Cardiac function, microvascular structure, and capillary hematocrit in hearts of polycythemic rats

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Rakusan, Karel, N. Cicutti, and F. Kolar. Cardiac function, microvascular structure, and capillary hematocrit in hearts of polycythemic rats. Am J Physiol Heart Circ Physiol 281: H2425–H2431, 2001.—The effect of polycythemia on the coronary microcirculation was studied in young male rats. Two experimental models of polycythemia were employed: cobalt-induced polycythemia, which mimics hypoxia-induced changes, and erythropoietin-induced polycythemia, which circumvents these changes. In both models, baseline left ventricular function was normal, whereas maximal systolic and developed pressures were decreased. In cobalt-treated rats the left ventricular functional reserve was also compromised. Morphometric analysis of the left ventricle confirmed previously described improved geometric conditions for oxygen supply at the distal portions of capillaries (smaller domain areas and shorter capillary segments). In cobalt-treated but not in erythropoietin-treated rats, increased capillary angiogenesis was also detected. In the hearts from rats with both types of polycythemia, a small but significant increase in the formation of arteries was found. Capillary linear hematocrit was within the normal range in both types of polycythemia despite sizeable increases in systemic hematocrit. Significant differences in red blood cell distribution within capillaries were found between proximal and distal portions in all experimental groups.

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RECENTLY, WE STUDIED the effect of anemia on the coronary microcirculation in young male rats (23). We hypothesized that chronic anemia would lead to coronary angiogenesis and microcirculatory restructuring as a response to hypoxia and/or increased coronary blood flow, both being recognized as powerful angiogenic stimuli (14, 21). We found evidence of angiogenesis in cardiomegaly induced by chronic anemia, which was accompanied by advantageous regulation of red blood cell (RBC) spacing within coronary capillaries. This resulted in linear capillary hematocrit higher than would be expected on the basis of systemic hematocrit values. The purpose of the present series of experiments was to investigate microcirculatory adjustments associated with changes at the opposite values of the hematocrit scale, i.e., in polycythemia.

Two different models of polycythemia were used. Cobalt-induced polycythemia is characterized by pathophysiological processes similar to hypoxic polycythemia, due to the specific effect of cobalt on oxygen sensors. Apparently, the divalent cation Co2+ displaces ferrous iron in tissue hemoporphyrin, or it changes iron’s redox state in the same fashion as in hypoxia. The final outcome of this process is the formation of hypoxia-inducible gene products such as vascular endothelial growth factor (VEGF), erythropoietin (EPO), and various glycolytic enzymes (5, 7, 9). On the other hand, polycythemia induced by the application of EPO circumvents this mechanism by acting directly on the erythropoietic mechanism, avoiding the remaining “hypoxia-induced” changes. Our hypothesis was that in both cases angiogenesis would be present, although the contribution of various stimuli would differ. Angiogenesis in both situations would result from increased mechanical stimuli due to increased RBC-capillary wall interactions. In the case of cobalt-induced polycythemia, the additional hypoxic angiogenic stimulus may be postulated. Application of EPO may be angiogenic as well: hematopoietic factors seem to act as angiogenic factors and vice versa (2).

Our previous experiments revealed a tendency to maintain coronary capillary hematocrit within normal limits: it does not change during the cardiac cycle in normal rats (26), and it decreases much less than large-vessel hematocrit in anemic rats (23). In the present series of experiments, we investigated whether the same tendency exists vis-à-vis an increased blood hematocrit, i.e., systemic polycythemia.

Two models of chronic polycythemia, one of the hypoxic type and one due to a direct increase in erythropoiesis from EPO application, were used to study their effect on cardiac function, which was followed by the morphometric analysis of coronary microvascular structure. Finally, RBC spacing in the coronary capillaries and capillary hematocrit was determined in hearts from rats with chronic polycythemia due to either co-
dP/dt pressure (LVDP), and the maximal rates of pressure development were derived using our computer program: left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure, left ventricular developed pressure (LVDP), and the maximal rates of pressure development (+dP/dt_max) and fall (−dP/dt_max). In addition, the time constant of relaxation (τ) was calculated on the basis of an exponential model of isovolumetric pressure decay as the time required for the pressure at −dP/dt_max to be reduced by 1/e (17). Heart rate was calculated from the pressure signal.

