

Original article

Thrombin increases cardiomyocyte acute cell death
after ischemia and reperfusionMaribel Mirabet, David Garcia-Dorado *, Marisol Ruiz-Meana,
José A. Barrabés, Jordi Soler-Soler*Servicio de Cardiología, Hospital Universitari Vall d'Hebron, Pg. Vall d'Hebron 119-129, 08035 Barcelona, Spain*

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Abstract

Thrombin exerts multiple actions on cardiomyocytes leading to increased intracellular Na^+ and Ca^{2+} concentrations, and to activation of a Ca^{2+} -independent PLA_2 , and has been proposed to favor the genesis of arrhythmias and ischemic injury in acute coronary syndromes. However, the influence of thrombin on cardiomyocyte cell death during ischemia–reperfusion has not been studied. A beneficial influence of low thrombin concentrations has been described in other cell types. HL-1 cardiomyocytes were subjected to simulated ischemia (SI) and reperfusion (SR) and cell death was assessed by means of LDH release to the incubation media. Thrombin dose-dependently increased cell death in normoxic cells, in cells subjected to SI, and in cells subjected to SR (by $20 \pm 8\%$, $95 \pm 32\%$ and $35 \pm 9\%$, respectively, at 100 U/ml). The effects of thrombin were associated to increased cytosolic Ca^{2+} overload, mimicked by 100 μM PAR-1 agonist peptide SFLLRNPNDDKYEPF, and reversed by the direct thrombin inhibitor lepirudin ($\text{IC}_{50} = 1.3 \pm 0.2 \mu\text{g/ml}$). The presence of thrombin during simulated ischemia–reperfusion increases cardiomyocyte cell death by a mechanism that involves activation of PAR-1 receptors and can be prevented by the direct thrombin inhibitor lepirudin.

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1. Introduction

Thrombin, an essential element of the blood coagulation cascade and a potent platelet activator [1], plays a central role in the pathophysiology of acute coronary thrombosis. Blockade of thrombin generation or inhibition of its activity have been shown to be beneficial in patients with acute coronary syndromes [2,3].

Thrombin also exerts direct actions in various cell types through activation of three (PAR-1, -3, and -4) of the four protease-activated receptors (PARs) so far identified [4].

Cardiomyocytes express PAR-1 and PAR-4 [5,6]. Activation of cardiomyocyte PAR-1 by thrombin stimulates phosphoinositide hydrolysis [7], increases intracellular $[\text{Ca}^{2+}]$ [7], and preferentially activates ERKs [5] and a calcium-independent PLA_2 [8], activating the sarcolemmal Na^+/H^+ exchanger [9] and increasing contractility [7]. Long-term

effects of PAR-1 activation by thrombin in cardiomyocytes include increased atrial natriuretic peptide synthesis and the induction of hypertrophy [10]. The final effects of PAR-4 activation, which requires higher thrombin concentrations and produces preferentially p38-MAPK and Src activation [6], are not well known. Thrombin also facilitates the activation of voltage-gated Na^+ channels in cardiomyocytes by a PAR-independent mechanism [11].

Thrombin has been described to influence cell survival and response to injury in different cell types, although the mechanisms involved have not been completely elucidated. It can prevent apoptosis secondary to serum deprivation in fibroblasts [12], monocytes [13], myoblasts [14], and embryonic myocytes [15]. It has also been shown to play an important role in modulating ischemic injury in the brain. Low to moderate thrombin concentrations protect astrocytes from death produced by hypoglycemia or oxidative stress [16] and hippocampal neurons or hippocampal slice cultures from death induced by hypoglycemia, hypoxia or growth factor depriva-

* Corresponding author. Tel.: +34 934 894 038; fax: +34 934 894 032.

E-mail address: dgdorado@vhebron.net (D. Garcia-Dorado).

tion [16,17], whereas high concentrations promote cell death both in astrocytes and neurons [16,17].

Although thrombin has been postulated to favor the generation of ventricular arrhythmias and cardiac ischemic injury [18], the potential direct effects of thrombin on cardiomyocyte survival during ischemia–reperfusion are not known. Thus, we aimed to investigate the effects of thrombin on viability of HL-1 cardiomyocytes subjected to simulated ischemia–reperfusion.

