Ca²⁺-induced PARP-1 activation and ANF expression are coupled events in cardiomyocytes

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The nuclear protein PARP-1 [poly(ADP-ribose) polymerase-1] is activated in cardiomyocytes exposed to hypoxia causing DNA breaks. Unlike this stress-induced PARP-1 activation, our results provide evidence for Ca²⁺-induced PARP-1 activation in contracting newborn cardiomyocytes treated with growth factors and hormones that increased their contraction rate, induced intracellular Ca²⁺ mobilization and its rhythmical and transient translocation into the nucleus. Furthermore, activated PARP-1 up-regulated the activity of phosphorylated ERK (extracellular-signal-regulated kinase) in the nucleus, promoting expression of the Elk1 target gene c-fos. Up-regulation of the transcription factor c-Fos/GATA-4 promoted ANF (atrial natriuretic factor) expression. Given that expression of ANF is known to be implicated in morphological changes, growth and development of cardiomyocytes, these results outline a PARP-1-dependent signal transduction mechanism that links contraction rate and Ca²⁺ mobilization with the expression of genes underlying morphological changes in cardiomyocytes.

Key words: atrial natriuretic factor (ANF) expression, calcium-induced poly(ADP-ribose) polymerase (PARP)-1 activation, calcium translocation, c-fos expression, newborn cardiomyocyte.

INTRODUCTION

Cardiomyocyte functioning is predominantly regulated by Ca²⁺ mobilization in the cytosol. During contraction the sarcoplasmic reticulum serves as a reservoir from which Ca²⁺ ions are released into the cytosol, whereas during relaxation Ca²⁺ ions from the cytosol are sequestrated by the Ca²⁺-ATPase pump and its regulatory protein phospholamban into the sarcoplasmic reticulum lumen [1–3]. Release of Ca²⁺ into the cytosol is dependent mainly on receptor-gated stores in the sarcoplasmic reticulum lumen, as well as on Ca²⁺ entry through Ca²⁺ channels in the cell membrane [4].

We have described previously in neuronal cells, activation of the DNA-bound protein PARP [poly(ADP-ribose) polymerase-1 by Ca²⁺ released from IP₃ (inositol trisphosphate)-gated stores located in the nuclear envelope [5,6], PARP-1 is the most abundant member of the PARPs [7], which catalyse a post-translational modification (polyADP-ribosylation) of nuclear proteins. This modification is initiated by ADP-ribose transferase activity and proceeds via polymerization of ADP-ribose moieties into long-branched negatively charged ADP-ribose polymers. PARP-1 activation takes part in DNA repair [8], as well as in chromatin remodelling and gene expression by a variety of mechanisms [7]. One of them is polyADP-ribosylation of a prominent PARP-1 substrate, linker histone H1, which causes relaxation of the highly condensed chromatin structure, rendering the DNA more accessible to transcription factors and DNA repair enzymes [9–11].

In addition to numerous findings indicating a pivotal role of DNA single-strand breaks in PARP-1 activation [7], our previous findings in cell-free systems and in neuronal cells have disclosed an alternative mechanism of PARP-1 activation, which operates in the absence of DNA damage, and even in the absence of DNA [5,6,12,13]. In cell-free systems, recombinant ERK2 (extracellular-signal-regulated kinase 2) and recombinant PARP-1 highly enhanced each other’s activity. In addition, the activity of phosphorylated ERK in the nuclei of neuronal cells was up-regulated by polyADP-ribosylated PARP-1 [12–14]. These findings actually implicated the activation of PARP-1 in networks of signal transduction mechanisms activated by the MEK (MAPK (mitogen-activated protein kinase)/ERK kinase)/ERK phosphorylation cascade [5,6,12–16]. In neuronal cells, activated PARP-1 up-regulated ERK-catalysed phosphorylation of the transcription factor Elk1, thereby inducing core histone acetylation and the expression of Elk1 target genes [12–14,17,18].

If PARP-1 activation in cardiomyocytes similarly up-regulates the Elk1 target gene c-fos, it may also up-regulate the formation of the transcription factor c-Fos/GATA-4, which is implicated in the response of cardiomyocytes to hormones and growth factors [19–21]. In the present paper, we examined this possibility. The enhancer/promoter region of the ANF (atrial natriuretic factor) gene contains several GATA-4-binding sites that are conserved among different species including rats, mice and humans [22]. ANF is expressed throughout embryonic and fetal development in both atrial and ventricular cardiac cells, and is essential for growth, differentiation and survival of cardiomyocytes [20–23]. The growth of cardiomyocytes has been associated with the expression of the immediate early gene c-fos, the activity of GATA-4 and ANF expression [23].

In the present study, we have examined the possibility that ANF expression in contracting cardiomyocytes of newborn rats

Abbreviations used: ANF, atrial natriuretic factor; AngII, angiotensin II; ARBP, acidic ribosomal phosphoprotein; AT₁, AngII type 1; BAPTA/AM, 1,2-bis-(o-aminophenoxylethane-N,N,N′,N′-tetra-acetic acid tetrakis(acetoxymethyl ester); CBP, CREB (cAMP-response-element-binding protein)-binding protein; ChIP, chromatin immunoprecipitation; dBcAMP, dibutyryl cyclic AMP; dBCAMP, dibutyryl cAMP; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular-signal-regulated kinase; Iso, isoprenaline; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PAR, poly(ADP ribose); PARP, poly(ADP-ribose) polymerase; rhod-2/AM, rhod-2 acetoxymethyl ester; siRNA, short interfering RNA; RT, reverse transcription.

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is mediated by the PARP-1-dependent up-regulation of c-fos expression, implicating Ca2⁺-induced activation of PARP-1 in a mutual up-regulation of PARP-1 and ERK activities in the nucleus of the cardiomyocyte.

