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Original Contributions

Capillary neoformation in the rat heart – stereological studies on papillary muscles in hypertrophy and physiologic growth

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Summary: Stereological investigations on myocardial capillaries provided evidence that the common estimator of capillarity, the capillary density (i.e., number of capillary profiles per unit transverse sectional area), underestimates the true capillary supply since the capillary axes are not oriented perfectly in parallel to the myofiber axes. Recently, we studied the "true" capillarity, i.e., the length density of capillaries (L_V = capillary length per capillary volume), in some experimental models of cardiac hypertrophy which have been published elsewhere. It has been shown that L_V decreases in renovascular hypertension, but is maintained in physical exercise and after chronic thyroxin application. However, the growth pattern of capillaries in hypertrophic hearts has not yet been analyzed. In the present paper it is demonstrated that important information on the capillary network can be derived from the two-dimensional capillary-to-fiber ratios (2D CFR: capillary profiles per myofiber profiles in transverse sections) and from the three-dimensional capillary-to-fiber ratios (3D CFR: capillary length per unit myofiber length). Increase in both suggests neoformation of additional capillary branches in parallel connection. Retrospective analysis of the quantitative data indicates that in hypertrophy induced by physical exercise or by chronic thyroxin application capillary neoformation in parallel connection counterbalances increase of oxygen diffusion distance due to myofiber enlargement. In renovascular hypertension, capillary neoformation in parallel connection does not occur. Studies on normal growth indicated both a slight decrease of L_V of capillaries, as well as a continuous neoformation of additional capillary branches.

Key words: myocardium; hypertrophy; capillaries; stereology

Introduction

During recent decades a long series of morphometric studies on experimental cardiac hypertrophy was performed. Analysis of capillaries provided evidence that hypertrophy induced by mild physical exercise and hypertrophy induced by thyroxin treatment is associated with proliferation of myocardial capillaries (2, 4, 6, 9, 23, 25, 27, 28) which counterbalances – at least in part – the increased width of hypertrophic fibers. However, hypertrophy induced by strenuous exercise and by chronic pressure overload leads to a decrease in capillary supply (1, 3, 7, 15, 19, 29, 30).

The common parameters of myocardial capillarization are the capillary density (i.e., number of capillary profiles in transverse sections per unit sectional area) and the capillary-to-fiber ratio (capillary profiles per myofiber profile in transverse sections). These two-dimensional parameters, however, which are obtained from planar sections do not give any information on the three-dimensional orientation distribution of capillaries, and they

underestimate the true capillary supply (22), i.e., the length of capillaries per unit volume (L_V).

Recently, we introduced a model-based stereological method in order to estimate L_V of myocardial capillaries, which has been described and applied by other groups on other organs (8, 20, 33). By means of this model, we studied some experimental models of cardiac hypertrophy and the physiologic growth process (19, 22, 24, 25). In doing so, mild physical exercise and chronic thyroxin application revealed unchanged L_V values (23, 25) whereas renovascular hypertension and normal growth induced a decrease of L_V (19, 24). However, up to now it has not been systematically analyzed as to whether capillary proliferation in hypertrophic hearts is realized by neoformation of additional capillary branches in parallel connection, by increase in transverse capillary branching, or by an overproportionate growth of capillary segments which would lead to an increased tortuosity of the capillary network. In a previous investigation on chronic dipyridamole effects on the heart, we found that an increase in both L_V of capillaries and capillary density {Q_A (α = 0); Q: number of capillary profiles, A: area, α = 0: transverse section} indicates neoformation of capillary segments in parallel connection, but this conclusion does not hold in the hypertrophic case (18). In the present study, we focused on capillary growth patterns in the hypertrophic heart.

Methods

The models of cardiac hypertrophy and normal growth described here have been published in detail elsewhere (19, 22, 23, 25).

