Phytomedicine 23 (2016) 1392–1400

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

Original Article

Cardioprotective actions of curcumin on the pathogenic NFAT/COX-2/prostaglandin E_2 pathway induced during *Trypanosoma cruzi* infection

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ARTICLE INFO

Article history:
Received 29 February 2016
Revised 18 May 2016
Accepted 25 June 2016

Keywords:
Curcumin
Trypanosoma cruzi
Cardiomyocyte
Cyclooxygenase type 2
Prostaglandin E_2
b-type natriuretic peptide

ABSTRACT

Background: Diverse cardiovascular signaling routes have been considered critical for Chagas cardiomyopathy caused by the protozoan parasite *Trypanosoma cruzi*. Along this line, *T. cruzi* infection and endothelin-1 (ET-1) have been shown to cooperatively activate the Ca^{2+}/NFAT cascade in cardiomyocytes, leading to cyclooxygenase type 2 (COX-2) induction and increased release of prostanoids and prohypertrophic peptides.
Purpose: To determine whether the well-known cardioprotective and anti-inflammatory effects of curcumin (Cur) could be helpful to interfere with this key machinery for pathogenesis of Chagas myocarditis.
Study Design: Cur treatment was evaluated through *in vivo* studies using a murine model of acute *T. cruzi* infection and *in vitro* experiments using ET-1-stimulated and parasite-infected mouse cardiomyocytes.
Methods: Cur-treated and untreated infected mice were followed-up to estimate survival postinfection and heart tissues from both groups were analyzed for inflammatory infiltration by histopathology, whereas parasite load, induction of arachidonic acid pathway and natriuretic peptide expression were determined by real-time PCR. Molecular analysis of Cur myocardial targets included intracellular calcium measurement, NFAT and COX-2 induction in transfected cells, and assessment of NFAT, COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1) levels by immunoblotting, prostaglandin E_2 (PGE_2) by ELISA, b-type natriuretic peptide (BNP) by real-time PCR, and PGE_2/EP4 receptor/BNP interaction by transwell experiments.
Results: Cur treatment of acute Chagas mice enhanced survival and proved to hinder relevant inflammatory processes in the heart, including leukocyte recruitment, activation of the eicosanoid pathway and BNP overexpression, without modifying parasite burden in the organ. Cur was capable of blocking Ca^{2+}-dependent NFATc1 transcriptional activity, COX-2 and mPGES-1 induction, and subsequent PGE_2 production in ET-1-stimulated and parasite-infected cardiomyocytes. Furthermore, the decline of cardiomyocyte-derived prostaglandin levels achieved upon Cur treatment impaired effective PGE_2/EP4 receptor interaction, resulting in attenuated expression of BNP, a strong indicator of cardiac pathogenesis in Chagas disease, in both infected and uninfected cells.
Conclusion: Our current study shows a putative mechanism of action of Cur involving inhibition of the Ca^{2+}/NFAT-dependent, pathogenic COX-2/mPGES-1/PGE_2 pathway in *T. cruzi*-infected myocytes, underlying cardioprotection achieved in Cur-treated infected mice. With a view to the limited therapeutic possibilities available, Cur represents a promising approach for the treatment of Chagas heart disease.

Introduction

Curcumin (Cur) [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a natural polyphenolic flavonoid isolated from the rhizomes of *Curcuma longa* L. (Zingiberaceae). Quantitative determination of curcuminoids in *C. longa* rhizomes has shown a Cur content of 1.87% (dry weight) (Lechtenberg et al., 2004). Research over the last decades demonstrates that Cur possesses antioxidant and anti-inflammatory effects, displaying

Abbreviations: $[Ca^{2+}]i$, intracellular calcium concentration; BNP, b-type natriuretic peptide; COX-2, cyclooxygenase type 2; Cur, curcumin; dnNFAT, dominant negative for all NFAT isoforms; DPI, days postinfection; EP, endoperoxide receptor; ET-1, endothelin-1; FCS, fetal calf serum; mPGES-1, microsomal prostaglandin E synthase-1; NFAT, nuclear factor of activated T cells; NFATc1, c1 isoform of NFAT; PGE_2, prostaglandin E_2; siRNA, small interfering RNA; Tc + ET-1, combined effect of ET-1 stimulation and *Trypanosoma cruzi* infection.

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http://dx.doi.org/10.1016/j.phymed.2016.06.017
0944-7113/

potential in prevention and treatment of diverse infectious, neoplastic and immune disorders (Fan et al., 2013). A host of studies in *in vitro* and *in vivo* models indicate that this natural product may also help preserve cardiovascular function following heart injury (Miriyala et al., 2007).

Accordingly, a recent report suggests that Cur could be a suitable drug for the amelioration of myocarditis caused by the protozoan parasite *Trypanosoma cruzi*, etiologic agent of Chagas disease (Nagajyothi et al., 2012). This condition is one of the most important and severe manifestations of the illness and represents the main cause of cardiac pathology in Latin America (Biolo et al., 2010). In *T. cruzi*-infected mice, oral therapy with Cur reduced parasitemia, mortality and tissue damage. These data are consistent with Cur modulating Chagasic infection-induced changes in signal transduction linked to inflammation and/or oxidative stress in myocardium, both central mechanisms of *T. cruzi*-driven pathogenesis (Machado et al., 2013; Nagajyothi et al., 2012).

Several cardiovascular signaling routes have been shown to participate in the pathophysiology of Chagas cardiomyopathy (Huang et al., 2003; Rigazio et al., 2014). Along this line, we previously found that *T. cruzi* infection and endothelin-1 (ET-1) cooperatively activate the Ca^{2+}/calcineurin/NFAT cascade in atrial myocytes, leading to cyclooxygenase type 2 (COX-2) induction and increased release of inflammatory mediators (Corral et al., 2013). As the anti-inflammatory actions of Cur largely stem from its ability to target key molecules (enzymes, cytokines, transcription factors) implicated in the etiology of different malignancies (Deguchi, 2015), we aimed to investigate whether Cur treatment of ET-1-stimulated and *T. cruzi*-infected cardiac cells could interfere with this pathway responsible for the production of inflammatory effectors relevant to the pathogenesis of Chagas heart disease.

Materials and methods

Materials

Cur (lot number 079K1756, purity ≥94% of curcuminoids and ≥80% of curcumin, high performance liquid chromatography) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Benznidazole (Bz, lot number 1614, purity ≥99%, high performance liquid chromatography), a reference standard drug against *T. cruzi*, was supplied by Laboratorio Elea, Buenos Aires, Argentina.

Cell ulture, primary cardiomyocytes and infection

Mouse HL-1 cardiomyocytes were plated onto gelatin/fibronectin pre-coated flasks and cultured at 37 °C, 5% CO_2 in Claycomb medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine as previously described (Corral et al., 2013). HL-1 cells were treated with 0.3 nM endotoxin-free ET-1 (Sigma-Aldrich) for 2 h and then infected for 3 h or 24 h with *T. cruzi* trypomastigotes (cell: parasite ratio 1:5), RA strain, in the presence and absence of Cur (0-13.5–27.0 µM).

Primary cardiomyocytes were isolated from adult BALB/c mice ($n = 10$) according to standard protocols (O' Connell et al., 2007). The purity and the viability (> 90% positive each) of the preparation were checked by immunocytochemical and dye exclusion assays, respectively. Myocytes (10^4 cells/cm^2) were seeded onto laminin-coated tissue cultureware in Dulbecco's modified Eagle's medium supplemented with 10% FCS and used within the next 24 h.

Intracellular calcium measurements

Agonist-induced changes in intracellular calcium concentration ($[Ca^{2+}]_i$) were detected using the Ca^{2+}-sensitive dye Fura-2/AM as described (Corral et al., 2013). Cells loaded with 1 µM Fura-2/AM were exposed to 0.3 nM ET-1 and infected with *T. cruzi* trypomastigotes, in the presence and absence of Cur (0-13.5–27.0 µM). HL-1 myocytes treated with Cur only were used as controls. At the indicated times, the fluorescence signal was recorded with an spectrofluorometer, with excitation and emission at 340 and 510 nm, respectively.

