Effects of chronic endothelin-1 stimulation on cardiac myocyte contractile function

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Submitted 25 June 2003; accepted in final form 31 October 2003

Zolk, Oliver, Felix Münzel, and Thomas Eschenhagen. Effects of chronic endothelin-1 stimulation on cardiac myocyte contractile function. Am J Physiol Heart Circ Physiol 286: H1248–H1257, 2004; 10.1152/ajpheart.00599.2003.—Endothelin-1 (ET-1) has acute positive inotropic effects, but consequences of chronically increased ET-1 on contractile function of cardiac myocytes are largely unknown. In the present study, effects of long-term treatment with ET-1 (10 nM) for 5 days on both force development [force of contraction (FOC)] and kinetics of contraction were determined in heart tissue reconstituted from rat cardiac cells. Isometric force was measured in response to cumulative concentrations of Ca2+ and isoprenaline. ET-1 augmented basal FOC by 64 ± 11% (P < 0.05), which was associated with a significantly blunted contractile response to Ca2+ and isoprenaline. Moreover, ET-1 significantly prolonged relaxation (62 ± 3 vs. 53 ± 2 ms). Selective ETα (BQ-123) and ETβ receptor blockade (BQ-788) demonstrated that effects of ET-1 on contractile function were mediated through the ETα receptor subtype. Effects of ET-1 were prevented by cotreatment with either Ro31-8425, a PKC inhibitor, or dimethylamiloride, an inhibitor of the Na+/H+ exchanger. In contrast to long-term ET-1 treatment, no changes in contractile parameters were observed after ET-1 treatment for 3 h before force measurement. These data suggest that chronic ET-1 stimulation has dual effects on contractility: improvement of basal force but impairment of twitch kinetics and inotropic responsiveness to β-adrenoceptor stimulation. The signaling pathways involved include ETα receptors, PKC, and the Na+/H+ exchanger. The present in vitro findings raise the possibility that ET-1 may exert both adaptive and maladaptive effects in the failing myocardium in which local accumulation of ET-1 is present.

engined heart tissue; Na+/H+ exchanger

PROGRESSION OF CONGESTIVE HEART FAILURE (CHF) is accompanied by left ventricular pump dysfunction and neurohumoral system activation. Specifically, augmented cardiac endothelin-1 (ET-1) concentrations and increased circulating levels of ET-1 have been identified as occurring with the development of left ventricular dysfunction (32, 33) and are related to the severity of heart failure (21). Activation of cardiac ET receptors in turn modulates a wide variety of biological processes including vascular tone, growth, and myocardial contractile function. In the past, the majority of studies investigating the effects of ET-1 on contractile performance concentrated on the acute actions of ET-1. These studies, performed in a multitude of cardiac preparations including isolated ventricular cardiomyocytes in vitro, have documented that acute exposure to ET-1 generally exerts positive inotropic effects (9, 22, 29). It remains unclear, however, whether chronic exposure to ET-1 is linked to a phenotype associated with improved or impaired contractile function.

From findings that ET-1 antagonism worsens contractile function in rat and hamster models of CHF (5, 24), it has been proposed that endogenous ET-1 may be involved in the maintenance of cardiac function in CHF (24). In contrast, other studies demonstrated overall beneficial effects of ET antagonists on ventricular remodeling, contractile performance, and survival in animal models of CHF (6, 17, 18, 23). One of the first studies that addressed the question of whether ET-1 supports cardiac contractility in humans was performed by MacCarthy et al. (14). In patients with reduced left ventricular function, intracoronary infusion of the selective ETα receptor antagonist BQ-123 increased contractility, whereas in patients with normal ejection fraction, a decrease in contractility occurred. From these results, the authors concluded that ET may have a supporting role on cardiac function in healthy subjects. In heart failure this supporting role appears to be lost.