Hypertrophy induced by physical training

Ten young female Sprague-Dawley rats performed an exercise program of 18 weeks' duration with gradually increasing intensity on a motordriven running device. In the final phase the animals exercised 90 min/day at a speed of 32 m/min; 10 animals served as sedentary controls (23).

Hypertrophy induced by renovascular hypertension (8 weeks)

Twelve male Wistar rats were subjected to moderate renovascular hypertension for 8 weeks with a surgical stenosis of the left renal artery; 10 animals served as sham-operated controls (19).

Hypertrophy induced by thyroxin application (3 weeks)

Fifteen male Wistar rats were treated with daily intraperitoneal injections of thyroxin (0.5 mg/kg); 10 animals served as controls (25).

Normal growth

Normal male Wistar rats (24) were investigated at the age of 5 weeks (n = 9), 7 weeks (n = 7), 13 weeks (n = 8), and 52 weeks (n = 9).

Tissue fixation

The viscera were fixed by retrograde vascular perfusion at a pressure of 110 mm Hg after catheterization of the abdominal aorta, as described elsewhere (16, 18).

Left ventricular papillary muscles were randomly cut, either longitudinally or transversely, with a tissue sectioner (14, 19). A minimum of two transversely cut, 200 μm thick slices and two longitudinally cut, 200 μm thick slices were randomly selected for stereology and embedded in Epon-Araldite. Semithin sections (1 μm) were stained with methylene blue and basic fuchsin (11), and examined by light microscopy using oil immersion and phase contrast. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 10 electron microscope.

Quantitative stereology

Capillaries and myofibers of the myocardium are oriented anisotropically. From a stereological viewpoint myofiber axes are perfectly anisotropic since they are oriented in parallel to the longitudinal axis of the muscle bundle. In contrast, capillary axes are partially, but not perfectly anisotropic.

Estimation of *length densities* (L_V: length per unit reference volume) from planar probes (sections) depends on the orientation of the length elements in space and the angle α between the section plane and the axis of anisotropy. Recently, we introduced stereological procedures to estimate L_V of myocardial capillaries (22) that are based on a mathematical model of directional statistics, the Dimroth-Watson orientation distribution (8, 20).

L_V is calculated according to the stereological equation (19):

$$L_V = c_1(K_L, \alpha = 0) * Q_A(\alpha = 0) \quad (1)$$

The $\{Q_A(\alpha = 0)\}$'s are the commonly used planar parameters: density of capillaries (Q_A) in transverse sections ($\alpha = 0$), e.g., number of profiles per unit transverse sectional area (≡ *capillary density).* They are to be multiplied with the coefficient of correction $c_1(K_L, \alpha = 0)$. α is the angle between the direction of the section plane (i.e., the normal to the plane) and the longitudinal axis.

The degree of anisotropy, i.e., the degree of concentration of the length elements around the longitudinal axis, is indicated by the constants of anisotropy K_L. It is estimated empirically from the ratio of counts of capillary profiles on transverse ($\alpha = 0$) and longitudinal sections ($\alpha = \pi/2$): $Q_A(\alpha = 0)/Q_A(\alpha = \pi/2)$, and the coefficients of correction $c_1(K_L, \alpha = 0)$ are calculated as described elsewhere (22).

Since myofiber axes are completely anisotropic, i.e., parallel to the longitudinal axis of the muscle bundles (25):

$$c_1(K_L, \alpha = 0) = 1. \quad (2)$$

In contrast, partial anisotropy of capillaries leads to

$$c_1(K_L, \alpha = 0) > 1, \text{ and } L_V > Q_A(\alpha = 0).$$

Myocardial capillarity has been frequently described as the ratio of myofiber profiles and capillary profiles in transverse sections (11). This capillary-to-fiber ratio corresponds to $Q_A(\alpha = 0)$ of capillaries/$Q_A(\alpha = 0)$ of myofibers, which we define as the *two-dimensional capillary-to-fiber ratio* (2D CFR). In addition, we consider the *3D capillary-to-fiber ratio* (3D CFR = L_V of capillaries/L_V of myofibers). Obviously, 3D CFR > 2D CFR.