Immunoblot analysis

For Western blotting experiments, isolation of subcellular fractions from ET-1-treated and *T. cruzi*-infected HL-1 cells was accomplished as reported (Rigazio et al., 2014). Purity of fractions was proven by analyzing marker proteins including α-tubulin (cytoplasmic), and topoisomerase IIβ and c-jun (nuclear). Immunoblotting was carried out as described elsewhere (Corral et al., 2013). Cardiac cells were disrupted and solubilized extracts (20 µg) were separated in 6% (only for analysis of NFAT translocation to the nucleus) or 10% sodium dodecyl sulfate-polyacrylamide gels, and transferred to nitrocellulose filters. After blocking, the membranes were probed 2 h at 37 °C with murine monoclonal antibodies against COX-2 (diluted 1:250 in blocking buffer, BD Biosciences), α-tubulin (1:1000, Sigma-Aldrich), and with rabbit polyclonal antibodies against the c1 isoform of NFAT (NFATc1, 1:200, Santa Cruz Biotechnology) and prostaglandin E synthase-1 [microsomal (mPGES-1), 1:500, Cayman]. The filters were washed and incubated with the corresponding secondary antibody coupled to horseradish peroxidase at 1:10,000 dilution, and the stained bands were visualized by a chemiluminescent peroxide substrate (Amersham Pharmacia).

Plasmid constructs

Human *Cox2* promoter luciferase constructs (P2-1900 and P2-274) in pXP2LUC plasmid have been described previously (Iñiguez et al., 2000). The pSH102CD418 expression vector encodes an NFATc1 deletion mutant (1–418) that functions as a dominant negative for all NFAT isoforms (dnNFAT) (Corral et al., 2013).

Transfection and luciferase assays

HL-1 cells were transfected by Lipofectamine (Invitrogen) following a routine protocol (Iñiguez et al., 2000). The total amount of DNA in each transfection was kept constant by using the empty expression vectors. Transfected cells were exposed to 0.3 nM ET-1 and *T. cruzi*-infected, in the presence and absence of Cur (0-13.5–27.0 µM), as indicated. Transfected myocytes treated with Cur only were used as controls. The myocytes were lysed and luciferase activity was determined by using a luciferase assay system (Promega) with a luminometer. Results were normalized for extract protein concentrations measured with a Bradford assay kit (Pierce, Thermo Fisher Scientific) and presented as fold induction with respect to untreated specimens.

Measurement of eicosanoid production

Serum-starved HL-1 cardiomyocytes were stimulated with 0.3 nM ET-1 for 2 h and subsequently infected with *T. cruzi* trypomastigotes, in the presence and absence of Cur (0-13.5–27.0 µM). After 24 h, media supernatants were collected and analysed for PGE_2 by ELISA (Cayman) according to manufacturer's specifications. The detection limit of the test is 7.8 pg/ml.

Transwell experiments

HL-1 atrial cells (5×10^5) were seeded onto the upper chamber of a Transwell polycarbonate microporous insert (0.1 µm membrane pore size, Corning), and subjected to ET-1 (0.3 nM) treatment plus *T. cruzi* infection, in the presence and absence of Cur (0–27.0 µM), as detailed above. After washing with Hank's balanced salt solution, the HL-1 cell-containing chamber was placed above the primary adult mouse cardiomyocyte monolayer in the bottom

compartment, allowing for potential diffusion of soluble mediators but preventing cell-cell contact. HL-1 atrial cells and BALB/c heart myocytes were then cocultured for 24 h in FCS-free media. In some wells, a selective EP2 (PF-0,441,8948, Cayman Chemical, 1 μM) or EP4 (L-161982, Cayman Chemical, 10 μM) receptor antagonist was added to the primary cardiomyocyte culture medium. BALB/c monolayers exposed to untreated and uninfected HL-1 cells served as negative controls. After the coculture period, primary mouse cardiomyocytes were removed from the plates, washed, and total cell RNA was extracted in Trizol® reagent (Invitrogen) following the instructions of the supplier.

Real-time RT-PCR analysis

RNA extracts were analyzed by quantitative real-time RT-PCR for mouse COX-2, mPGES-1 and B-type natriuretic peptide (BNP) gene expression, as previously published (Dhiman and Garg, 2011; Kubota et al., 2005). The primer sequences (forward and reverse) were as follows:

5′-ACACTCTATCACTGGCACCC-3′ and
5′-GAAGGGACACCCCTTCACAT-3′ (COX-2)
5′-GAGCCCACCGCAACGACATG-3′ and
5′-CAGATGGTGGGCCACCTCCC-3′ (mPGES-1)
5′-CTGAAGGTGCTGTCCCAGATG-3′ and
5′-GACGGATCCGATCCGGTC-3′ (BNP).

RNA interference

RNA interference in murine cardiomyocytes was carried out according to Abdelalim and Tooyama (2009). The sequence of BNP small interfering RNA (siRNA) was as follows: sense, 5′-CCCAGAGACAGCUCUUGAATT-3′; antisense, 5′-UUCAAGAGCUGUCUCUGGGTT-3′. The BNP siRNA and control siRNA were transfected at a final concentration of 40 μM for 24 h in triplicate for each treatment. At 48 h post-transfection, BNP knockdown was confirmed by RT-PCR.

Experimental T. cruzi infection in mice

Six- to 8-week-old female BALB/c mice were obtained from the Centro Nacional de Energía Atómica (Buenos Aires, Argentina) and maintained under standard conditions at the Hospital de Niños 'Dr. Ricardo Gutiérrez' (Buenos Aires, Argentina) animal facility. All experiments in this study were performed in strict accordance with the recommendations of the European Council Directive 2010/63/EU from the Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The study protocols were approved by the Research Ethics Committee of Hospital de Niños 'Dr. Ricardo Gutiérrez' (report #012-2405). Mice were infected intraperitoneally with 50 trypomastigotes of *T. cruzi* RA strain. Acute infection was checked by detection of parasites in direct blood examination at 14 days postinfection (DPI) (Cutrullis et al., 2009). One set (12 mice in each group) of infected mice was treated with Cur dissolved in corn oil (25, 50, or 100 mg/kg body weight, 35 days), administered daily by oral gavage. The dose was chosen based on a previous oral study of the anti-inflammatory effect of Cur in murine *T. cruzi* infection (Nagajyothi et al., 2012). Another set of chagasic mice received oral therapy with Bz at 100 mg/kg body weight/day over the same period. Untreated and uninfected mice were included as controls. Six mice from each group were sacrificed on day 21 of infection and the heart tissue was analyzed for parasite load, leukocyte infiltration, and COX-2, mPGES-1 and BNP mRNA expression, as previously described (Dhiman and Garg, 2011; Guerrero et al., 2015). The remaining mice were observed for 14 more days and mortality was recorded daily. Each experiment has been repeated thrice.

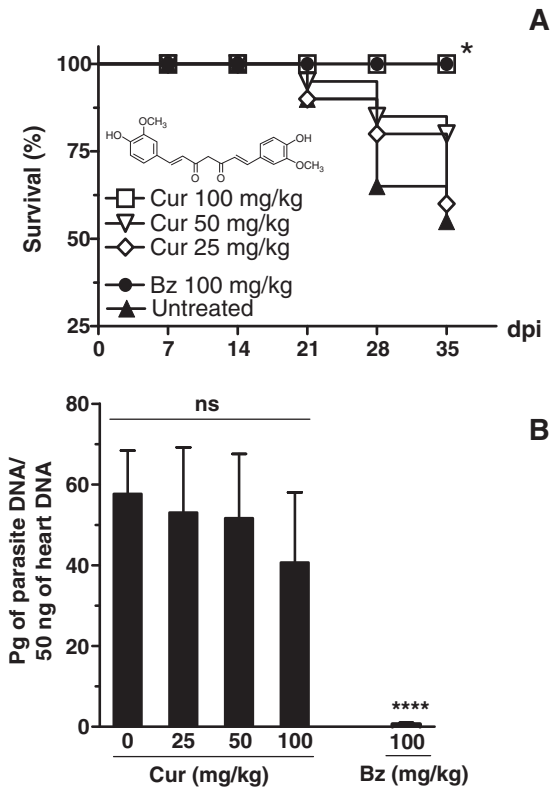

Fig. 1. Survival rate and parasite burden in the heart from *T. cruzi*-infected mice treated or not with curcumin (Cur, 25, 50 or 100 mg/kg body weight/day orally) or benznidazole (Bz, 100 mg/kg body weight/day orally) for 35 days. (A) Mortality of Cur-/Bz-receiving and untreated mice infected with the parasite. dpi, days postinfection. $^{*}P < 0.05$ *vs* the untreated group. (B) DNA from heart tissue was isolated and quantitative PCR using *T. cruzi* DNA standard was performed to determine parasite load in Cur-/Bz-treated or untreated infected mice at 21 dpi. Means ± s.e.m. from three independent experiments are shown ($n = 6$). ns, non-significant. $^{****}P < 0.0001$ *vs* the untreated group.