**EXPERIMENTAL**

**Primary cultures of newborn cardiomyocytes**

Cultures were prepared from cardiomyocytes obtained from the cardiac ventricles of 1–2-day-old Sprague–Dawley rats, essentially as described previously [24]. The cultures were seeded in collagen-coated six-well dishes and incubated in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% fetal bovine serum, 10 μg/ml transferrin, 10 μg/ml insulin, 100 μg/ml BSA and 1% penicillin/streptomycin (Biological Industries), at 37 °C in a humidified atmosphere of 5% CO₂ and 95% O₂. The cardiomyocytes were spontaneously contracting. Cells were exposed to growth factors or hormones in this buffer, after being kept for 24 h in DMEM deprived of all supplements except for 0.5% fetal calf serum (starvation).

The newborn rats were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Approval for the present study was granted by the Tel-Aviv University Ethics Review Board.

**Isolation of crude nuclei**

Crude nuclei were isolated from lysed cardiomyocytes as described previously for brain cortical neurons [5,25]. Briefly, cultured cardiomyocytes were washed with ice-cold PBS and homogenized on ice in isotonic sucrose solution containing 0.1 mM PMSF. Cells were lysed in a hypotonic solution (50 mM Tris/HCl, pH 7.4) containing 0.1 mM PMSF. Pellets obtained after centrifugation (900 g for 10 min at 4 °C) contained isolated crude nuclei (nuclei containing remnants of endoplasmic reticulum).

[32P]polyADP-ribosylation of proteins in isolated nuclei of cardiomyocytes

Isolated nuclei were incubated for 5–15 min with 1000 Ci/mmol [32P]NAD (1 μCi/sample; Amersham Biosciences) and 2.3 mM MgATP at 37 °C in a solution containing 60 mM Tris/HCl (pH 7.4), 1 mM MgCl₂ and 0.8 mM DTT (dithiothreitol) in de-ionized water [5]. Solutions used in these experiments contained 25–30 mM Ca²⁺ (determined by atomic absorption). Nuclear proteins were extracted by high-salt protein extraction buffer. The proteins were separated by electrophoresis on 0.1% SDS/polyacrylamide gels. After electrophoresis, the gels were fixed with 10% methanol/10% acetic acid, immersed in 0.3% agarose, then the DNA was removed by treatment with 0.5 M NaOH for 30 min. The proteins were then transferred to nitrocellulose membranes. [32P]polyADP-ribosylated proteins on nitrocellulose membranes were detected by autoradiography and immunolabelled for detection of PARP-1 (MC1522; Serotec) and PAR [poly(ADP-ribose)] polymers (Alexis Biochemicals #96-10-04).

**In situ immunofluorescent labelling of proteins in cultured cardiomyocytes**

Cardiomyocytes were cultured on coverslips coated with collagen/gelatin. Anti-PAR polymer polyclonal antibody, diluted 1:300 (96-10-04; Alexis), was applied to cardiomyocytes that had been rapidly fixed by treatment for 10 min with ice-cold methanol/acetone (1:1, v/v) and thoroughly washed. After incubation for 2 h with the primary antibody at room temperature (25 °C), the cardiomyocytes were washed with PBS containing 0.1% Tween 20 and exposed for 1 h at room temperature to the secondary fluorescent antibody [Jackson Dylight (green) and Cy3™-3 (red)]. PAR polymers formed on the nuclear proteins and phosphorylated ERK (Cell Signaling #9106) were detected by confocal microscopy.

**Live imaging of intracellular Ca²⁺ migration by a cell-permeant fluorescent Ca²⁺ chelator (fluorophore)**

The Ca²⁺ fluorophore rhod-2/AM (rhod-2 acetoxyethyl ester) form was loaded (4.5 μM, incubation for 30 min at 25 °C in the dark) into cultured cardiomyocytes on collagen-coated glass coverslips. The fluorescence signals of Ca²⁺-bound rhod-2 (excitation wavelength 540 nm, emission wavelength >570 nm) were collected through appropriate filters above 520 nm and monitored by inverted confocal microscopy using a Zeiss LSM 410 equipped with a 25 mW krypton/argon laser (488 nm and 568 nm lines) and a 10 mW helium/neon laser (633 nm line). For imaging we used a Zeiss C-Apochromat 403 NA (numerical aperture) 1.2/water-immersion lens (Axiovert 135 M).

**Examination of single-strand DNA breaks by alkaline gel electrophoresis**

Examination of single-strand DNA breaks by alkaline gel electrophoresis provides a sensitive and rapid method for direct quantification of breaks in single strands of DNA [26]. DNA was isolated from the nuclei of cardiomyocytes using the Hirt procedure [27]. The migration of equivalent amounts of single-stranded DNA in 1% alkaline agarose gel by electrophoresis [27] was detected by staining with 1 μg/ml ethidium bromide. Stained gels were photographed under UV illumination.

**Hypoxic conditions**

Cardiomyocyte cultures (5–7 days old) were washed from the medium with PBS containing 5 mM Hepes (pH 7.4) and transferred to glucose-free DMEM before exposure to hypoxia at 37.8 °C for 2–3 h. The cultured cardiomyocytes were placed in a hypoxic incubator in which the atmosphere was replaced by 100% argon [28,29]. In the present study, hypoxic damage in the treated cardiomyocytes was characterized by DNA single strand breaks.

**Contraction frequency in treated cardiomyocytes**

Contractility of the cardiomyocytes was measured by placing the culture dishes in a specially designed Plexiglas chamber, as described previously [30]. The chamber was placed on the stage of an inverted phase-contrast microscope (Olympus). Contractions of the cardiomyocytes, without or during stimulation, were measured using a video motion detector system. Analogue tracing was recorded with an oscilloscope connected through a specially designed interface to an IBM computer, and kinetic data were analysed as described previously [30].
Silencing of PARP-1 by siRNA (short interfering RNA)

The nucleotide sequences of the siRNA target sites in the PARP-1 gene corresponded to the sequences of rat PARP-1 cDNA. Two sequences comprising nucleotides 800–807 (5'-AAGAUAGA-GCCUAGACCCGAA-3') and 890–897 (5'-AAGCCUCGCCGCGAUAAAU-3') in the rat PARP-1 catalytic domain near the conserved active site were used to silence the expression of PARP-1. As a control we used the non-targeting siRNA #2 (Dharmacon #D-001810-10-05). In all experiments the cells were transfected with siRNA (100 nM) in serum-deprived medium using the X-tremeGENE siRNA transfection reagent (Roche Diagnostics #04-476-093-001). Changes in the expression of PARP-1 were measured 48–72 h after siRNA insertion.