Stereological analysis was performed on transverse and longitudinal sections of the left ventricular papillary muscles at a light microscopic magnification of 1000×. Papillary muscles were completely dissected, either longitudinally or transversely into small tissue slices by means of a tissue sectioner, as described elsewhere (16). Randomly selected slices were embedded in Epon-Araldite and cut for stereological investigations. In eight systematically subsampled test areas per section the capillary profiles were counted. Reference volume was the total myocardial tissue of the left ventricular papillary muscles.

In our previous studies, we did not focus on the mean sarcomere length, which may be slightly variable, even under the standardized conditions of perfusion fixation. In longitudinally cut semithin sections of papillary muscles, we measured the length of 10 sarcomeres at six sites (60 sarcomeres) at a magnification of 1000:1, and the mean sarcomere length was calculated.

Two transversely embedded probes per animal were chosen for ultrathin sectioning. Myofiber profiles were counted under electron microscopy at a magnification of 4000:1 as described elsewhere (20).

Stereological models of capillary growth
(cf. Appendix)

In order to get information on the growth process of capillaries in cardiac hypertrophy the CFR's were considered as useful parameters since they are independent of mean cross-sectional areas of myofibers. Three models of capillary growth (Fig. 1) can theoretically be discriminated on the base of the CFR's. However, interpretation of CFR's requires a constant number of myofibers, i.e., absence of myofiber necrosis and proliferation. In the experimental models under study myofiber necroses and scarring was not observed. With respect to myofiber hyperplasia it is generally assumed that myocyte proliferation ceases by the age of weaning in the rat (26) and cardiac growth occurs primarily through

hypertrophy (5). However, recently published studies on rats indicate that in advanced stages of hypertensive cardiac hypertrophy, which are associated with cardiac failure and progressive scarring, myocyte proliferation may develop (5). But the present models of hypertrophy were short-term experiments, and myofiber necroses and scarring were completely absent (19, 23, 25).

Enlargement of myofibers in normal growth and cardiac hypertrophy corresponds regularly to an increased mean cross-sectional area and to an increased total length of myofibers. If lengthening of the capillary network is proportionate to the length increase of myofibers 3D CFR and 2D CFR are not changed. This type of capillary growth is now designed as *elongation of capillaries*. Obviously, it leads to