Statistical data evaluation

Statistical analysis was performed by using GraphPad Prism 5.0 software. Arithmetics means and standard error of the means (s.e.m.) were calculated. Significant differences among groups were made by using the one-way analysis of variance test followed by Tukey's test. The chi-square logrank test was conducted for the analysis of survival curves. A difference between groups of $P < 0.05$ was considered significant.

Results

Cur treatment reduces mortality and ameliorates inflammatory pathology and BNP induction in acutely T. cruzi-infected mice

Cur treatment (100 mg/kg body weight/day orally) of parasite-infected mice significantly ($P < 0.05$) reduced mouse mortality. Infected mice receiving Cur, as well as the group treated with Bz at 100 mg/kg/d, had a 100% survival whereas control untreated infected mice showed a 55% survival rate (Fig. 1A).

On the other hand, in Chagasic mice given Cur at lower doses (25 and 50 mg/kg/d) mortality rates did not significantly differ from those recorded in the Cur-free group. Using Cur at 100 mg/kg/d, survival was enhanced in spite of the fact that therapy did not result in substantially decreased parasitism in the myocardium. Independently of the dose administered (25, 50 or 100 mg/kg/d), quantitative PCR analysis revealed that Cur-receiving infected animals (21 DPI) displayed a slight, albeit not significant ($P \geq 0.06$), reduction of parasite burden in the heart compared with

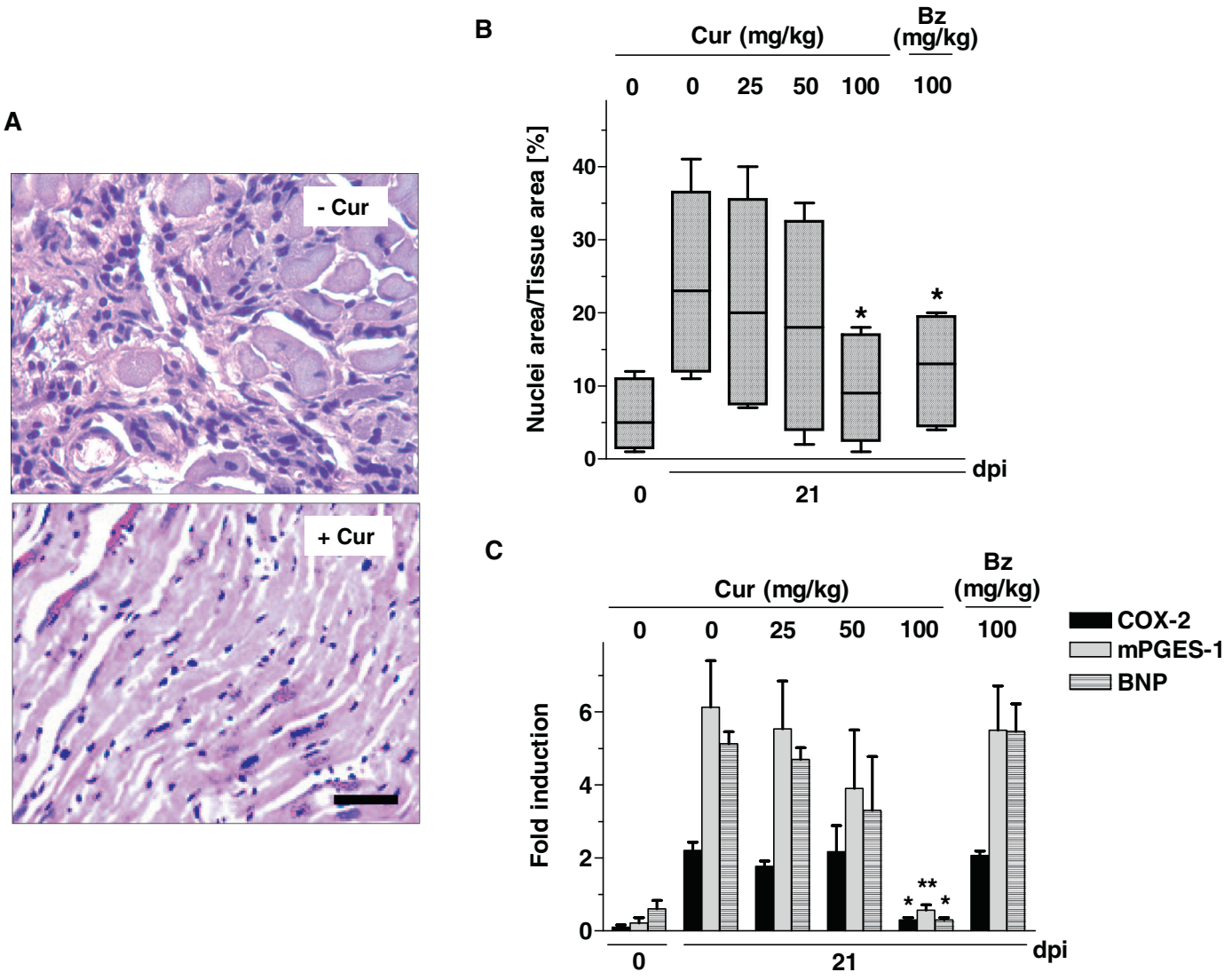

Fig. 2. Inflammation and expression of proinflammatory mediators in the heart from *T. cruzi*-infected mice (21 dpi) treated or not with curcumin (Cur, 25, 50 or 100 mg/kg body weight/day orally) or benznidazole (Bz, 100 mg/kg body weight/day orally). (A) Representative microphotographs of histological analysis (hematoxylin and eosin staining) of cardiac tissue specimens from untreated (upper panel) or Cur (100 mg/kg/d)-receiving (lower panel) mice acutely infected with *T. cruzi* are displayed. Scale bar is 100 µm. (B) Heart inflammation in untreated or Cur-/Bz-treated infected mice. Specimens from uninfected mice without Cur or Bz treatment were also included. Inflammatory cell infiltration in tissue sections was quantified as described in Materials and methods. $^{*}P < 0.05$ *vs* the untreated infected group. (C) Myocardial COX-2 (*Cox2*), mPGES-1 (*Ptges*) and BNP (*Nppb*) gene expression during the acute phase of infection in mice receiving or not oral Cur-or Bz-based therapy . RNA from both uninfected and infected heart tissues was used to perform quantitative RT-PCR analysis as indicated in Materials and methods. Values are expressed as means ± s.e.m. from 3 independent infections, each performed with 6 mice per group. $^{*}P < 0.05$ and $^{**}P < 0.01$, *versus* the untreated infected group.

the untreated infected controls (Fig. 1B). Oppositely, treatment with Bz at 100 mg/kg/d resulted in a highly significant ($P < 0.0001$) drop in myocardial parasite load to nearly undetectable levels. Early infection of BALB/c mice with the RA strain of *T. cruzi* is characterized by severe cardiac inflammatory damage (Cutrullis et al., 2009). Inflammatory infiltrates were analyzed and quantified in all experimental groups. A diffuse and multifocal inflammatory infiltrate consisting mainly of lymphoplasmocytic mononuclear cells and macrophages, with fewer polymorphonuclear and mast cells, could be observed in the infected myocardial tissue. Inflammatory cells were present throughout the organ and affected different heart structures (endocardium, myocardium and epicardium). We observed less inflammatory cells in the heart tissue of Cur (100 mg/kg/d)-treated infected mice than in those infected only. Representative images corresponding to the extent of leukocyte infiltration in each group are exhibited in Fig. 2A. Inflammatory and parasite foci were mainly identified in the ventricular wall, also involving the autonomic ganglia and subpericardial tissues. As shown in Fig. 2B, the percentage of nuclei per 50 µm section of heart tissue was 2.4-fold lower in acute Chagas mice under the Cur (100 mg/kg/d) regimen (24.0 ± 5.7) than in infected rodents without treatment (9.7 ± 3.4). At this dose, the anti-inflammatory effect was even more marked with Cur than with Bz. However, the decline observed for Cur at 25 and 50 mg/kg/d was not statistically significant ($P = 0.10$ and $P = 0.08$, respectively). We next studied the expression level of enzymes involved in prostanoid production in parasite-harboring hearts (Fig. 2C). Heightened *Cox2* gene expression was observed in cardiac tissue during the acute stage of murine Chagas disease. In addition, *T. cruzi* infection strongly boosted mPGES-1 (*Ptges*) mRNA expression in the heart. *Cox2* and *Ptges* expression in the infected myocardium was over 20 and 25-fold higher than uninfected controls, respectively. Conversely, mice receiving oral Cur therapy displayed 7 and 10-fold lower transcript levels for myocardial *Cox2* ($P < 0.05$) and mPGES-1

