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TGF-β1 prevents simulated ischemia/reperfusion-induced cardiac fibroblast apoptosis by activation of both canonical and non-canonical signaling pathways

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ABSTRACT

Ischemia/reperfusion injury is a major cause of myocardial death. In the heart, cardiac fibroblasts play a critical role in healing post myocardial infarction. TGF-β1 has shown cardioprotective effects in cardiac damage; however, if TGF-β1 can prevent cardiac fibroblast death triggered by ischemia/reperfusion is unknown. Therefore, we test this hypothesis, and whether the canonical and/or non-canonical TGF-β1 signaling pathways are involved in this protective effect. Cultured rat cardiac fibroblasts were subjected to simulated ischemia/reperfusion. Cell viability was analyzed by trypan blue exclusion and propidium iodide by flow cytometry. The processing of procaspases 8, 9 and 3 to their active forms was assessed by Western blot, whereas subG1 population was evaluated by flow cytometry. Levels of total and phosphorylated forms of ERK1/2, Akt and Smad2/3 were determined by Western blot. The role of these signaling pathways on the protective effect of TGF-β1 was studied using specific chemical inhibitors. Simulated ischemia over 8 h triggers a significant cardiac fibroblast death, which increased by reperfusion, with apoptosis actively involved. These effects were only prevented by the addition of TGF-β1 during reperfusion. TGF-β1 pretreatment increased the levels of phosphorylated forms of ERK1/2, Akt and Smad2/3. The inhibition of ERK1/2, Akt and Smad3 also blocked the preventive effects of TGF-β1 on cardiac fibroblast apoptosis induced by simulated ischemia/reperfusion. Overall, our data suggest that TGF-β1 prevents cardiac fibroblast apoptosis induced by simulated ischemia–reperfusion through the canonical (Smad3) and non canonical (ERK1/2 and Akt) signaling pathways.

1. Introduction

Ischemia and reperfusion (I/R) injury is a major cause of myocardial death. Ischemia causes extensive biochemical changes in the cytoskeleton and subcellular organelles, leading to the cell death [1]. Although reperfusion initially restores cardiac tissue function, providing nutrients and oxygen to deprived ischemic tissue, and limits the extent of myocardial necrosis [2], this process also may induce additional cell death associated to the generation of reactive oxygen species (ROS), producing oxidative modification and proteolysis of several proteins [3–5]. I/R trigger necrosis and apoptosis depending on the intensity and time duration of injury. The latter type of cell death is especially important during the reperfusion stage [6].

Cardioprotective effects of TGF-β1 have been widely reported [7–9]. TGF-β1 binds to specific receptors with serine–threonine kinase activity, leading to intracellular signaling through the activation of the canonical pathway Smad 2/3 [10]. However, TGF-β1 also signals through the non-canonical signaling pathways, including ERK1/2 and PI3K-Akt [11–13]. In rat cardiac fibroblasts (CFs), the activation of TGF-β1-dependent Smad2/3 pathway positively correlated with fibrosis. This growth factor promotes the secretion of extracellular matrix (ECM) proteins, as well as the CF differentiation to cardiac myofibroblasts [14,15]. In CF, the activation of ERK1/2, PI3K-Akt and Smad2/3 by TGF-β1 is linked to increased lysyl oxidase expression, a key enzyme controlling cardiac fibrosis [16]. However, TGF-β1-dependent ERK1/2 and PI3K-Akt signaling pathways can mediate the proapoptotic effects in epithelial cells or anti-apoptotic actions in neuronal and osteoclast cells [17–19].

CFs are the major non-myocyte cell constituent in the myocardium and actively participate in the maintenance of myocardial structure by controlling the homeostasis of the ECM in normal tissue, but they

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actively participate in the healing after a cardiac injury, through the secretion of cytokines, growth factors and metalloproteases [20]. Despite the extensive literature on the effects of I/R on cardiac myocytes, studies on the effects of I/R in CF death are scarce, as well as how TGF-β1 could exert its cardioprotective effect [21]. In the present work we determine the effect of simulated I/R (sI/R) on CF death by apoptosis and/or necrosis; as well as to identify the signaling pathways activated by this cytokine to exert its cytoprotective effect on CF death. We hypothesize that TGF-β1 through the canonical and noncanonical signaling pathways prevents CF apoptosis induced by sI/R.

2. Methods

2.1. Reagents

The following reagents were from Sigma Chemical Co (St. Louis, MO, USA): trypan blue and PD98059 (PD); from Gibco BRL (Carlsbad, CA, USA): primary antibody for β-tubulin, trypsin/EDTA, pre-stained molecular weight standards, fetal bovine serum (FBS) and fetal calf serum (FCS). All organic and inorganic compounds were from Merck (Darmstadt, Germany). The enhanced chemo-luminescence reagent was from Perkin Elmer Life Sciences, Inc. (Boston, MA, USA). TGF-β1 was from Millipore (Billerica, MA, USA). The primary antibodies for caspases 9, 8 and 3, ERK1/2, p-ERK1/2, Akt, p-Akt, p-Smad2, Smad2, p-Smad3 and Smad3 were from Cell Signaling Technology (Boston, MA, USA), LY294002 (LY) was from Cayman Chemicals (Ann Arbor, MI, USA). Secondary antibodies, TGF-β1 signaling pathway inhibitors were from Sigma-Aldrich: PD for ERK1/2, SB431542 (SB) (inhibitor TβR1-Smad2/3), SIS3 for smad3, LY for PI3K-Akt and Akti for Akt.

2.2. Isolation and culture of cardiac fibroblasts

Rats were obtained from the Animal Breeding Facility of the Faculty of Chemical and Pharmaceutical Sciences, University of Chile. All studies were conformed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and experimental protocols were approved by our Institutional Ethics Review Committee. Neonate rat CFs were isolated from the ventricles of 1–3 day-old Sprague–Dawley rats as described previously [22]. In brief, ventricles were minced and digested in 0.05% collagenase (Gibco, Invitrogen, Carlsbad, CA, USA) and 0.05% pancreatin (Sigma, St. Louis, MO, USA) containing solution at 37 °C. The digestion yield was pre-cultured for 2 h at 37 °C in culture media containing 5% FBS and 10% FCS. Differential adhesion on plastic allows the isolation of CF from cardiac myocytes. After 2 h, culture media was replaced for DMEM-F12 containing 10% FBS media and CFs were let to proliferate to confluence. Cellular passages were performed using 0.05% trypsin/EDTA (passage 2 as maximum). After passage 2, cultures were maintained with DMEM-F12.