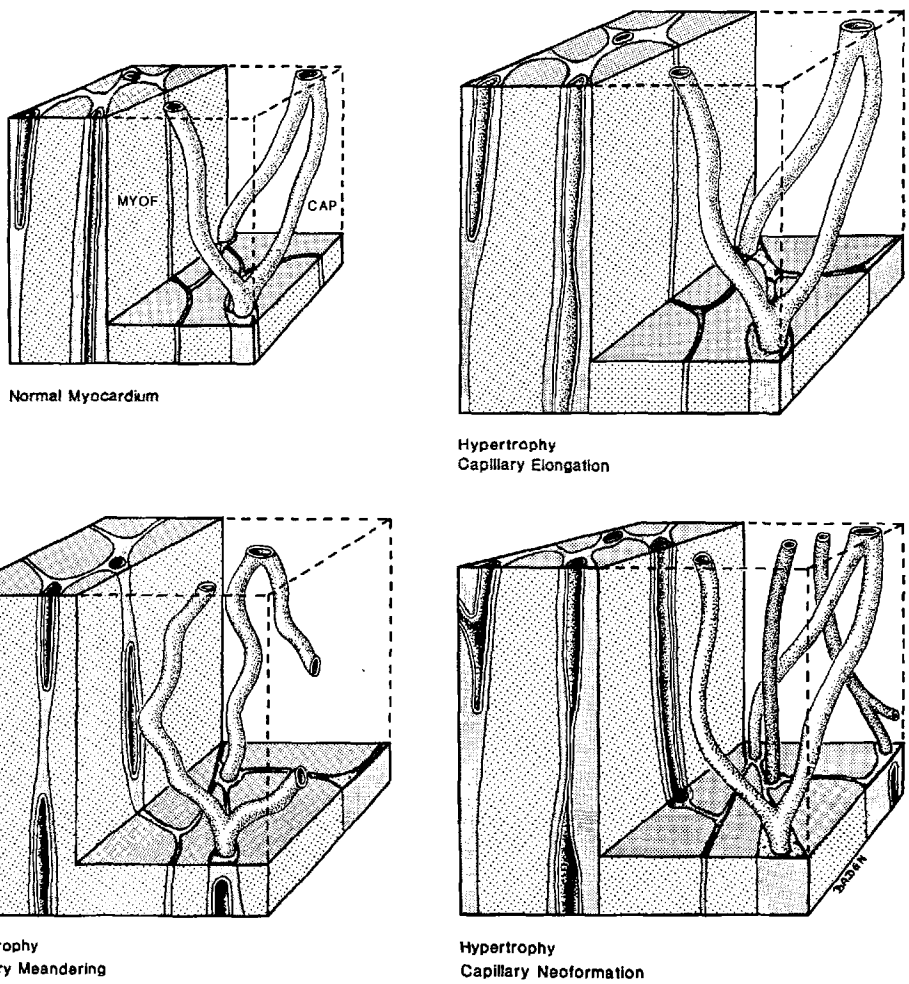


Fig. 1. Capillary growth patterns in cardiac hypertrophy that can be discriminated on the base of the CFR's. Fig. 1a shows the nonhypertrophic case. Figs. 1b–d illustrate enlargement of myofibers (increase in length and cross-sectional area) and three different patterns of capillary growth: Fig. 1b) capillary elongation without additional capillary segments in parallel connection. The spatial orientation of capillary axes is not changed. Fig. 1c) more marked length increase of the capillary branches which would lead to an increased tortuosity (meandering). Fig. 1d) neoformation of additional capillary branches in parallel connection.

a decrease in capillary supply which depends on the degree of hypertrophy (Figs. 1a, b). In appendix 1 it is shown that elongation can be realized, either by elongation of individual capillary branches or by addition of new capillary branches in series-connection. If the process of hypertrophy is not associated with length increase of myofibers unchanged CFR's indicate absence of any capillary growth.

Elongation of capillary branches that exceeds length extension of myofibers would necessarily lead to a more tortuous arrangement of capillaries, and the length of capillaries per unit length of myofibers (3D CFR) would increase, but the 2D CFR would not be changed (Figs. 1a, c). This growth pattern is designed as *meandering of capillary branches*. The third growth pattern is characterized as *neoformation of capillary branches in parallel connection*. It is correlated with an increase of both 3D CFR and 2D CFR (Figs. 1a, d).

Results

3D CFR and 2D CFR were increased in hypertrophy induced by physical exercise and by thyroxin treatment ($p < 0.01$, Student's t-test for unpaired data) and in normal growth ($p < 0.001$, one-way analysis of variance), but were not significantly changed in renovascular hypertension (Table 1). Thus, the marked capillary growth that has been observed after mild physical exercise and after chronic thyroxin application is caused by neoformation of additional capillary branches in parallel connection. Furthermore, compensatory neoformation of capillary segments occurs also in normal cardiac growth. However, the unchanged CFR's in renovascular hypertension correspond to the absence of compensatory capillary neoformation. Spatial distribution of capillary axes showed only slight variations between

Table 1. Left ventricular muscle mass and stereological parameters (means ± SD).

