expressed two types of 17kDa MLC (MLC_{17}) designated with increasing acidity as MLC_{17b}, and MLC_{17a} (Fig. 5; 2D-PAGE analysis).

Taking both MLC_{17} isoforms as 100%, SMC of rat uterus expressed 67% MLC_{17a} which decreased significantly ($P < 0.01$) to 47% upon ovarectomy. Estrogen, but not testosterone, normalized MLC_{17a} expression (Fig. 6).

SMC of rat aorta expressed 78% MLC_{17a} (N). Neither ovarectomy nor hormonal substitution changed MLC_{17} isoform expression in the aorta (Fig. 7).

DISCUSSION

The expression of different subunit isoforms of myosin modulate the contractile and energetic behaviour of all muscle types. It is, therefore, of great interest to study the regulation of myosin expression. Steroid hormones are important candidates since they are involved in the

TABLE I. †Tissue and Body Weights of Experimental and Control Animals

	N	S	E	T
BW	218.3 ± 20.2	321.7 ± 28.9	202.5 ± 24.4	315.8 ± 13.6
HW	0.76 ± 0.04	1.06 ± 0.05	0.73 ± 0.06	1.11 ± 0.04*
UW	0.54 ± 0.03	0.1 ± 0.01**	0.51 ± 0.02	0.42 ± 0.07

†Body weight (BW, in g), heart weight (HW, in g), uterus weight (UW, in g) of 12-weeks-old sham-operated/sham-treated (N), ovarectomized/sham-treated (S), ovarectomized/estrogen substituted (E), and ovarectomized/testosterone subsituted (T) rats. Values are means ± SD.

*$p < 0.05$.

