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Inhibition of Crossbridge Function in the Normal Human Heart by Hypoxic Endothelial Superfusate

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Endothelial cells release diffusible substances which modulate myocardial function. Oxygen pressure is one important factor for stimulation and modulation of endothelial function. Here we investigated the effects of a superfusate obtained from hypoxic (pO_2 40–50 mmHg) porcine endothelial cell culture on human myocardial crossbridge cycling rate. Isometric force development and the rate constant for tension development of demembranated multicellular fibers from the left myocardium of a normal human heart were determined from the low-tension rigor by photolytic release of ATP from caged-ATP. Incubation with hypoxic or normoxic superfusates did not change maximal isometric force development. However, rate constant of tension development of the normal human heart fibers significantly decreased to 43.3% upon incubation with the hypoxic but not normoxic endothelial cell superfusate. © 1999 Academic Press

Key Words: **endothelium; human heart; cross-bridge kinetics; caged-ATP.**

Endothelial cells modulate vascular tone and blood flow by the release of contracting and relaxing factors (1–3). Furthermore, endocardial endothelium (4) and coronary vascular endothelium (5) release substances that induce positive or negative inotropic effects on the myocardium. Substance P and bradykinin enhance left ventricular relaxation, attributed to the paracrine release of nitric oxide in isolated ejecting guinea pig hearts (6). Bicoronary infusion of substance P modulates left ventricular function in humans, probably through the release of cardioactive agents from the coronary endothelium (7). The contractile performance may also be influenced by nitric oxide production within cardiac myocytes themselve (8).

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Oxygen pressure is one of the most important factors for stimulation and modulation of endothelial cell function. Thus, cultured endothelial cells superfused with hypoxic physilogical buffer released an unidentified substance(s) that inhibited myocardial function (9). The activity of the (reoxygenated) hypoxic superfusate was present in low molecular weight fractions ($M_r <$ 500) and remained after heating (95°C). Hypoxic superfusate induced rapid, potent, and reversible inhibition of isolated cardiac myocyte contraction without reducing the cytosolic Ca^{2+}- transient. Furthermore, it reduced unloaded shortening velocity of skinned fibers from human skeletal muscle and depressed in vitro actin motility and the rate of actin-activated myosin ATPase activity (9).

Here we characterized cross-bridge cycling kinetics of normal human heart fibers and investigated the effects of a superfusate from hypoxic endothelial cell culture on human myocardial crossbridge cycling rate. The rate constant for tension development of demembranated multicellular fibers from the left myocardium of a normal human heart were determined from the low-tension rigor by photolytic release of ATP from caged-ATP (10).

Here we describe for the first time that myosin crossbridges of normal human myocardial fibers revealed decreased rate constants for tension development after incubation with the superfusate of hypoxic cultured endothelial cells.

MATERIALS AND METHODS

Patient population. Normal left ventricle derived from a donor heart (NH) which could not be transplanted due to technical reasons. Tissue was supplied by Prof. E. Erdmann from the III. Medical Clinic of the University Cologne, Germany.

Tissue preparation. The mechanical experiments were performed with chemically demembranated multicellular heart fibers (skinned fibers). The muscle tissue was excised and dissected into small fiber bundles (about 1 mm thick, 5–10 mm length) and demembranated by

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incubation in a solution containing 20 mM imidazole, 5 mM ATP, 5 mM $MgCl_2$, 4 mM EGTA, 2 mM DTE, 10 mM NaN_3, 50% glycerol, 1% Triton X-100, pH 7 at 4°C for 18-20 hours. Subsequently, the fibers were transfered into the same solution without Triton X-100 and stored at −20°C not longer than 4 weeks.

Mechanical analysis. For the mechanical analysis, fibers were dissected into bundeles of 150–250 μm diameters and 4–5 mm length under a preparation microscope. Fiber bundeles were mounted horizontally between two micro clamps in a 10 μl quartz cuvette with relaxation solution (20 mM imidazole, 10 mM ATP, 10 mM creatinphosphate, 380 U/ml creatine kinase, 5 mM NaN_3, 5 mM EGTA, 12.5 mM $MgCl_2$, 1 mM DTT, pH 7). One of the clamps was connected with a force transducer, the other one with a micrometer screw (Scientific Instruments Heidelberg, Germany). Length was adjusted by the micrometer screw such that resting tension was threshold. Sarcomere length at resting tension was always between 1.95 and 2.0 μm as detected by laser diffraction. Subsequently fibers were incubated in Ca^{2+}-free rigor solution (20 mM imidazole, 5 mM NaN_3, 5 mM EGTA, 12.5 mM $mgCl_2$, 1 mM DTT, 100 mM BDM) for 30 min and a "low-tension rigor" developed. The fibers were then transferred in Ca^{2+}-containing (pCa 4.5) rigor solution (same as rigor solution except that EGTA was substituted by 5 mM Ca^{2+}-EGTA and without BDM) for 5 min and then in Ca^{2+}-rigor solution plus 2.5 mM caged ATP (Calbiochem, San Diego, CA, USA) and 10 mM DTT for 3 min.