RT (reverse transcription)–PCR profiling of relative expression levels of c-fos in stimulated rat cardiomyocytes

Expression of the c-fos gene was assessed by RT of nucleotides 1734–1853 in c-fos, using the forward primer 5'-GTCCTGGCATTAGGTTGTC-3' and the reverse primer 5'-GCTGAAGAGCTACAGTAGT-3'. RNA was prepared (RNaseasy Mini Kit, Qiagen) 15, 30, or 60 min after treatment with 1 μM AngII (angiotensin II) for 10 min at 37°C. RNA samples were treated with DNase-I (Ambion #1906) to avoid the presence of DNA residues. cDNA was prepared from the RNA samples with the aid of SuperScript II kit (Invitrogen, #48-90-01). Primer pairs underwent preliminary testing by conventional RT–PCR on one strand of the cDNA samples. RT–PCR was assayed (cycling program: 95°C for 15 s, 60°C for 30 s and 72°C for 30 s) with the aid of the 7300 PCR System and the SYBR Green PCR Master Mix (Applied Biosystems). After RT–PCR, the dissociation curve was analysed to confirm that a single gene product was synthesized. The relative quantities of c-fos per sample were calculated in relation to each of two reference genes in rat S14 (hepatic lipogenic S14 protein [31]; 5'-TCTCTCCTCGATGC-3' and 3'-ACCTTCTCTGGTCGTCA-5'), ARBP (acidic ribosomal phosphoprotein [32]; 5'-CTGACTAC-ACCTTCCACCTG-3' and 3'-TCCGACTCTTTGGCTTC-5') and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; 5'-CTGGAAAGCTGTGGCGTGATGG-3' and 3'-CTGGAAAGCTGTGGCGTGATGG-5') using the ΔΔCt formula as described previously [34].

ChIP (chromatin immunoprecipitation) assay

Using the ChIP assay [35] we were able to detect c-Fos, GATA-4, CBP and acetylated core histone H4 adjacent to the promoter of the expressed ANF gene in myocytes treated by stimulation activating PARP-1 and MEK/ERK phosphorylation. In our protocol, proteins in the chromatin of cultured cardiomyocytes were cross-linked to DNA by formaldehyde (1% formaldehyde for 10 min). This reaction was stopped by addition of glycine (125 mM for 5 min). Cardiomyocytes were then scraped, dispersed in solution containing 1% SDS and protease (Sigma #P8340), phosphatase (sodium orthovanadate) and deacetylase (sodium butyrate) inhibitors, and sonicated (Probe sonicator 1000 W, 20 kHz, 15 cycles of 15 s, power set 3, 1 min intervals) on ice to obtain DNA fragments (400–600 b.p. average size). Proteins and transcription factors cross-linked to promoters in the DNA fragments were detected by immunoprecipitation with acetylated histones. The co-immunoprecipitated promoter of ANF with acetylated histone H4 was identified by PCR amplification. The transcription factor c-Fos/GATA-4 recruited to the ANF promoter was identified by co-immunoprecipitation with acetylated histone H4 and PCR amplification. Two nucleotide sequences in the ANF promoter were co-immunoprecipitated with acetylated histone H4: 5'-GCCCTTTGTCGTCACGTCT-3' and 5'-GAGCGCCCGGG-AAGATAACC-3' for the promoter’s proximal region and 5'-AAAGGCTTTTCTCCTCCAGGC-3' and 5'-ACAGGCTCTTAAAGATTCGCACAGC-3' for its distal region.

RESULTS

Mobilization of intracellular Ca\(^{2+}\) induces PARP-1 activation in stimulated contracting cardiomyocytes of newborn rats

Confocal scanning of live cardiomyocytes was performed after application of 1 μM AngII to primary cell cultures of cardiomyocytes on collagen-coated coverslips. These cardiomyocytes were first exposed to the cell-membrane-permeant calcium fluorophore rhod-2/AM. Live confocal scanning revealed fluorescent sparks in the cells, indicating mobilization of Ca\(^{2+}\) from intracellular stores during an accelerated contraction rate (2–3-fold the contraction rate of untreated cardiomyocytes). The stimulatory treatment with 1 μM AngII caused a transient repetitive rhythmical shreddling of Ca\(^{2+}\) from perinuclear stores into the nucleoplasm (Figure 1, and Supplementary Movies S1 and S2 at http://www.BiochemJ.org/bj/438/bj4380337add.htm). Furthermore, after 60 min of incubation with AngII (1 μM) or Iso (isoprenaline; 10 μM); an enhanced fluorescence of rhod-2/AM was measured in the nuclei of the contracting cardiomyocytes, apparently indicating a tendency of Ca\(^{2+}\) accumulation in the nucleoplasm (Figure 2, and Supplementary Figure S1 at http://www.BiochemJ.org/bj/438/bj4380337add.htm).

In view of previously reported evidence for Ca\(^{2+}\)-induced PARP-1 activation in neuronal cells [5] and the rhythmical transient translocation of Ca\(^{2+}\) into the nuclei of contracting cardiomyocytes (Supplementary Movies S1 and S2), we were interested in determining whether similar activation of PARP-1 occurs in the nuclei of the treated cardiomyocytes.