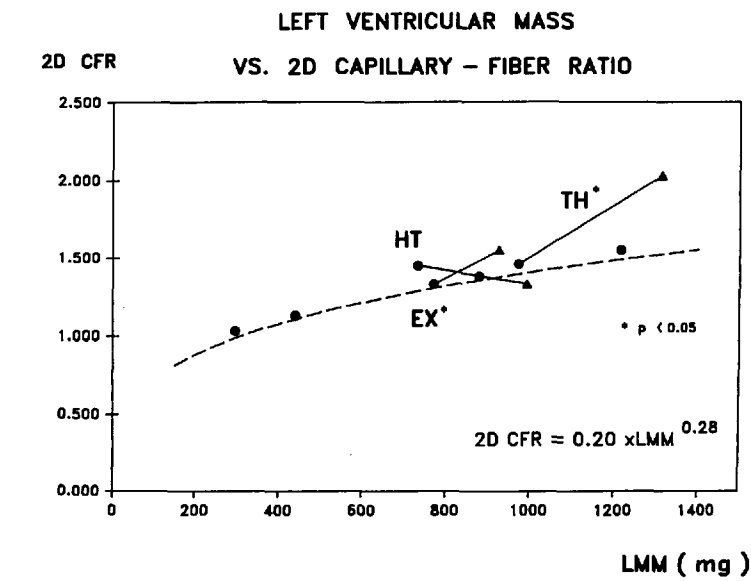
Groups	LMM (mg)	MCSA (μm^2)	2D CFR	3D CFR (mm/mm)	SL (μm)	c_1
Growth 5 weeks	295 ± 18	190 ± 21	1.01 ± 0.12	1.09 ± 0.12	2.03 ± 0.02	1.07 ± 0.02
Growth 7 weeks	439 ± 26	246 ± 21	1.07 ± 0.15	1.19 ± 0.14	2.11 ± 0.03	1.09 ± 0.02
Growth 13 weeks	877 ± 65	354 ± 31	1.34 ± 0.16	1.48 ± 0.18	2.06 ± 0.02	1.09 ± 0.02
Growth 52 weeks	1214 ± 111	459 ± 48	1.48 ± 0.22	1.63 ± 0.21	2.06 ± 0.02	1.09 ± 0.02
Control (Exercise)	769 ± 66	310 ± 41	1.17 ± 0.15	1.28 ± 0.14	2.13 ± 0.03	1.07 ± 0.02
Physical Exercise	924 ± 73	369 ± 33	1.42 ± 0.14	1.53 ± 0.14	2.17 ± 0.03	1.05 ± 0.02
Control (Hypert.)	731 ± 76	374 ± 26	1.33 ± 0.12	1.44 ± 0.10	2.18 ± 0.02	1.05 ± 0.02
Renovasc. Hypert.	991 ± 114	436 ± 43	1.30 ± 0.22	1.40 ± 0.18	2.02 ± 0.03	1.07 ± 0.02
Control (Thyroxin)	971 ± 66	414 ± 63	1.43 ± 0.32	1.57 ± 0.28	2.03 ± 0.02	1.09 ± 0.02
Thyroxin Applicat.	1313 ± 101	591 ± 103	1.98 ± 0.39	2.17 ± 0.38	2.04 ± 0.02	1.09 ± 0.02

Abbreviations

LMM: Left ventricular muscle mass
MCSA: Mean cross-sectional area of myofibers
2D CFR: Two-dimensional capillary-to-fiber ratio
3D CFR: Three-dimensional capillary-to-fiber ratio
SL: Mean sarcomere length
c_1: Coefficient of correction (degree of anisotropy) (MCSA corrected for variable SL)

Statistics (Student's *t*-test, one-way analysis of variance)

LMM: $p < 0.001$ in all experiments and in normal growth
MCSA: $p < 0.001$ in all experiments and in normal growth
2D CFR: $p < 0.01$ after exercise, thyroxin application and in normal growth, but not in renovascular hypertension
3D CFR: $p < 0.01$ after exercise, thyroxin application and in normal growth, but not in renovascular hypertension