2.3. Simulated ischemia/reperfusion protocol

CFs were maintained for 24 h in DMEM-F12, and then they were washed with phosphate buffer saline (PBS) before ischemic protocol. The cells were exposed to a balanced salt solution (ischemic medium), (composition in mM: NaCl 115, KCl 12, $MgCl_2$ 1.2, $CaCl_2$ 2, HEPES 25, lactic acid 20 mM and 2-deoxy-D-glucose 20 mM), for different times. Hypoxia was achieved in a customized chamber by N_2 flow at 37 °C. A reperfusion protocol was developed replacing the ischemic medium with DMEM-F12 in a 95/5% air/CO_2 incubator (37 °C) for the indicated times. Control (C) cells were incubated in DMEM F-12 for different ischemia times in a 95/5% air/CO_2 incubator.

2.4. Cell viability

CF cultures with a 2×10^4 cells/mm^2 density in 35 mm plastic dishes in DMEM-F12 media were stimulated under the conditions indicated for each experiment. After the stimulation, cells were treated with trypsin/EDTA 1× for detachment and the action of this protease was inhibited by adding DMEM-F12 plus 10% FBS. After detachment, a 20 μL aliquot plus 20 μL of trypan blue were transferred to a Neubauer camera and cell counting was performed by microscopy. Experiments were performed in triplicate and repeated five times. At least 400 cells were counted in each well.

2.5. SubG1 determination by flow cytometry

After treatment, cells were collected, permeabilized with ice-methanol overnight, and treated with RNAase in PBS for 1 h, and 3 μL propidium iodide was included before flow cytometry (PI, 25 mg/mL). Finally, the DNA fragmentation (sub-population G1) was determined by flow cytometry analysis. A total of 5000 cells/sample were analyzed.

2.6. Western blot analysis

50 μg of total protein extracts was separated by SDS-PAGE. Then, proteins were electrotransferred to a PVDF membrane, which was blocked with non-fat milk 5% (w/v). Antibodies against p-ERK1/2, p-Akt, p-Smad2, p-Smad3, caspases 3, 8 and 9 (dilution 1:1,000) or tubulin (dilution 1:10,000) were incubated overnight at 4 °C. Secondary antibody (dilution 1:5,000), was incubated for 2 h at room temperature. After washing, membranes were exposed to enhanced chemoluminiscent (ECL) reagent. BioMax film was used for western blot determinations. Blots were quantified by laser scanning densitometry. Total ERK, Akt, Smad2, Smad3 and tubulin antibodies were used to control equal loading of all blots.

2.7. Immunofluorescence studies

CFs were seeded onto coverslips in six-well cell culture dishes and were then serum starved by washing twice with PBS, adding starvation media, and incubating for 24 h. After the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. Cells were then washed three times with PBS and permeabilized with 0.1% Triton X-100 followed by three additional washes. Slides were blocked with 3% bovine serum albumin in PBS for 30 min, washed, and incubated overnight with primary antibody (Smad2) diluted in 1% BSA in PBS. Again, cells were washed three times with PBS and incubated with Alexa Fluor-conjugated secondary antibody for 1.5 h followed by another wash cycle. Coverslips were dried, using a vacuum, and mounted on slides using Dako with Hoechst mounting media and then sealed with nail polish. The final images were obtained by confocal microscopy.

2.8. Necrosis by flow cytometer

Cells were collected, centrifuged at 1500 rpm for 5 min and the soluble fraction recovered and kept on ice. Cells remaining on the plates (live cells) were released dilution with 0.3 mL of 1× trypsin-EDTA in PBS (37 °C). Immediately, the cells were added propidium iodide (PI). Finally cellular necrosis was determined by the incorporation of necrotic cardiac fibroblast IP flow cytometry (FACS CANTO, Becton Dickinson).

2.9. LDH activity

After completion of the experimental time 500 μL collected DMEM-F12 from each well and stored at −20 °C for subsequent analysis. The disappearance of the absorbance of NADH (10 mg/mL) in the presence of lactate (2.5 mg/mL), phosphate buffer (pH 7.0) and

100 μL of DMEM-F12 was measured spectrophotometrically, for 2 min at a temperature of 37 °C. After which the enzymatic activities were obtained (AAbs/min). The obtained values are interpolated on a calibration curve previously designed, which relates the LDH enzyme activity with the number of necrotic cells.

2.10. Statistical analysis

All data are presented as means 4–8 independent experiments ± S.E.M. The differences in each parameter were evaluated by a one-way ANOVA of the increase or decrease of each variable measured. One-way ANOVA was followed by the Tukey test to compare the effect of different conditions on these parameters. Statistical significance was accepted at $P<0.05$.

3. Results

3.1. I/R induces CF death by apoptosis

Ischemia is a major damaging event affecting cardiac cells. Fig. 1A, shows that simulated ischemia induces the loss of CF viability in a time-dependent manner, being significant from 12 h. For reperfusion studies, 8 h of ischemia was chosen because there are no changes in cell viability at this time. Fig. 1B shows that reperfusion by 16, 24 and 48 h induces the loss of CF statistically significant of simulated ischemia (8 h), and peaking at 48 h. The injurious effect caused by reperfusion was mediated primarily through apoptosis. The main players of the apoptotic program are caspases. Fig. 1C depicts that simulated ischemia (8 h) induces caspases 9 and 3 activation, but not in caspase 8. However, reperfusion significantly activated these three caspases. Also in Fig. 1D we show by DNA fragmentation (sub-population G1), that simulated ischemia (8 h) does not induce apoptosis; however, reperfusion (8/24 h) induces a significant apoptosis. Supplementary Fig. 1 shows that simulated ischemia (24 h) induces CF death over 80%, whereas that sI/R (8/16; and therefore a total time of 24 h), CF death was approximately 37%. Supplementary Fig. 2A shows that simulated ischemia (8 h) does not produce necrosis; whereas reperfusion induced necrosis from 24 h with respect to the control (CI), control of reperfusion (C/R) and simulated ischemia (sI). Simulated ischemia (8 h) did not increase the release of LDH into the extracellular medium; whereas reperfusion produced a significant release of LDH into the extracellular medium (see Supplementary Fig. 2B).