**$p < 0.01$.

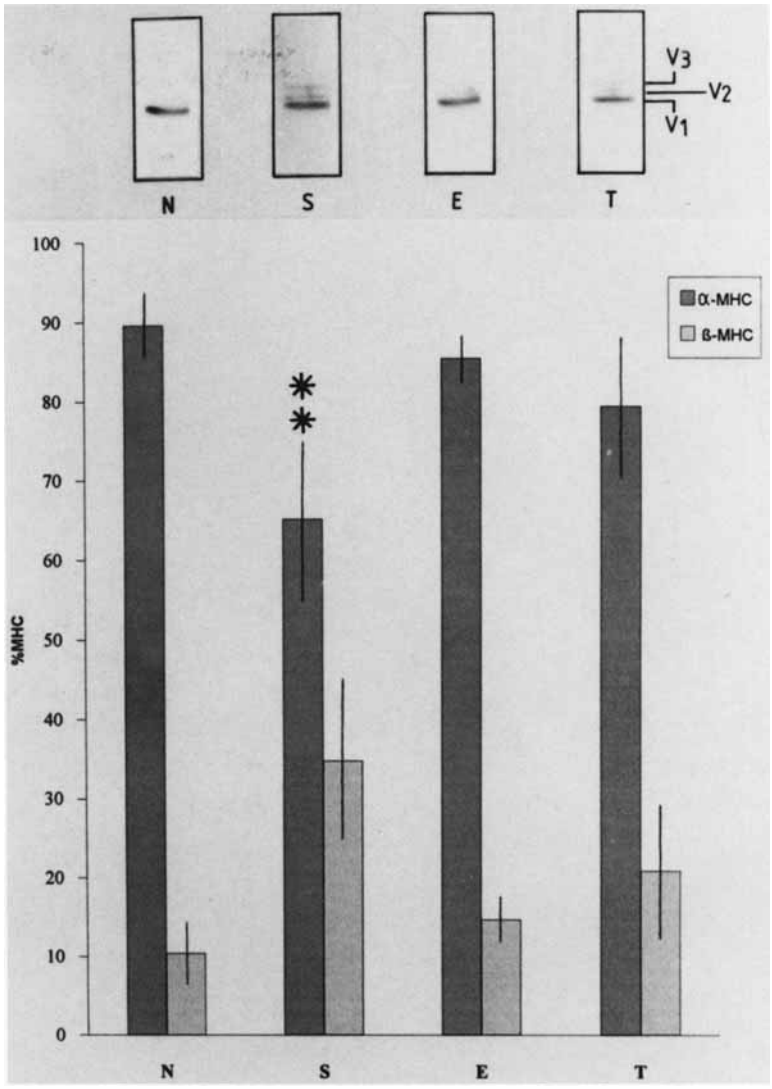

Fig. 1. Analysis of in vivo expression of cardiac myosin isoenzymes by PP-PAGE. V1, V2, and V3 correspond to the alpha-alpha-homodimer, alpha-beta-heterodimer, and beta-beta-homodimer, respectively, of 12-weeks-old sham-operated/sham-treated (N), ovarectomized/sham-treated (S), ovarectomized/estrogen substituted (E), and ovarectomized/testosterone substituted (T) rats. **Top:** Representative original gels. **Bottom:** Statistical analysis of the densitometrically scanned protein bands (%alpha-MHC = %V1 + 0.5%V2; %beta-MHC = %V3 + 0.5%V2). Values are means ± SD. $^{**}P < 0.01$ (compared with N).

regulation of cell differentiation and mediate responsiveness of cells to different environmental demands. Thus, it has been demonstrated that long-term treatment (8–10 weeks) of rats with testosterone and estrogen increased alpha-MHC expression and contractility of the rat heart [Scheuer et al., 1987; Schaible et al., 1984]. In this study we investigated whether short-term treatment of ovarectomized rats (2 weeks) with steroid hormones affects the expression of myosin subunits in cardiac and two smooth muscle types.

To prove the effectiveness of operation and treatment we determined steroid hormone levels as well as body, heart and uterus weights. In fact, ovarectomy decreased steroid hormone levels and uterus weight while both steroid hormones normalized uterus weight. Body and heart weight increased upon ovarectomy, an observation which is in accordance with previous reports [Scheuer et al., 1987; Schaible et al., 1984].

Short term application (two weeks) of testosterone increased cardiac alpha-MHC expression on the protein level. This is in accordance with