First, we examined the possibility of a Ca\(^{2+}\)-induced activation of PARP-1 in isolated nuclei of cardiomyocytes by measuring Ca\(^{2+}\)-induced dose-dependent changes in PARP-1 auto-[\(^{32}\)P]polyADP-ribosylation in isolated crude nuclei of cardiomyocytes exposed to increasing concentrations of extranuclear Ca\(^{2+}\) in the presence of [\(^{32}\)P]NAD (1000 Ci/mmol; 1 μCi/sample). Autoradiograms of [\(^{32}\)P]polyADP-ribosylated PARP-1 showed a dose-dependent activation of PARP-1 by extra-nuclear Ca\(^{2+}\) (Figure 3). A possible effect of ryanodine-dependent Ca\(^{2+}\) mobilization on PARP-1 activation was examined as well. We did not observe any significant change in PARP-1 activation induced by ryanodine applied to isolated nuclei (Supplementary Figure S2).

We then examined the possible effect of treatments inducing Ca\(^{2+}\) mobilization from internal Ca\(^{2+}\) stores on autopolyADP-ribosylation of PARP-1. In these experiments, cardiomyocytes were treated with AngII and α- and β-adrenergic agonists, as well as with dbcAMP (dibutyryl cAMP) and forskolin causing cAMP elevation. Immediately after treatment, their nuclei were isolated and incubated with [\(^{32}\)P]NAD, so that their [\(^{32}\)P]polyADP-ribosylation, could be measured. Autoradiograms of the extracted nuclear proteins indicated AngII-dependent [\(^{32}\)P]polyADP-ribosylation of PARP-1. Addition of a modified AngII peptide (H-1725; Bachem) that interferes with the binding of AngII with AT\(_1\) (AngII type 1) receptors [36] disrupted the AngII-induced PARP-1 [\(^{32}\)P]polyADP-ribosylation, in line with the assumption that PARP-1 was activated via AngII induced stimulation of AT\(_1\) receptors in the cell membrane (Figure 4A).

In addition, AngII-induced PARP-1 activation was suppressed by...
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Figure 1. Ca\textsuperscript{2+} is rhythmically translocated into nuclei of AngII-treated contracting cardiomyocytes of newborn rats

The cell-permeant fluorescent calcium fluorophore rhod-2/AM (4.5 μM) was incubated (25°C for 30 min in the dark) with primary cell cultures of cardiomyocytes prepared from newborn rats. Live confocal scanning of rhod-2 fluorescence during contraction of cardiomyocytes treated for 5–10 min with 1 μM AngII (red, excitation wavelength 540 nm and emission wavelength 570 nm) indicated Ca\textsuperscript{2+} translocation is shown. (A) Upper panels, Ca\textsuperscript{2+} translocated into the nucleus of a randomly selected AngII-treated cardiomyocyte. Lower panels, monitoring by a video motion detector system (see the Experimental section) twice-to-three times accelerated contraction rate of the AngII-treated cardiomyocytes. (B) A rhythmical Ca\textsuperscript{2+} translocation into the nucleus accompanied the contraction rhythm of a randomly selected cardiomyocyte in the AngII-treated cell culture. The live confocal imaging is shown in the Supplementary Movies S1 and S2 at http://www.BiochemJ.org/bj/438/bj4380337add.htm.

Figure 2. Evidence for Ca\textsuperscript{2+} accumulation in the nuclei of AngII-treated cardiomyocytes

Time-dependent enhancement of the fluorescence of rhod-2/AM in the nuclei of contracting cardiomyocytes indicates a tendency of Ca\textsuperscript{2+} to accumulate there during 80 min of exposure to 1 μM AngII. Changes in the background-corrected normalized fluorescence emission of the membrane-permeant calcium fluorophore rhod-2/AM (red) relative to its fluorescence emission just before AngII was applied to the cells [(F – F\textsubscript{0})/F\textsubscript{0}] were measured by live confocal imaging. DNA in the nuclei was labelled (blue) by Hoechst 33258. Similar results were observed in 20 randomly selected cardiomyocytes in four independent experiments. Arrows indicate two of the sampled nuclei. Inset, calculated average values of normalized fluorescence [(F – F\textsubscript{0})/F\textsubscript{0}] are presented. Results are means ± S.E.M. calculated from the averaged results. Additional results in Iso-treated cardiomyocytes are displayed in Supplementary Figure S1 at http://www.BiochemJ.org/bj/438/bj4380337add.htm.

Figure 3. Dose-dependent activation of PARP-1 by extra-nuclear Ca\textsuperscript{2+} in isolated nuclei of cardiomyocytes

Cultured cardiomyocytes were treated (30 min at 37°C) with the cell-impermeant Ca\textsuperscript{2+} chelator BAPTA (15 μM) to minimize Ca\textsuperscript{2+} influx into the cells. After the cells were thoroughly washed, their nuclei were isolated and exposed to increasing Ca\textsuperscript{2+} concentrations in the presence of [\textsuperscript{32}P]NAD (1000 Ci/mmol; 1 μCi/sample). polyADP-ribosylation of PARP-1 was measured by autoradiography of nuclear proteins prepared from the isolated crude nuclei, separated by SDS/PAGE and electrotransferred on to nitrocellulose membranes by Western blotting. The presented autoradiograms identify Ca\textsuperscript{2+}-dependent [\textsuperscript{32}P]polyADP-riboseylation of immunolabelled PARP-1 (MCA1522, Serotec). Results are means ± S.E.M. calculated from three independent experiments and are presented in the histogram. See additional results in Supplementary Figure S2.