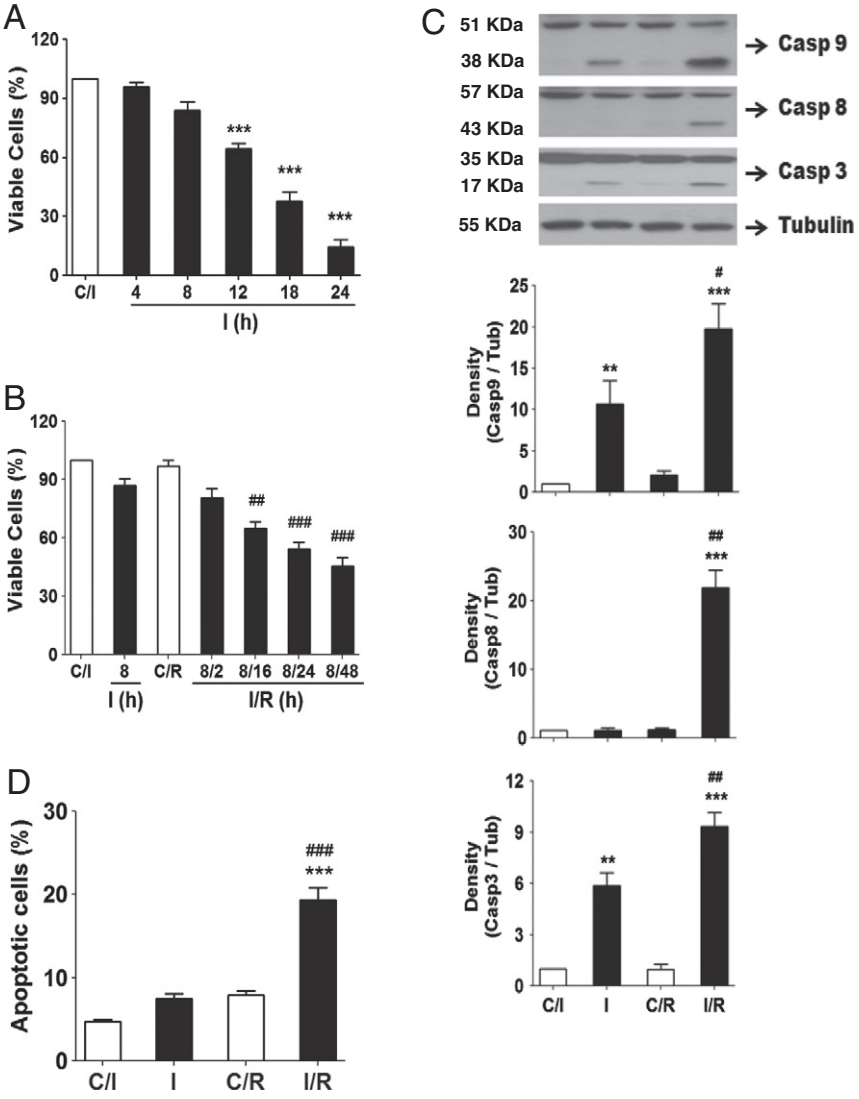

Fig. 1. Deleterious effects of ischemia and I/R in cardiac fibroblasts. Time course of loss of viability in cardiac fibroblasts subjected to ischemia (A) (***$p<0.001$ *vs* C/I, n = 6) and I/R (B) (**$p<0.01$ and ***$p<0.001$ *vs* I 8 h, n = 6), as measured by the quantification of trypan blue exclusion. (C) Densitometry of representative immunoblot of activation of caspases 9, 8 and 3 (**$p<0.01$ and ***$p<0.001$ *vs* C/I, #$p<0,05$ and ##$p<0.001$ *vs* I 8 h. n = 6). (D) Graphic analysis of apoptosis measured by flow cytometry in cardiac fibroblasts undergoing ischemia (8 h) and I/R (8 h/24 h) (***$p<0.001$ *vs* I 8 h). The results are means (± S.E.M.) of 6 independent experiments.

3.2. Antiapoptotic effect of TGF-β1

To test cytoprotective effect of TGF-β1, CFs were subjected to sI/R in the presence or absence of TGF-β1 (5 ng/mL). Incubation with TGF-β1 for the entire period of sI/R (8 h/24 h), induced significant protection against the loss of cell viability (Fig. 2A), as well as apoptosis (Fig. 2C). On the other hand, simulated ischemia (8 h) triggers the processing of procaspases 9 and 3 but not caspase 8 (Fig. 2B), which was not prevented by TGF-β1. Additionally, reperfusion significantly triggers caspases 8, 9 and 3 fragmentations, effects which were attenuated by TGF-β1. This cytokine alone did not induce caspase fragmentation during simulated ischemia.

In order to analyze whether the cytoprotective effect of TGF-β1 takes place during simulated ischemia, reperfusion, or sI/R; experiments were conducted according to the scheme shown in Fig. 3A. Incubation with TGF-β1 during simulated ischemia alone (+/−) did not induce protection in the loss of cell viability (Fig. 3B), caspase 3 fragmentation (Fig. 3C) or apoptosis (Fig. 3D) compared with sI/R. TGF-β1 addition during reperfusion (−/+) or sI/R (+/+) stimulated a significant protection in the loss of CF viability (Fig. 3B), apoptosis (Fig. 3C) and procaspase 3 fragmentation (Fig. 3D) compared to sI/R. Finally, we test the effects of TGF-β1 in adult rat CF (Supplementary Fig. 3), and we show a similar cell behavior against sI/R injury; and similarly, TGF-β1 shows cytoprotective effects similar to those observed in neonatal CF against sI/R.