Photolysis of caged ATP was achieved using a xenon flash lamp system (Dr. Rapp Optoelektronik, Hamburg, Germany). The lamp deliverd UV ligth pulses with a duration of about 1 ms and was focused through a UG-11 filter. The signals of force development were displayed on a storage digital oscilloscope (HM 408, HAMEG Instr., Frankfurt/M, Germany) and analyzed with an IBM compatible PC. After registration the fiber was incubated in relaxing solution. Temperature was 21°C.

To investigate the influence of the endothelial superfusates the procedure was exactly the same as describted, except that 10 μl of a 1:10 diluted hypoxic endothelial superfusate or 10 μl normoxic endothelial superfusate (diluted 1:10) were present in the Ca^{2+}-rigor and the caged ATP-Ca^{2+}-rigor solution.

Hypoxic endothelial cell superfusates. Endothelial cells cultured from porcine aorta were superfused for 1–5 hours with hypoxic (PO_2 40–50 mmHg) or normoxic ($PO_2 \approx 160$ mmHg) physiological buffer solution as recently described by Shah *et al.* (9). Single-pass superfusates were passed through a 500 Da molecular weight cutoff filter (Centricon, Millipore Corp., Massachuetts, USA) and re-equilibrated for pO_2, pH and ionic composition prior to testing.

Statistical analysis. Half times of tension development ($t_{1/2}$) were determined from the recorded force transient with "Proscope"-software (HAMEG Instr., Frankfurt/M., Germany). The rate constants for tension development (k_{td}) were calculated using the formula: $t_{1/2} = \ln 2/k_{td}$ assuming a monoexponential tension rise (10). The statistical analysis was performed using commercially available statistical programs (Epistat) on an IBM compatible PC. Values are expressed as means ± SEM; n = number of fibers investigated. Significance analysis was performed using the Student's t-test for unpaired values.

RESULTS

Figure 1 shows an original record of a representative experiment (NH-fiber) registrated by y/t-recorder. Rigor solution incubated fibers developed "low-tension rigor" after average 23 min. Rigor force being 8.6 ± 1.2% of the tension obtained upon photolytical release of ATP from caged-ATP at maximal Ca^{2+}-activation. Fast tension development was elicited upon a light flash releasing ATP from caged-ATP.

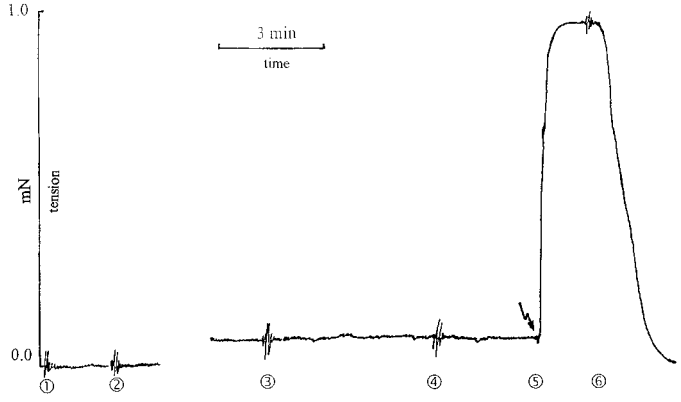

FIG. 1. y/t-recorded experiment of normal heart fiber: ① relaxing solution; ② rigor solution (with BDM); ③ rigor solution (pCa 4.5, without BDM); ④ caged-ATP solution (pCa 4.5); ⑤ flash; ⑥ relaxing solution.

Figure 2 shows an original registration of tension development after photolytical release of ATP from caged ATP in low tension rigor of a demembranated fiber from a normal human heart with high time resolution. Half time of tension development of control fibers (i.e. without superfusate incubation) was 466 ms ± 122 (n = 8), equivalent a rate constant of 1,58 s^{-1}. The rate constants were found to be significantly lower upon incubation with the hypoxic superfusate: rate constant of force development of the normal heart significantly ($p < 0.05$) decreased to 43.3% ± 15 (n = 8). The normoxic superfusate however, had no influence on kinetics of tension development of fibers from normal human heart (Fig. 3).

The levels of maximal force (pCa 4.5) of the normal human heart control fibers (without superfusate incubation) after photolytical release of ATP from caged ATP were 11,01 ± 0,66 mN/mm^2 (n = 8). Hypoxic endothelial cell superfusate caused no statistical significant change in maximal tension (9,57 ± 2,01 mN/mm^2, n = 8). Similary, the normoxic superfusate had no statistical significant influence on the maximal force of the normal heart fibers (9,39 ± 2,01 mN/mm^2; n = 8).