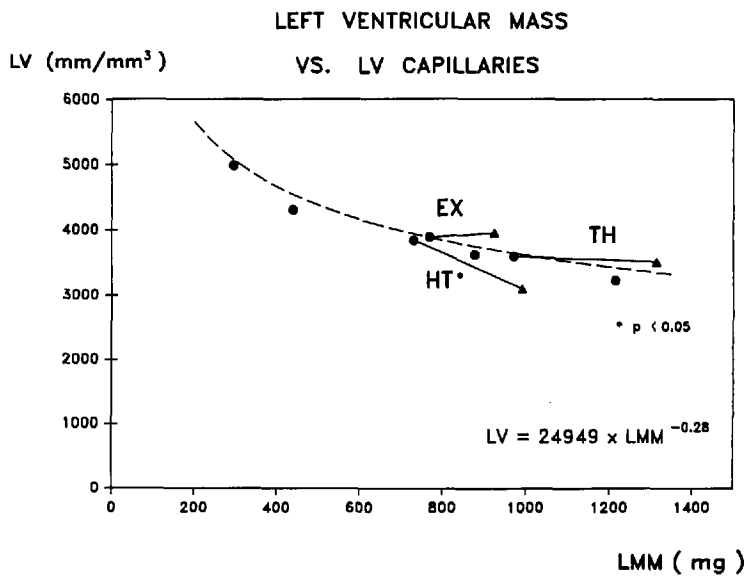


Fig. 2. Circles correspond to group means of non-treated groups, triangles to group means of treated groups (EX: exercise; HT: hypertension; TH: thyroxin). Fig. 2a shows an increase of 2D CFR in EX, TH and in normal growth, which corresponds to neoformation of capillary branches in parallel connection. Thus, length densities of capillaries (L_V) are not reduced in EX and TH (Fig. 2b) despite fiber hypertrophy, and only a mild reduction is observed in normal growth (Fig. 2b). However, the unchanged 2D CFR in HT leads to a considerable decrease in capillary supply (Figs. 2a, b). The nonlinear regressions are derived from the group means of non-treated animals (circles).

the different experiments, which is indicated by the coefficients of correction c_l (Table 1). Mean sarcomere lengths were significantly correlated with the c_l values, i.e., the degree of capillary tortuosity ($r = 0.67$; $p < 0.05$) is essentially determined by the state of contraction.

Discussion

Analysis of the CFR's and L_V of capillaries provided evidence that mild physical exercise, chronic thyroxin treatment, and normal growth induce capillary neoformation in parallel connection which either *completely* or *partially* counterbalances the increased myofiber width (Fig. 2). In contrast, hypertrophy induced by renovascular hypertension was not correlated with significant neoformation of capillary branches in parallel connection, which causes a decrease in capillary supply (Fig. 2).

Capillary proliferation is generally assumed to be a rare event in the adult mammalian heart (14). Early studies on hypertrophic human hearts revealed constant capillary-to-fiber ratios (cf. (13) for detailed discussion) which are associated with a decrease in capillary supply. During normal growth, the myocardial capillary density slightly decreases (4, 6, 28) despite the slight capillary neoformation that was observed in our study. However, right or left ventricular hypertrophy induced by mild physical exercise (2, 4, 6, 27, 28), hypertrophy following chronic hypobaric hypoxia (12) and hypertrophy following chronic treatment with thyroxine (9) were correlated either with higher capillary-to-fiber ratios or with normal or even increased levels of capillary density, despite fiber hypertrophy. Recently, Crisman and coworkers (10) described an exercise-induced myocardial capillary growth in the spontaneously hypertensive rat.

