Pflugers Arch - Eur J Physiol (2002) 445:246–256
DOI 10.1007/s00424-002-0921-y

ORIGINAL ARTICLE

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Chronic exposure to interleukin 1β induces a delayed and reversible alteration in excitation–contraction coupling of cultured cardiomyocytes

Received: 2 April 2002 / Revised: 5 July 2002 / Accepted: 15 July 2002 / Published online: 13 September 2002

Abstract While proinflammatory cytokines can depress cardiac contractility, the mechanism by which this occurs remains unclear. To clarify the cellular effects of interleukin (IL)-1β, we assessed contractility, calcium homeostasis, and gene expression in cardiomyocytes exposed to this proinflammatory cytokine. Neonatal rat cardiomyocytes were exposed to IL-1β in the presence or absence of an inhibitor of nitric oxide (NO) synthase. Videomicroscopy was used to follow calcium transients (Fura-2 fluorescence) and amplitude of contraction, both unstimulated and after isoproterenol challenge. Gene expression was assessed by Northern and Western blot analyses. Both basal contractility (amplitude of contraction, maximum speed of contraction and relaxation) and amplitude of calcium transients were decreased, respectively, ca. 60% ($P \leq 0.05$) and ca. 40% ($P \leq 0.05$) after 3 days of IL-1β exposure. Contractile function and amplitude of calcium transients returned to control values when cells where cultured an additional 3 days in the absence of IL-1β. IL-1β-treated cells had reduced responses to isoproterenol as evidenced by a lack of enhanced amplitude of contraction and a reduction in cAMP production. IL-1β decreased the expression of genes important to the regulation of calcium homeostasis (phospholamban, sarcoplasmic reticulum calcium ATPase) at both the transcript and protein level. Alterations in contractile function did not occur through NO-mediated pathways. These results support the hypothesis that IL-1β may play an important role in contractile dysfunction through alterations in calcium homeostasis.

Keywords Calcium transients · Cardiomyocyte · Contractile function · Gene expression · Proinflammatory cytokine

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Introduction

Proinflammatory cytokines (including IL-1β and TNF-α), classically characterized by their mediation of inflammatory processes after synthesis by macrophages and other immunoregulatory cells, are elevated in the serum and cardiac tissue of patients with endstage heart failure [13, 27, 50, 52]. Furthermore, they are believed to depress cardiac function in heart failure, transplant rejection, myocarditis, sepsis, and burn shock [1, 12, 15, 25, 27, 36, 50]. In experimental models, IL-1β and TNF-α have been shown to depress contractile function in intact animals, isolated hearts, and papillary muscles [11, 35, 43]. A more explicit demonstration of the potential role of proinflammatory cytokines in the development of heart failure arises from murine models in which cardiac-specific overexpression of TNF-α leads to cardiac hypertrophy, fibrosis, reduced ejection fraction, loss of adrenergic responsiveness, and induction of a fetal gene program [24].

In order to understand the cellular biology of proinflammatory cytokine exposure, investigators have studied the effects of a variety of cytokines in neonatal and adult myocytes in culture. Although myocytes challenged with TNF-α or IL-1β uniformly show depressed contractility, the various studies have also yielded conflicting results [17, 57]. For example, contractile responses and alterations in adrenergic responsiveness are alternatively reported to be either dependent or independent of inducible nitric oxide synthase (iNOS) induction and NO production [34, 49, 57].

Additionally, proinflammatory cytokines have been reported to (1) enhance or depress the spontaneous contraction rates of neonatal cardiomyocytes [37, 42]; (2) enhance, depress, or leave unchanged the basal contractile activity of neonatal and or adult cardiomyocytes [3, 4, 25, 49]; (3) increase or decrease the peak systolic intracellular calcium level [4, 57]; (4) increase or leave unchanged the level of transcripts for sarcomeric proteins [39, 40, 51]; and (5) induce or not induce cardiomyocyte protein synthesis [30, 39, 51]. These disparities may be partly attributable to differences in culture techniques and inherent differences between adult and neonatal cardiomyocytes. However, the existing inconsistencies might also be attributable to various doses and durations of cytokine treatment, a reliance on transcript levels without assessing the effects of cytokines on protein expression, and a failure to correlate changes in gene expression with functional responses. Indeed, most studies evaluating the effects of IL-1β on myocyte contractility and/or calcium homeostasis have focused on short-term (min to a few h) effects of the cytokine on heart cells and IL-1 concentrations used in these studies were extremely high, ranging from 10 ng ml^{-1} to 500 ng ml^{-1} [4, 8, 25, 49, 54]. However, lower IL-1 plasma concentration have been measured in pathological conditions such as heart failure (up to 250 pg ml^{-1} [50]) or severe septic shock (up to 500 pg ml^{-1} [7]) even if the presence in the plasma of IL-1sRII, the soluble form of type II IL-1 receptor, reduces the detection of IL-1β in clinical samples by at least 50% [7].

Therefore, to clarify the effects of prolonged (3 days) cytokine exposure on myocyte cell biology, we assessed the effects of physiological concentrations of IL-1β on contractile function, calcium homeostasis, and transcript and protein expression in neonatal cardiac myocytes.

Materials and methods

Preparation and treatment of neonatal rat cardiomyocytes

Cardiomyocytes were prepared from 1- to 2-day-old Sprague-Dawley rats and cultured in DF-5% on Pronectin (Biosource)-coated tissue culture plates or glass coverslips as previously described [30]. Cells were cultured in DF-5% for 24–48 h before the initiation of experiments. In preliminary studies, we evaluated the dose response of amplitude of contraction and maximal amplitude of calcium transients to 3 days of treatment with various concentrations of IL-1β. As shown in Fig. 1, the effects of IL-1 β treatment on myocyte contractility (Fig. 1B) and amplitude of calcium transients (Fig. 1D) showed a concentration dependence, with a significant decrease observed at 0.1 ng ml^{-1} and a maximal effect with concentrations ≥2 ng ml^{-1}. Therefore, in all the experiments described we used 2 ng ml^{-1} of IL-1β, a concentration which is in the range of that described in human pathological conditions (0.5–1 ng ml^{-1}) [7]. Cells were treated with murine IL-1β (2 ng ml^{-1}) from Biosource, or vehicle (phosphate buffered saline; PBS). Cells were evaluated at three time points: after 24 h or 3 days of cytokine exposure, and 3 days after cessation of cytokine challenge. Fresh media containing the same cytokines was added every 24–36 h, similarly fresh vehicle was added to the control cells every 24–36 h and cytokine free media was added every other day during the recovery period. Dexamethasone (3 μmol l^{-1}, Sigma), and IL-1β neutralizing antibody (Biosource) were used as reported [30]. For studies on the role of NO production, cells were cultured in DF-5% for 24 h followed by arginine-free media (Select Amine, Life Technologies) for 4 h prior to the addition of either 1 mmol l^{-1} L-NMMA (N^G-monomethyl-L-arginine, see below) or 1 mmol l^{-1} arginine as previously described [26]. Spontaneous rates of contraction were determined from cells grown on plastic dishes in DF-5% in the presence or absence of IL-1β. Cells were observed with an inverted phase microscope at either 100× or 200× magnification. Rates of contraction were determined over a 20-s interval and contractions per minute calculated.