The controversial results of these studies underscore the need to address the question of whether chronic exposure to ET-1 improves contractile function in cardiomycocytes and thereby participates in the maintenance of cardiac function or, on the contrary, impairs contractility and therefore could provide a mechanism for progressive functional deterioration in CHF. Part of the controversy may derive from the fact that in vivo studies were often complicated by a negative inotropic effect of ET-1 secondary to coronary artery vasoconstriction. It is well established that decreased coronary flow rate results in a decrease in cardiac oxygen consumption and contractility, a phenomenon first described by Gregg (7). Therefore, we decided to use an in vitro model that 1) is independent from hemodynamic mechanisms and 2) allows long-term treatment studies. Rat ventricular cardiac cells were reconstituted in a collagen matrix to obtain engineered heart tissue (EHT) (30), which was tested for the effects of chronic, i.e., 5-day ET-1 exposure on twitch tension and twitch kinetics. Selective ET receptor antagonists were used to discriminate ETα and ETβ receptor-mediated effects. Moreover, specific inhibitors of intracellular effector proteins activated by ET-1, notably PKC and the Na+/H+ exchanger (NHE), were used to investigate the relevance of these signaling pathways for contractile performance.

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MATERIALS AND METHODS

Reagents. Recombinant human ET-1 and the inhibitor of the NHE dimethylamiloride (DMA) were purchased from Sigma. The PKC inhibitor Ro31-8425 and the ET<sub>A</sub> receptor antagonist BQ-123 were bought from Calbiochem. The ET<sub>B</sub> receptor antagonist BQ-788 was from Novabiochem.

EHT. All care and treatment of animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, Revised 1985) and were subjected to prior approval by the local animal protection authority. EHT was prepared as described earlier in detail (30). Cardiac cells isolated from hearts of 1- to 3-day-old neonatal Wistar rats (2.5 × 10<sup>6</sup>/EHT) were mixed with collagen type I (0.9 mg/ml), 10% Matrigel, DMEM, 10% horse serum, 2% chick embryo extract, 100 U/ml penicillin, and 100 µg/ml streptomycin. The optimal reconstitution mix has been the result of testing the effects of the cell number, and the concentrations of collagen type I, Matrigel, and supplements (serum, chick embryo extract) on tissue formation and force development. Matrigel, a complex mixture of extracellular matrix proteins and growth factors, was essential for any tissue development. In addition, even a small reduction in horse serum concentrations was found to prevent condensation of the cell-matrix mix and almost completely abolishing contractile function.

The reconstitution mix was pipetted into circular casting moulds and incubated for 45 min to let the reconstitution mixture gel. Medium (DMEM, 10% horse serum, 2% chick embryo extract, 100 U/ml penicillin, and 100 µg/ml streptomycin) was added, and EHTs were cultured as described earlier (30). After 6 days in culture, ring-shaped EHTs were transferred to a holder in which EHTs were exposed to unidirectional static stretch secondary to collagen elasticity and further collagen condensation. Culture medium was changed 12 h after EHT casting and then every other day while the culture was performed in casting molds. After transfer to the holder, the culture medium was changed every day.

EHTs were stimulated with 10 nM ET-1 (3 nM for experiments with ET receptor antagonists) for the indicated time. Time courses for the different experimental setups are shown in Fig. 1. For some experiments, EHTs were preincubated with specific ET receptor inhibitors for 20 min before ET-1 treatment was started. The antagonist concentrations were chosen from knowledge of their binding affinities, so that these concentrations could be expected to cause maximum inhibitory effects while remaining specific for the ET receptor subtype. Concentrations used were 1 µM for the ET<sub>A</sub> receptor antagonist BQ-123 [dissociation constant (K<sub>d</sub>) ET<sub>A</sub> 1.18 nM, K<sub>d</sub> ET<sub>B</sub> 1370 µM (19)] and 30 nM for the ET<sub>B</sub> receptor antagonist BQ-788 [K<sub>d</sub> ET<sub>A</sub> 318 nM, K<sub>d</sub> ET<sub>B</sub> 0.6 nM (15)]. Medium containing fresh ET-1 and the respective antagonists was changed every day.

Force measurement. EHTs were transferred into gassed organ baths (37°C, modified Tyrode solution with 0.2 mM Ca<sup>2+</sup>). The standard salt solution had the following composition (in mM): 119.8...
NaCl, 5.4 KCl, 1.2 MgCl2, 0.42 NaH2PO4, 22.6 NaHCO3, 5.05 glucose, 0.05 Na2EDTA, 0.28 ascorbic acid, and 0.2 CaCl2. The solution was gassed with 95% O2-5% CO2 and had a pH of 7.4. After 30-min equilibration without pacing, EHTs were electrophically stimulated with rectangular pulses (2 Hz, 5 ms, 100–120 mA). The preparations were gradually stretched to a length in which twitch force reached nearly its maximal value (90–95%). Isotonic and lusitropic responses to cumulative concentrations of Ca2+ (0.2–2.8 mM) and isoprenaline (0.1–1.0 mM) were recorded. Effects of isoprenaline were determined in the presence of 0.2 mM Ca2+. Twitch tension, contraction duration (T1: time from 50% of peak contraction to peak contraction), relaxation duration (T2: time to 50% relaxation), and the first derivative of developed tension (+dF/dt) and −dF/dt) were evaluated by BMON software (Jäckel; Hanau, Germany). Maximum twitch tension achieved with cumulative Ca2+ concentrations was used to correct for differences in the number of cardiomyocytes reconstituted in individual EHTs. Concentrations inducing 50% of the maximal effect were expressed as pD2 values (where pD2 is log EC50) and calculated by using GraphPad Prism.