After recording baseline parameters, the maximal isovolumetric performance of the left ventricle was assessed in open-chest animals on the basis of a previously described method (3). Tracheostomy was performed, and the animals were connected to a Harvard rodent ventilation pump (70 strokes/min). The sternum was opened by a lateral incision above the base of the heart, and a ligature was placed on the ascending aorta. Acute clamping of the aorta served as a loading test for ventricular function. The pressure signal was recorded during the 10 s after the clamp and stored on computer. The LVSP, LVDP, and +dP/dt_max were derived, and their means were calculated from regular beats per each second after the clamp. This approach significantly reduced the influence of rhythm disturbances induced by the clamp. The highest mean values of these parameters were considered to represent maximal isovolumetric performance of the ventricle. The difference between the values after and before the aortic clamp was considered as the functional reserve.

**Morphometric Analysis**

Samples of frozen myocardial tissue were used for morphometric studies, studies of RBC spacing within the capillaries, and determination of capillary hematocrit. In each experimental group, 10 animals were used.

Frozen sections provided us with an opportunity to use differential staining, which allows us to distinguish between the proximal (arteriolar) and distal (venular) portions of the capillary bed. The staining protocol has been described elsewhere (22). Briefly, tissue sections (16 μm) were incubated in a solution sensitive to dipeptidyl peptidase IV, which stained the distal portions of the capillaries red. Next, the sections were transferred to a solution sensitive to alkaline phosphatase, which stained the proximal portions of capillaries blue. This approach was used for determination of the capillary domain, an index of the heterogeneity of capillary spacing (SD log) and capillary segment length. A capillary domain is defined as the tissue cross-sectional area that is closer to a given capillary than to any other. It was derived in the following fashion: first, the positions of the center of all capillaries in the field were recorded by an image analyzer as pairs of coordinates. Then perpendicular lines were drawn in the middle between the capillary whose domain was being calculated and all neighboring capillaries. The lines intersect and form a polygon, the actual capillary domain. Measurements were done in each heart on ~600 capillaries in cross sections from subendocardial portions of the left ventricular free wall. Distribution of capillary domain areas was log-normal. Therefore, the SD of this log-normal distribution (SD log) was used as an index of the heterogeneity of capillary spacing. The higher the index is, the more variable is the capillary spacing, and vice versa. Longitudinally cut midsections were used for measuring capillary segment length, defined as the distance between two consecutive branching points (400–600 measurements per heart were done). Finally, the capillary supply unit was calculated; the capillary supply unit is the smallest tissue supply volume that can be modeled in three dimensions. It is defined as the product of the average capillary domain area and average capillary segment length. The use of our double-staining method enabled us to distinguish between the capillary supply units derived from proximal and distal portions of the capillaries.

The same sections were also used for determination of the fraction of immature arterioles, using the double-immunolabeling method, based on expression of vascular smooth muscle cell (SMC)-related proteins. The immunohistochemical protocol described by Price and colleagues (19) was used to detect SMC α-actin, which is expressed from the earliest stages of SMC differentiation, and SMC myosin heavy chains (SM-1 and SM-2 MHCs), which are expressed solely in mature, fully differentiated SMCs. The same approach was also used in our recent study of postnatal development of coronary arterioles, which also contains typical micrographs (11). Briefly, tissue sections were washed in PBS and incubated with a hybridoma cell supernatant containing antibodies reactive to SM-1/SM-2 MHCs (antibodies kindly donated by Dr. Gary Owens). Lissamine rhodamine sulfonyl chloride (LRSC)-labeled goat anti-mouse IgG (H + L) Fab fragments (Jackson ImmunoResearch Laboratories,) were applied to sections. After washing, FITC-labeled SMC α-actin monoclonal antibodies (clone 1A4; Sigma) were added to sections. Coverslips were placed on slides using glycerol. For each heart, the entire tissue section (1–2 sections/heart) was scanned using a Zeiss fluorescence microscope with FITC and LRSC filters. All vessels within a tissue section exhibiting

**METHODS**

**Experimental Design**

Two experimental models of polycythemia were used in two series of experiments on rats: the first series was used for evaluation of left ventricular function, and the second was used for morphometric analysis of myocardial tissue.

