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Ras inhibition attenuates myocardial ischemia–reperfusion injury

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ABSTRACT

Myocardial injury, developed after a period of ischemia/reperfusion (I/R) results in the destruction of functional heart tissue, this being replaced by scar tissue. Intracellular signaling pathways mediating cardiomyocyte death are partially understood and involve the activation of Ras. p38–MAPK, JNK and Mst-1 are downstream effectors of Ras protein. We hypothesized that S-farnesylthiosalicylic acid (FTS), a synthetic small molecule that detaches Ras from the inner cell membrane, consequently inhibiting Ras activity, reduces I/R myocardial injury *in vitro* and *in vivo*.

Wistar rat hearts were isolated, mounted on the Langendorff apparatus and subjected to ischemia (30 min, 37 °C) and reperfusion. During the reperfusion period, the hearts were perfused with FTS (1 μM) solution or control buffer. Left anterior descending (LAD) ligation and subsequent reperfusion was performed in two groups of Wistar rats. Rats received 5 mg/kg FTS or PBS according to two protocols: (A) FTS or PBS were administered daily 7 days prior, immediately before and 14 days (every other day) after LAD occlusion or (B) every other day for 14 days post-I/R. Hearts from FTS-treated rats (Langendorff) and FTS-treated rats (protocol A) showed a significant improvement in myocardial performance and smaller scar tissue compared with the PBS group. Infarct size in the FTS-treated group was 12.7 ± 2% vs. 23.7 ± 4% in the PBS-treated (*in vitro*) group and 17.3 ± 2.5% vs. 36 ± 7% compared with control I/R rats (*in vivo*) $p < 0.05$. These effects may be associated with the down regulation of JNK as a short-term effector and with Mst-1 in the long-term remodeling process.

1. Introduction

Myocardial injury developed after a period of ischemia–reperfusion, may have many causes. In the past, most research concentrated on the mechanisms causing cellular injury during ischemia and on protective measures to reduce development of ischemic injury [1]. It is now recognized that the resumption of oxygenated blood into previously ischemic myocardium can initiate a cascade of events that paradoxically produces additional myocardial cell dysfunction and cell death [2]. The cellular mechanisms involved in the pathogenesis of myocardial ischemia/reperfusion (I/R) injury are complex and involve the interaction of a number of cell types, including coronary endothelial cells, circulating blood cells, and cardiac myocytes [3], most of which are capable of generating reactive oxygen species (ROS). ROS play an important role in the progression and aggravation of heart failure, and can induce contractile dysfunction and myocardial structural damage [4].

Apoptosis is an active gene-directed cell death process that plays a key role in myocardial reperfusion injury [5]. Cardiac myocyte cell death triggered by I/R can occur by apoptosis or necrosis. While cell death after prolonged periods of ischemia is ascribed to necrosis, apoptosis occurs in cells and tissues exposed to reoxygenation after ischemia [6]. The intracellular signaling pathways that mediate stress responses of cardiomyocytes have not been fully delineated.

It has been shown that Ras protein can be activated by ROS and by oxidative stress. This activation may lead to cardiac hypertrophy by the activation of downstream effectors such as ERK 1/2 [7]. Other signaling pathways which appear to play central roles in myocyte damage, are the two 'stress-responsive' mitogen-activated protein kinase (MAPK) subfamilies, c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (p38–MAPKs) [6]. It has recently been demonstrated that myocardial I/R, with contribution of ROS, activates JNK and P38 pathways resulting in apoptosis [6,7]. These signaling pathways are downstream effectors of Ras protein [8].

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Mammalian sterile 20-like kinase 1 (Mst-1) is a ubiquitously expressed serine/threonine kinase [9], which belongs to a mammalian sterile 20-like (STE 20-like) kinase family. Increasing lines of evidence suggest that Mst-1 and other STE20-like family kinases play an important role in mediating apoptosis [9,10]. Mst-1 is both a caspase-3 cleavage target and an enhancer of caspase-3 activation [11]. Similar to Erk, P38 and JNK, Mst-1 is also a downstream effector of Ras protein [11,12] Inhibition of Mst-1 attenuated apoptosis and cardiac dysfunction following MI [13]. Cardiac-specific overexpression of Mst-1 causes dilated cardiomyopathy in mice and the inhibition of endogenous Mst-1 prevents cardiac myocyte apoptosis and reduces the size of MI in response to I/R [14].

Ras dependent signaling requires both GTP binding and inner cell membrane anchorage. Specific anchorage of Ras is promoted by the carboxy terminal S-farnesyl cysteine carboxy methylester and by the upstream hypervariable region typical of each Ras isoform [15,16]. Previous studies have shown that prevention of Ras membrane anchorage by the Ras inhibitor S-farnesylthiosalicylic acid (FTS, Salirasib) results in the effective inhibition of Ras signaling [17,18]. FTS dislodges active Ras from its membrane anchorage sites [17]. Animal studies [18] and human trials (http://www.concordiapharma.com/index.htm) have shown that FTS has a good safety profile.