Some variability in the data derived from different experimental sub-studies was observed. These quantitative changes are most likely attributable to batch differences of enzymes used to isolate cardiomyocytes or collagen and Matrigel used to reconstitute the heart tissue. Such variability is not unique to EHTs and is well known for other cell culture systems. Control groups have been included in each substudy. To demonstrate that ET-1-evoked changes in contractile performance are robust and well reproducible independent from batch variability of materials used, the data are shown without further standardization.

Gene expression analysis by real-time RT-PCR. Total RNA (2 μg) isolated from EHTs was reverse transcribed with Moloney murine leukemia virus reverse transcriptase, and cDNA was subsequently amplified with the TaqMan system (Prism 7700, PE Biosystems) as described previously (31). Primers and probes (Table 1) for α-myosin heavy chain (α-MHC), β-MHC, sarco(endo)plasmic reticulum Ca2+-ATPase 2a (SERCA2a), phospholamban, NHE-1, atrial natriuretic peptide (ANP), calsequestrin, and GAPDH were designed to cross an intron/exon boundary; thereby the possibility of chromosomal DNA artifacts in the PCR was eliminated. The level of GAPDH in each sample was used to normalize for the variability in RNA quantity or differences in the efficiency of the RT reaction.

Western blot analysis. EHTs were rinsed with cold PBS and homogenized in lysis buffer (30 mM Tris-HCl, pH 8.8, 3% SDS, 10% glycerol, 5 mM EDTA, 30 mM sodium fluoride, and 2 ng/ml aprotinin) in a glass/Teflon homogenizer. For immunoblotting, aliquots of denatured protein were subjected to SDS-PAGE on a 10% polyacrylamide gel, and separated proteins were electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell). Nonspecific binding was blocked by incubation with 5% nonfat dry milk for 1 h at room temperature. Membranes were then incubated with the anti-SERCA2 polyclonal antibody (1:1,000; Santa Cruz Biotechnology, table: Table 1. Primers and probes for real-time RT-PCR

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ANP, atrial natriuretic peptide; SERCA2a, sarco(endo)plasmic reticulum Ca2+-ATPase 2a; PLB, phospholamban; CSQ, calsequestrin; MHC, myosin heavy chain; NHE-1, Na+/H+ exchanger-1.

Fig. 2. Arrhythmias induced by ET-1. A: concentration-dependent effects of Ca2+ on force of contraction in control EHTs, EHTs after acute ET-1 administration (n = 8), and EHTs treated with ET-1 for 5 days (n = 5) or 3 h (n = 6). With low extracellular Ca2+ concentration (0.2 mM), active force of contraction was significantly increased in EHTs after acute and long-term ET-1 treatment (0.83 ± 0.03 and 0.78 ± 0.05 mN, respectively) compared with controls or short-term ET-1 treatment (0.46 ± 0.09 and 0.48 ± 0.07 mN, respectively). A breakdown of active force due to arrhythmias was observed in EHTs after acute ET-1 treatment when Ca2+ concentrations exceeded 1 mM. B: representative twitch recordings (in the presence of 2.4 mM Ca2+) from a control EHT and a ET-1-treated EHT.
RESULTS

Contractile properties of EHTs treated with ET-1. Figure 1 summarizes the effects of ET-1 (10 nM) on isometric force and twitch kinetics in EHTs. After long-term treatment for 5 days, twitch tension was significantly increased to 0.78 ± 0.05 mN (n = 5) versus 0.46 ± 0.09 mN (n = 8) in controls. In contrast, the positive inotropic effects to cumulative concentrations of isoprenaline were significantly diminished (Fig. 1B). Maximum isoprenaline-induced increase in twitch tension from basal force (change in force of contraction) amounted to 0.05 ± 0.01 mN (n = 5) in the ET-1-treated group compared with 0.29 ± 0.05 mN (n = 8) in controls. pD2 values for the effect of isoprenaline were significantly decreased in ET-1-treated EHTs (7.61 ± 0.25 vs. 9.00 ± 0.16; P < 0.05), associated with a rightward shift of the concentration-response curve. Moreover, T2 was significantly prolonged after ET-1 treatment (basal: 91 ± 2 ms, n = 5, vs. 73.0 ± 4.3 ms, n = 8).