**Cobalt-induced polycythemia.** Cobalt-induced polycythemia was initiated in young Sprague-Dawley male rats, with initial body mass between 110 and 125 g. We used a protocol that we have applied previously (24): rats were injected intraperitoneally, three times per week for 5 wk, with an aqueous solution of cobalt chloride (7.5 mg CoCl₂/100 g), while control rats received saline injections.

**EPO-induced polycythemia.** As above, animals were also used in producing experimental polycythemia by subcutaneous injection of recombinant human EPO (kindly supplied by Jansson Ortho, Toronto, Canada), while control rats received aqueous solution of cobalt chloride (7.5 mg CoCl₂/100 g), while control rats received saline injections. In this case, 200 units of EPO diluted in saline were injected subcutaneously per animal three times per week for 4 wk.

**Evaluation of Left Ventricular Function**

In this series of experiments, one control group was used for comparison with two types of experimental polycythemia. Blood pressure in the left ventricle of anesthetized rats (pentobarbital sodium, 50 mg/kg ip) was measured using a Millar catheter-tipped transducer connected to a Grass polygraph and a personal computer. The catheter was inserted into the left ventricular cavity via the right carotid artery under continuous pressure monitoring. Baseline measurements were recorded after a stabilization period of 10 min; the mean from three consecutive recordings was calculated.

The analog pressure signal was digitized with a sampling frequency of 1 kHz and stored on computer for later processing. The following parameters were derived using our computer program: left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure, left ventricular developed pressure (LVDP), and the maximal rates of pressure development (+dP/dt_max) and fall (−dP/dt_max). In addition, the time constant of relaxation (τ) was calculated on the basis of an exponential model of isovolumetric pressure decay as the time required for the pressure at −dP/dt_max to be reduced by 1/e (17). Heart rate was calculated from the pressure signal.

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SMC α-actin immunoreactivity were counted; thus for each heart approximately 500–700 arterioles were analyzed. Of these the proportion of arterioles not exhibiting SM-1/SM-2 MHC immunoreactivity (new, immature arterioles) was determined; those arterioles expressing both SMC α-actin and SM-1/SM-2 MHC immunoreactivity were considered fully developed, mature arterioles. The proportion of new, immature arterioles determined for each heart was used to calculate the overall average percentage of new, immature arterioles.

Finally, 16-μm longitudinal sections were used for the determination of RBC spacing and capillary hematocrit, as described in detail previously (23, 26). In this case, sections were incubated for 40 min in Papanicolaou OG-6, which stains RBCs orange, and double staining of capillaries, as described above, followed. Image Analyzer (Bioquant Meg V) was used for the measurements of linear capillary hematocrit and RBC spacing in proximal and distal portions of the coronary microvascular bed using longitudinally cut midsections of the anterior left ventricular wall. Linear capillary hematocrit was defined as the aggregate RBC length per total capillary bed. Total capillary length, of course, included also capillaries perfused by plasma only. In capillary segments with two or more RBCs, the edge-to-edge distance between two adjacent RBCs was measured. Mean RBC spacing values were determined from 400–500 individual measurements. A RBC spacing value was not measured if capillary branching was present between two RBCs or if a RBC was located within a branching point. Two extreme RBC spacing patterns were also recorded: the frequency of occurrence of RBC spacing values equal to 0 μm, representing “touching” RBCs, and RBC spacing values exceeding 40 μm, representing extremely large values.

Statistics

Results are expressed as means ± SE. They were evaluated by a simple unpaired t-test or by one-way ANOVA with subsequent Bonferroni post hoc tests when applicable (more than 2 comparisons).