Several investigators found growth of myocardial capillaries in non-hypertrophic hearts, too. Chronic bradycardial pacing (32), chronic treatment with vasodilating drugs (14, 21, 31, 34), and chronic ethanol feeding (17) were associated with proliferation of endothelial cells and growth of capillaries. In a recently published paper, we reported stereological evidence of capillary neoformation after dipyridamole treatment (18). It has been suggested that the common denominator of all experimental conditions which were associated with proliferation of myocardial capillaries is the enhanced myocardial blood flow (14, 18, 21). In terms of capillary growth patterns, neoformation of capillary branches in parallel connection may be stimulated by increase in mechanical stress following the enhancement of capillary blood flow (14).

It should be emphasized that meandering of capillaries was not detected in the experimental models under study. The slight differences in the degree of capillary tortuosity are, in part, caused by slightly variable sarcomere lengths. One may suggest that the strikingly constant directional distribution of capillaries is a physiologically determined phenomenon. It should be noted that this finding also precludes the possibility that capillary proliferation was associated with a significant, overproportionate increase in transverse capillary anastomoses.

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Appendix

Capillary growth patterns in hypertrophy

The following parameters be defined:

L_V myofibers: Length of myofibers per unit volume$^+$
L_V capillaries: Length of capillaries per unit volume$^+$

$Q_A(\alpha = 0)$ myofibers: Number of myofiber transects per unit transverse sectional area$^+$
$Q_A(\alpha = 0)$ capillaries: Average number of capillary transects per unit transverse sectional area$^+$
($^+$ Unit volume and unit transverse sectional area in the nonhypertrophic case)

$Q_A(\alpha = 0)$ of myofibers can be derived from the area fraction of myofibers A_A and the mean cross-sectional area of myofibers MCSA:

$$Q_A(\alpha = 0) \text{ myofibers} = A_A \text{ myofibers/MCSA myofibers} \quad (1)$$

Since myofibers are oriented in parallel (= perfect anisotropy) the following equation holds (23):

$$Q_A(\alpha = 0) \text{ myofibers} = L_V \text{ myofibers} \quad (2)$$

A capillary segment can now be defined as individual capillary branch between two ramifications with length l, the number of segments being n. The probability to hit an individual segment by transverse sectioning is proportionate to $l * \cos \alpha$ (α: angle between axis of anisotropy and capillary axis, $\alpha \leq \pi/2$). Now we introduce $l(p) = l * \cos \alpha$ as the projected length of a segment (projected on the axis of anisotropy) and $n * l(p) = L(p)$ as total projected length per unit tissue volume, i.e., $\{n * l(p)\}_V$ and $L(p)_V$.

L(p) per definition corresponds to a set of parallel length elements and we obtain:

$$Q_A \text{ capillaries} = L(p)_V \text{ capillaries.} \quad (3)$$

From $L_V = c_1(K_L\ \alpha = 0) * Q_A(\alpha = 0)$ capillaries, we derive

$$L_V \text{ capillaries} = c_1(K_L\ \alpha = 0) * L(p)_V \text{ capillaries.} \quad (4)$$

Consider now the hypertrophic case: the hypertrophy process may expand the unit tissue volume by the constant factor of hypertrophy H. Then we obtain for the expanded unit volume $V(H) = V * H$. Unit cross-sectional area in hypertrophy will obviously be increased to $A(H) = A * H^{2/3}$ and face length of the unit volume cube to $FL(H) = FL * H^{1/3}$.

If we assume constant volume and area fraction, as well as number of myofibers in the hypertrophic case (which was approximately the case in our experiments), total volume and mean volume of myofibers will increase H-fold. Since enlargement of myofibers can be realized by both MCSA increase and length increase, we introduce the variable (x), depending on H in order to describe the length and MCSA changes: length increase be H^x-fold and MCSA increase be H^{1-x}-fold (if growth of myocytes is harmonic x = 1/3).