Acute administration of ET-1 (10 nM) to untreated EHTs during force measurement affected twitch tension and twitch kinetics similar to long-term ET-1 treatment (Fig. 1B). In contrast, contractile parameters did not differ from those of untreated controls when EHTs were exposed to 10 nM ET-1 for 3 h before contractile parameters were recorded in Tyrode solution free of ET-1. Thus washout and clearance of ET-1 from EHTs occurs rapidly. These results exclude the possibility that ET-1 remaining in EHTs might be responsible for the observed effects on contractility after long-term treatment.

After acute treatment with ET-1 (i.e., in the continued presence of ET-1), arrhythmias were observed when extracellular Ca2+ was increased above 1 mM in 6 of 8 EHTs (Fig. 2).
Arrhythmias were not observed in control EHTs or in EHTs treated with ET-1 for 3 h or 5 days followed by a short washout period before contractile parameters were recorded. Representative twitch recordings (in the presence of 2.4 mM Ca^{2+}) from a control EHT and an EHT after acute ET-1 treatment are shown in Fig. 2B.

We also studied the concentration dependence of long-term ET-1 stimulation on contractile function. ET-1 was tested at concentrations between 1 and 10 nM. These ET-1 concentrations have been demonstrated to be effective, for example, to activate and translocate PKCζ (EC_{50} 11–15 nM) and PKCε (EC_{50} 1.4–1.7 nM) (3) and to induce protein synthesis and cell hypertrophy in isolated cardiomyocytes (10). As shown in Fig. 3, the contractile phenotype of EHTs gradually changed with increasing ET-1 concentrations.

Figure 4 summarizes the effects of long-term ET-1 stimulation (10 nM) on kinetics of contraction. Figure 4A shows representative twitch recordings illustrating the profound effects of ET-1 on the twitch shape mainly under basal conditions. T1 and T2 were significantly prolonged after ET-1 exposure. This effect was more pronounced in the absence of β-agonists and was minor in the presence of isoprenaline (1 μM). Although duration of contraction and relaxation was significantly prolonged, ET-1 treatment did not significantly change the rate of contraction (+dF/dt) and relaxation (−dF/dt).

Effects of ET-1 on morphology and [14C]phenylalanine incorporation. Formation of longitudinally oriented cellular aggregates of cardiomyocytes and myofilament organization within the cells was not affected in EHTs treated with ET-1 for 5 days. Representative photographs of actin immunofluorescence are shown in Fig. 5A. Quantification of the cell diameter demonstrated no significant difference between control and ET-1-treated EHTs (Fig. 5B). Moreover, ET-1 treatment did not change the rate of protein synthesis, as assessed by [14C]phenylalanine incorporation.

Gene expression. To characterize alterations of gene expression, which could explain the functional consequences of long-term ET-1 exposure, transcript levels of genes known to be regulated during cardiac hypertrophy and heart failure were quantified by real-time RT-PCR analysis. Among these were genes that encode proteins involved in intracellular calcium handling (SERCA2a, phospholamban), sarcomeric proteins (MHC isoforms), the cardiomyocyte marker protein caldesmon, and ANP as a surrogate marker of myocyte hypertrophy. Figure 6A summarizes the results. Data are normalized to GAPDH expression and are displayed as relative expression, related to untreated controls. ANP mRNA and the β-MHC mRNA-to-α-MHC mRNA ratio markedly increased by 257 and 118%, respectively, whereas gene expression of NHE-1, phospholamban, and caldesmon was not changed. SERCA2a mRNA expression was significantly decreased by 38% after ET-1 treatment. Corresponding to decreased transcript expression, SERCA protein was significantly diminished by 28%, as shown in Fig. 6B.