RESULTS

Injections of either EPO or cobalt were successful in inducing polycythemia, characterized by significant (P < 0.001) increases in both hematocrit and hemoglobin levels compared with control animals (+51 and 44%, respectively, in EPO-treated rats and +32 and 28%, respectively, in rats injected with cobalt; see Table 1).

Functional data are summarized in Table 2 and Fig. 1. Baseline left ventricular hemodynamic parameters in both types of experimental polycythemia did not differ from those in control animals. On the other hand, maximal left ventricular function after aortic clamping was characterized by small but significant decreases in systolic pressure and developed pressure in both models of polycythemia. In the case of cobalt-induced polycythemia, a decrease in functional reserve was also observed (see Fig. 1). Maximal rates of pressure development were similar in all three experimental groups.

Injections of EPO did not influence the body mass of rats in the experimental group, while cobalt treatment resulted in significantly lower values of body mass (see Table 1). In the Table 1, cardiac mass measurements are also included. Once again, treatment of rats with EPO did not influence any of these parameters, whereas in cobalt-treated animals the left ventricular mass was significantly lower because of lower body mass. Right ventricular mass was the same in both control and experimental groups. Thus relative mass of the right ventricle was significantly (P < 0.001) increased in cobalt-induced polycythemia.

Results from capillary morphometry based on analysis of frozen tissue samples are summarized in Table 3 and Fig. 2. In this case, our double-staining method allowed us to distinguish between proximal (arteriolar) and distal (venular) portions of capillary bed. We confirmed previously reported differences between proximal and distal portions: proximal capillaries supplied a larger tissue area on cross section (capillary domain), they had longer capillary segment length, and therefore the capillary supply unit (domain × segment length) was increased.

### Table 1. Basic data

<table>
<thead>
<tr>
<th>Erythropoietin</th>
<th>Body Mass, g</th>
<th>Cardiac Mass, mg</th>
<th>Cardiac Mass, mg</th>
<th>Cardiac Mass, mg</th>
<th>Hematocrit, %</th>
<th>Hemoglobin, g/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Both ventricles</td>
<td>Right ventricle</td>
<td>Left ventricle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>312 ± 6</td>
<td>836 ± 17</td>
<td>188 ± 4</td>
<td>648 ± 15</td>
<td>44.2 ± 0.4</td>
<td>13.2 ± 0.2</td>
</tr>
<tr>
<td>Experimental</td>
<td>310 ± 5</td>
<td>831 ± 15</td>
<td>176 ± 5</td>
<td>655 ± 12</td>
<td>66.9 ± 1.0‡</td>
<td>19.0 ± 0.2‡</td>
</tr>
<tr>
<td>Cobalt Controls</td>
<td>332 ± 7</td>
<td>874 ± 30</td>
<td>194 ± 7</td>
<td>680 ± 24</td>
<td>45.6 ± 0.4</td>
<td>13.8 ± 0.3</td>
</tr>
<tr>
<td>Experimental</td>
<td>263 ± 9‡</td>
<td>785 ± 14*</td>
<td>195 ± 8</td>
<td>590 ± 9†</td>
<td>60.3 ± 1.0‡</td>
<td>17.7 ± 0.3‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. Significantly different from control values: *P < 0.02, †P < 0.01, and ‡P < 0.001.

### Table 2. Baseline left ventricular function

<table>
<thead>
<tr>
<th></th>
<th>Polythemia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>n</td>
<td>13</td>
</tr>
<tr>
<td>Systolic pressure, mmHg</td>
<td>137 ± 3</td>
</tr>
<tr>
<td>End-diastolic pressure, mmHg</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>Developed pressure, mmHg</td>
<td>133 ± 3</td>
</tr>
<tr>
<td>+dP/dt max, mmHg/s</td>
<td>8,018 ± 230</td>
</tr>
<tr>
<td>-dP/dt max, mmHg/s</td>
<td>6,633 ± 262</td>
</tr>
<tr>
<td>Constant τ, ms</td>
<td>8.9 ± 0.3</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>372 ± 12</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of rats. +dP/dt max, maximal rate of pressure development; −dP/dt max, maximal rate of pressure fall; τ, time constant of relaxation.
length) was significantly larger in proximal capillaries ($P < 0.001$). In addition, hearts from rats with cobalt-induced polycythemia had, compared with controls, lower values of domain area, segment length, and capillary supply unit. This reached statistical significance ($P < 0.01$) in the case of domain area in the proximal portion of the capillary bed, in the case of segment length in the distal portion, and in capillary supply units from both portions. We found no differences in heterogeneity of capillary spacing ($SD \log$) between proximal and distal segments and between experimental and control groups.