Analysis of cytosolic calcium and contraction–relaxation

To provide high-contrast spots for tracking contractile activity, glass beads (2.1±0.5 μm; Duke Scientific, Palo Alto, Calif., USA) were added to the neonatal myocytes. The preparation was illuminated with red light through a dichroic mirror, and a video edge-detection system (VAD 104; Crescent Electronics, Sandy, Utah, USA) was used to record the motion of glass beads attached to the surface of contracting myocytes. Calcium transients were followed in cells loaded with the acetoxymethyl ester form of Fura-2 (Molecular Probes). Cardiomyocytes were placed in Tyrode's solution containing (in mmol l^{-1}): NaCl 137, KCl 5, glucose 15, $MgSO_4$ 1.3, NaH_2PO_4 1.2, HEPES 20, $CaCl_2$ 1, Fura-2-AM (3 μmol l^{-1}) and D-Pluronic (Molecular Probes; 3 μl of 25% wt/wt in dimethyl sulfoxide) for 20 min. Myocytes were then rinsed with Tyrode's solution and maintained for 15 min at room temperature to allow for de-esterification of the dye. Coverslips were transferred to a temperature regulated chamber (33°C) mounted on a Nikon Diaphot 300 inverted microscope stage and cells perfused with prewarmed Tyrode's solution. While the cultures contained a spontaneously contracting monolayer of cells, they were paced by electrical field stimulation at 1 Hz (15 V/4 ms pulse duration, Grass S11 Stimulator, Grass Instruments) using platinum electrodes embedded in the wall of the perfusion chamber [30]. Fluorescence of intracellular Fura-2 was determined by illuminating the cells at 360 nm (the isosbestic wavelength for Fura-2) at both the beginning and end of the time interval in which cells were continuously exposed to 380-nm light, which stimulates fluorescence emission from Fura-2 that is complexed with Ca^{2+}. Light emission at 520 nm was continuously recorded (Ionoptix), and the fluorescent light due to calcium transients calculated from the ratio between that stimulated by 380-nm excitation, and that calculated as a linearly interpolated numerator from the two 360-nm excitations (Ionoptix). The sampling rate for collection of ratio values was 100 Hz. Because the Fura-2 fluorescence ratio is an extremely sensitive indicator of changes in free calcium levels, and significant uncertainties remain in calibrating the Fura-2 fluorescence ratio to absolute intracellular free calcium levels, the fluorescence ratio (generated from the light intensity emitted by 360-nm and 380-nm excitations) was used to monitor changes in intracellular free calcium levels. A data analysis program (IonWizard 4.3, Ionoptix) was used to measure Fura-2 parameters (diastolic and peak systolic calcium) and calculate maximum speed of contraction, maximum speed of relaxation, and peak amplitude of contraction. Amplitude of calcium transients were reported both as the fluorescence ratio at the peak calcium transient as well as the difference between the diastolic baseline ratio and that observed at the calcium transient peak. Calibration of contractile distance was determined by using Cell-VU grid coverslips (Erie Scientific). Assessment of cellular contractile responsiveness to 0.1 mmol l^{-1} isoproterenol challenge was performed as previously described with one cell recorded per coverslip [30].

Media nitrite measurements

Cells were cultured in arginine-free media as described above supplemented with 1 mmol l^{-1} arginine (control), 1 mmol l^{-1} arginine and 2 ng ml^{-1} IL-1β, or 1 mmol l^{-1} NMMA and 2 ng ml^{-1} IL-1β.

Briefly, 100 µl of cell culture supernatant was added to 100 µl of Griess reagent (0.75% sulfanilamide in 0.5 mol l^{-1} HCl/0.075% naphtylethylenediamidine) and absorbance at 550 nm determined [16]. A standard curve was constructed with known concentrations of sodium nitrite over the linear range of the assay (1–128 µmol l^{-1} nitrite) and the results were expressed as nanomoles of nitrite produced per 10^6 cells per 36 h. The detection threshold for NO_2 in this assay is 1 µmol l^{-1}.

cAMP measurements

Cells were cultured in arginine-free media supplemented with L-arginine or L-NMMA (1 mmol l^{-1}) and either IL-1β (2 ng ml^{-1}) or vehicle (PBS). After 3 days cells received 0.1 mmol l^{-1} 3-isobutyl-1 methylxanthine (Sigma) for 30 min followed by either 1 µmol l^{-1} isoproterenol or solvent (0.1 mmol l^{-1} HCl). After 15 min media was removed, cells rinsed in PBS, scraped into 0.1 mol l^{-1} HCl in PBS, and samples boiled for 10 min. Cell extracts were further processed and protein concentrations determined using a modified Bradford reaction (BioRad) exactly as described previously for cGMP assays [30]. Cellular cAMP levels were determined with a standard radioimmunoassay kit (Biomedical Technologies). Results are reported as picomoles cAMP per milligram protein.

Immunohistochemistry

Control and IL-1β-treated cardiomyocytes were grown on glass coverslips, permeabilized and fixed in 2% paraformaldehyde/0.1% Triton X-100, and washed with PBS. Cells were stained [1 mmol l^{-1} TRITC-phalloidin (Molecular Probes) in PBS] in the dark for 1 h, and washed sequentially with 0.5% BSA/0.15% glycine in PBS (BSA solution), and 0.5% normal goat serum in PBS. Cells were then incubated for 1 h in a 1:2 dilution of an anti-sarcomeric myosin monoclonal antibody, MF20. The MF20 antibody was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City [2]. After washing in the BSA solution, cells were interacted with a 1:200 dilution (in the BSA solution) of Alexa-conjugated anti-mouse antibody (Molecular Probes) for 1 h. Coverslips were then washed sequentially with the BSA solution followed by PBS. Coverslips were mounted and viewed with a Leica TCSNT confocal microscope at 60× magnification, 1024×1024 scan resolution and integrated over 8 frames.