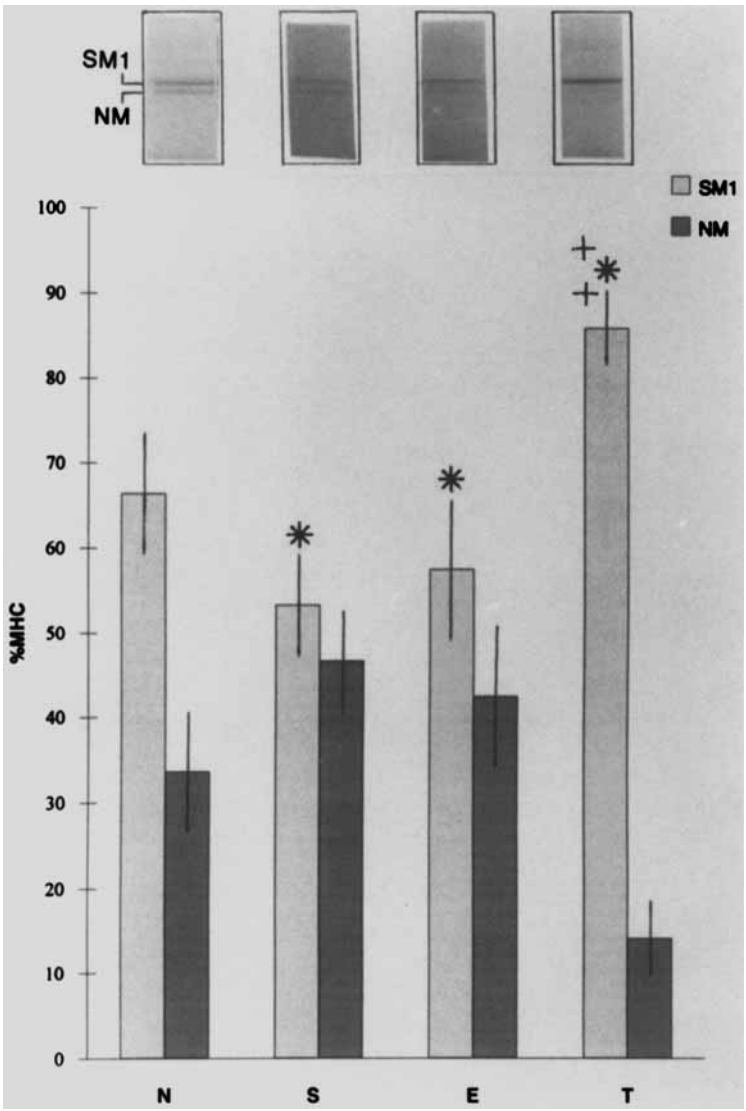

Fig. 2. In vivo expression of myosin heavy chains of the uterus of 12-week-old sham-operated/sham-treated (N), ovarectomized/sham-treated (S), ovarectomized/estrogen substituted (E), and ovarectomized/testosterone substituted (T) rats. **Top:** Representative original gels. **Bottom:** Statistical analysis of the densitometrically scanned protein bands expressed in % of whole MHC.

long-term studies of steroid hormone actions on cardiac myosin expression (8–10 weaks of treatment) [Scheuer et al., 1987; Schaible et al., 1984]. Likewise, alpha-MHC expression increased upon testosterone application of orchiectomized spontaneously hypertensive rats despite a concomitant increase in blood pressure [Lengsfeld et al., 1988] which is known to favour increased beta-MHC expression [Lompré et al., 1979]. Steroid hormones, therefore seem to be a dominant factor in the regulation of MHC gene expression. This could be due to a direct upregulation of the rate of alpha-MHC gene transcription [Morano et al., 1992] and alpha-MHC mRNA expression [Morano et al., 1990]. It is believed that the testosterone-receptor-complex binds to the promoter region of the alpha-MHC gene, thus increasing the transcription rate of the gene [Morano, et al., 1991]. Since calcium- and actin-activated myosin ATPase activity [Pope et al., 1980] and shortening velocity of heart fibers with alpha-MHC [Schwartz et al., 1981] is higher than that of fibers with beta-MHC, steroid hormones could be considered as important factors