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Ca²⁺-induced PARP-1 activation mediates ANF expression

Figure 4 PARP-1 polyADP-ribosylation in cardiomyocytes treated with agents inducing intracellular Ca²⁺ mobilization

Cultured cardiomyocytes were treated for 10 min with AngII, dbcAMP, Iso or phenylephrine (Phen) at the indicated concentrations. Activation of PARP-1 was assayed in the stimulated cardiomyocytes by its auto-[³²P]polyADP-ribosylation. PARP-1 was immunolabelled in each sample (MCA1522, Serotec). (A) Autoradiograms depict dose-dependent [³²P]polyADP-ribosylation of PARP-1 in nuclear protein extracts prepared from cardiomyocytes treated with 1 μM AngII. The modified AngII peptide H-1725 that interferes with the binding of AngII to AT₁ receptors reduced PARP-1 activation, as did treatment of the cardiomyocytes with the membrane-permeant Ca²⁺ chelator BAPTA/AM (50 μM) or with EGTA (3 mM). Results are means ± S.E.M. calculated from the results of four independent experiments and are presented in the histogram. (B) Effect of elevated cAMP on PARP-1 activation in cultured cardiomyocytes, assayed by [³²P]polyADP-ribosylation of PARP-1 in the nuclear protein extracts. Cardiomyocytes were treated for 30 min with the cell-permeant derivative of cAMP (dbcAMP) at the indicated concentrations or with 25 μM forskolin (Forsk.), both elevating cAMP-induced Ca²⁺ mobilization. [³²P]polyADP-ribosylated PARP-1 in the nuclear protein extracts was autoradiographed and immunolabelled. Treatment with BAFT/AM suppressed cAMP-induced PARP-1 [³²P]polyADP-ribosylation. The histogram shows the means ± S.E.M. calculated from the results of three independent experiments. (C) Effects of α- and β-adrenergic agonists on PARP-1 activation in cultured cardiomyocytes, assayed by PARP-1 [³²P]polyADP-ribosylation in nuclear protein extracts. Nuclei were isolated from cardiomyocytes that were exposed for 10 min to the indicated α- or β-adrenergic ligands: the α-adrenergic agonist phenylephrine (Phen) and the specific β-agonist Iso, in the absence or presence of the α-adrenergic antagonist prazosin (Praz), or the β-adrenergic antagonist propranolol (Prop). [³²P]polyADP-ribosylated PARP-1 was autoradiographed and immunolabelled in each sample. Treatment with 50 μM BAFT/AM interfered with PARP-1 auto-[³²P]polyADP-ribosylation by both adrenergic agonists. The histogram shows the means ± S.E.M. calculated from the results of three independent experiments. (D) DNA damage was not detected in the treated cardiomyocytes. DNA was isolated from cardiomyocytes treated with the indicated agents. Single-stranded DNA prepared from the nuclei of treated cells (DNA mounted on denaturating alkaline agarose gel electrophoresis) was analysed for nick formation. For comparison, a DNAse-treated DNA sample was mounted on the alkaline agarose gel as indicated. DNA fragments were detected after staining with ethidium bromide (1 μg/ml) under UV light. Markers: 1 kb DNA ladder (Sigma). Cont., control.

measured in cardiomyocytes treated with the cell-permeant derivative of cAMP dbcAMP [40]. PARP-1 was [³²P]polyADP-ribosylated in the nuclei of cardiomyocytes treated with dbcAMP (Figure 4B) and in nuclei of cardiomyocytes treated with α- or β-adrenergic agonists (Figure 4C). Application of specific adrenergic antagonists suppressed the activity of PARP-1 (Figure 4C). Also, PARP-1 activation was suppressed by intracellular Ca²⁺ depletion, achieved either by insertion of the cell-permeant Ca²⁺ chelator BAPTA/AM or by exposing the cells to the Ca²⁺ chelator EGTA [41]. These results further supported a linkage between stimuli that induce mobilization of intracellular Ca²⁺ and activation of the nuclear protein PARP-1 in contracting cardiomyocytes (Figures 3 and 4).

Treatments with AngII, α- and β-adrenergic agonists or compounds inducing cAMP elevation were not accompanied by single-strand breaks in the DNA (Figure 4D). This excluded a
PARP-1 in each sample (lower panel) are presented. Autoradiographed and immunolabelled. The autoradiogram (upper panel) and immunolabelled from the treated cardiomyocytes, incubated with \([ ^{32}P \text{NAD} \text{Ci/sample} ]\) respectively, and from cardiomyocytes treated with AngII in the absence or in the presence of the \(\beta\)-adrenergic antagonist propranolol (Prop; 10 \(\mu\)M) (lanes 3 and 4 respectively), and from cardiomyocytes treated with AngII in the absence or in the presence of the modified AngII peptide H-1725 (100 \(\mu\)M) (lanes 5 and 6 respectively). Nuclei were isolated from untreated cardiomyocytes (control, lane 1), from cardiomyocytes exposed to hypoxia without (lane 2), or while treated with Iso alone (lane 1), from cardiomyocytes treated with Iso after staining with ethidium bromide (1 \(\mu\)M) (lanes 2 and 4 respectively), double-strand and single-stranded breaks (lanes 1 and 2, and 3 and 4 respectively) were identified under UV light after staining with ethidium bromide (1 \(\mu\)M) or AngII (1 \(\mu\)M) (lanes 3 and 4 respectively). Nuclei were isolated from untreated cardiomyocytes (control, lane 1), from cardiomyocytes exposed to hypoxia without (lane 2), or while treated with Iso alone or in the presence of the \(\beta\)-adrenergic antagonist propranolol (Prop; 10 \(\mu\)M) (lanes 3 and 4 respectively), and from cardiomyocytes treated with AngII in the absence or in the presence of the modified AngII peptide H-1725 (100 \(\mu\)M) (lanes 5 and 6 respectively). Nuclei were isolated from the treated cardiomyocytes, incubated with \([ ^{32}P \text{NAD} \text{Ci/sample} ]\) and autoradiographed and immunolabelled. The autoradiogram (upper panel) and immunolabelled PARP-1 in each sample (lower panel) are presented.

Possible activation of PARP-1 by these compounds due to DNA nicks formation in the treated cardiomyocytes.

Treatments with AngII, \(\alpha\)- and \(\beta\)-adrenergic agonists and compounds inducing cAMP elevation activate signal transduction mechanisms targeting PKC (protein kinase C) and MAPK [42,43]. On the basis of our results in a cell-free system, indicating an alternative mechanism of PARP-1 activation by phosphorylated ERK2, which operates only in the absence of DNA damage [12,13], we examined the possibility of a similar mechanism operating in cardiomyocytes. We first examined the effects of AngII and Iso in cardiomyocytes exposed to 3 h of hypoxia, which induced single-strand breaks in their DNA (Figure 5A). As expected, PARP-1 was activated in nuclei of cardiomyocytes exposed to hypoxia (Figure 5B). However, neither Iso nor AngII induced a further activation of PARP-1 (Figure 5B), in accordance with our findings in cell-free systems and in cerebral neurons, where DNA damage interfered with PARP-1 activation by phosphorylated ERK2 [12]. We therefore examined whether AngII- or Iso-induced PARP-1 activation is mediated by ERK phosphorylation in contracting newborn cardiomyocytes.