RNA isolation and Northern blot analysis

Total RNA isolation, Northern blot hybridizations, and preparation of phospholamban cDNA, sarcoplasmic reticulum calcium ATPase cDNA (SERCA), glyceraldehyde phosphate dehydrogenase cDNA, and 18S ribosomal RNA oligonucleotide probes were performed as previously reported [30]. Additional probes were prepared from oligomers complementary to rat alpha-myosin heavy chain (α-MHC) (5'-ttcctcatcgtgcattttct-3' complementary to bases 5832–5851 of GenBank X15938) and β-MHC (5'-ggtctcagggcttcacaggc-3' complementary to bases 5845–5864 of GenBank X15939), rabbit calcium release channel cDNA, rat atrial natriuretic factor cDNA, and rat cardiac calsequestrin cDNA. MHC probes were radiolabelled with T4 polynucleotide kinase and hybridized overnight (51°C α-MHC; 55°C β-MHC; 6X SSC, 1X Denhardt's, 0.1% SDS, 0.05% sodium pyrophosphate, 100 µg ml^{-1} sheared denatured salmon sperm DNA), with a final filter wash in 6X SSC-0.1% SDS (45°C α-MHC; 50°C β-MHC). Images of the radioactivity from hybridizations were obtained with a Phosphorimager (Molecular Dynamics) and quantified with ImageQuant software (Molecular Dynamics). Filters were then rehybridized with the 18S oligonucleotide. Hybridizations signals were normalized to that of the 18S probe to correct for differences in RNA mass and efficiency of transfer. Data were in turn normalized to the mean of the control samples, arbitrarily set at 100%.

Protein isolation and Western blot analysis

Neonatal cardiac myocytes (6×10^6) were washed with PBS, scraped into homogenization buffer (all in mmol l^{-1}; potassium phosphate 50, sucrose 300, NaF 10, EDTA 1, dithiothreitol 0.5, phenyl methyl sulfonyl fluoride, PMSF 0.3), and homogenized with a Dounce homogenizer. Proteins were quantified as described above. Serial dilutions of the lysates were subjected to SDS polyacrylamide (4–20% gradient) gel electrophoresis and proteins electrophoretically transferred to nitrocellulose membranes. Filters were blocked (1 h in 5% non-fat dry milk/PBS at room temperature) and subsequently incubated for 1 h at room temperature with either 1 µg ml^{-1} anti-phospholamban monoclonal antibody (Upstate Biotechnology), or 5 µg ml^{-1} rabbit polyclonal anti-SERCA antibody. The SERCA antibody (Zymed) was raised against a peptide previously utilized to generate anti-SERCA antibodies [23]. Membranes were washed and incubated for 1 h with either goat anti-mouse IgG (phospholamban) or goat anti-rabbit IgG (SERCA) conjugated to horseradish peroxidase. Visualization was achieved using a chemiluminescence Western blotting detection system (Pierce) and X-ray film. Under our conditions phospholamban was detected predominantly as a pentamer. Developed film images were digitized using a Hewlett Packard Scan Jet 4C and quantified by ImageQuant software (Molecular Dynamics). Data are calculated as pixel value per microgram of protein for each of three to five protein masses per sample (control and IL-1β treated) and normalized to the mean of the control samples, arbitrarily set at 100%. Results are the average of four to seven experimental sets of data.

Statistical analyses

Results are presented as the mean±SE. Statistical analysis was performed using Student's *t*-test (for two groups) or one-way ANOVA (greater than two groups). Where appropriate, post-hoc multiple comparison testing was performed (Student-Newman-Keuls test) to test for differences between groups. A value of $P<0.05$ was accepted as significant.

Results

IL-1β induces a delayed alteration of the contractile properties and calcium transients of neonatal rat cardiomyocytes

We previously reported that neonatal rat cardiomyocytes exposed to IL-1β for 20 h (2 ng ml^{-1}) do not show significant changes in the peak contractile amplitude, maximal speed of contraction and maximal speed of relaxation, but are no longer responsive to isoproterenol [30]. However, when exposed to IL-1β for 3 days, cardiomyocytes exhibit marked alterations in basal contractile parameters (Fig. 1A), with reductions in amplitude of contraction, maximum speed of contraction and maximum speed of relaxation (Table 1).

Calcium transients were also followed as a contributor to alterations in contractile activity. Peak systolic calcium was measured as the peak Fura-2 fluorescence ratio. After 1 day of IL-1β treatment (2 ng ml^{-1}) we did not observe any modification in peak systolic Fura-2 fluorescence ratio (1.72±0.03 and 1.74±0.03; n=65 and 67, P=NS, for control and IL-1β-treated cells, respectively). However after 3 days, both the peak systolic calcium as well as the difference between diastolic baseline and peak systolic calcium were significantly decreased in the IL-1β-treated

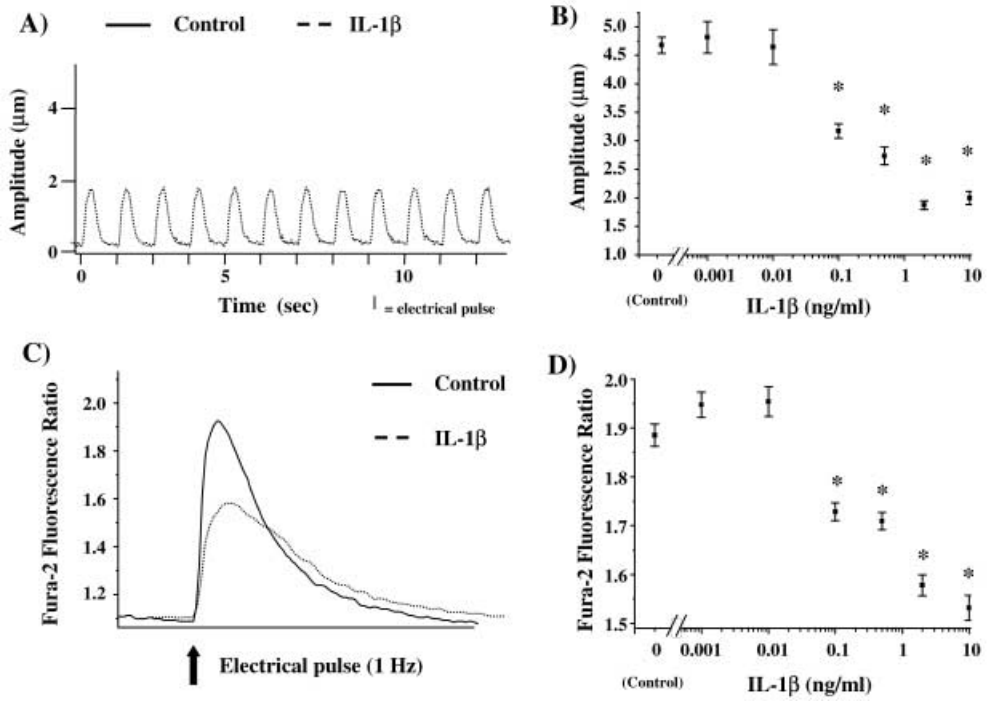

Fig. 1A–D Proinflammatory cytokine IL-1β decreases amplitude of contraction and calcium transients in neonatal rat cardiomyocytes. **A** Representative contraction trace from one control cardiomyocyte and one cardiomyocyte treated with IL-1β for 3 days (2 ng ml^{-1}). **B** Dose response of amplitude of contraction to 3 days treatment with various concentrations of IL-1β. n=44–71 cells per group; *P<0.05 by ANOVA (post hoc comparison) versus control. **C** Representative trace of calcium transient (ascertained from ratio of Fura-2 fluorescence) from one control cardiomyocyte and one cardiomyocyte treated with IL-1β for 3 days (2 ng ml^{-1}). **D** Dose response of maximum amplitude of calcium transient (ratio of Fura-2 fluorescence) to 3 days treatment with various concentrations of IL-1β. n=44–70 cells per group; *P<0.05 by ANOVA (post hoc comparison) versus control