3.3. TGF-β1 signaling pathways involved in cardiac fibroblast protection

TGF-β1 activates several signaling pathways in CF, including the canonical (Smad2/3) and non-canonical (ERK1/2 and Akt) pathways. TGF-β1 activated Smad2/3, ERK1/2 and Akt in normal CF (Supplementary Fig. 4A–B, respectively). Next we study whether these TGF-β1 signaling pathways were associated with its protective effects. As shown in Fig. 4A, simulated ischemia (8 h) and reperfusion do not activate Smad2/3, but TGF-β1 caused a significant activation of Smad2/3 during reperfusion. In parallel, simulated ischemia (8 h) decreased the levels of phospho-ERK1/2, but not for Akt (Fig. 4B). A significant activation of both kinases was detected during the first 10 min reperfusion with respect to control and ischemia (8 h). However, TGF-β1 treatment for 10 min triggers a higher activation of ERK1/2 and Akt during reperfusion with respect to sI/R. In addition, TGF-β1 also stimulated a significant Akt activation after 24 h compared with sI/R. We also studied whether these TGF-β1 signaling pathways are activated in simulated ischemia. Our results (Supplementary Fig. 5), show that ERK1/2 is activated at 10 min after ischemia starts; however, after 1 and 8 h of ischemia ERK1/2 was inactivated. In the same manner, simulated ischemia dephosphorylates Akt. However, TGF-β1 did not have any additional effect on the activation of these signaling pathways, although TGF-β1 triggers Smad2 phosphorylation.

Finally, we used specific inhibitors to investigate the role of these signaling pathways on CF viability and apoptosis. As shown in Fig. 4C;

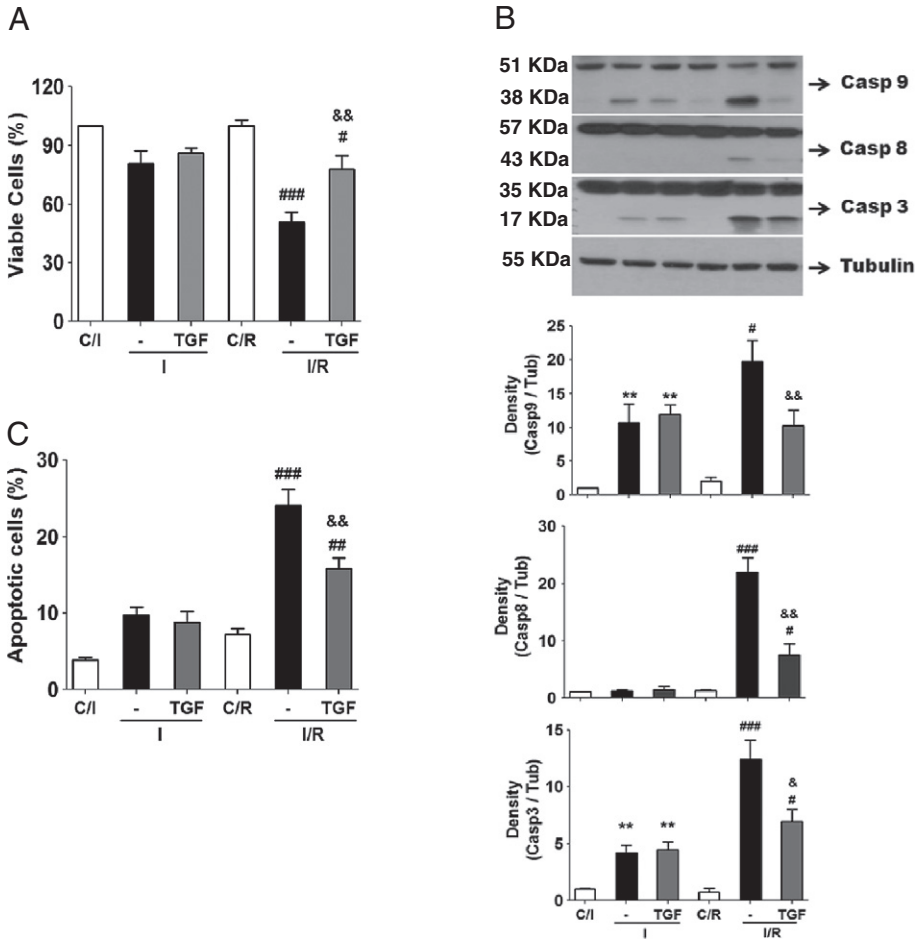

Fig. 2. TGF-β1 promotes cytoprotection in cardiac fibroblasts subject to I/R. TGF-β1 (TGF: 5 ng/mL) exerts protection against both (A) cell viability lost measured by the quantification of trypan blue exclusion (#p<0,05 and ###p<0,001 *vs.* I 8 h; &&p<0,01 *vs.* I/R), and (B) the activation of caspases 9, 8 and 3 produced by I/R (8 h/24 h), but not ischemia (8 h) measured by the densitometry of representative immunoblots (**p<0.01 *vs.* C/I, #p<0.05 and ###p<0.001 *vs.* I 8 h, &p<0.05 *vs.* &&p<0.01 *vs.* I/R n = 6). (C) apoptosis by flow cytometry triggered in cardiac fibroblasts subject to I/R (8 h/24 h) (**p<0.01 and ***p<0.001 *vs.* I 8 h, ##p<0.01 *vs.* I/R + TGF). The results are means (± S.E.M.) of 6 independent experiments.