(*Ptges*, $P < 0.01$), respectively, relative to those observed in the untreated infected group (Fig. 2C). Further, Cur abrogated the 8-fold increase in BNP (*Nppb* gene) expression induced by parasitization of cardiac muscle. In contrast to Cur, elevated mRNA expression of these cardiopathogenic mediators could not be reverted upon oral treatment with Bz at 100 mg/kg/d. This outcome suggests that, compared with Bz, high-dose Cur treatment is more capable of down-modulating inflammatory myopathic processes, including leukocyte recruitment, activation of the arachidonic acid pathway and BNP overexpression, triggered in the heart at the beginning of Chagas disease.

Cur interferes with COX-2- and mPGES-1-mediated biosynthesis of cardiomyocyte-derived PGE2 elicited by endothelin-1 stimulation plus T. cruzi infection

Cur produced a dose-dependent inhibition of COX-2 protein expression in HL-1 cardiomyocytes stimulated with ET-1 and infected with *T. cruzi* trypomastigotes (Tc + ET-1), reaching almost complete inhibition in cells treated with Cur at 27.0 µM (Fig. 3A). We next analysed whether the effect of Cur on COX-2 expression was taking place at the transcriptional level. For this, HL-1 myocytes were transfected with a promoter/luciferase construct spanning from nucleotide −1796 to +104 bp relative to the human *Cox2* gene transcription start site (P2-1900-LUC) (Iñiguez et al., 2000). As shown in Fig. 3B, (Tc + ET-1) promoted a fivefold increase ($P < 0.01$) in luciferase activity in transiently transfected cells compared to uninfected and untreated controls (mock). Cur treatment significantly ($P < 0.05$ and $P < 0.01$ at 13.5 and 27.0 µM, respectively) reduced (Tc + ET-1)-mediated induction of *Cox2* promoter. Also, we found that Cur (13.5–27.0 µM) acts as an inhibitor of the expression of mPGES-1, a terminal synthase that functions downstream to COX-2 for eicosanoid production, cooperatively induced by ET-1 and the pathogen in heart muscle cells (Fig. 3C). To assess whether Cur-dependent down-regulation of both COX-2 and mPGES-1 expression was associated with decreased enzymatic activity, PGE_2 release by HL-1 cardiomyocytes was measured. Compared to mock-treated cells, stimulation of myocytes with the combination of ET-1 and *T. cruzi* potently induced a time-varying secretion of PGE_2 over a 24-h period (Fig. 3D). PGE_2 synthesis was significantly ($P < 0.05$ and $P < 0.01$ at 13.5 and 27.0 µM Cur, respectively) attenuated in endothelin-stimulated and parasite-infected HL-1 cells after one day of incubation in the presence of Cur, further confirming the inhibitory effect of this natural product on COX-2/mPGES-1 signaling pathway for PGE_2 generation.

Cur blocks the activation of the myocardial Ca^{2+}-dependent NFAT intracellular signaling pathway and inhibits Cox2 *transcription cooperatively induced by T. cruzi infection and endothelin-1*

In a previous work (Corral et al., 2013), we demonstrated that the combined effect of ET-1 treatment and *T. cruzi* infection provoked a sustained increase in $[Ca^{2+}]_i$ in atrial cells. Coupled to Ca^{2+} entry, NFAT is overexpressed, dephosphorylated by calcineurin, and translocates to the nucleus in cardiomyocytes activated by the vasoactive peptide and subsequent parasite infection (Corral et al., 2013). Oppositely, upon the addition of Cur at increasing concentrations (13.5–27.0 µM), we observed a dose-dependent reduction of the $[Ca^{2+}]_i$ response, reaching levels akin to those recorded in myocytes devoid of (Tc + ET-1) stimulation (Fig. 4A). Also, immunoblot analysis showed that cell treatment with Cur (13.5–27.0 µM) prevented (Tc + ET-1)-dependent NFATc1 migration, thereby resulting in an increasing accumulation of cytoplasmic NFATc1 protein (Fig. 4B). To further examine the downstream cascade of NFAT activation promoted by (Tc + ET-1) in HL-1 cells, we co-transfected the P2-274-LUC *Cox2* vector -carrying two NFAT binding sites- together or not with a dominant-negative dnNFAT plasmid. Expression of dnNFAT abrogated (Tc + ET-1)-induced transcription of the reporter, supporting the involvement of NFAT signaling in the regulation of myocardial *Cox2* gene expression (Fig. 4C). More important, Cur treatment significantly ($P < 0.05$ and $P < 0.01$ at 13.5 and 27.0 µM, respectively) diminished NFAT-dependent P2-274-LUC up-regulation in response to (Tc + ET-1). Taken together, the above results suggest that Cur is capable of abolishing the $[Ca^{2+}]_i$-associated induction of the cardiac NFATc1 isoform, as well as its modulatory activity on *Cox2* transcription, initiated by (Tc + ET-1).

Cur inhibits PGE2-mediated induction of BNP mRNA expression in both infected and uninfected cardiomyocytes

Cur treatment of HL-1 myocytes significantly ($P < 0.05$ and $P < 0.01$ at 13.5 and 27.0 µM, respectively) attenuated the BNP response raised upon endothelin stimulation and parasite infection (Fig. 5A). BNP silencing by specific siRNA further provided strong evidence for the induction of this natriuretic peptide by (Tc + ET-1). In order to expand our observation in a more pathophysiologically relevant model pertinent to *T. cruzi* infection in the heart, we went on to study the ability of Cur to ameliorate the stimulatory effect of (Tc + ET-1)-elicited PGE_2 on myocardial BNP production (Qian et al., 2006). For this, a coculture experiment of primary BALB/c mouse-derived cardiomyocytes with infected HL-1 cells incubated in a transwell system was assayed (Fig. 5B). We speculated that PGE_2 released from HL-1 cells in response to ET-1 stimulation and parasite infection could bind EP receptors on non-stimulated and non-infected cardiomyocytes leading to BNP overexpression.

Our results suggest that PGE_2 secreted from (Tc + ET-1)-stimulated HL-1 cells is a central inducer of BNP mRNA expression in the BALB/c heart myocytes (Fig. 5C). To determine whether this effect was due to PGE_2/EP interaction, coculture experiments in the presence of selective EP2 (PF-04418948) or EP4 (L-161982) receptor antagonists were conducted. Naïve BALB/c cardiomyocytes displayed similar levels of surface expression for both subtypes of endoperoxide receptors (data not shown). The observed elevation of cardiac BNP transcript levels significantly ($P < 0.01$) declined upon addition of the antagonist for EP4, but not for EP2, prostaglandin receptor, lending support to the notion that BNP increment was triggered at least in part by interaction of HL-1 cell-derived PGE_2 and EP4. Specificity of the result was confirmed by RNA interference studies, in which transfection of BNP siRNA abolished the myocardial expression of natriuretic peptide in response to the activity of PGE_2 liberated from HL-1 myocytes.

When (Tc + ET-1)-stimulation of HL-1 cells was performed in the presence of Cur (27.0 µM), coincubation of atrial cells with the BALB/c cardiomyocyte monolayer resulted in marked ($P < 0.05$) inhibition of BNP mRNA expression compared to that recorded in HL-1 cultures stimulated in the absence of phytochemical (Fig. 5C). Altogether, our findings suggest that Cur therapy results in significant down-modulation of (Tc + ET-1)-triggered BNP and PGE_2 synthesis in cardiac myocytes that contributes to prevent PGE_2/EP4-dependent induction of BNP in untreated and uninfected myocardial cells.