Effects of ETA and ETB selective antagonists on contractility. Concomitant treatment with the selective ETA receptor antagonist BQ-123 prevented the effects of ET-1 (3 nM) on isometric force and twitch kinetics (Fig. 7). Basal twitch tension amounted to 0.31 ± 0.04 mN (n = 8) and was not significantly different from untreated EHTs. In addition, concentration-response curves for the inotropic effects of isoprenaline were not significantly different from controls. Finally, negative lusitropic effects observed in ET-1-treated EHTs were completely reversed by BQ-123 (T2: 58 ± 2 ms, n = 8, P < 0.05 vs. ET-1-treated EHTs). In contrast, the selective ETB receptor antagonist BQ-788 was not able to reverse alterations in contractile function induced by ET-1. Neither basal twitch tension (0.48 ± 0.05 mN, n = 8) nor inotropic responses to isoprenaline were significantly different from EHTs treated with ET-1 alone (Fig. 5). In addition, there was no significant effect on ET-1-induced negative lusitropy (T2: basal: 64.6 ± 1.4 ms, n = 8, P < 0.05 vs. control).

Effects of NHE and PKC blockade on contractile performance. Whereas there are a number of intracellular events that occur after ETA receptor stimulation, activation of PKC and the NHE may have particular relevance with regard to myocyte contractility (4). Thus we studied the effects of PKC and NHE inhibition on contractile function in EHTs exposed to ET-1. Results are summarized in Fig. 8. Concomitant PKC inhibition abolished the effect of ET-1 on contractile responses to cumu-
relative concentrations of isoprenaline and normalized, in part, twitch kinetics (T2: 63 ± 3 ms, n = 7, vs. 78 ± 5 ms, n = 5, P < 0.05). The PKC inhibitor alone had no effect. Coadministration of the NHE inhibitor DMA similarly reversed the effects of ET-1 on contractility. Baseline force was decreased even below control levels (0.40 ± 0.08 mN, n = 6, vs. 0.59 ± 0.13 mN, n = 5), whereas twitch tension under maximally effective isoprenaline concentration was unchanged (0.99 ± 0.05 mN, n = 6, vs. 1.04 ± 0.05 mN, n = 5). Effects of ET-1-DMA cotreatment on force of contraction did not significantly differ from the effects of DMA treatment alone. Moreover, time to 50% relaxation was partially normalized but did not reach the control level (82 ± 5 ms, n = 6, vs. 91 ± 2 ms, n = 5, P < 0.05).

DISCUSSION

In vitro studies have shown that acute administration of ET-1 exerts positive inotropic effects on isolated cardiac myocytes or multicellular preparations. The objective of the present study was to determine whether long-term exposure to elevated levels of ET-1, which more closely reflects the situation for example in heart failure, similarly changes cardiac contractile performance. Our main novel findings, obtained in rat EHT, are that 1) long-term (5 days) ET-1 stimulation markedly

Fig. 5. Morphology and [14C]phenylalanine incorporation. A: immunofluorescent staining of actinin in EHTs treated with ET-1 (10 nM) for 5 days or in control EHTs. Shown are representative sections from 3 different EHTs per group investigated. Left, actinin (red); right, actinin (green) and nuclear propidium iodide (red) counterstain. Photomicrographs were taken of randomly chosen fields at a magnification of ×400. B: ET-1 does not affect cell size and rate of protein synthesis in EHTs. Cardiac myocytes in whole mount preparations of EHTs were stained with an antibody against actinin. Twenty myocytes per group (from 4 different EHTs per group) were chosen randomly and the maximum cell diameter was determined (left). EHTs were stimulated with 10 nM ET-1 for 24 h and then labeled by [14C]phenylalanine for 24 h before harvest. Total radioactivity of incorporated [14C]phenylalanine into proteins was determined by liquid scintillation counting. The relative phenylalanine uptake was normalized to the cell count and is shown as means ± SE of 6 independent experiments (right).