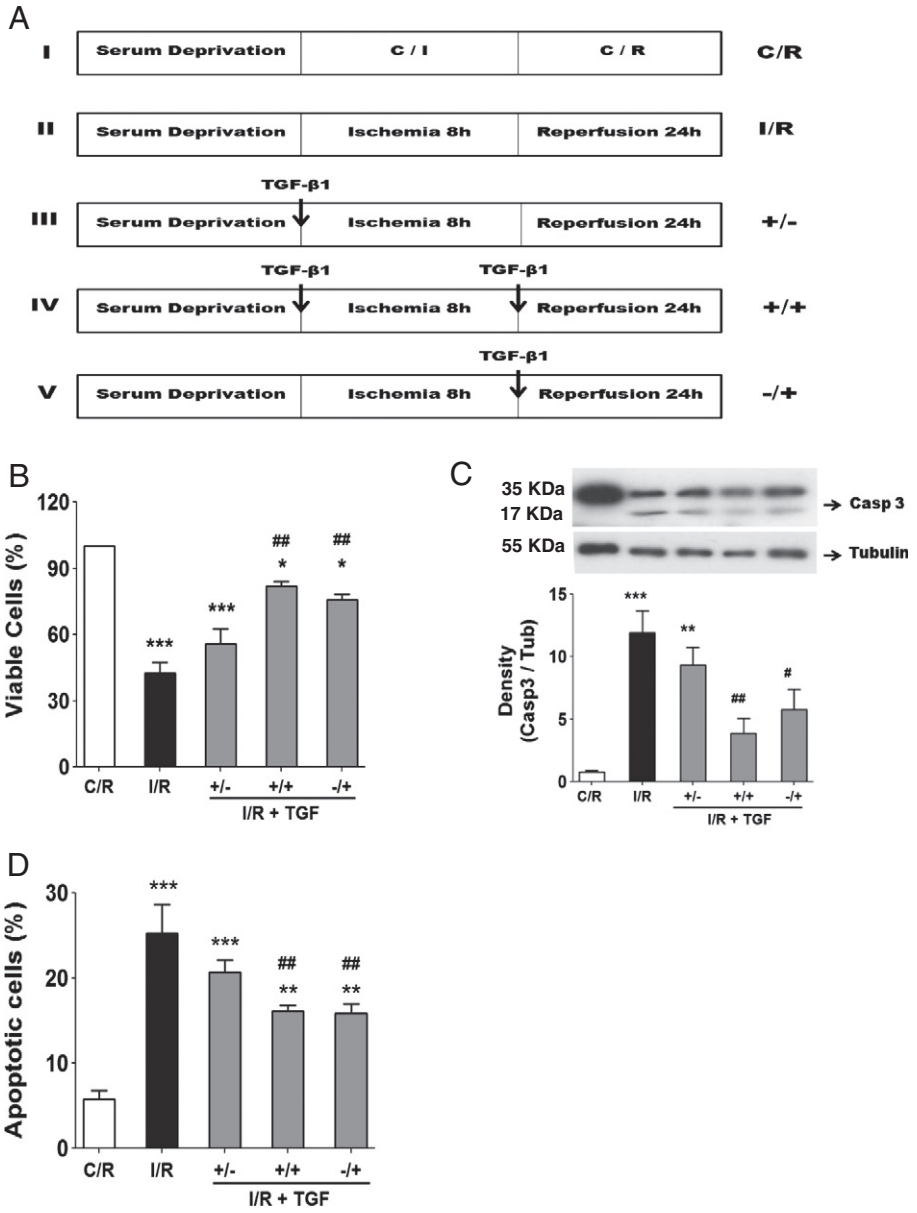

Fig. 3. Time course of cytoprotective effects by TGF-β1 in cardiac fibroblast subject to I/R. (A) representative diagram of TGF-β1(TGF: 5 ng/mL) treatment during ischemia (+/−), only in reperfusion (−/+) and in both instances (+/+). (B) Graphic analysis of cytoprotection afforded by TGF-β1 on loss of cell viability quantified by trypan blue exclusion, (C) the activation of caspase 3, measured by the densitometry of representative immunoblots, and (D) apoptosis by flow cytometry (*p<0.05, **p<0.01 and ***p<0.001 *vs.* C/R, #p<0.05 and ##p<0.01 *vs.* I/R). The results are means (±S.E.M.) of 4 independent experiments.

PD, LY and SB inhibited completely the respective signaling pathways activated by TGF-β1, and we did not observe cross-modulation of the inhibitors between these signaling pathways. Supplementary Fig. 6 depicts that Smad2 is localized in the cytosol in unstimulated CF. However, TGF-β1 (1 h) stimulated Smad2 migration to the nucleus, being affected by SB but not by PD and LY. As shown earlier, ERK1/2 and Akt, but not Smad2/3, were activated by reperfusion in CF. Thus, we evaluated whether these protective signaling pathways are involved in the prevention of CF death after sI/R. To this aim, CFs were subjected to sI/R in the presence of PD and LY. Both inhibitors potentiated the injured events caused by sI/R, increasing the loss of cell viability (Supplementary Fig. 7A), activation of caspases 8, 9 and 3 (Supplementary Fig. 7B) and apoptosis (Supplementary Fig. 7C) whereas, SB did not change the effects induced by I/R.

Fig. 5A shows that the protection in cell viability, induced by TGF-β1, was completely blocked by PD, LY and SB. Similar results were observed on the activation of caspases 8, 9 and 3 (Fig. 5B) and apoptosis (Fig. 5C). Finally, we evaluated the role of Akt and Smad3 on the cytoprotective effects triggered by TGF-β1 using the specific inhibitors Akti and SIS3. Our results show that both inhibitors completely prevented the cytoprotective and anti-apoptotic effects of TGF-β1 (Fig. 6A–B), suggesting that Akt and Smad3 participate in the cytoprotective effects of TGF-β1.

4. Discussion

The main result of this study was that TGF-β1 activated canonical and non-canonical signaling pathways and produce cytoprotective effects on cultured neonate and adult rat CF under sI/R. According to the literature ischemia causes cell damage in a time dependent manner, as demonstrated both by increased cell death under ischemia in *in vitro* assays [23], as well as in *in vivo* models of cardiac ischemia