Discussion

Among diverse inflammation-promoting moieties, bioactive lipids have been implicated in development of Chagas heart disease (Machado et al., 2011). In particular, the arachidonic acid pathway is now emerging as a fundamental participant in the cardiopathogenesis of this life-threatening illness (Guerrero et al., 2015; Maya et al., 2010). Hence, there is a growing interest in pharmacological modulation of eicosanoid production in attempt to

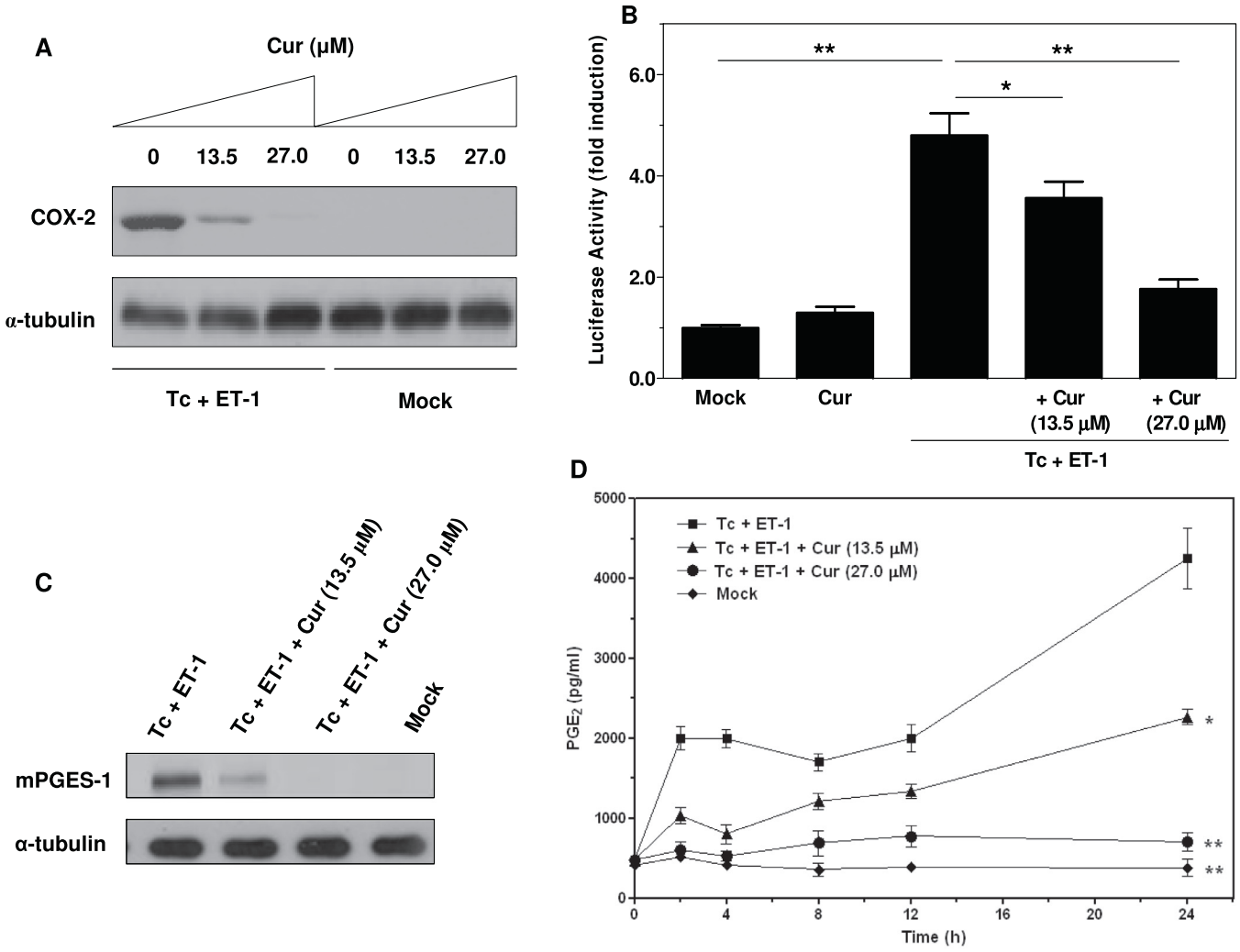

Fig. 3. Cur inhibits COX-2 and mPGES-1 expression, and PGE_2 production triggered upon ET-1 stimulation plus *T. cruzi* infection of atrial HL-1 cells. (A) Dose effect of Cur on COX-2 protein expression. HL-1 cardiomyocytes were incubated with 0.3 nM ET-1 for 2 h and then infected with RA strain trypomastigotes (Tc + ET-1), in the presence or in the absence of Cur (0,13.5 or 27.0 μM), for 3 h. COX-2 and α-tubulin (loading control) proteins were visualized by Western blotting as described under Materials and methods. Unstimulated and non-infected cells (Mock) were also analyzed. (B) Regulatory effect of Cur on the inducibility of the *Cox2* gene promoter activated upon ET-1 pre-treatment and parasite infection of HL-1 cardiomyocytes. Cells were transiently transfected with the P2-1900-LUC reporter construct, and then stimulated with 0.3 nM ET-1 for 2 h and *T. cruzi*-infected, in the presence or in the absence of Cur (13.5–27.0 μM), for 3 h. Cell preparations from transfected (Tc + ET-1)-free myocytes, either untreated (Mock) or treated with the phytochemical (Cur), were included in the assay. Luciferase activity is expressed as fold induction relative to the transfection with empty expression vector. Data are the means ± s.e.m. of three independent experiments, each performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$. (C) Dose-dependent Cur inhibition of mPGES-1 protein expression. Lysates from (Tc + ET-1)-treated cardiac cells, either incubated or not with Cur (13.5–27.0 μM), were analyzed by immunoblotting for detection of mPGES-1 and α-tubulin proteins. Unstimulated and non-infected cell preparation was included as control (Mock). (D) Amelioration of PGE_2 synthesis in infected HL-1 cardiac myocytes in response to Cur treatment at different doses. Atrial cells were ET-1-primed and subsequently infected, with or without addition of Cur at 13.5 or 27.0 μM for additional 24 h. Unstimulated and non-infected cells (Mock) were also studied. PGE_2 levels were determined by enzyme immunoassay in the supernatants collected at indicated times. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* (Tc + ET-1).

control the evolution of *T. cruzi*-driven myocarditis (Malvezi et al., 2014).

In the present study, we found that i*n vivo* oral treatment with Cur, a natural COX-2/mPGES-1 inhibitor (Koeberle et al., 2009), conferred 100% survival after infection and also proved effective to attenuate inflammatory leukocyte infiltration in the heart of acutely infected mice, but did not modify cardiac parasitism. COX-2 deficiency does not seem to alter the protozoan pathogen burden in heart tissues (Guerrero et al., 2015). In the treated group, the lack of reduction in parasite load is most likely attributable to the reported inability of Cur to act directly as a trypanocidal agent in the rodent model of infection, as achievable serum and tissue levels are much lower than drug concentrations required for *in vitro T. cruzi* killing (Nagajyothi et al., 2012). Nevertheless, it is worth to highlight that Cur significantly impaired the generation of inflammatory infiltrate, widely accepted as the ultimate effector of heart injury and a potent source of inflammatory agents mediating organ dysfunction in Chagas disease. The decrease in inflammatory damage observed upon Cur therapy was further associated with down-modulation of key myocardial enzymes (COX-2, mPGES-1) for local eicosanoid production and deeply attenuated BNP mRNA expression. Coincidently, using a different mouse model of *T. cruzi* infection, Nagajyothi and co-workers (2012) demonstrated COX-2 transcripts present at low levels in cardiac tissues from Cur-receiving rodents. According to our experience, Cur seems to be more efficient than the standard trypanocidal drug Bz in preventing overproduction of infected heart-derived inflammatory mediators.