Analysis of the formation of new arterioles disclosed a small but significant increase in the fraction of immature arterioles in the hearts from both types of experimental polycythemia. This fraction increased from $0.36 \pm 0.10$ to $2.53 \pm 0.47\%$ in the heart from rats with EPO-induced polycythemia ($P < 0.003$) and from $0.19 \pm 0.08$ to $1.56 \pm 0.11\%$ in the hearts from rats with cobalt-induced polycythemia ($P < 0.001$) (see Fig. 3). This finding was accompanied by a moderate but significant increase in arteriolar numerical density in the hearts from rats with cobalt-induced polycythemia ($4.2 \pm 0.2$ vs. $4.9 \pm 0.2$, $P < 0.05$), whereas in EPO-induced polycythemia it was essentially the same as in the control group ($4.9 \pm 0.4$ vs. $4.8 \pm 0.3$).

Finally, our results on RBCs within the coronary microvascular bed are summarized in Figs. 4 and 5. Increases in the systemic hematocrit were not followed by similar changes at the microvascular level. Linear capillary hematocrit was similar in both control and experimental groups: $25.9$ and $27.0\%$ in proximal and distal capillaries of EPO controls vs. $25.3$ and $27.2\%$ in EPO experimental animals; in cobalt-treated rats it was $27.7$ and $28.9\%$ vs. $28.0$ and $30.3\%$. Therefore, the capillary-to-systemic hematocrit ratio was significantly lower ($P < 0.001$) in both proximal and distal capillaries from hearts of rats with both types of experimental polycythemia (see Fig. 4). This was accompanied by basically the same values of RBC spacing, the same percentage of RBCs with spacing of $0 \mu m$ (touching RBCs), and the same percentage of RBCs with spacing $> 40 \mu m$ in hearts from normal and polycythemic rats. In these parameters, however, we noticed significant differences between proximal and distal portions of capillaries present in all experimental

Table 3. Frozen tissue: capillary morphometry

<table>
<thead>
<tr>
<th></th>
<th>Domain Area, $\mu m^2$</th>
<th>SD log $\times 1,000$</th>
<th>Capillary Segment Length, $\mu m$</th>
<th>Capillary Supply Unit, $10^3 \mu m^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proximal</td>
<td>Distal</td>
<td>Proximal</td>
<td>Distal</td>
</tr>
<tr>
<td><strong>Erythropoetin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>395 ± 17</td>
<td>365 ± 15</td>
<td>62 ± 3</td>
<td>59 ± 2</td>
</tr>
<tr>
<td>Experimental</td>
<td>424 ± 20</td>
<td>392 ± 16</td>
<td>61 ± 1</td>
<td>60 ± 2</td>
</tr>
<tr>
<td><strong>Cobalt</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>376 ± 12</td>
<td>317 ± 11</td>
<td>60 ± 2</td>
<td>57 ± 2</td>
</tr>
<tr>
<td>Experimental</td>
<td>323 ± 9*</td>
<td>290 ± 13</td>
<td>61 ± 3</td>
<td>61 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE. Significant difference, control vs. experimental: *$P < 0.01$. Significant difference, arteriolar (proximal) vs. venular (distal): †$P < 0.01$ and ‡$P < 0.001$. 

Fig. 1. Left ventricular maximal systolic (A) and developed pressure (B), the maximal rate of pressure development ($+dP/dt_{max}$; C), and their respective reserves in control (normal) animals ($n = 11$) and in rats with polycythemia induced by either cobalt ($n = 10$) or by erythropoietin (EPO) ($n = 8$). Reserve means the difference between values after and before the aortic clamp. Values are means ± SE.
groups. Proximal portions were characterized by larger distances between RBCs (RBC spacing), a lower fraction of touching RBCs (RBC spacing $<0.01\, \mu m$), and a higher fraction of distant RBCs (RBC spacing $>40\, \mu m$) than the same parameters in the distal portions of the capillary bed (Fig. 5).