Interplay between PARP-1 activation and ERK phosphorylation in contracting cardiomyocytes

We examined by confocal imaging of fixed cells whether the fluorescent immunolabelling of activated PARP-1 co-localizes with the fluorescent labelling of phosphorylated ERK in nuclei of cardiomyocytes treated with AngII, Iso or dbcAMP. Co-localization of PAR polymers (indicating PARP activity, mainly the activity of PARP-1 [8]) and phosphorylated ERK co-localized in the nuclei of treated cardiomyocytes (Figure 6A). Furthermore, PARP-1 activation was suppressed by MEK inhibitors (Figure 6B), in line with the previously reported activation of recombinant PARP-1 by recombinant phosphorylated ERK in cell-free systems [12]. Moreover, phosphorylated ERK decayed rapidly after suppressing the expression of PARP-1 by targeted siRNA in nuclei of AngII-treated cardiomyocytes (Figures 6C), in line with a suggested up-regulation of the activity of phosphorylated ERK in the nuclei by polyADP-ribosylated PARP-1, possibly acting as an anchoring protein of phosphorylated ERK [12,13]. This suggested mechanism was supported further by down-regulation of c-Fos protein levels after PARP-1 silencing with PARP-1-targeted siRNA (Figure 6C).

ERK-catalysed phosphorylation of the transcription factor Elk1 up-regulates the HAT (histone acetyltransferase) activity of p300/CBP, resulting in acetylation of core histones and the expression of several immediate early genes including \(c-fos\). The non-stable product c-Fos is mainly stabilized by ERK-induced phosphorylation [17,18]. In view of the effect of PARP-1 silencing on phosphorylated ERK1/2 in the nucleus (Figure 6C), we examined the possibility that PARP-1 and PARP activation also affect the expression of the Elk1 target gene \(c-fos\) in cardiomyocytes.

PARP-1 activation mediates the expression of the immediate early gene \(c-fos\) in AngII-treated cardiomyocytes

Enhanced \(c-fos\) expression was measured within 30 min after stimulation in cardiomyocytes exposed to 1 \(\mu\)M AngII for 10 min. As expected, \(c-fos\) expression, which is mainly induced by the ERK-catalysed phosphorylation of the transcription factor Elk1, was down-regulated in cardiomyocytes treated with the specific MEK inhibitor U0126 which suppresses the phosphorylation of ERK1/2 (Figure 7A). However, the expression of \(c-fos\) was also suppressed by PARP inhibition or by silencing of PARP-1 with PARP-1-targeted siRNA (Figure 7), providing evidence for PARP-1 in the ERK/Elk1-induced signal transduction mechanism. Furthermore, \(c-fos\) expression was down-regulated by BAPTA/AM, implicating \(Ca^{2+}\) mobilization in \(c-fos\) expression. These findings suggest a mechanism up-regulating the expression of \(c-fos\) by simultaneous \(Ca^{2+}\) mobilization, and ERK and PARP-1 activation.

In cardiomyocytes, a long-lasting ERK activity in the nucleus enabling ERK-induced phosphorylation (and thereby stabilization) of c-Fos protein [17] could up-regulate the activity of the transcription factor c-Fos/GATA-4 [20–23]. We next examined the possible role of PARP-1 and PARP activity in c-Fos/GATA-4-induced gene expression.

PolyADP-ribosylation mediates c-Fos interaction with GATA-4 and with the ANF promoter in AngII-treated cardiomyocytes

Relatively large amounts of c-Fos were detected 80 min after treatment of cardiomyocytes with 1 \(\mu\)M of AngII (Figure 8A). Also, depending on PARP activity (polyADP-ribosylation is mainly attributable to PARP-1 activation [8]), c-Fos co-immunoprecipitated with GATA-4 in nuclear protein extracts isolated from AngII-treated cardiomyocytes; PARP inhibition down-regulated the amount of c-Fos co-immunoprecipitated with GATA-4 (Figure 8B), presumably interfering with the formation of the transcription factor c-Fos/GATA-4 [23,44].
Ca\(^{2+}\)-induced PARP-1 activation mediates ANF expression

We examined further whether polyADP-ribosylation is required for the expression of the c-Fos/GATA-4 target gene ANF. This possibility was examined by using a ChIP assay. Cross-linkage of c-Fos with GATA-4 and with the ANF promoter was examined 1 h after application of 1 \(\mu\)M AngII to cardiomyocytes that were than treated with 1% formaldehyde to achieve cross-linking of DNA to proteins in the chromatin (see the Experimental section). These experiments revealed that the proximal region of the ANF promoter, which contains binding sites for GATA-4 [19, 44, 45], co-immunoprecipitated with c-Fos in the cross-linked chromatin segments (400–600 b.p.) prepared from nuclear extracts of the treated cardiomyocytes. Inhibition of PARP activity by 10 \(\mu\)M PJ-34 interfered with their co-immunoprecipitation (Figure 8B).