Fig. 6. Effects of ET-1 on gene expression in EHTs. A: expression of atrial natriuretic peptide (ANP), β- and α-myosin heavy chain (β-MHC/α-MHC), sarco(endo)plasmic reticulum Ca2+/ATPase (SERCA2a), phospholamban (PLB), Na+/H+ exchanger-1 (NHE-1), and calsequestrin mRNA in EHTs treated with ET-1 (10 nM) for 5 days assessed by real-time RT-PCR. Vehicle-treated EHTs (Ctr) served as controls. n = 12, *P < 0.05. B: Western blot analysis of SERCA2a expression in control EHTs and EHTs treated with ET-1 (10 nM, 5 days). The internal standard protein calsequestrin (CSQ) was used to normalize for equal protein loading. n = 8, *P < 0.05.
improved basal force but impaired responsiveness to β-adrenoceptor agonists and prolonged relaxation; 2) antagonists of the ET<sub>A</sub> receptor but not ET<sub>B</sub> receptor abolished these effects; and 3) effects of ET-1 on force of contraction and twitch kinetics were prevented, at least in part, by cotreatment with either Ro31-8425, a PKC inhibitor, or DMA, an inhibitor of the NHE.

In the first set of experiments, long-term treatment of EHTs was compared with the effects of either short-term treatment for 3 h or acute administration of ET-1 during force measurement. Short-term administration of ET-1 for 3 h did not change contractile performance compared with untreated controls, indicating that ET-1 is effectively washed out in the equilibrium period before force measurement. Interestingly, acute administration of ET-1 and long-term treatment with ET-1 for 5 days similarly affected twitch tension and twitch kinetics. It is noteworthy that EHTs became prone to arrhythmias after acute treatment with ET-1 when extracellular Ca<sup>2+</sup> exceeded 1 mM. In contrast, arrhythmias did not occur after prolonged ET-1, i.e., when ET-1 was washed out before force measurement. A role for ET-1 in arrhythmogenesis has been proposed early after its discovery (28). Previous in vivo studies (2, 27) tried to define a direct arrhythmogenic role of ET-1 that is not solely attributable to myocardial ischemia secondary to its vasoconstrictor properties. However, these studies could not exclude the possibility that coronary ET-1 infusion caused some degree of accompanying regional, if not generalized, ischemia. Our study performed in EHTs provides more conclusive data supporting a direct role for ET-1 in the development of arrhythmias, although the mechanisms remain to be determined.

It is well known that ET-1 significantly increases the cell surface area and also concentration dependently stimulates the synthesis of protein without cell proliferation in quiescent, serum-deprived cardiomyocytes in vitro (13). In contrast, the phenotype of EHTs was not significantly changed by long-term treatment with ET-1 for 5 days, as judged by total cell count, rate of protein synthesis, and overall cell morphology. Especially cell diameters, formation of multicellular aggregates, and organization of sarcomeres were not changed. This discrepancy might be attributable to the different culture conditions. In the case of EHTs, these include 10% serum and 2% chick embryo extract, which are essential for any tissue development, condensation of the cell matrix and contractile function. Growth factors supplied together with serum or chick embryo extract most likely mask the stimulatory action of ET-1 on protein synthesis and cellular growth. However, other features of the ET-1-induced hypertrophic response consistently observed in serum-deprived cardiomyocytes, such as an upregulation of ANP mRNA expression and a switch of MHC isoform expression pattern in favor of the fetal isoform (10), were also observed in EHTs treated with ET-1.

On the basis of previous structure-function and transgenic studies, there is some rationale to hypothesize that altered α-MHC-to-β-MHC ratio in EHTs might decrease rate of relaxation (−dF/dt) without affecting average force generation (13, 26). However, we do not know whether changes in α- and β-MHC transcript levels observed in our study translate into similar changes in α- and β-MHC protein concentrations. Our results, demonstrating that dF/dt was unchanged, whereas iso-

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**Fig. 7. Effects of ET receptor blockade on force of contraction and twitch kinetics.** Concentration-dependent effects of isoprenaline on force of contraction, contraction duration, and relaxation duration were determined in control EHTs (n = 8), in EHTs treated with ET-1 (3 nM, n = 8), and in EHTs treated with ET-1 in the presence of the ET<sub>A</sub> receptor antagonist BQ-123 (1 μM, n = 8) or the ET<sub>B</sub> receptor antagonist BQ-788 (30 nM, n = 8). *P < 0.05 vs. control.
metric force (in the absence of β-agonists) was markedly augmented, suggest that mechanisms other than MHC isoform transition become relevant to determine the final contractile phenotype. A candidate mechanism might be activation of the NHE, as suggested by NHE inhibitor studies.