In our *T. cruzi* infection model, a high-dose Cur therapy exhibited promising results but limiting factors such as its poor aqueous solubility and low oral bioavailability hamper the potential application of Cur as therapeutic agent. Hydrophobic nature of this polyphenolic compound along with its rapid metabolism

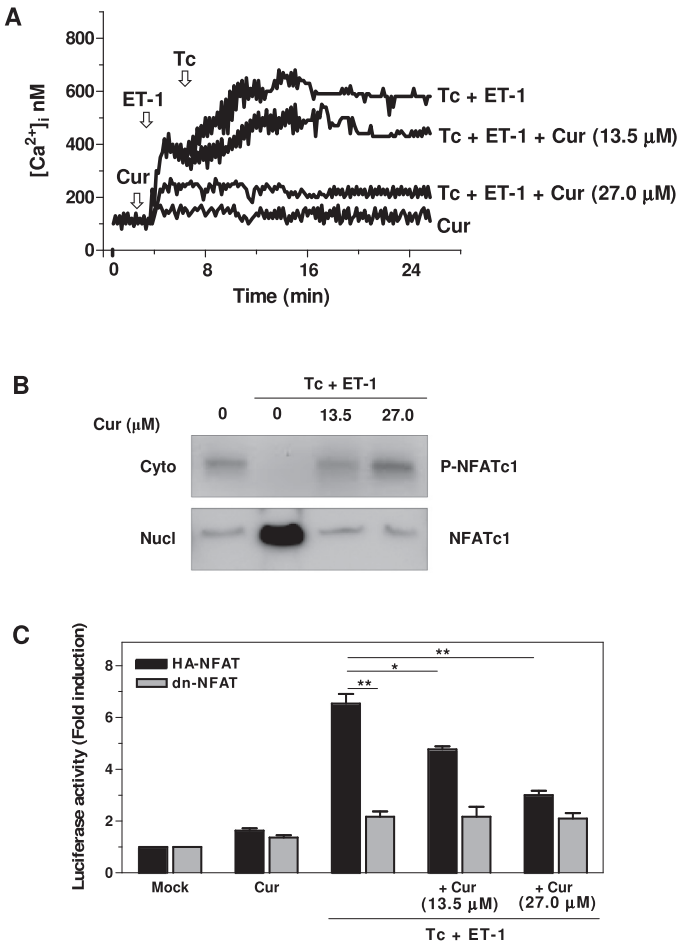

Fig. 4. Cur-mediated blocking of the Ca^{2+}-dependent NFAT intracellular signaling pathway in ET-1-stimulated and *T. cruzi*-infected cardiomyocytes. (A) Calcium influx modulated by Cur. HL-1 cells, exposed to 0.3 nM ET-1, were loaded with the Ca^{2+} indicator Fura-2/AM and changes in $[Ca^{2+}]_i$ upon *T. cruzi* infection, performed in the presence or in the absence of Cur (13.5–27.0 µM), were recorded. Uninfected cells incubated with Cur at the highest dose only were used as a control. Arrows indicate the time (min) when the peptide, the parasite and/or the phytochemical were added. The results presented are representative of three independent experiments. (B) NFAT migration prevented by Cur. Cardiomyocytes were stimulated with ET-1 and then infected with trypomastigotes, with or without addition of Cur at 13.5 or 27.0 µM, for 3 h. Fractionated extracts from both untreated and Cur-treated infected cells, as well as uninfected and untreated controls, were analyzed by immunoblotting. The phosphorylated cytosolic (P-NFATc1) or dephosphorylated nuclear (NFATc1) forms of the transcription factor are indicated. Cyto, cytosolic extracts; Nucl, nuclear extracts. (C) Cur-dependent attenuation of NFAT-mediated *Cox2* induction. HL-1 myocytes were transiently transfected with the P2-274-LUC (HA-NFAT) reporter plasmid alone or co-transfected along with a dominant-negative version of NFAT (dn-NFAT). Transfected cells were then stimulated with ET-1 (0.3 nM) for 2 h and infected with *T. cruzi* parasites, in the presence or in the absence of Cur (13.5–27.0 µM), for 3 h. Transfected cells without treatment or infection (Mock), or incubated with drug only (Cur), were also assayed. Luciferase activity is expressed as fold induction (mean ± s.e.m.) relative to that achieved in empty vector-loaded cells. One out of three separate experiments performed is shown. $^{*}P < 0.05$ [respect to the P2-274 construct, between (Tc + ET-1) and (Tc + ET-1 + Cur at 13.5 µM)]; $^{**}P < 0.01$ [P2-274 construct, between (Tc + ET-1) and (Tc + ET-1 + Cur at 27.0 µM); and P2-274 construct *versus* dn-NFAT, for the (Tc + ET-1) set].

and physicochemical/biological instability contribute to impaired bioavailability. Due to reduced oral absorption, turmeric extract administered in large doses should be required to achieve significant pharmacological effects. Long-term therapy with high doses of Cur might increase the risk of toxicity. To overcome these problems several approaches are being developed like encapsulation of Cur in liposomes and polymeric micelles, formation of inclusion complexes and polymeric conjugates, and synthesis of new heterocyclic Cur derivatives and analogs (Liu et al., 2016). An efficacious solution to the bioavailability issue is needed to ensure appropriate tissue exposures of this natural product in clinical investigation.

In view of our findings in infected mice, we next analyzed *in vitro* the molecular mechanisms underlying the inhibitory effect of Cur on myocardial COX-2 and mPGES-1 induction early triggered by parasite infection. For this, we used HL-1 cardiomyocytes stimulated with ET-1 and infected with *T. cruzi* that display prompt induction of the arachidonic acid pathway as well as enhanced release of natriuretic peptides (Corral et al., 2013; Rigazio et al., 2014). In agreement with documented observations (Kliem et al., 2012), addition of the phytochemical to cultures prevented Ca^{2+} mobilization and NFATc1 activation, thereby reducing COX-2 and mPGES-1 expression and activities in (Tc + ET-1)-treated cardiomyocytes. Consequently, the presence of Cur resulted in decreased levels of proinflammatory PGE_2, usually elevated in infected myocardial tissue (Guerrero et al., 2015). Altogether, these findings reveal that Cur interferes with the transcriptional control of *Cox2* expression, the rate-limiting step in prostaglandin biosynthesis, mediated by the Ca^{2+}/NFAT signaling route in stimulated and infected heart myocytes.

We found that Cur exerts cardioprotective activity in Chagas heart disease partly through the regulation of genes involved in inflammatory signaling routes. Beyond this targeted effect, the pleiotropic nature of the biological actions of this molecule is becoming increasingly apparent. Cur has been shown to elicit a broad range of cell responses by simultaneous targeting of multiple genes and pathways (Phuah and Nagoor, 2014). Natural agents such as Cur may act via induction and inhibition of microRNAs which are capable of up/downregulating the expression of many genes. Thus, modulation of microRNA biogenesis and function by phytochemicals might bring major consequences on cardiovascular health and disease. Further studies should be conducted to provide mechanistic evidence for the beneficial effects of Cur through regulation of defined microRNAs in *T. cruzi* cardiomyopathy.

Linked to the reduction of PGE_2 secretion achieved upon treatment of endothelin-primed and parasite-infected cardiac cells, Cur was able to down-modulate the expression of myocardial BNP, which has been tightly associated with progressive left ventricular disorders in patients with Chagas disease (Sherbuk et al., 2015). Even though ventricular wall stress and cardiac fibrosis have been postulated as critical triggers for natriuretic peptide release in *T. cruzi* infection (Garcia-Alvarez et al., 2010), PGE_2 is the main eicosanoid produced in cardiomyocytes and, acting through the EP4 receptor, may boost BNP gene expression (Qian et al., 2006). In addition, PGE_2 could up-regulate COX-2 and mPGES-1 expression in an autocrine/paracrine manner via EP receptor signaling (Díaz-Muñoz et al., 2012). To the best of our knowledge, our current investigation demonstrates for the first time that Cur-mediated decline in myocardial PGE_2 levels may further lead to effective inhibition of BNP expression in uninfected cardiac cells, thus preventing amplification of pathogenic response in the Chagasic heart.