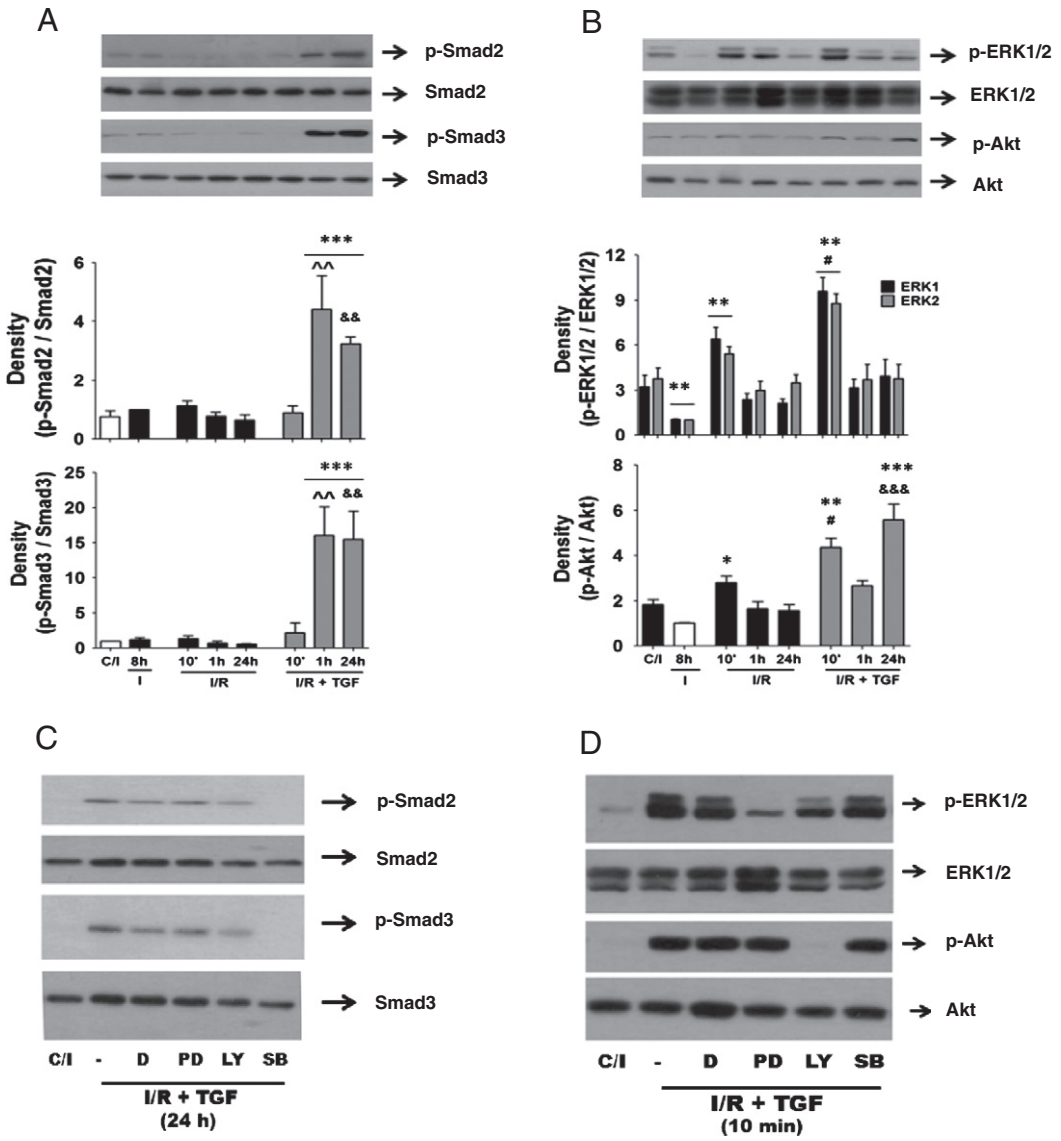

Fig. 4. Activation of ERK1/2, Akt and Smad2/3 signaling pathways by I/R and TGF-β1. I/R and TGF-β1 (TGF: 5 ng/mL) activate the (A) canonical (Smad2 and Smad3) and (B) non-canonical (ERK1/2 and Akt) signaling pathways, measured by densitometry of representative immunoblots for each protein (*p<0.05, **p<0.01 and ***p<0.001 *vs.* C/I, #p<0,05, and ###p<0.001 *vs.* I/R 10 min; &&p<0.01 and &&&p<0.001 *vs.* I/R 24 h). The results are means (±S.E.M.) of 8 independent experiments. (C) Representative immunoblot of p-ERK1/2, p-Akt and p-Smad2 in cardiac fibroblast subject to I/R plus TGF-β1, in the presence of specific inhibitors, PD98059 (PD) (25 μM), LY294002 (LY) (10 μM) and SB431542 (SB) (10 μM) plus DMSO as vehicle (V).

by the occlusion of left ventricular artery [24]. We recognize that our *in vitro* work is far from those performed *in vivo*; however, several investigators have shown a relationship between the damage caused by I/R *in vivo* with those performed in *in vitro* assays [21,25,26]. Our data are in accordance with the previous observations because simulated ischemia induced significant CF death from 12 h. However, Chen et al. have reported that CF proliferate and become more active under anoxia [27]. These differences could be explained by differences in methodology (anoxia *vs* hypoxia). Our model of simulated ischemia includes hypoxia, low pH, nutrient deprivation and the inhibition of glycolysis, making it more deleterious than hypoxia itself. Our data also show that non-lethal ischemia (less than 8 h) stimulated the processing of procaspases 9 and 3 but without DNA fragmentation (Fig. 1C). These data are somewhat contradictory; it could be explained by the different energy states experienced by cells during the ischemic period, in which ATP levels do not completely decrease [23]. Some reports have shown that cells exposed to apoptotic stimulus activated caspase 3, but are unable to activate the final stage of apoptosis (DNA fragmentation and condensation) [23,28]. Reperfusion is a double edged sword because although initially it may attenuate cell damage generated by ischemia, thereafter this process may increase cell death [29,30]. Our data show that CF death caused by simulated ischemia 24 h is higher than those triggered by sI/R (8 h/16 h); however the reperfusion (by 16 h) also generates additional CF death compared to control and non-lethal simulated ischemia (Supplementary Fig. 1). Deleterious events generated by reperfusion are usually attributed to apoptosis [31–34]. Cardiomyocytes exposed to I/R die by activating both the intrinsic and extrinsic pathways of apoptosis [35]. In this regard, our data show that simulated ischemia activated caspases 3 and 9 but without DNA fragmentation. However, reperfusion triggers a robust apoptotic process by activating the intrinsic (caspase 9), extrinsic (caspase 8) pathways including the executing caspase 3 and subsequently increasing sub-G1 population. Collectively, these results suggest that reperfusion could also stimulate the release of proapoptotic factors involved in the activation of the extrinsic pathway of apoptosis. However, other types of cell death were triggered in the CF