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**Figure 6** Mutual effects on ERK and PARP-1 activation in cardiomyocytes

(A) Co-localization of polyADP-ribosylated proteins and phosphorylated ERK in the nucleus. Confocal scanning of fixed cardiomyocytes indicated co-localization of immunolabelled polyADP-ribosylated nuclear proteins (detected by the secondary fluorescent antibody Dylight; green) and immunolabelled phosphorylated ERK1/2 (detected by secondary fluorescent antibody Cy3; red) in the nuclei of randomly selected newborn cardiomyocytes treated with AngII (1 \(\mu\)M, 10 min), Iso (10 \(\mu\)M for 10 min) or dbcAMP (10 \(\mu\)M for 30 min). (B) Inhibition of polyADP-ribosylation by MEK inhibitors. Upper panel, confocal images displaying cardiomyocytes treated with AngII (1 \(\mu\)M) or Iso (10 \(\mu\)M) applied without or in the presence of the specific MEK inhibitor U0126 (10 \(\mu\)M for 30 min) or the PARP inhibitor PJ-34 (10 \(\mu\)M, 30 min), as indicated. PolyADP-ribosylated proteins were immunolabelled by antibody directed against PAR polymers (secondary fluorescent antibody Dylight; green). Phosphorylated ERK1/2 (pERK) were immunolabelled (fluorescent secondary antibody Cy3; red) in the cytoplasm and nucleus of the cardiomyocytes. Representative results of three independent experiments are displayed. Lower panel, \(^{32}\)P-labeling of PARP-1 and ERK phosphorylation in nuclear protein extracts of cardiomyocytes treated with AngII (1 \(\mu\)M for 10 min) or Iso (10 \(\mu\)M for 10 min). The activity of PARP-1 and ERK phosphorylation were both suppressed by the MEK inhibitor U0126 (10 \(\mu\)M for 30 min). Autoradiographed \(^{32}\)P-labeling of polyADP-ribosylated PARP-1 and phosphorylated ERK were immunolabelled in each sample. Means ± S.E.M. were calculated from the results of three independent experiments (as shown in the histograms). (C) Down-regulation of phosphorylated ERK1/2 and c-Fos in nuclei of cardiomyocytes after PARP-1 silencing with PARP-1-targeted siRNA. Suppression of PARP-1 expression by PARP-1 targeted siRNA caused decay in ERK1/2 phosphorylation and c-Fos levels in nuclei of cardiomyocytes treated with 1 \(\mu\)M AngII. Nuclei were isolated 15, 30, 45, 60 and 90 min after AngII application [lanes 2–6, untransfected; and lanes 7–12, transfected with siRNA (100 nM, 72 h) respectively]. In lanes 13 and 14, AngII-treated cardiomyocytes were transfected with non-targeting siRNA, and their nuclei were isolated 45 and 75 min after AngII (1 \(\mu\)M) application. Extracted nuclear proteins and cytoplasmic proteins were separated by SDS/PAGE, electrotransferred to nitrocellulose membranes (Western blots) and immunolabelled. PARP-1, ERK2, phosphorylated ERK1/2, c-Fos protein and histone H1 were immunolabelled in each sample as indicated. Representative results of three independent experiments are displayed. Means ± S.E.M. were calculated from the results of three independent experiments (as shown in the histograms).
Evidence for PARP-1 activation mediating the formation of the transcription factor c-Fos/GATA-4 and promoting ANF expression (Figures 7 and 8) outlines a pattern of signal transduction mechanisms activated by Ca^{2+} mobilization and Ca^{2+}-induced PARP-1 activation in the absence of DNA damage (Figures 1–5), causing a mutual up-regulation of PARP-1 and ERK activities in the nucleus (Figure 6). Evidence for a positive-feedback mechanism keeping both PARP-1 and ERK2 activated were detected in cell-free systems [12]. The suggested signal transduction mechanisms in cardiomyocytes linking ANF expression to Ca^{2+}-induced PARP-1 activation are shown in Figure 9.

DISCUSSION

The present study provides evidence indicating the operation of signal transduction pathways that couples Ca^{2+}-induced PARP-1 activation to ANF expression in stimulated contracting cardiomyocytes of newborn rats. Brief exposure of cultured cardiomyocytes to 1 μM AngII induced an accelerated rate of contraction accompanied by intracellular Ca^{2+} mobilization and a rhythmic transient translocation of Ca^{2+} into the cell nuclei accompanying their contractions (Figures 1 and 2 and Supplementary Figure S1 and Movie S1). We also found that PARP-1 is auto-polyADP-riboseylated in isolated nuclei of cardiomyocytes exposed to elevated extra-nuclear Ca^{2+} concentrations (Figure 3), suggesting a Ca^{2+}-mediated AngII-induced PARP-1 activation (Figure 4A). PARP-1 was similarly activated by treatments inducing mobilization of intracellular Ca^{2+}, including exposure to adrenergic stimulation (with α- and β-adrenergic agonists) or elevated cAMP, and Ca^{2+} chelators interfered with the activation of PARP-1 (Figures 4B and 4C). A similar activation of PARP-1 via Ca^{2+} release from perinuclear stores into the nucleoplasm was reported previously in depolarized cerebral neuronal cells [5]. Mobilization of Ca^{2+} did not induce DNA damage in either of these cell types (Figure 4D), excluding a possible PARP-1 activation by AngII and Iso, and elevated cAMP due to nick formation in the DNA [5,12]. Furthermore, PARP-1 activation in cardiomyocytes exposed to hypoxia-inducing DNA single-strand breaks was not activated by AngII (1 μM) or Iso (10 μM) (Figure 5), in accordance with previously reported DNA-independent mechanism of PARP-1 activation in cell-free system [12].

In addition, our results provided evidence for ERK phosphorylation coupled with the ensuing PARP-1 activation, which mediates the expression of the ERK target gene c-fos (Figures 6 and 7). In cardiomyocytes, this linkage may further induce the up-regulation of ANF expression [20–22,44]. By using a ChIP assay, we showed that activated PARP-1 up-regulated the formation of the transcription factor c-Fos/GATA-4 and ANF expression (Figures 8A and 8B). These findings are consistent with a signal transduction mechanism activated by signals inducing Ca^{2+} mobilization from intracellular stores (Figure 9).

In cardiomyocytes, variations in the amplitude, frequency and compartmentalization of Ca^{2+} signals are decoded by enzymes, ion channels and transcription factors operating via diverse signal transduction mechanisms [4,40,44,46]. Previous results have suggested a variety of Ca^{2+}-dependent mechanisms associating the rhythmic mobilization of Ca^{2+} from intracellular stores with the regulation of cardiomyocyte contractility and growth [46–48].