DISCUSSION

Decrease of coronary blood flow (11), dysfunction of the subendocardial myocardium after subendothelial hypoxia (12) and insufficient perfusion (13, 14) were described to diminished myocardial function. The underlying mechanism of this "myocardial hibernation" phenomenon is still unknown. Recent studies suggest that a diffusible factor released by hypoxic endothelial cells could be involved. We therefore investigated the direct effects of a superfusate of hypoxic porcine cultured endothelial cells on human myocardial cross-bridge kinetics. The rate constants of tension development of demembranated fibers derived from a ventricle

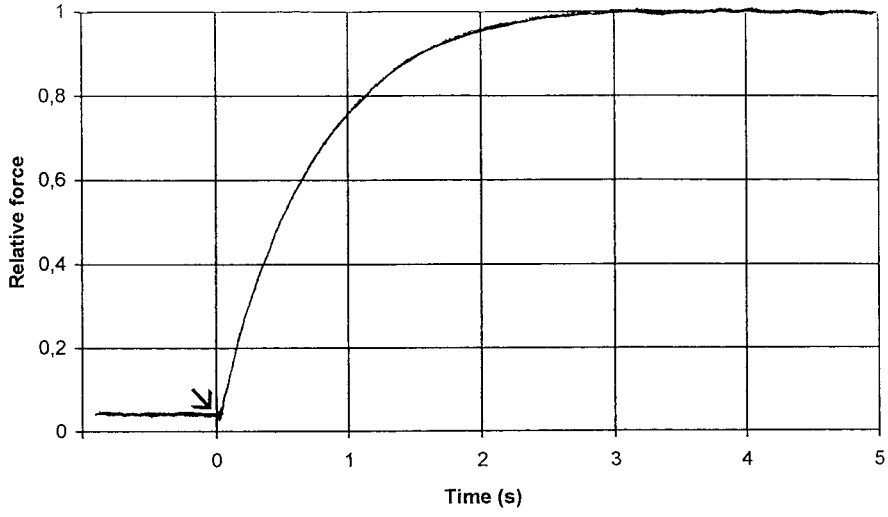

FIG. 2. Representative isometric tension development of a normal heart fiber after photolytical release of ATP from caged-ATP at low-tension rigor; (↘) light flash.

of a normal human heart were determined from the low-tension rigor by photolytic release of ATP from caged-ATP. To guarantee selective analysis of myosin cross-bridge function, and to exclude changes in regulatory protein expression, we investigated rate of tension development at maximal Ca^{2+}-activation (pCa 4.5). Different expression of myosin heavy chains (MHC) in the analyzed tissue could be excluded since only β-MHC is expressed in the normal human ventricle (15). Rate constants of tension development of normal human heart fibers were consitent with results obtained with pig heart fibers (10). The present study demonstrated that myosin cross-bridge kinetics and, therefore the "contractile state" of normal human myocardium was inhibited by a superfusate from hypoxic endothelial cells. ATP consumption could be considered as the ratio between $[(f \times g)/(f + g)]$ (16), where f is the rate-constant for the transition from non-force into force-generating states (attachment rate), and g is the rate-constant for the transition of cross-bridges from force into non-force generating states (detachment rate). Since the rate of tension development equals (f + g) (16), ATP consumption of the cardiomyocytes may be decreased upon the influence of the hypoxic superfusate.

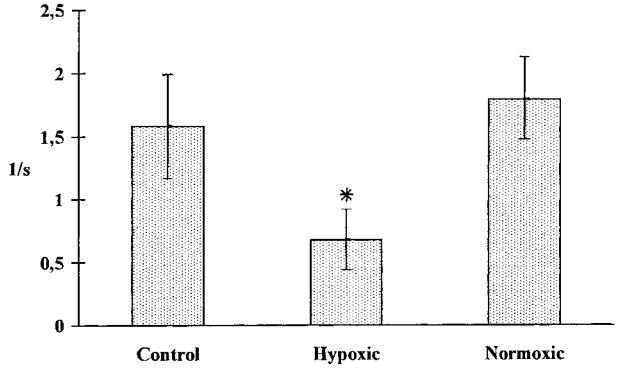

FIG. 3. Rate constants for tension development (1/s) after photolytical release of ATP from caged-ATP of normal human heart fibers; Control = normal human heart fibers without endothelial superfusate incubation; Hypoxic = normal human heart fibers with hypoxic endothelial superfusate incubation; Normoxic = normal human heart fibers with normoxic endothelial superfusate incubation; *$p < 0,05$ compared to control.

Generated isometric force F is determined by the force elicited by a single cross-bridge F' and the fraction of force-generating cross-bridges, n, which itself is determined by the ratio f/(f + g) (16). The fraction of force-generating cross-bridges and hence force generation, therefore should decrease upon incubation with the hypoxic superfusate. However the generated maximal tension was not influenced by hypoxic or normoxic superfusates, which is in accordance with a previous report (9). We may, therefore speculate that the hypoxic superfusate decreased the rate of cycling kinetics but increased F', i.e. tension output per cross-bridge thus balancing the decrease of the fraction of force-generating cross-bridges keeping force-generation at a normal level.

Our data suggest that a diffusible factor produced by endothelial cells upon low oxygen pressure directly inhibited the motor protein of human cardiomyocytes thus decreasing the contractile state and energy consumption but conserving the normal force production of the myocardium. A recent report confirm the presence of an identical activity in the coronary effluent of isolated hearts subjected to brief hypoxia (17). A decrease of regional contractility may lead to a downregulation of oxygen demand (18) and may preserve recovery of contractile function.

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