Our current study has exclusively assessed Cur therapy for amelioration of early cardiac pathology by *T. cruzi*. Regarding that the severity of prolonged myocardial involvement appears to be related to the magnitude of acute Chagasic myocarditis (Cutrullis et al., 2009), it would be interesting to observe if the cardioprotective effects of Cur continue over longer periods of time thereby limiting disease progression. Since the most serious and frequent manifestations of *T. cruzi*-elicited cardiomyopathy occur during the chronic stage of infection, future directions in our research will surely include the evaluation of Cur effectiveness in reducing inflammatory damage in a reliable animal model of long-term Chagas heart disease.

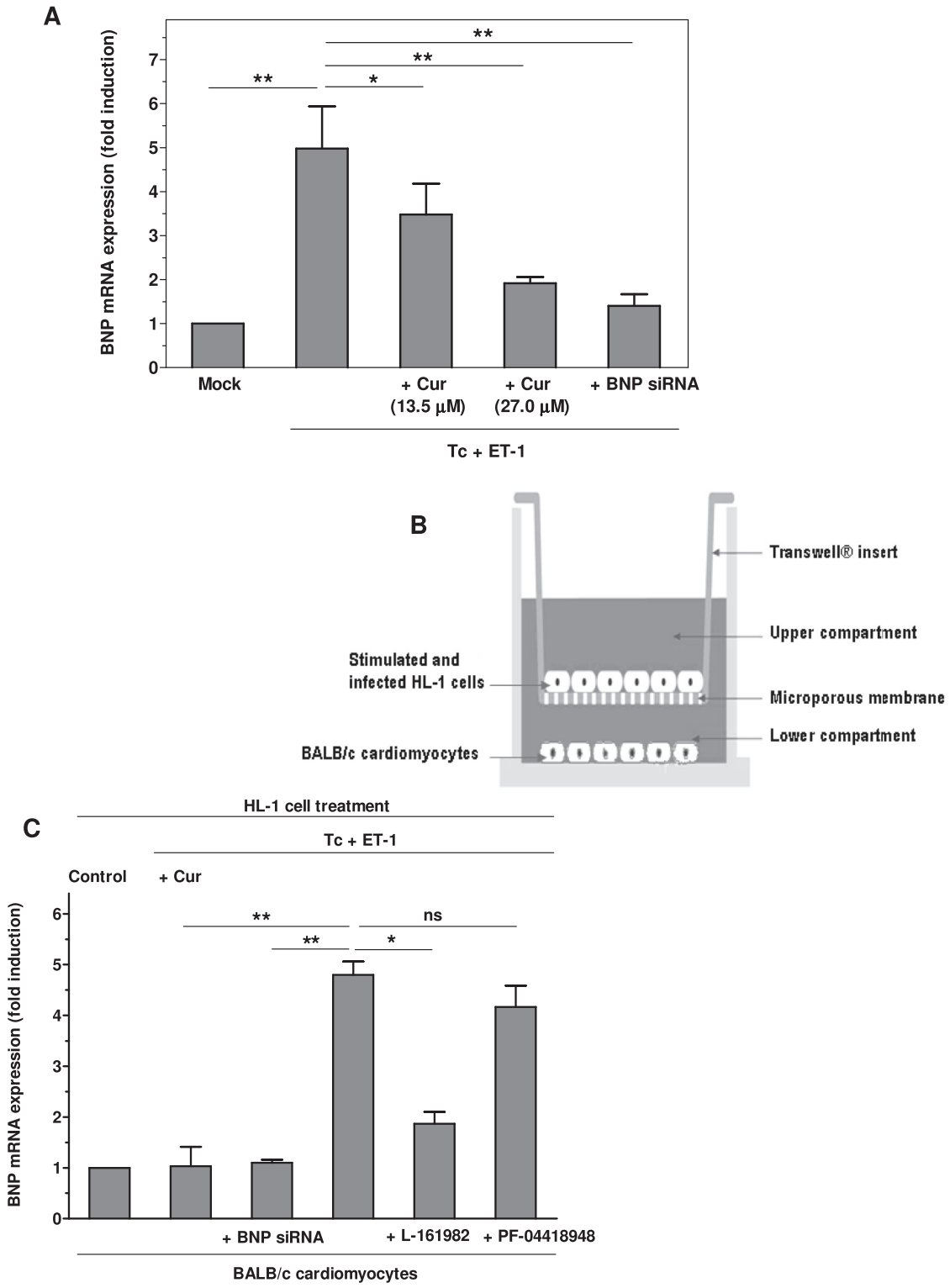

Fig. 5. Cur interferes with PGE_2/EP4-dependent myocardial BNP induction cooperatively promoted by ET-1 and *T. cruzi*. (A) Cur-mediated down-modulation of BNP (*Nppb*) gene expression. HL-1 cells were stimulated with ET-1 for 2 h and infected with trypomastigotes for 3 h, in the presence or absence of Cur (13.5–27.0 μM), and the mRNA levels of BNP were measured by RT-PCR as described in Materials and methods. Untreated/uninfected samples (Mock) were also assayed. In some experiments, transfection of small interfering RNA was used for BNP silencing (BNP siRNA). Results (mean ± s.e.m. of fold induction) from three independent experiments performed in triplicate are shown. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Schematic representation of the coculture system, consisting of a transwell insert with a base made of microporous membrane separating the well into upper and lower compartments that impedes cell migration. (C) Interference with the PGE_2/EP4 pathway resulting in attenuated BNP response. HL-1 cells were ET-1-stimulated and *T. cruzi*-infected, with or without treatment with Cur at 27.0 μM, and seeded in the upper compartment. Untreated and uninfected preparations were used as a control. Primary adult mouse cardiomyocytes were added to the lower chamber. HL-1 and BALB/c cardiac cells were then cocultured for 24 h. In some experiments, transfection of BNP siRNA into primary cardiomyocyte cultures, or incubation of BALB/c myocytes with selective EP2 (PF-04418948,1 μM) or EP4 (L-161982,10 μM) receptor antagonist, was further accomplished. BNP mRNA levels were measured by quantitative PCR. Data represent mean ± s.e.m. of fold induction determined in three independent experiments performed in quadruplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; ns, non-significant.

Conclusion

The outcome of our study shows a putative mechanism of action of Cur involving inhibition of the Ca^{2+}/NFAT-dependent, cardiopathogenic COX-2/mPGES-1/PGE_2 pathway in *T. cruzi*-infected myocytes, which could, at least in part, explain the significant reduction of heart inflammatory damage and BNP down-regulation observed in acutely infected mice subjected to oral Cur therapy. These findings provide considerable support for rational exploration of the usefulness of this multifunctional phytochemical for amelioration of parasite-driven myocarditis. Despite the morbidity and mortality inflicted by Chagas disease, the etiologic treatment currently relies on only two drugs, nifurtimox and benznidazole. Furthermore, due to their well known toxicity and limited effect in prolonged symptomatic infection, the research and identification of new and non-hazardous medication of enhanced efficacy is urgently necessary for the large population infected with the pathogen (Bahia et al., 2014). Over the last decade, numerous molecules, both natural and synthetic, have been postulated for Chagas chemotherapy, but their unsuitable and undesirable structural and pharmacokinetic properties preclude further development (Soeiro et al., 2009). Therefore, the potent anti-inflammatory and cardioprotective actions, low cost, advantageous oral administration and outstanding safety profile evidenced by Cur deserve consideration as a complementary tool to the limited array of therapies so far available for treatment of Chagas cardiomyopathy.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgments

RSC is a Member of Research Career from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). SW thanks Ministerio de Educación (San Luis, Argentina) for fellowship granted. This study was supported in part by a grant from CONICET (112-201201-00518CO) to RSC. The funding source had no role in study design; collection, analysis and interpretation of data; writing of the report and in the decision to submit the article for publication. We acknowledge Stella González Cappa for providing the RA strain of *T. cruzi* and the assistance of Ruth Zimmerman for RNA interference assays. We are grateful to Miguel Iñiguez and Gerald Crabtree for plasmid constructs used in transfection experiments, and to William Claycomb for HL-1 cell line.