Table 1 Effect of proinflammatory cytokine IL-1β on neonatal rat cardiomyocyte contractile properties and calcium transients. Data presented as mean±SE (*DEX* 3 μmol l^{-1} dexamethasone; *Ab* anti-IL-1β antibody; *NMMA* 1 mmol l^{-1} L-*N*-monomethyl arginine; *isop.* 1 μmol l^{-1} isoproterenol)

	Control	IL1β	IL1β+DEX	IL1β+Ab	IL1β+NMMA
Amplitude of contraction (μm)	4.67±0.14	1.87±0.08*	3.86±0.24	3.91±0.28	2.10±0.09*
Maximum speed of contraction (μm s^{-1})	46.7±2.33	24.0±0.99*	41.7±2.33	47.7±4.51	28.1±1.57*
Maximum speed of relaxation (μm s^{-1})	37.5±2.40	19.8±0.92*	34.4±3.00	33.2±2.97	20.3±0.98*
Peak systolic calcium (ratio units)	1.88±0.02	1.61±0.02*	1.88±0.09	1.81±0.02	1.61±0.02*
Peak systolic/diastolic calcium difference (ratio units)	0.742±0.022	0.453±0.019*	0.709±0.078	0.652±0.027	0.454±0.022*
n	70	69	19	20	57
Adrenergic response (maximum amplitude of control +isop/–isop)	1.32±0.08 (n=10)	1.03±0.02* (n=10)			1.02±0.014* (n=10)

*P<0.05 by ANOVA (post hoc comparison) versus control

myocytes (Fig. 1C and Table 1). Furthermore, it should be noticed that no significant differences were observed between experimental groups in the diastolic or baseline fluorescence ratios.

Additional evidence for the ability of IL-1β to alter cardiomyocyte contractile parameters in a time-dependent fashion was obtained from determining the rates of spontaneous contraction. While the above studies were performed under programmed electrical stimulation, the cardiomyocytes contract synchronously and spontaneously within 48 h of plating. After an additional 24 h of culture in control media, IL-1β was added and the spontaneous rates of contraction monitored over the next 3 days. A significant decrease in the spontaneous rates of contraction was evident beginning with 2 days exposure to IL-1β (Fig. 2).

Alterations in contractile activity and adrenergic responsiveness do not require no production and are reversible

The contractile response to IL-1β was blocked by preincubation of the cytokine with anti-IL-1β antibody, or by

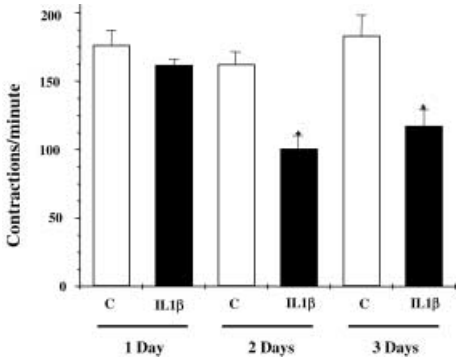

Fig. 2 IL-1β decreases rates of spontaneous contraction. Spontaneous rates of contraction were determined (see Materials and methods) in neonatal rat cardiomyocytes, control (*C*) and IL-1β (*IL1*β; 2 ng ml^{-1}) treated for 1–3 days. *n*=17–22 cells each day and treatment

co-treatment with dexamethasone (Table 1), consistent with other known effects of IL-1β [8, 30]. While it is well established that IL-1β can activate iNOS expression in cultured cardiomyocytes [46, 49], the contractile effects of IL-1β were not dependent upon NO production as culture of cells in L-NMMA blocked the level of nitrate production induced by IL-1β (Fig. 3A) but did not block the reduction in the amplitude of contraction, calcium transient, or adrenergic responsiveness (Table 1). Further evidence that the loss of adrenergic responsiveness was not mediated through an NO-dependent pathway was obtained by assessing the isoproterenol induction of cAMP in control and IL-1β-treated cells grown in the presence of arginine or L-NMMA (Fig. 3B). As previously observed, cells treated with IL-1β showed a marked reduction (relative to untreated cells) in the level of cAMP produced after 1 μmol l^{-1} isoproterenol challenge [6]. Cardiomyocytes grown in 1 mmol l^{-1} L-NMMA showed a similar reduction in the amount of cAMP produced in response to isoproterenol, suggesting that NO-dependent pathways do not mediate the reduced cAMP production in response to catecholamine challenge. Interestingly, the effects of IL-1β on contraction amplitude, amplitude of calcium transients, and adrenergic responsiveness were fully reversible after cells were cultured in cytokine-free media for an additional 3 days (Table 2), suggesting that IL-1β does not have an irreversible, grossly cytotoxic effect on these cells.

IL-1β does not alter the organization of contractile elements in neonatal rat cardiomyocytes

Alterations in contractile activity can arise from multiple perturbations in the mechanism affecting either calcium transients or sarcomere structure or function. To test the hypothesis that alterations in sarcomere structure mediate IL-1β-induced changes in contractile activity, we used anti-myosin heavy chain and phalloidin immunohistochemistry to examine sarcomeric organization in neonatal rat cardiomyocytes after 3 days treatment with control media or 2 ng ml^{-1} IL-1β. Both control and treated cells were observed to have well organized sarcomeric structures filling the majority of the cytoplasmic area. Figure 4 demonstrates a cell treated with IL-1β for 3 days, with control cells appearing indistinguishable from treated cells. Thus, the alterations in contractile activity arising from IL-1β exposure are unlikely to be arise from gross alterations in sarcomere number or organization.