**DISCUSSION**

Both experimental models were successful in producing significant increases in hematocrit and hemoglobin concentration in blood, i.e., polycythemia. The increase in these two parameters was more pronounced in the case of EPO-treated rats compared with increases found in cobalt-treated animals (see Table 1). At the same time, the dose of cobalt utilized was relatively high, as documented by diminished body growth of cobalt-treated rats. On the other hand, lower dosage of chronic oral administration of cobalt as described in a recent publication (6) resulted in only marginal increase of hematocrit: 13% increase compared with 32% in our cobalt-treated animals and 51% in our EPO-treated rats.

**Functional Studies**

In both models of polycythemia, baseline heart rate and contractile parameters of the left ventricle did not differ from the respective values in controls. Maximum values of systolic and developed pressure achieved after acute pressure load were slightly but significantly decreased in both EPO- and cobalt-treated animals, whereas the functional reserve was compromised in the latter group only. It seems conceivable that increased viscosity of the blood due to elevated hematocrit may have contributed to these changes. Decreased functional reserve was also observed in other experimental models of severe polycythemia, such as that resulting from chronic exposures to high-altitude hypoxia (1) or to carbon monoxide (18). We are not aware of any study indicating that EPO can negatively influence cardiac contractile function. On the contrary, numerous reports described toxic effects of cobalt, resulting in cardiomyopathy after long-term exposure to excessive amounts of this trace element (reviewed in Ref. 25). Cobalt appears to accumulate mainly in the mitochondria and sarcoplasmic reticulum of cardiac myocytes (4). The precise mechanism of its adverse influence on myocardial function is unclear; it may involve the inhibition of mitochondrial respiration, altered calcium handling, or reduced capacity of scavengers of reactive oxygen species (4, 10, 25). Thus it cannot be excluded that the lower cardiac functional reserve in cobalt-treated rats compared with that of control and EPO-treated animals in our study is, at least partially, due to direct myocardial effects of this element.
Oxygen supply to tissue is influenced not only by the density of capillaries but also by the number of RBCs within these capillaries, i.e., capillary hematocrit and RBC spacing. Several theoretical studies (8, 12, 13) have shown that as the distance between the RBCs increases, the capillary ceases to be a continuous source of oxygen. Thus oxygen flux from plasma gaps between the RBCs becomes negligible at a certain distance from the erythrocyte. In our previous study (23), we found a smaller decrease in the RBC distribution within the coronary capillary bed than the decrease in the systemic hematocrit, associated with acute and chronic anemia. This reaction may be considered as an adaptive response to acute or chronic hemodilution. According to Federspiel and Popel (8), the fraction of total oxygen transport resistance that

Morphometric Studies: Angiogenesis

Results from capillary morphometry confirmed our previous finding of significant differences between capillaries located within proximal or distal portions of the capillary bed, i.e., close to or far from their feeding arterioles (22). Distal portions have a shorter capillary segment length and smaller capillary domain areas, and therefore the volume of tissue supplied by the distal segment (capillary supply unit) is significantly smaller than the capillary supply units on the proximal side ($P < 0.001$). Such modifications in microvascular design theoretically provide improved geometric conditions for oxygen supply on the venous side of the capillary network, where $P_{O_2}$ values are lower. Furthermore, these results would be consistent with the view that the local architecture of the capillary net adjusts to local oxygen supply conditions.