References

Abdelalim, E.M., Tooyama, I., 2009. BNP signaling is crucial for embryonic stem cell proliferation. PLoS One 4, e5341.
Bahia, M.T., Diniz, L.deF., Mosqueira, V.C., 2014. Therapeutical approaches under investigation for treatment of Chagas disease. Expert Opin. Investig. Drugs 23, 1225–1237.
Biolo, A., Ribeiro, A.L., Clausell, N., 2010. Chagas cardiomyopathy—where do we stand after a hundred years? Prog. Cardiovasc. Dis. 52, 300–316.
Corral, R.S., Guerrero, N.A., Cuervo, H., Gironès, N., Fresno, M., 2013. *Trypanosoma cruzi* infection and endothelin-1 cooperatively activate pathogenic inflammatory pathways in cardiomyocytes. PLoS Negl. Trop. Dis. 7, e2034.
Cutrullis, R.A., Postan, M., Petray, P.B., Corral, R.S., 2009. Timing of expression of inflammatory mediators in skeletal muscles from mice acutely infected with the RA strain of *Trypanosoma cruzi*. Pathobiology 76, 170–180.
Deguchi, A., 2015. Curcumin targets in inflammation and cancer. Endocr. Metab. Immune Disord. Drug Targets 15, 88–96.
Dhiman, M., Garg, N.J., 2011. NADPH oxidase inhibition ameliorates *Trypanosoma cruzi*-induced myocarditis during Chagas disease. J. Pathol 225, 583–596.
Díaz-Muñoz, M.D., Osma-García, I.C., Fresno, M., Iñiguez, M.A., 2012. Involvement of PGE2 and the cAMP signalling pathway in the up-regulation of COX-2 and mPGES-1 expression in LPS-activated macrophages. Biochem. J. 443, 451–461.
Fan, X., Zhang, C., Liu, D.B., Yan, J., Liang, H.P., 2013. The clinical applications of curcumin: current state and the future. Curr. Pharm. Des. 19, 2011–2031.
Garcia-Alvarez, A., Sitges, M., Pinazo, M.J., Regueiro-Cueva, A., Posada, E., Poyatos, S., Ortiz-Pérez, J.T., Heras, M., Azqueta, M., Gascon, J., Sanz, G., 2010. Chagas cardiomyopathy: the potential of diastolic dysfunction and brain natriuretic peptide in the early identification of cardiac damage. PLoS Negl. Trop. Dis. 4, e826.
Guerrero, N.A., Camacho, M., Vila, L., Iñiguez, M.A., Chillón-Marinas, C., Cuervo, H., Poveda, C., Fresno, M., Gironès, N., 2015. Cyclooxygenase-2 and prostaglandin E2 signaling through prostaglandin receptor EP-2 favor the development of myocarditis during acute *Trypanosoma cruzi* infection. PLoS Negl. Trop. Dis. 9, e0004025.
Huang, H., Petkova, S.B., Cohen, A.W., Bouzahzah, B., Chan, J., Zhou, J.N., Factor, S.M., Weiss, L.M., Krishnamachary, M., Mukherjee, S., Wittner, M., Kitsis, R.N., Pestell, R.G., Lisanti, M.P., Albanese, C., Tanowitz, H.B., 2003. Activation of transcription factors AP-1 and NF-kappa B in murine Chagasic myocarditis. Infect. Immun. 71, 2859–2867.
Iñiguez, M.A., Martínez-Martínez, S., Punzón, C., Redondo, J.M., Fresno, M., 2000. An essential role of the nuclear factor of activated T cells in the regulation of the expression of the cyclooxygenase-2 gene in human T lymphocytes. J. Biol. Chem. 275, 23627–23635.
Kliem, C., Merling, A., Giaisi, M., Köhler, R., Krammer, P.H., Li-Weber, M., 2012. Curcumin suppresses T cell activation by blocking Ca^{2+} mobilization and nuclear factor of activated T cells (NFAT) activation. J. Biol. Chem. 287, 10200–10209.
Koeberle, A., Northoff, H., Werz, O., 2009. Curcumin blocks prostaglandin E2 biosynthesis through direct inhibition of the microsomal prostaglandin E2 synthase-1. Mol. Cancer Ther. 8, 2348–2355.
Kubota, K., Kubota, T., Kamei, D., Murakami, M., Kudo, I., Aso, T., Morita, I., 2005. Change in prostaglandin E synthases (PGESs) in microsomal PGES-1 knockout mice in a preterm delivery model. J. Endocrinol. 187, 339–345.
Lechtenberg, M., Quandt, B., Nahrstedt, A., 2004. Quantitative determination of curcuminoids in *Curcuma* rhizomes and rapid differentiation of *Curcuma domestica Val.* and *Curcuma xanthorrhiza Roxb.* by capillary electrophoresis. Phytochem. Anal. 15, 152–158.
Liu, W., Zhai, Y., Heng, X., Che, F.Y., Chen, W., Sun, D., Zhai, G., 2016. Oral bioavailability of curcumin: problems and advancements. J. Drug Target. Mar 17, 1–9. doi:10.3109/1061186X.2016.1157883.
Machado, F.S., Mukherjee, S., Weiss, L.M., Tanowitz, H.B., Ashton, A.W., 2011. Bioactive lipids in *Trypanosoma cruzi* infection. Adv. Parasitol 76, 1–31.
Machado, F.S., Tanowitz, H.B., Ribeiro, A.L., 2013. Pathogenesis of chagas cardiomyopathy: role of inflammation and oxidative stress. J. Am. Heart Assoc. 2, e000539.
Malvezi, A.D., Panis, C., da Silva, R.V., de Freitas, R.C., Lovo-Martins, M.I., Tatakihara, V.L., Zanluqui, N.G., Neto, E.C., Goldenberg, S., Bordignon, J., Yamada-Ogatta, S.F., Martins-Pinge, M.C., Cecchini, R., Pinge-Filho, P., 2014. Inhibition of cyclooxygenase-1 and cyclooxygenase-2 impairs *Trypanosoma cruzi* entry into cardiac cells and promotes differential modulation of the inflammatory response. Antimicrob. Agents Chemother 58, 6157–6164.
Maya, J.D., Orellana, M., Ferreira, J., Kemmerling, U., López-Muñoz, R., Morello, A., 2010. Chagas disease: Present status of pathogenic mechanisms and chemotherapy. Biol. Res. 43, 323–331.
Miriyala, S., Panchatcharam, M., Rengarajulu, P., 2007. Cardioprotective effects of curcumin. Adv. Exp. Med. Biol 595, 359–377.
Nagajyothi, F., Zhao, D., Weiss, L.M., Tanowitz, H.B., 2012. Curcumin treatment provides protection against *Trypanosoma cruzi* infection. Parasitol. Res 110, 2491–2499.
O'Connell, T.D., Rodrigo, M.C., Simpson, P.C., 2007. Isolation and culture of adult mouse cardiac myocytes. Methods Mol. Biol. 357, 271–296.
Phuah, N.H., Nagoor, N.H., 2014. Regulation of microRNAs by natural agents: new strategies in cancer therapies. Biomed. Res. Int 2014, 804510.
Qian, J.Y., Leung, A., Harding, P., LaPointe, M.C., 2006. PGE_2 stimulates human brain natriuretic peptide expression via EP4 and p42/44 MAPK. Am. J. Physiol. Heart Circ. Physiol. 290, H1740–H1746.
Rigazio, C.S., Hernández, M., Corral, R.S., 2014. Cardiopathogenic mediators generated by GATA4 signaling upon co-activation with endothelin-1 and *Trypanosoma cruzi* infection. Microb. Pathog 73, 47–52.
Sherbuk, J.E., Okamoto, E.E., Marks, M.A., Fortuny, E., Clark, E.H., Galdos-Cardenas, G., Vasquez-Villar, A., Fernandez, A.B., Crawford, T.C., Do, R.Q., Flores-Franco, J.L., Colanzi, R., Gilman, R.H., Bern, C., 2015. Biomarkers and mortality in severe Chagas cardiomyopathy. Glob. Heart 10, 173–180.
Soeiro, M.deN., Dantas, A.P., Daliry, A., Silva, C.F., Batista, D.G., de Souza, E.M., Oliveira, G.M., Salomão, K., Batista, M.M., Pacheco, M.G., Silva, P.B., Santa-Rita, R.M., Barreto, R.F., Boykin, D.W., Castro, S.L., 2009. Experimental chemotherapy for Chagas disease: 15 years of research contributions from *in vivo* and *in vitro* studies. Mem. Inst. Oswaldo Cruz 104, 301–310.