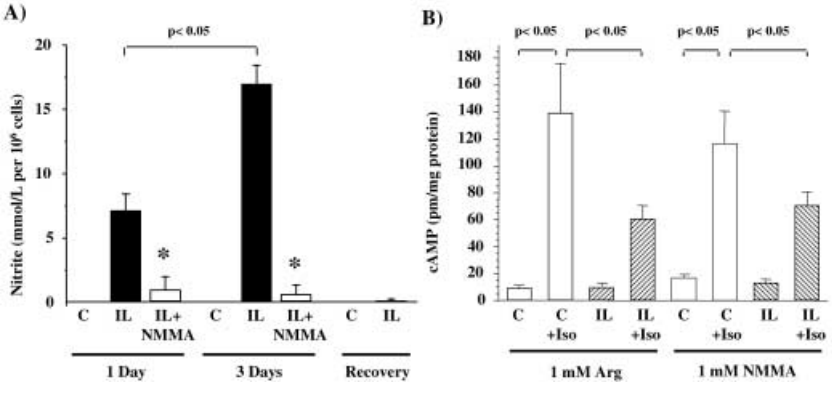

Fig. 3A, B IL-1β inhibits adrenergic responses in a nitric oxide (NO)-independent fashion. **A** One and three days treatment with IL-1β (2 ng ml^{-1}) induces NO production as measured by media nitrites and this production is blocked by growth in media in which 1 mmol l^{-1} arginine is replaced with 1 mmol l^{-1} N^{G}-monomethyl-L-arginine (NMMA). Nitrite concentration was below the detection threshold in control groups at all three time points. Nitrite production returns to control levels after a 4-day recovery period in IL-1β-free media. *n*=3–9 samples from 1–3 preparations of cells. *P<0.05 for IL-1β+NMMA versus IL-1β treated cells for the same treatment duration. **B** IL-1β inhibits production of cAMP in response to 1 mmol l^{-1} isoproterenol (cells grown in media containing 1 mmol l^{-1} arginine or 1 mmol l^{-1} NMMA plus solvent or IL-1β (2 ng ml^{-1} 3 days) prior to a 15-min challenge with 1 mmol l^{-1} isoproterenol and processing as described in Materials and methods). This inhibition is not blocked by culture in NMMA. *n*=6 (3 replicates from 2 preparations of cells) per treatment group; P<0.05 by ANOVA (post hoc comparison) versus control (*C*) plus isoproterenol (*Iso*). *IL* Treatment with 2 ng ml^{-1} IL-1β

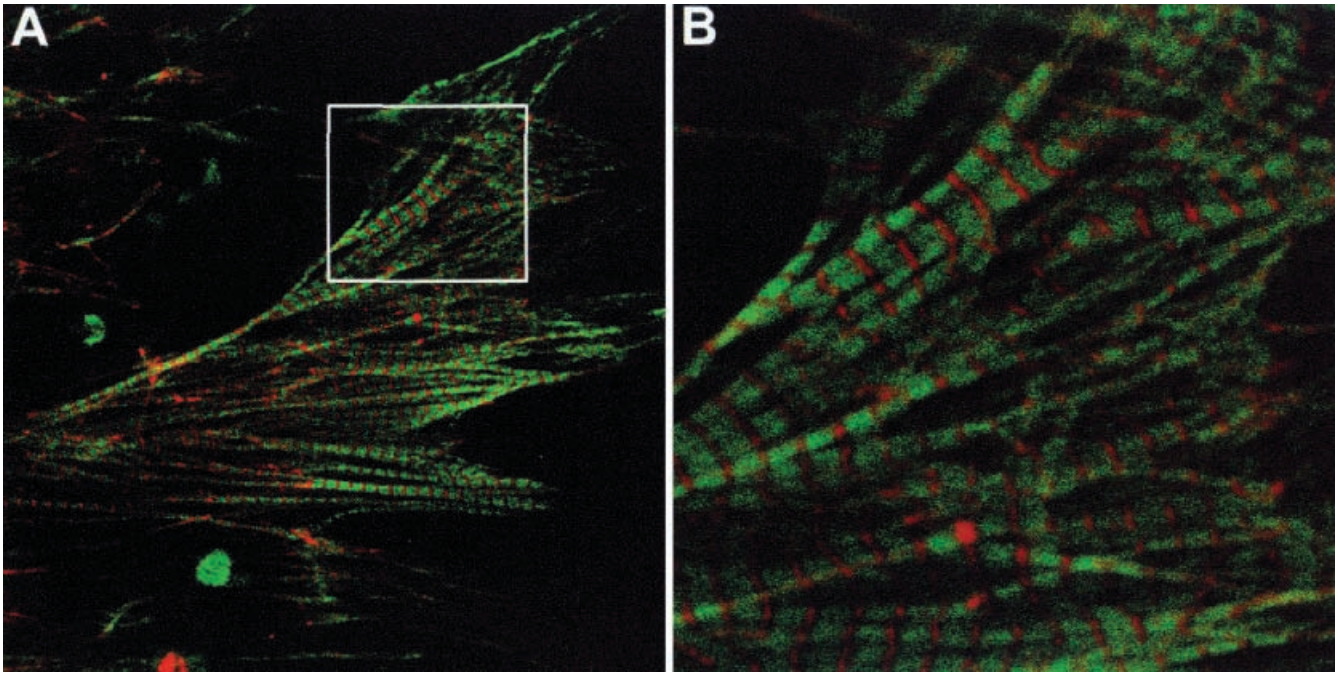

Fig. 4A, B IL-1β does not alter sarcomere structure. Cardiomyocytes treated with IL-1β (2 ng ml^{-1}, 3 days) and processed for visualization of filamentous actin (phalloidin, *red*) and sarcomeric myosin (MF20 antibody, *green*) show well-organized sarcomeres filling the cytoplasm and extending to the periphery of the cells. Control cells were indistinguishable from IL-1β-treated cells in terms of extent and organization of sarcomeres. **A** Photographed at 60× magnification. **B** High magnification showing predominantly parallel organization of sarcomeres with regular striations of alternating actin and myosin-rich regions

Table 2 Effect of 3 days recovery from IL-Iβ on contractile properties and calcium transients. Data presented as mean±SE (*IL1β* 3-day culture in control media after 3-day culture in media containing IL-1β). All parameters NS versus control

	Control	IL1β
Amplitude of contraction (μm)	3.39±0.12	3.33±0.12
Maximum speed of contraction (μm s^{-1})	38.68±2.85	36.90±1.71
Maximum speed of relaxation (μm s^{-1})	27.27±2.00	27.03±1.52
Peak systolic calcium (ratio units)	1.78±0.02	1.77±0.02
Peak systolic/diastolic calcium difference (ratio units)	0.623±0.021	0.616±0.021
n	44	44
Adrenergic response (maximum amplitude of control +isop/–isop)	1.30±0.09	1.29±0.06
n	10	10

IL-1β decreases the expression of genes regulating calcium transients in neonatal rat cardiomyocytes

The alterations in contractile activity and calcium transients suggest that IL-1β may alter function or expression of proteins that regulate calcium flux. Indeed, we have previously reported that IL-1β (2 ng ml^{-1}, 20 h) decreases phospholamban and SERCA mRNA transcripts levels and phospholamban protein in rat neonatal cardiomyocytes, whereas others have reported down-regulation of transcripts encoding other proteins which regulate calcium transients [30, 51]. We extended these studies to Northern and Western blot analysis of calcium homeostasis regulating genes in cardiomyocytes exposed to IL-1β for 3 days.