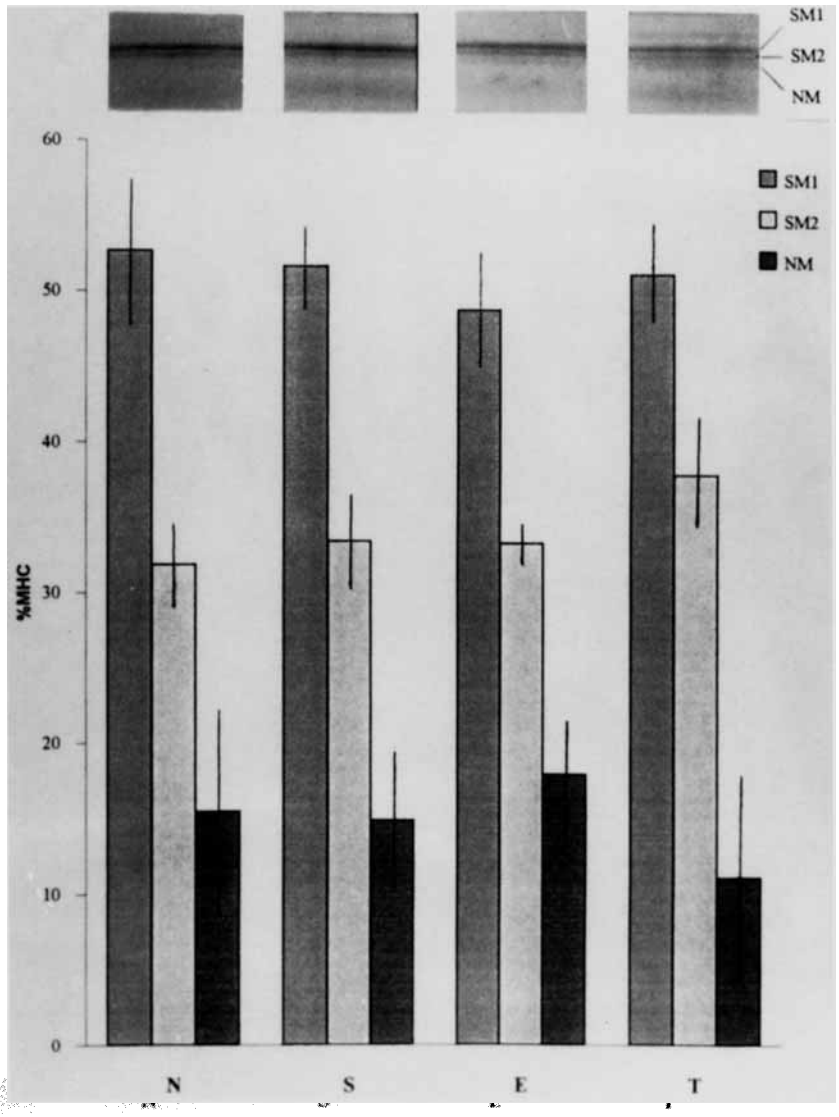

Fig. 3. In vivo expression of myosin heavy chains of rat aorta of 12-week-old sham-operated/sham-treated (N), ovarectomized/sham-treated (S), ovarectomized/estrogen substituted (E), and ovarectomized/testosterone substituted (T) rats. **Top:** Representative original gels. **Bottom:** Statistical analysis of the densitometrically scanned protein bands. $*P < 0.05$ (compared with N); $++P < 0.01$ (compared with S).

in the regulation of cardiac performance. An involvement of hormones other than steroids in the regulation of myosin expression in this study seems to be unlikely since it has already been demonstrated that thyroid indexes, plasma insuline and blood glucose did not change upon ovarectomy and steroid substitution [Scheuer et al., 1987].

MHC expression in smooth muscle was tissue-specific: uterus cells expressed no detectable amounts of SM2 while aorta cells did. The cause of this differential expression pattern is not clear. Steroid hormones were unable to induce expression of 3′-spliced SM-MHC, i.e., did not induce SM2 expression in the uterus.

However, steroid hormones regulated SM1A and SM1B expression. Thus, decreasing steroid hormone levels caused by ovarectomy decreased whole SM1 (i.e., the sum of SM1A and SM1B) expression but had no influence on the SM1B isoenzyme which contains the 5′-insert, suggesting a selective down-regulation of the SM1A isoenzyme. Interestingly, steroid hormones affected the expression of MHC differentially. Thus, testosterone increased the expression of