This model leads to the following equations in the hypertrophic case (H):

$$Q(H)/A(H) \text{ myofibers} = A_A \text{ myofibers}/(\text{MCSA} * H^{1-x})$$

$$Q(H)/A(H) \text{ myofibers} = Q_A \text{ myofibers} * H^{x-1} \quad (5)$$

$$L(H)/V(H) \text{ myofibers} = (L * H^x/(V * H)) \text{ myofibers}$$

$$L(H)/V(H) \text{ myofibers} = L_V \text{ myofibers} * H^{x-1} \quad (6)$$

Since it cannot be presupposed that the myofiber length increase is paralleled by a proportionate increase of the projected capillary length L(p), neither can it be assumed that the spatial distribution of capillary axes will be constant in hypertrophy, we introduce the variables (y) and (z) in order to describe the capillary growth: L(p) increase be H^y-fold and $c_1(K_L\ \alpha = 0)$ increase be H^z-fold, which leads to the following equations:

$$L(H)/V(H) \text{ capillaries} = L \text{ cap.} * H^{y+z}/(V * H)$$

$$L(H)/V(H) \text{ capillaries} = L_V \text{ capillaries} * H^{y+z-1}$$

$$L(H)/V(H) \text{ capillaries} = L(p)_V * c_1(K_L\ \alpha = 0) \text{ capillaries} * H^{y+z-1} \quad (7)$$

$$Q(H)/A(H) \text{ capillaries} = L(p) \text{ cap.} * H^y/(V * H)$$

$$Q(H)/A(H) \text{ capillaries} = L(p)_V \text{ cap.} * H^{y-1} \quad (8)$$

From (5), (6), (7), (8):
2D CFR = Q_A capillaries/Q_A myofibers

2D CFR (H) = Q(H)/A(H) capillaries/Q(H)/A(H) myofibers

2D CFR (H) = (L(p) capillaries/L myofibers) $* H^{y-x}$ (9)

3D CFR = L_V capillaries/L_V myofibers

3D CFR (H) = L(H)/V(H) capillaries/L(H)/V(H) myofibers

3D CFR (H) = (L(p) cap. $* c_1$ (K_L $\alpha = 0$)/L myof.) $* H^{y+z-x}$ (10)

Note that 3D CFR(H)/2D CFR(H) = $c_1(K_L \alpha = 0)) * H^z$.

Consider now the relations between the capillary growth patterns in hypertrophy and the CFR's:

1) 2D CFR and 3D CFR are not changed. Thus $y-x = 0$, $y+z-x = 0$, and $z = 0$. Capillary length increase (y+z) is proportionate to myofiber length increase (x), and the degree of capillary anisotropy is not changed ($z = 0$). This growth pattern is designed as *elongation*.

It is evident that an H^x-fold capillary length increase can be obtained either by *length increase of capillary segments* ($l(p) * H^x$) or by *addition of new segments in series-connection* ($n * H^x$). However, if myofiber length extension is absent ($x = 0$) capillary growth does not occur ($y = 0$).

2) 2D CFR is not changed and 3D CFR is increased. Thus $y-x = 0$ and $z > 0$. In addition to the process of elongation, as described above, an additional length increase of capillary segments occurs and leads to an increased tortuosity *(meandering)* of the capillary network which is associated with an increased c_1 value ($z > 0$). It should be emphasized that meandering of capillaries does not change $l(p) * n$ when compared with the elongation pattern $l(p) * n * H^x$}.

3) 2D CFR and 3D CFR are increased after myocyte enlargement. Thus $y > x$, $y+z > x$, whereby $z \geq 0$. In addition to the process of elongation, we obtain further increases in L(p). Length increase of individual segments that is associated with an increase of the mean *projected* segment length {$l(p) * H^y$; $y > x$} would necessarily cause a complete remodeling of capillary connections. Though this reaction pattern cannot be ruled out mathematically, it seems to be biologically meaningless. Alternatively, *neoformation of capillary segments in parallel connection* ($n * H^y$; $y > x$) can easily be realized by capillary sprouting or by division of individual branches.

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