In accordance with our previous data, we observed a significant decrease in the level of phospholamban and SERCA transcripts relative to untreated controls after both 1 day and 3 days of IL-1β treatment, whereas GAPDH transcript levels remained unchanged [30] (Fig. 5). Relative to control cultures maintained in parallel, the decrease in phospholamban and SERCA transcripts was reversed after an additional 3 days of culture in the absence of cytokine. Three days exposure to IL-1β also decreased the transcript levels for the calcium-release channel, whereas transcript levels for calsequestrin were not altered (Fig. 6). We also examined the expression of genes typically responsive to hypertrophic stimuli. Transcript levels for both atrial natriuretic factor (Fig. 6D) and β-MHC (Fig. 6E) remained unchanged after 3 days treatment with IL-1β (2 ng ml^{-1}). In contrast, α-MHC transcript levels were significantly reduced after 3 days treatment with IL-1β (Fig. 6F). The selective alteration in transcript levels, as well as the normalization of transcript levels after cessation of cytokine treatment again suggests the lack of a gross cytotoxic effect of IL-1β on cultured cardiomyocytes.

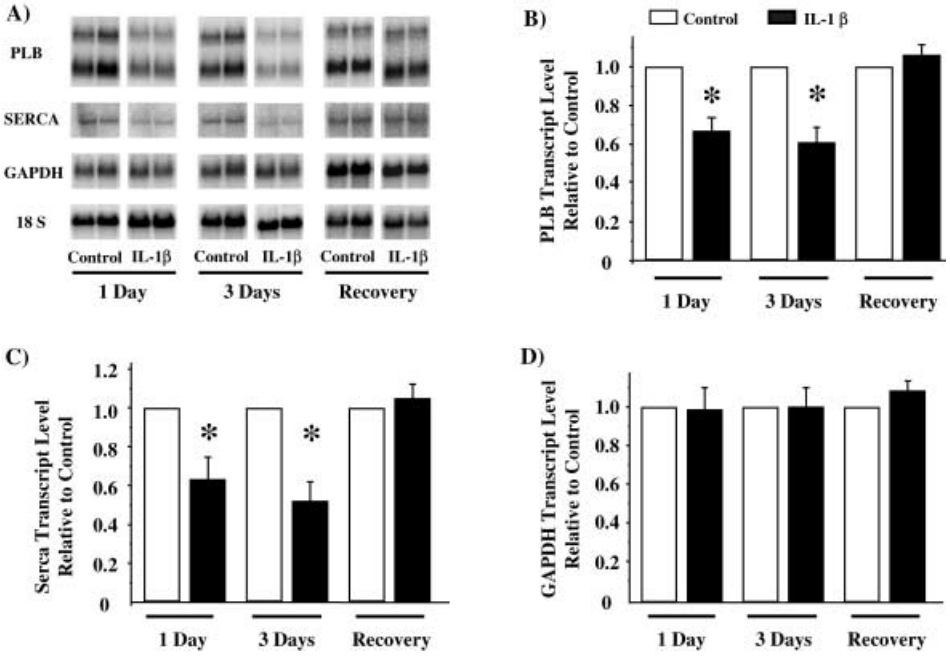

Fig. 5A–D IL-1β induces a reversible decrease in transcripts for phospholamban and sarcoplasmic reticulum calcium ATPase (SERCA). **A** Representative Northern blot image of RNA isolated from cardiomyocytes treated with solvent (PBS, *Control*) or IL-1β (2 ng ml^{-1}) for three different intervals; *1 day* or *3 days*, or 3 days followed by a 4 day recovery in media lacking IL-1 (*Recovery*). Hybridization probes include phospholamban (PLB), SERCA, glyceraldehyde phosphate dehydrogenase (*GAPDH*), and 18S ribosomal RNA (*18S*). Quantitative measurement (performed as described in Materials and methods) of transcript levels for **B** phospholamban, **C** SERCA, and **D** GAPDH transcript levels during three treatment intervals. For all three hybridizations n=18 (6 replicates from 3 preparations of cells) for 1-day and 3-day treatments, and n=9 (3 replicates from 3 preparations of cells) for recovery treatments. *P<0.03 (or less) versus control of same treatment interval

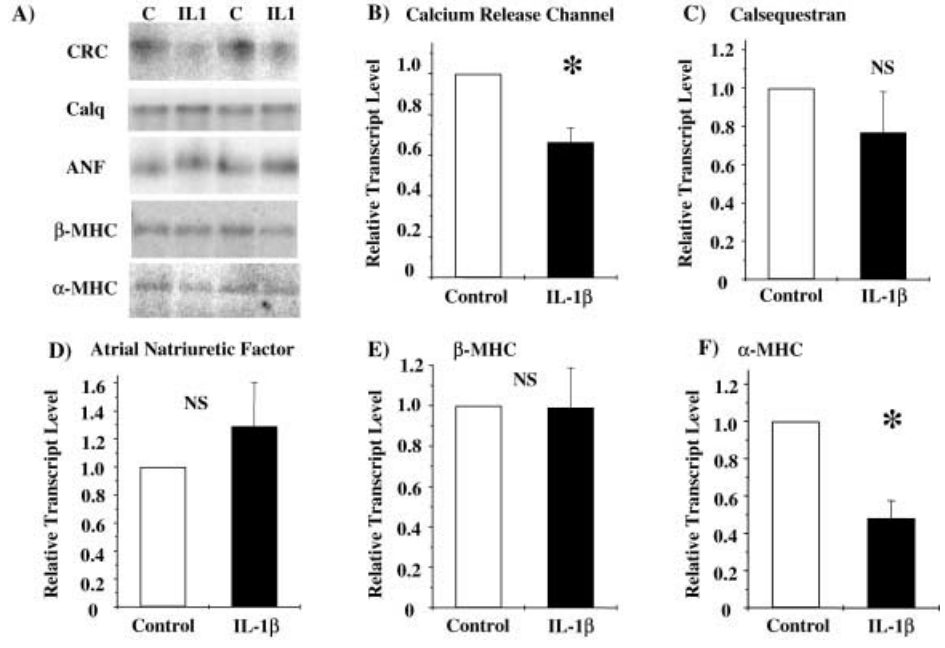

Fig. 6A–F IL-1β selectively alters neonatal rat cardiomyocyte gene expression. **A** Representative Northern blot image of RNA isolated from cardiomyocytes treated with solvent (PBS; control) or IL-1β (2 ng ml^{-1}) for 3 days. Hybridization probes include calcium-release channel (*CRC*); cardiac calsequestrin (*Calq*); atrial natriuretic factor (*ANF*); beta-myosin heavy chain *(β-MHC)*, and alpha-myosin heavy chain (α-*MHC*). Control (*C*); IL-1β treated (*IL1*) Quantitative measurements of transcripts for **B** calcium release channel, **C** calsequestrin, **D** atrial natriuretic factor, **E** β-MHC, and **F** α-MHC performed as described in Materials and methods. n=5 cell preparations per group (control or IL-1β treated) except for β-MHC where n=4 per group; *P<0.04 (or less) versus control. *NS* not significant