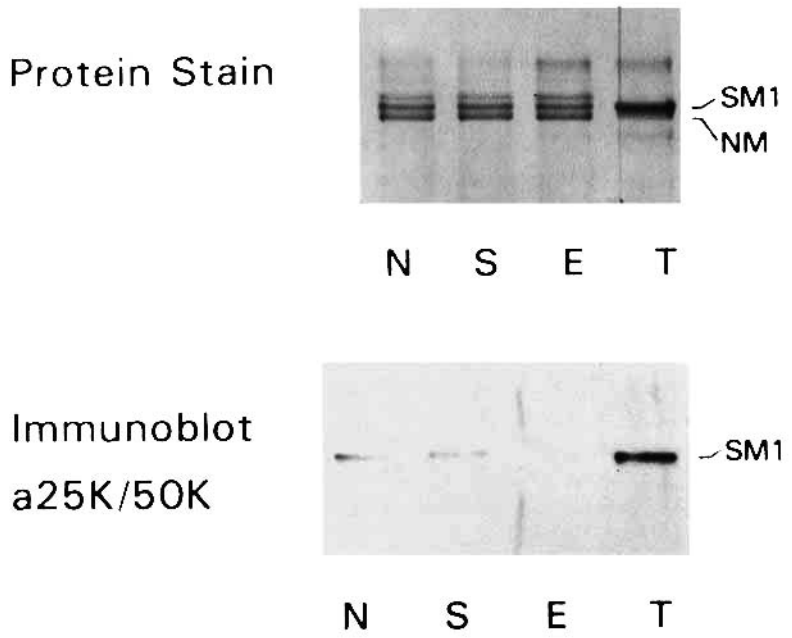

Fig. 4. Analysis of in vivo expression of myosin heavy chains (MHC) with the 25K/50K insert by Western-blot using a peptide-directed antibody. **Top:** MHC as detected by protein staining (Coomassie-blue). **Bottom:** Western-blot analysis (ECL-staining). 12-week-old sham-operated/sham-treated (N), ovarectomized/sham-treated (S), ovarectomized/estrogen substituted (E), and ovarectomized/testosterone subsituted (T) rats.

both, whole SM1 as well as expression of SM1B. Estrogen, in contrast reduced SM1B to an undetectable level while whole SM1 did not change. An interpretation of this observation would be that estrogen increases SM1A and decreases SM1B to about similar extents. Either, the estrogen effect on SM-MHC must be comparably small to the testosterone effect since in the myometrial cell only very low basal levels of SM1B are expressed [White et al., 1993].

Differential steroid hormone effects could also be observed for MLC expression. Estrogen but

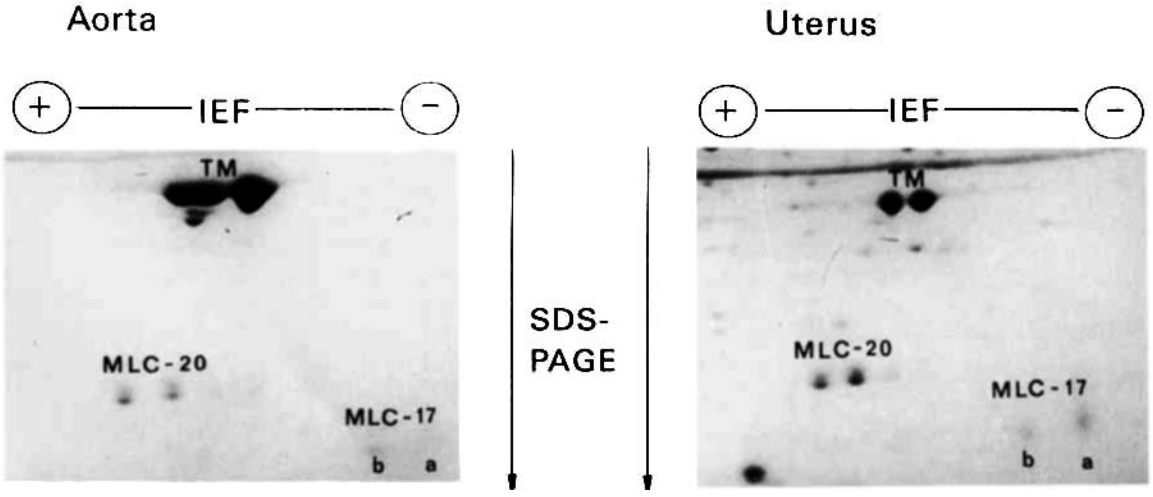

Fig. 5. Analysis of myosin light chains (MLC) in vivo by 2D-PAGE of rat uterus and aorta (original gels). MLC20, LC17a, and MLC17b correspond to the 20 kDa (phosphorylatable) and 17 kDa (essential) MLC isoforms. TM = tropomyosin.