In ET-1-treated EHTs, SERCA2a was significantly reduced at transcript and protein level. This finding confirms results from a previous study. Hartong et al. (8) demonstrated that hypertrophic agonists including ET-1 induce downregulation of SERCA2a mRNA in neonatal rat cardiomyocytes. Although ET receptors are known to be linked to PKC activation by stimulation of the hydrolysis of phosphoinositide and generation of diacylglycerol, the signaling events leading to SERCA downregulation remained unclear. Most recently Porter et al. (20) demonstrated that the PKC isoenzymes PKCδ and PKCe selectively regulate SERCA2a gene expression in cultured neonatal rat ventricular myocytes. Altogether, abnormally prolonged myocardial relaxation in response to chronic ET-1

Fig. 8. Effects of PKC inhibition and NHE inhibition on force of contraction and twitch kinetics. A: concentration-dependent effects of isoprenaline on force of contraction, contraction duration, and relaxation duration were determined in control EHTs (n = 11), in EHTs treated with ET-1 (10 nM, 5 days, n = 5), PKC inhibitor Ro31-8425 (1 μM, n = 10), or ET-1 in the presence of Ro31-8425 (1 μM, n = 9). *P < 0.05 vs. control, #P < 0.05 vs. ET-1. B: concentration-dependent effects of isoprenaline on force of contraction and twitch kinetics were determined in control EHTs (n = 5) and in EHTs treated with ET-1 (10 nM, 5 days, n = 5), the NHE inhibitor DMA (10 μM, n = 7), or ET-1 in the presence of DMA (n = 6). *P < 0.05 vs. control; #P < 0.05 vs. ET-1.
stimulation, as demonstrated here in EHTs, seems to be at least partly attributable to the downregulation of SERCA2a.

The second part of our study aimed to explore signal transduction pathways mediating the effects of chronic ET-1 stimulation on contractile performance in EHTs. Two major ET receptor subtypes, ET_A and ET_B receptors that bind ET-1, have been identified in heart tissues and in cultured heart cells. Whereas the ET_A receptor represents the predominant ET receptor subtype in cardiomyocytes (15, 1), the ET_B receptor is predominant in cardiac fibroblasts (11). The specific ET_A receptor antagonist BQ-123 completely reversed changes in contractile function induced by ET-1, whereas the specific ET_B receptor antagonist BQ-788 had no effect. Our data thus suggest that particularly ET_A receptor stimulation is of importance in modulating cardiac contractility by chronically increased ET-1.

Whereas there are a number of intracellular events that occur after ET-1 exposure, evidence has accumulated that implicates PKC and the NHE in the signal transduction cascade after ET_A receptor activation [review by Sugden (25)]. The NHE has been demonstrated to alter intracellular pH and calcium concentration, both potentially influencing myocyte contractility (12). PKC activation may also be associated with abnormally slowed myocardial relaxation due to the downregulation of SERCA2a. Moreover, PKC has been linked to ERK activation, which in turn represents one of the strongest NHE activating kinases (16). However, the relationship of PKC and NHE signaling to contractile function of heart tissue after chronic ET receptor stimulation remains unexplored. Accordingly, the second goal of this study was to interrupt either the PKC or the NHE to determine whether the PKC and/or the NHE are obligatory for the effects of ET-1 on myocyte contractility. Effects of ET-1 on both basal force of contraction and contractile response to β-adrenergic stimulation were completely reversed by selective inhibition of the PKC or the NHE. These findings suggest that both downstream targets of ET receptor activation are decisively involved in regulation of myocyte contractility on exposure to ET-1. It is noteworthy that PKC or NHE inhibition completely reversed the effects of ET-1 on twitch tension, but changes in twitch dynamics, i.e., broadening, were only partially reversed. These findings suggest that inotropic effects of ET-1 are mainly mediated by PKC/NHE activation, whereas additional signaling pathways downstream of ET_A receptor activation are relevant in the regulation of twitch dynamics.

The present study highlights the chronic effects of ET-1 on contractile performance of the heart tissue independent from hemodynamic mechanisms. Basal force of contraction is improved. However, drawbacks are decreased responsiveness to β-adrenergic stimulation and impaired twitch kinetics. Our in vitro findings, obtained in reconstituted heart tissue, raise the possibility that ET-1 may exert both adaptive and maladaptive functions in the failing myocardium in which ET-1 concentrations are chronically increased.

ACKNOWLEDGMENTS

Beate Endress and Sven Engmann provided expert technical assistance for this study.

GRANTS

This work was supported by the Deutsche Forschungsgemeinschaft Grant Es88/8-2.

REFERENCES