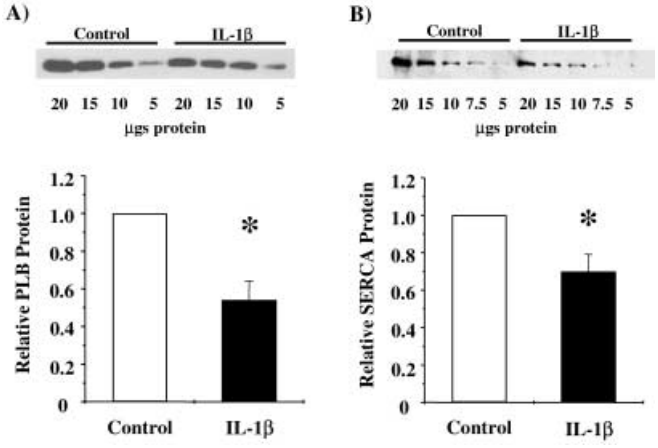

Fig. 7A, B IL-1β decreases phospholamban and SERCA protein levels. **A** *Top panel* Representative Western blot image of serial dilutions of protein isolated from control or IL-1β-treated (2 ng ml^{-1}; 3 days) cardiomyocytes and challenged with anti-PLB antibody as described in Materials and methods. *Bottom panel* Quantitative measurement of Western blot images from five cell preparations per group. **B** *Top panel* Representative Western blot image of serial dilutions of proteins isolated from control or IL-1β-treated (2 ng ml^{-1}; 3 days) cardiomyocytes and challenged with anti-SERCA antibody as described in Materials and methods. *Bottom panel* Quantitative measurement of Western blot images from seven cell preparations per group. *P<0.04 (or less) versus control

Since some reports suggest that a lack of parallel changes can occur in the proteins and transcripts encoding calcium regulatory genes, we also performed Western blot analyses to assess alterations in protein levels for phospholamban and SERCA in neonatal rat cardiomyocytes treated with control media or IL-1β for 3 days [32, 44]. Similar to transcript levels, both phospholamban and SERCA proteins were decreased in cells exposed to IL-1β for 3 days (Fig. 7).

Discussion

In this study we used a well-characterized model of spontaneously contracting neonatal rat cardiomyocytes to assess the effect of chronic (3-day) exposure to physiologic levels of the pro-inflammatory cytokine IL-1β. We hypothesized that responses occurring after continued exposure to physiologically relevant doses of cytokines would mirror the changes that occur in the myocardium of animals with prolonged exposure to proinflammatory cytokines. To perform these analyses we correlated the cytokine effects on cell structure, contractile activity, calcium transients, adrenergic responsiveness, and expression of relevant genes at both the transcript and protein level in order to assess the physiologic significance of changes in gene or protein expression. These studies have resulted in three principal findings.

First, 3-day exposure to IL-1β induced a delayed decrease of basal contractile properties and calcium transients of neonatal rat cardiomyocytes, and the effects were reversible upon removal of IL-1β. Previous reports observed a decreased contractile response with acute (minute) exposure to IL-1β [4, 8, 25, 49, 54], or alternatively a significant increase in spontaneous beating rates of cardiomyocytes [37]. In these studies, extremely high IL-1β concentration were used (range 10–500 ng ml^{-1}) and acute effects of the cytokine were studied. Furthermore, the response of IL-1-treated cells to adrenergic challenge was either a delayed loss of adrenergic response [17], or an enhanced isoproterenol contractile response (18 h exposure) [4]. By contrast, in the present study we used longer exposure to more physiologically relevant IL-1 concentration, in an attempt to mimic pathologic conditions such as advanced heart failure. Our studies show that short (1 day) exposure led to no alterations in basal contractile profiles with a loss of adrenergic responses, but that 3 days exposure led to a reversible decrease in basal contractile profiles and calcium transients with a continued loss of contractile response to isoproterenol [30]. This reversible loss of adrenergic response confirms earlier reports [17]. However, the method used in those studies (attachment of cells to free-floating membranes) made it impossible to directly compare the basal contractile properties of treated and untreated cell preparations, although their relative response to isoproterenol challenge could be determined [18].

A second significant finding is that the changes in contractile activity and adrenergic response did not occur through NO-dependent pathways. We and others have previously observed that while IL-1β increases iNOS activity and cGMP production, changes in gene expression or cardiomyocyte hypertrophy did not require iNOS activity [30, 51]. Similarly, although a decrease in basal contractile and calcium transient parameters was apparent after 3 days of IL-1β treatment, the inclusion of the NOS inhibitor NMMA in the culture media (which blocked both the increase in cGMP [30] and media NO_2 production) did not alter the loss of basal contraction, calcium transients, or responsiveness to isoproterenol. While we confirmed that prolonged IL-1β exposure markedly inhibited (but did not prevent) the isoproterenol stimulation of cAMP production [17], a novel observation was that NMMA did not abrogate this inhibition. These results contrast with reports that in adult cardiomyocytes, products of activated macrophages stimulated NO production and produce a loss of adrenergic contractile responsiveness and an inhibition of cAMP production that is prevented with the iNOS inhibitor NMMA [21]. However, our results are consistent with reports that the negative inotropic effects of other proinflammatory cytokines (such as TNF-α) on adult feline cardiomyocytes are not NO mediated [57], and that physiologic concentrations of NO do not elicit a negative inotropic effect [55]. While the diverse conclusions concerning the role of NO in alterations of basal contractile parameters may arise partially from acute versus prolonged exposure, culture conditions [53], or developmental stage of cardiomyocytes, our neonatal cardiomyocyte system does not support a role for NO in IL-1β-induced alterations of basal contraction or adrenergic responsiveness.