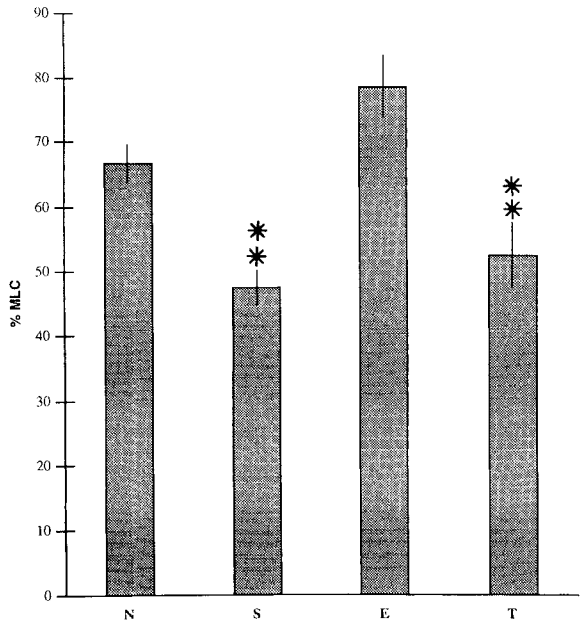

Fig. 6. In vivo expression of MLC17 isoforms of rat uterus of 12-week-old sham-operated/sham-treated (N), ovarectomized/sham-treated (S), ovarectomized/estrogen substituted (E), and ovarectomized/testosterone subsituted (T) rats. $^{**}P < 0.01$ (compared with N).

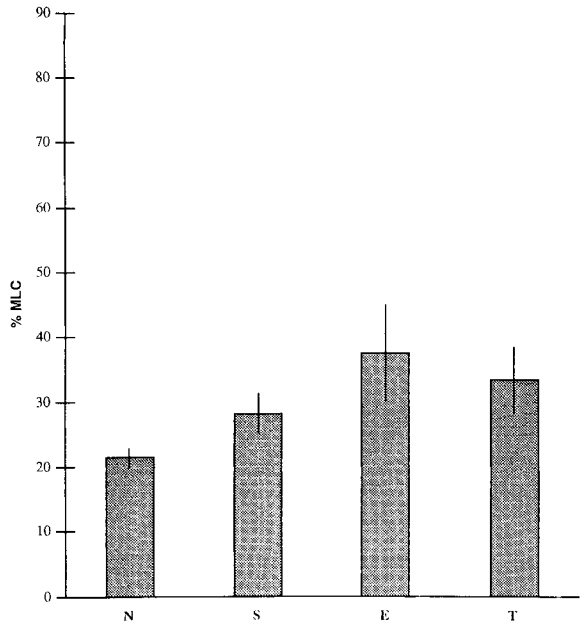

Fig. 7. In vivo expression of MLC17 isoforms of rat aorta of 12-week-old sham-operated/sham-treated (N), ovarectomized/sham-treated (S), ovarectomized/estrogen substituted (E), and ovarectomized/testosterone subsituted (T) rats. **Top:** Representative original gels. **Bottom:** Statistical analysis of the densitometrically scanned protein bands.

not testosterone increased MLC_{17a} expression in the rat uterus.

These observations could have some important functional consequences: enhanced MLC_{17a} was associated with increased myosin ATPase activity [Helper et al., 1988] and maximal shortening velocity [Morano et al., 1993; Malmqvist and Arner, 1991] of smooth muscle fiber preparations. MHC with the 25K/50K insertion revealed an increased ATPase activity and higher actin filament sliding velocity in the in vitro motility assay [Kelley et al., 1993]. Hence, one can speculate that a simultaneous increase of both steroid hormones, estrogen and testosterone, should elevate smooth muscle contractility (increase of MLC_{17a} by estrogen, increase of 5′-inserted MHC by testosterone). In fact, uterus contractility increased significantly only during late pregnancy [Morano et al., 1993], i.e., in a state where both estrogen and testosterone revealed peak concentrations [Legrand et al., 1984].

In summary, steroid hormones regulate myosin gene expression in smooth and cardiac tissues thus controlling and modulating performance of muscle contraction.

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