Comparison of control and EPO-treated animals did not reveal any differences in the quantitative morphology of coronary capillaries. On the other hand, capillary supply units were significantly smaller in cobalt-treated hearts than in control rats. These significant differences ($P < 0.01$) were found in both proximal and distal portions of the capillary bed. Our finding may be interpreted as indirect evidence for the formation of new capillary material (angiogenesis) in this experimental situation. In the introduction, we discussed the role of cobalt in mimicking a hypoxic stimulus. VEGF has been demonstrated in vivo and in vitro to be the principal mediator of hypoxia-induced angiogenesis. We may speculate that the mechanism of capillary angiogenesis in our experimental situation follows the same pathway. Ladoux and Frelin (15) reported that cobalt stimulates the expression of VEGF mRNA in rat cardiomyocytes. Moreover, Endoh and co-workers (6) found an in vivo induction of VEGF mRNA in cardiac tissue of rats chronically treated with low concentration of cobalt. Hearts of these rats exhibited improved contractile functions in hypoxia-reoxygenation as a sign of increased hypoxic tolerance.

Both experimental situations seem to be associated with a significant increase in the percentage of immature coronary arterioles: ~7-fold in the case of EPO-induced polycythemia ($P < 0.003$) and 8-fold in cobalt-induced polycythemia ($P < 0.001$). The overall percentage, however, remained relatively low in both models of polycythemia (2.53 and 1.56%, respectively). It seems that in the case of EPO-polycythemia, the effect results only in higher turnover of arteriolar structures, with the arteriolar density unchanged. In the hearts from chronic cobalt-induced polycythemia, a small (17%) but significant ($P < 0.05$) increase in arteriolar numerical density was found.

In the introduction we postulated potential angiogenesis in both experimental models of polycythemia. In the case of EPO-induced polycythemia, morphometric characteristics of both coronary arterioles and capillaries were similar to those in the control group. Possible angiogenesis, as reflected by a higher percentage of immature arterioles, is probably accompanied by an involution of these vessels to a similar degree, resulting in unchanged microvascular structure. On the other hand, in cobalt-induced polycythemia a small but significant increase in arteriolar and capillary supply was found. Thus a hypoxic stimulus, which was apparently present in cobalt-induced polycythemia, was, in this experimental situation, more effective than the mechanical stimuli present in both models of polycythemia.

Capillary Hematocrit and RBC Spacing

In the proximal and distal segments of coronary capillaries in control (normal) animals ($n = 10$) and in rats with polycythemia induced either by cobalt ($n = 8$) or by EPO ($n = 10$). Values are means ± SE.
resides inside the capillary is influenced significantly by the discreet nature of blood and can account for 30–70% of the total resistance to oxygen transport from blood to tissue.

Results from the present study seem to indicate that capillary hematocrit and RBC spacing are preserved within a certain optimal range even in the case of systemic polycythemia. This is in agreement with predictions of Vicaut and coworkers (27), that changes in RBC flow in individual capillaries are not parallel to changes in entering RBC flow. Increased capillary hematocrit results in increased viscosity and increased chances for rouleaux formation and possible plugging of these vessels. Therefore, a potential increase in the capillary oxygen content due to higher hematocrit may be counterbalanced by decreases in the capillary blood flow. At the same time, capillary blood flow and oxygen content would influence the tissue Po2 to the same degree (20).

In addition, we have found in all groups significant differences between proximal and distal portions of capillaries in the distribution of RBCs within the capillary bed. The distances between the RBC (RBC spacing) were larger in the proximal segments, the percentage of RBCs touching was lower, and the percentage of RBC spacing larger than 40 μm was higher. This is in agreement with our previous results based on RBC spacing in rat coronary capillaries during the cardiac cycle (26). Such an arrangement favors oxygen transfer in the distal portions of the capillary bed by reducing the intracapillary resistance to oxygen transport. Smaller distances between RBCs, together with shorter intercapillary distances in distal capillaries, would improve oxygen supply to tissue in the regions of distal capillaries where the capillary blood Po2 is lower.

Conclusion

Only rats with cobalt-induced polycythemia had an increased coronary capillary and arteriolar supply. Chronic polycythemia induced by EPO injections was characterized by even larger increases in hematocrit and hemoglobin concentration, but the coronary microvascular bed remained unchanged. Therefore, a hypoxic stimulus seemed to be more effective in inducing angiogenesis than mechanical stimuli. Finally, capillary hematocrit was considerably lower than the systemic hematocrit in both types of polycythemia.

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REFERENCES