A third finding is that IL-1β-induced alterations in contractile properties may arise from changes in expression of genes encoding proteins important to calcium cycling. Chronic exposure to IL-1β was associated with a decreased amplitude of calcium transients that occurred concomitantly with the alterations in contraction. Decreased expression of transcripts encoding phospholamban and SERCA, already apparent after 1 day of IL-1β exposure, persisted through 3 days of treatment and was accompanied by a decrease in phospholamban and SERCA protein levels as well [30]. Intracellular calcium homeostasis and excitation–contraction coupling are regulated mainly by the function of the sarcoplasmic reticulum (SR). During relaxation, Ca^{2+} is transported from the cytosol into the lumen of the SR by the SR Ca-ATPase (SERCA). The activity of SERCA is regulated by phospholamban: decreases in the levels of phospholamban or increases in its phosphorylation state results in an increase of SERCA Ca^{2+} affinity and augment contractile parameters. Subsequently, in response to Ca^{2+} influx through L-type Ca^{2+} channels, Ca^{2+} loaded in the SR is released through the calcium release channel (ryanodine receptor) and initiate muscle contraction. The IL-1β-induced alterations in contractile properties and changes in expression of SR proteins described in the present work parallel that of end-stage human heart failure where reduced Ca^{2+} transients, alterations in SR proteins expression and lower levels of SR Ca^{2+} uptake have been associated with contractile abnormalities [19, 33, 41]. Specifically, reduced SERCA and phospholamban gene and protein expression have been described in these patients [31, 32]. The decrease in SERCA activity results in a decrease of cytosolic calcium removal and a subsequent decrease in calcium release from the SR, with reduced amplitude of calcium transients and reduced systolic and diastolic performance [20]. Alternatively, the decrease in phospholamban expression is expected to relieve inhibition of the SR Ca^{2+} pump, which should enhance and not diminish contractility. In fact, a human heart failure study reported a greater decrease of SERCA protein level as compared to that of phospholamban [32], leading to an increased ratio of phospholamban to SERCA, with a resultant greater inhibition of SERCA activity. In the present work, we observed a similar (roughly 50%) decrease in phospholamban and SERCA protein expression. Therefore, additional pathophysiological processes might participate in the altered calcium homeostasis we observed. Indeed, the decreased myocardial performance of terminal human heart failure has been recently associated with reduction in the phosphorylation state of phospholamban [45]. Cytokine challenge can also decrease the phosphorylation levels of phospholamban in spontaneously beating rat neonatal cardiac myocytes [58] and may participate in our observations of IL-1-induced alterations of intracellular Ca^{2+} cycling and force of contraction.

It should be noticed that the alterations in contractility and calcium homeostasis were observed only after 3 days of cytokine treatment in the present work, when decreased expression of SERCA and phospholamban were present after 20 h of IL-1 challenge. As stated above, cytokine-induced reduction in the phosphorylation state of phospholamban may partly explain this delayed alteration of SR function and contractility. Indeed, in a model of septic shock induced by cecal ligation in rats, where high levels of proinflammatory cytokine (including IL-1β) are released, phospholamban phosphorylation increased in the early hyperdynamic phase of sepsis and subsequently was markedly decreased in the late hypodynamic phase of sepsis. This late decrease in protein phosphorylation was accompanied by a significant decrease in SERCA activity and SR Ca^{2+} uptake [56]. Second, the half-life of SERCA has been estimated to be 14 days [10]. Therefore, it may take several days until the IL-1β-induced decrease in protein expression becomes functionally significant. Third, recent studies have implicated oxygen-derived free radicals as possible mediators of inflammatory cytokine-induced cell injury: IL-1β exposure induced marked and prolonged myocardial dysfunction in dogs in vivo with simultaneous production of superoxide anion [5]. Furthermore, reactive oxygen species can inactivate the SERCA through oxidation of protein cysteine residues [22]. Progressive oxidation and inactivation of the SERCA might also participate in the delayed alteration in contractility and calcium homeostasis we observed. Additionally, the observed reduction in rates of spontaneous contraction which becomes significant only after 2 days of cytokine challenge is also consistent with the delayed alteration in EC-coupling observed in the present study. However, we cannot exclude that other mechanisms such as differential expression of other proteins involved in calcium homeostasis or in the contractile apparatus of the cell might explain the observed delayed alteration in contractility.

While changes in contractile properties could arise from alterations in the expression of proteins that constitute the myofiber or the organization of the sarcomere, IL-1β did not cause gross alterations in sarcomere structure. Whereas cells with minimal contractile activity grown in the absence of serum show an enhanced organization of myofibers in response to IL-1β, we observed that contracting cultured cells already posses well-organized sarcomeres and lacked a hypertrophic response to IL-1β [30, 39]. Nonetheless, under both conditions a decrease in α-MHC transcript levels was observed and could indicate an altered myofiber protein composition, contributing to modified contractile properties [29, 40].

Although many studies support a role for the proinflammatory cytokine TNF-α in cardiac diseases such as heart failure [9], evidence that IL-1β also mediates alterations in cardiac function remains less definitive but suggestive. Elevated plasma levels of IL-1β have been observed in patients with heart failure consequent to coronary artery disease or hypertension [50], while enhanced IL-1β RNA and protein is detected in the myocardium and coronary arteries of patients with idiopathic dilated cardiomyopathy [13], and in the coronary arteries of patients with ischemic heart disease

[14]. Animal studies also suggest a possible role for IL-1β in experimental heart failure arising from chronic hypertension [48], in cardiac remodeling after coronary artery ligation [38], and in dilated cardiomyopathy arising from viral myocarditis [47]. Previous in vitro studies also suggest that IL-1β can induce alterations in cultured neonatal rat cardiomyocytes [17, 28, 30, 39, 40, 51] and the present work demonstrate that this cytokine can recapitulate in vitro some of the major phenotypic modifications observed in the failing myocardium.

In summary, these results suggest that IL-1β has a potential role in regulating myocardial contractile activity through altered expression of calcium handling genes. Although neonatal cardiomyocytes are a convenient and well characterized model system in which to identify pathways through which cytokines alter contractile properties, we recognize that culture conditions and developmental maturation of cardiomyocytes may be important parameters in determining the cardiomyocyte response to proinflammatory cytokines. Therefore, the role of IL-1β in modifying adult cardiomyocyte function needs further confirmation. Despite these limitations, the present study suggests that IL-1β may mediate pathophysiological changes in various cardiovascular diseases.

Acknowledgements Dr. Combes was supported by the AMSTER association, the Fédération Française de Cardiologie and the Laboratories SERVIER, and funds from the Bill Hillgrove Cardiology Fellowship. We are grateful to Ms. Debbie Williams for assistance with nitrite measurements; Ms. Yi Qin Feng, Ms. Beth Will, and Mr. George Bounoutas for preparation of cardiomyocytes; and Dr. Andrzej Janczewski for comments on the manuscript. All experimental procedures were carried out under sterile conditions and were in accordance with the *Guide for the Care and Use of Laboratory Animals* (7th edn) of the National Research Council and approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

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