In the present study, our results identified a mechanism that couples ANF expression, which has a major role in cardiomyocyte development and survival [20–23], with Ca^{2+} translocation and Ca^{2+}-mediated signal transduction mechanisms activating...
PARP-1 and ERK interplay in the nucleus of newborn cardiomyocytes that are not exposed to hypoxia (Figures 5 and 9). Both PARP inhibition and PARP-1 silencing suppressed the expression of c-fos, thereby down-regulating the formation of the transcription factor c-Fos/GATA-4 and suppressing ANF expression in the stimulated cardiomyocytes (Figures 7 and 8). This suggested mechanism is in line with other findings indicating the role of perinuclear Ca$^{2+}$ in gene expression [39,49–51] and with evidence for AngII-induced enlargement of cardiomyocyte size [52], which was dependent on PARP activity [53].

Figure 8  PolyADP-ribosylation is required for co-immunoprecipitation of c-Fos and acetylated histone H4 with the ANF promoter in cross-linked chromatin segments of AngII-treated cardiomyocytes

(A) Upper panel, c-Fos protein was immunoprecipitated by an anti-c-Fos antibody (Cell Signaling Technology) from nuclear protein extracts of cardiomyocytes 80 min after treatment of the cardiomyocytes for 10 min with 1 μM AngII, and before or after treatment for 30 min with 10 μM PJ-34. Lower panel, co-immunoprecipitation of c-Fos with GATA-4 in the nuclear extracts of the AngII-treated cardiomyocytes before and after their treatment with PJ-34. (B) The proximal region of the ANF promoter was co-immunoprecipitated with the anti-c-Fos antibody from cross-linked chromatin segments prepared by sonication from the AngII-treated cardiomyocytes (ChIP assay, see the Experimental section). Following protein digestion, DNA segments carrying the proximal region of the ANF promoter were detected after PCR amplification in the presence of the primers 5′-GCCTTTGCTGGTGACTCT-3′ and 5′-GAGCGCCCAGGAAGATAACC-3′ for the proximal region in the ANF promoter (similar results were observed in three independent experiments). (C) Co-immunoprecipitation of both the proximal and the distal regions of the ANF promoter with a monoclonal antibody directed against acetylated histone H4 (Upstate Biotechnology) in cross-linked chromatin segments of the AngII-treated cardiomyocytes. Treatment (as above) with the PARP inhibitor PJ-34 interfered with the co-immunoprecipitations. Both the proximal and the distal regions of the ANF promoter were detected in the immunoprecipitates after protein digestion by PCR amplification in the presence of the primers 5′-GCCTTTGCTGGTGACTCT-3′ and 5′-GAGCGCCCAGGAAGATAACC-3′ for the proximal region, and 3′-AAACCGCTTCACGCTCCTACAG-5′, and 5′-ACACGGCTCTAAGAATTCAGCTACG-3′ for the distal region in the ANF promoter. (D) PARP-1, phosphorylated ERK1/2, c-Fos, GATA-4, and CBP were co-immunoprecipitated with acetylated histone H4 after AngII treatment. PARP-1 inhibition with PJ-34 (10 μM for 30 min) interfered with their co-immunoprecipitation. Proteins were separated on Tris/Tricine gel, electrotransferred (Western blots) and immunolabelled on nitrocellulose membranes. The immunolabelled proteins are indicated. (E) ANF protein was immunodetected in cell lysates of newborn rat cardiomyocytes treated as described above with AngII, without (lanes 2 and 3) or after the transient (trans.) silencing of PARP-1 with siRNA (100 nM, 72 h; lanes 4 and 5), as indicated, immediately after silencing (lane 4), or 48 h after PARP-1 silencing by directed siRNA (lane 5). ANF levels were not affected in cardiomyocytes treated with the transfection reagent (lane 3) or with non-targeting siRNA (100 nM, 72 h; lane 6). Representative results of three independent experiments are displayed.
Figure 9 Proposed signal transduction mechanisms that couple the expression of c-fos and ANF to PARP-1 activation by stimuli inducing intracellular Ca$^{2+}$ mobilization and ERK phosphorylation

These mechanisms are evidently part of a network of signal transduction mechanisms activating mutual effects of PARP-1 and phosphorylated ERK that were reported previously in cell-free systems [12]. GPCR, G-protein-coupled receptor; PARP, PAR glycohydrolase; pERK, phosphorylated ERK; PKA, protein kinase A; PLC, phospholipase C.

Accumulating findings in animal models and in humans indicate that accelerated pacing induces remodelling of the cardiac atria and ventricles [54], sometimes leading to pathologies with poorly understood molecular mechanisms and high resistance to currently available drug therapy. The presented molecular mechanisms provide additional information on the cascade of events leading from malfunctioning to pathological changes in the morphology of cardiomyocytes.

AUTHOR CONTRIBUTION

Ilona Geistrikh, Rodica Klein, Leonid Visochek and Liron Miller performed the experiments. Leonid Mittelman performed the confocal measurements. Asher Shainberg participated in the design and proofreading of the paper prior to submission. Malka Cohen-Armon designed the experiments and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Ca^{2+}-induced PARP-1 activation and ANF expression are coupled events in cardiomyocytes

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Figure S1 Evidence for Ca^{2+} accumulation in the nucleus of cardiomyocytes treated with Iso (10 μM, 80 min)

Arrows indicate three of the sampled nuclei.

Figure S2 Ca^{2+}-dependent [32P]polyADP-ribosylation of PARP-1 in isolated nuclei of cardiomyocytes in the presence of ryanodine (100 nM) measured by autoradiography

Nuclei were isolated and exposed to increasing [Ca^{2+}] in the presence of [32P]NAD (1000 Ci/mmol; 1 μCi/sample). Nuclear proteins were extracted and analysed by SDS/PAGE, electrotransferred (Western blot), autoradiographed and immunolabelled for PARP-1 in each sample. Representative results of three independent experiments are displayed. Means ± S.E.M. for three independent experiments are shown in the histogram.

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