2. Materials and methods

2.1. Chemicals

Experiments were performed with two different thrombin preparations: Sigma T-1063 and Calbiochem 605206. These thrombin preparations were reconstituted or diluted with H₂O to achieve a concentration of 1000 U/ml. All other thrombin concentrations were prepared by sequential dilution from these stock preparations. Vehicle solutions were prepared equivalent in salt composition to the 1000 U/ml thrombin stock solutions, as provided by the manufacturers, and sequentially diluted. In all conditions studied, the same number of experiments was performed with each of these two thrombin preparations and corresponding vehicles and, after checking for homogeneity, results were averaged. In addition, a different thrombin preparation (Sigma T-9135), reconstituted in a BSA and high [NaCl]-containing vehicle, was used in a separate series of experiments. Lepirudin (Refludin®) was obtained from Schering AG, and thrombin receptor agonist peptide SFLLRNPNDKYEPF (TRAP) from Sigma.

2.2. Cells and culture conditions

The HL-1 cell line derives from tumoral atrial cardiac myocytes from transgenic mice [19], and was a gift from Dr. W.C. Claycomb (Louisiana State University Medical Center). HL-1 cells were cultured in Claycomb medium (JRH Biosciences Ltd.) supplemented with 10% FBS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all from GIBCO), and 10 µM norepinephrine (Sigma) at 37 °C in a humid atmosphere of 5% CO₂. Cells were split onto 0.02% gelatin (Becton Dickinson) pre-treated multiwell plates at a cell density of 30,000 per cm² and were allowed to reach 90% confluence before beginning the experiments.

2.3. Simulated ischemia–reperfusion

Cells were washed with normoxic buffer (140 mM NaCl, 3.6 mM KCl, 2 mM CaCl₂, 1.2 mM MgSO₄, 20 mM HEPES, 5 mM glucose, pH 7.4) and incubated for 90 min in this buffer (normoxic cells) or subjected to 90 min of simulated ischemia (SI) in the same buffer lacking glucose and containing 2 mM NaCN and 20 mM 2-deoxyglucose at pH 6.4, as described [20]. Simulated reperfusion (SR) was performed by removing this solution and adding normoxic buffer to treated cells.

2.4. LDH activity

LDH activity was quantified spectrophotometrically [21]. Samples were obtained after 90 min-incubation in normoxic buffer or SI solution, and 30 min after SR. The amount of LDH release in extracellular buffer was expressed as percentage of the total activity in wells, determined by lysing cells in untreated wells with 25 µM digitonin.

2.5. Propidium iodide staining

After treatment, cells were incubated with 1 µg/ml propidium iodide (PI, Sigma) for 10 min at 37 °C. PI fluorescence was measured in a Spectra Max GeminiXS microplate fluorimeter (Molecular Devices Corporation, Sunnyvale, CA, USA; excitation: 430 nm; emission: 615 nm). Control cells kept in culture media were used as blanks and cell death was expressed as percentage of fluorescence obtained with 25 µM digitonin-treated wells.

2.6. Analysis of intracellular [Ca²⁺]

Changes in cytosolic [Ca²⁺] were monitored by a ratio-fluorescence imaging system (Visitech, UK) in HL-1 cardiomyocytes loaded with Fura-2 acetoxymethyl ester (Molecular Probes), as described [22]. Color-coded 340/380 nm ratio images were generated and the average ratio was calculated for regions of interest within the cells and analyzed throughout time.

2.7. Statistical analysis

Dose–effect of thrombin and vehicle on LDH release were analyzed by repeated measures ANOVA, and the effects of TRAP were assessed by means of paired *t*-tests, both by using SPSS software. Data for the inhibitory dose–effect of lepirudin were fitted to a sigmoid curve by using Sigmaplot software. *P* values < 0.05 were considered significant. All values are expressed as mean ± S.E.M.

3. Results

3.1. Effect of thrombin on cell death in HL-1 cardiomyocytes

In order to validate the LDH release method for the measurement of cell death, HL-1 cardiomyocytes were treated with different concentrations of digitonin (0, 5, 7.5, and 25 µM) for 30 min and the percentage of dead cells was determined both by LDH release to the extracellular buffer and by fluorescent PI labeling of nuclei. A close linear correlation between the measurements obtained by both methods was observed (Fig. 1).

Incubation of HL-1 cardiomyocytes with thrombin in normoxic buffer for 90 min induced a modest, although signifi-

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