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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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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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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