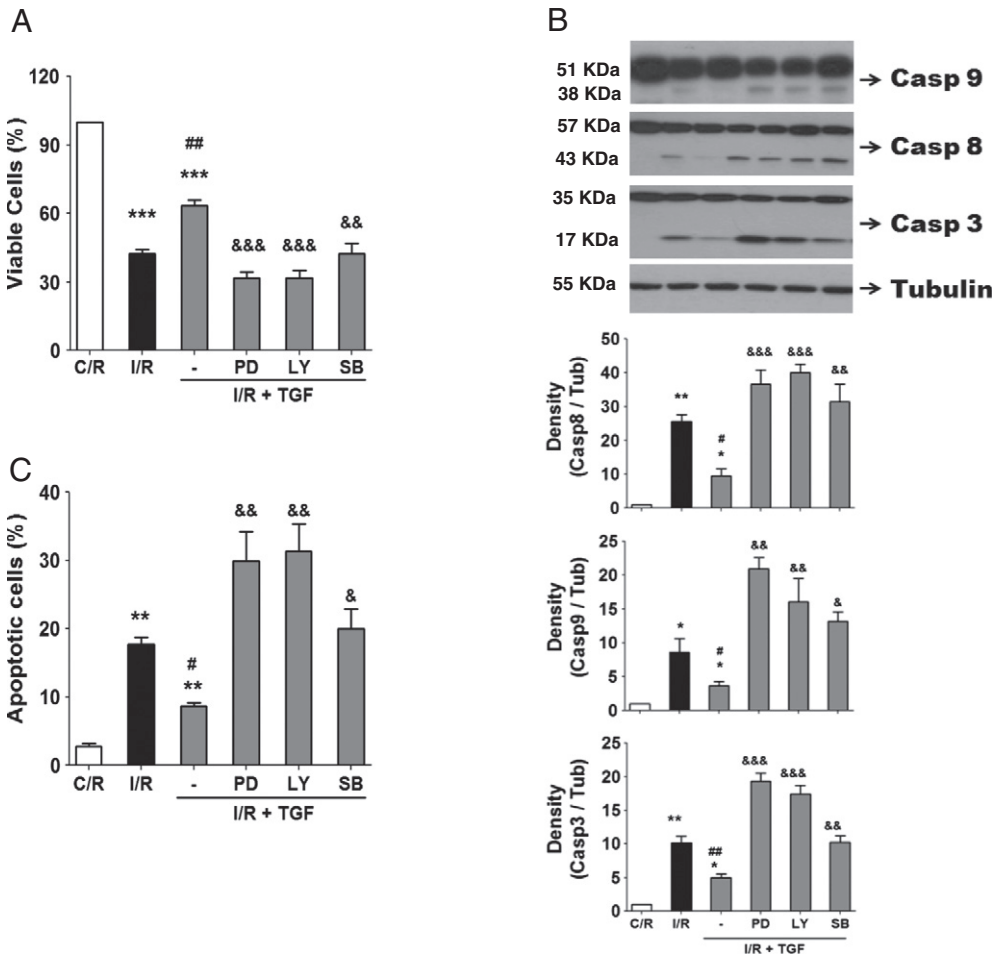

Fig. 5. Cytoprotective effects of TGF-β1 by the activation of ERK1/2, PI3K-Akt and Smad2/3 signaling pathways. (A) Cell viability loss measured by the quantification of trypan blue exclusion, (B) the activation of caspases 9, 8 and 3, measured by the densitometry of representative immunoblots and, (C) apoptosis by flow cytometry in cardiac fibroblast subject to I/R (8 h/24 h) in the presence of TGF-β1 (5 ng/mL) plus the specific inhibitors of ERK1/2 (PD: 25 μM), PI3K-Akt (LY: 10 μM) and Smad2/3 (SB: 10 μM) signaling pathways (*p<0.05, **p<0.01 and ***p<0.001 *vs.* C/R, #p<0.05 and ##p<0.01 *vs.* I/R, &p<0.05, &&p<0.01 and &&&p<0.001 *vs.* I/R + TGF). The results are means (± S.E.M.) of 6 independent experiments.

subjected to sI/R. Our data also showed sI/R induced necrosis assessed by two independent techniques (Supplementary Fig. 2A and B).

It is well known that TGF-β1 promotes CF proliferation and clearly its differentiation to myofibroblast [36]. We show here that TGF-β1 also exerted cytoprotective effects rather than proliferative actions on stressed neonate (Supplementary Fig. 8) and adult rat CF. At cardiac level, TGF-β1 has shown to exert anti-apoptotic effects both *in vitro* [7–9] and *in vivo* [37]. In CF subjected to sI/R, TGF-β1 decreased the activation of the intrinsic and extrinsic apoptotic pathways. Several evidences have shown that TGF-β1 decreases and/or inactivates some key pro-apoptotic proteins such as Bax and caspases whereas in parallel this growth factor induces anti-apoptotic protein like Bcl-2 [9,17,18]. TGF-β1 may also promote cell survival by preventing the release of TNF-α [38], a cytokine involved in the activation of caspases 8 and 3

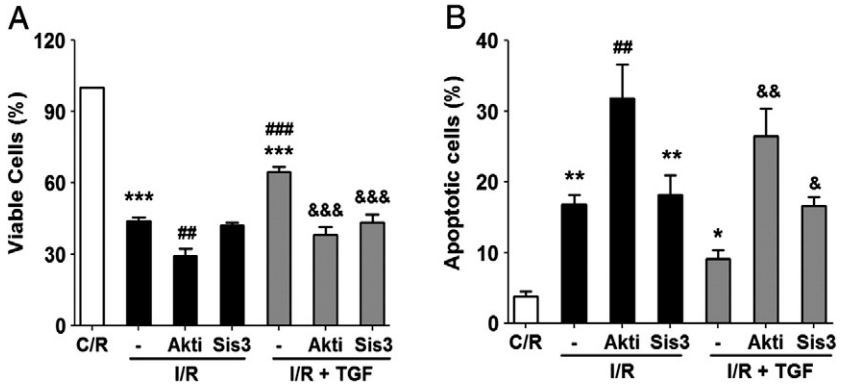

Fig. 6. TGF-β1 activates Akt and Smad3 to prevent the loss of CF viability and apoptosis I/R-induced. (A) Cell viability loss measured by the quantification of trypan blue exclusion and, (B) apoptosis measured by flow cytometry of cardiac fibroblasts subject to I/R (8 h/24 h) treated with TGF-β1 (5 ng/mL) in the presence of specific inhibitors of Akt (Akti1/2: 50 μM) and Smad3 (SIS3: 1 μM), respectively (*p<0.05, **p<0.01 and ***p<0.001 *vs.* C/R; #p<0.05, ##p<0,01 and ###p<0.001 *vs.* I/R, &p<0.05, &&p<0.01 and &&&p<0.001 *vs.* I/R + TGF). The results are means (± S.E.M.) of 6 independent experiments.

[39]. Our results depicted that the non-lethal simulated ischemia-dependent activation of caspases 9 and 3 was not prevented by TGF-β1. These results suggest that TGF-β1 is not enough to counteract the damage triggered by a prolonged simulated ischemia. In this regard, some maneuvers like cardiac preconditioning reduce cardiac damage produced by ischemia [40–42], but a previous window of time is required to prevent cardiac damage. In this sense, our results show that TGF-β1 may also require time to exert cytoprotection. Future work should explore if TGF-β1 regulates CF survival stimulating gene transcription by the activation of Smad3.

Our results are the first to demonstrate that phospho-ERK1/2 levels decreased in CF subjected to simulated ischemia (8 h), while no changes in the levels of phospho-Akt and phospho-Smad2/3 were observed. In this sense, our results agree with literature because the exposure of cardiomyocytes to ischemia also decreased MAPK and Akt activities [43]. In addition, our results show that ERK1/2 was activated early at the beginning of simulated ischemia, perhaps as a consequence of trigger survival mechanism induced by ischemic conditions, whereas Akt was inactivated during all ischemic period. Thus, the late inactivation of ERK1/2 and Akt is probably indicating that cell death mechanisms were activated. However, other groups have shown that the ischemia increased the activation level of ERK1/2 and Akt in cultured cardiomyocytes [44]. On the other hand, reperfusion increased the activation of salvage kinases such as ERK1/2 and Akt and maneuvers of survival in sI/R, such as pre- and post-conditioning, significantly activate all these pathways [45,46]. Our results show that cytoprotective activity of ERK1/2 and Akt was increased in reperfused CF. Other authors have highlighted the importance of the activation of these survival pathways as well as their inhibition at the time of reperfusion may exacerbate damage caused by sI/R. In this sense, Yue et al., showed that reperfusion of infarcted rat hearts in the presence of inhibitor PD, increases damage caused by I/R [47]. Our data demonstrate that the inhibition of ERK1/2, induced greater activation of extrinsic and intrinsic pathways of apoptosis, and it has been described in the literature that caspases are inhibited by survival proteins such as ERK1/2 [48]. Similarly, the presence of the inhibitor LY potentiated the CF apoptosis effects of sI/R. In this regard, there are several mechanisms by which Akt could be inhibiting apoptosis, including their ability to inhibit pro-apoptotic protein Bad and inhibition of the fragmentation of caspase 9 [49,50].

As discussed above, TGF-β1 and reperfusion activated ERK1/2 and Akt signaling pathways, suggesting that these pathways are partially involved in the survival mechanism activated by both reperfusion and TGF-β1. However, under ischemic conditions TGF-β1 does not triggers ERK1/2 and Akt activation, although Smad2 was activated. We do not know why under ischemic conditions this growth factor does not induce the activation of ERK1/2 and Akt; however, we believe that ischemic conditions (hypoxia, low pH, nutrient deprivation and inhibition of glycolysis), could hamper the activation of these kinases, or probably under ischemic conditions the specific phosphatases are activated. These studies will be performed further.

On the other hand, in the presence of PD and LY, the anti-apoptotic effects of TGF-β1 were prevented and they agree with results observed in the cells of neuronal origin [17]. In CF, Smad2/3 regulates collagen secretion and differentiation to cardiac myofibroblasts [14]. However, our results show for the first time that Smad2/3 has cytoprotective effects on CF subjected to I/R. It is also known that PI3K-Akt signaling pathway activated through TGF-β1receptor type 1 (TβR1) may have other downstream targets of Akt, including cAbl [51,52]. However, Akti completely inhibited the cytoprotective effects of TGF-β1, showing that Akt is involved in CF survival exposed to sI/R. Finally, SIS3 significantly inhibited anti-apoptotic effects of TGF-β1 on CF under sI/R, showing that Smad3 is important for the cytoprotective effects of TGF-β1. SIS3 had similar effects to that produced by SB; therefore, the activation of Smad2/3 is crucial for cytoprotection of TGF-β1 in sI/R. Although we cannot conclude that Smad2 is directly involved in the anti-apoptotic action of TGF-β1, most of the genes regulated by the TGF-β1 canonical pathway require both Smad2 and Smad3 [14,53], and has been shown that Smad2 may have a role in the effects of both pro- and anti-apoptotic TGF-β1 in cancer cells [54]. Finally, it has been shown that TGF-β1 modulates cross-talk between ERK1/2 and Akt with Smad2/3 signaling pathways, suggesting that maybe the activation of these pathways together could account for its cytoprotective effect. However, with our results, we suggest that these pathways play independent cytoprotective roles, since the specific activation of Smad2/3 by TGF-β1 was inhibited only by SB and not by specific inhibitors of ERK1/2 and Akt.

The main limitation of this work was that: TGF-β1 through TβR1 may activate other signaling pathways apart from Smad2/3, ERK1/2 and Akt, such as p38 or JNK, which have proven to be both pro-apoptotic and anti-apoptotic [23,55]. This question should be resolved in further studies.

In conclusion, CF subjected to simulated ischemia and sI/R experienced a loss of viability and apoptosis in a time dependent manner, although sI/R resulted in additional cell death and apoptosis than that produced by ischemia alone. Cytoprotective effects of TGF-β1 were mediated by the activation of ERK1/2, Akt and Smad2/3 signaling pathways.

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