In the present study, we tested, using *in vitro* and *in vivo* models, the hypothesis that the inhibition of Ras protein can attenuate the cardiac damage triggered by I/R injury, Down stream targets such as MAPK, that could be activated in the immediate phase of I/R and Mst-1 were involved 2 weeks following MI. FTS cardioprotection from I/R injury was associated with down regulation of JNK as a short-term effector and the remodeling process in the long-term.

2. Materials and methods

2.1. Animals

Male Lewis rats were purchased from Harlan and maintained in a local animal house under conventional conditions. Experimental procedures were approved by the Institutional Ethics Committee for Animal Experimentation.

2.2. Reagents

Most of the materials were purchased from Sigma Chemical Co. (St. Louis, MO). FTS, synthesized as previously described [19], was a gift from Concordia Pharmaceuticals Inc. (Fort Lauderdale, FL). Western immunoblotting was conducted using: pan-anti-Ras Ab (Ab03; Santa Cruz, CA, USA) and all the other antibodies were from Cell Signaling Technology, USA.

2.2.1. FTS preparation for isolated heart perfusion

FTS was stored in chloroform, which was evaporated under a stream of nitrogen immediately before use. The powder was dissolved in DMSO 10%. This solution was diluted with Krebs–Henseleit bicarbonate (KHB) buffer solution to yield a 1 μM drug solution. The DMSO concentration in the KHB was negligible (0.001%).

2.2.2. Isolated heart perfusion

Rats were heparinized (500 U/kg) and anesthetized (I.P.) with ether. The hearts ($n = 7$, from each group) were quickly removed, the aorta cannulated and the heart perfused in retrograde according to Langendorff at a pressure of 96 cm H_2O with oxygenated KHB containing (mM): 118 NaCl, 2.4 KCl, 1.2 $MgSO_4$, 7× H_2O, 2.5 $CaCl_2$, 5 EDTA, 1.2 KH_2PO_4, 25 $NaHCO_3$, 4 glucose at 37 °C [20]. A latex balloon filled with water was inserted into the left ventricular cavity through a small incision in the left atrium and connected to a Statham Medical P132284 pressure transducer (Mennen Medical, Inc., Clarence, NY). The balloon was tied and inflated to a volume producing a diastolic pressure of 0–5 mm Hg. Left ventricular developed pressure (LVP) was continuously monitored during the experiment using AT-CODAS Software (Dataq Instr. Inc., Akron, OH). The isolated heart was stabilized for 20 min at a constant perfusion pressure and then subjected to 30 min of ischemia followed by 30 min reperfusion. Ischemia was created by clamping the aortic cannula. During reperfusion, hearts were given KHB with FTS 1 μM or KHB only. The temperature of the heart (sensed in the right ventricle) was maintained at 37 ± 0.2 °C throughout the experiment by a micro thermocouple connected to a digital thermometer (Webster Laboratories Altadena, CA, USA). Left ventricular systolic pressure was continuously monitored and recorded every 10 min. The rate of pressure development and relaxation ($\pm dP/dt$ max) and the heart rate (HR), were measured using AT-CODAS Software (Dataq Instr. Inc., Akron, OH). The LVP was calculated by subtracting end-diastolic pressure from the peak end-systolic pressure, rate pressure product (RPP), an index of myocardial workload, was calculated by multiplying LVP by HR. Coronary effluent was collected at 1-min intervals before and after ischemia, at various time points (1, 10 and 30 min reperfusion) and analyzed for CK activity (Boehringer Mannheim).

We conducted a dose-finding pilot study before the main experiments, in which we tested different concentrations of FTS and chose the dose with the best results of LVP, CF, and CK release.

2.3. Measurement of irreversible ischemic injury

After 30 min reperfusion, hearts were weighed and then frozen. Hearts were sliced in parallel to the AV groove into approximately 2-mm sections, which were incubated with 2,3,5-triphenyl tetrazolium chloride (TTC 1%) in phosphate buffer at 37 °C for 30 min. TTC stained the viable tissue with red while the necrotic tissue remained discolored. Sections were fixed overnight in 2% paraformaldehyde and then placed between two cover slips and digitally photographed using a Fugi Finepixs1pro camera, with a resolution of 1400 × 960 pixels and quantified with IMAGE J 5.1 software. Risk zone areas and infarct-to-risk ratios were determined by computerized planimetry. From each slice an image was obtained from both sides, and all calculations from one heart were averaged into one value for statistical analyses [21].

2.4. Left anterior descending (LAD) artery ligation

At the age of 12 weeks, rats ($n = 5$ from each group) were anesthetized (mixture of 8 mg/100 g ketamine, 5 mg/100 g xylazine), intubated, and ventilated with a Harvard Rodent Ventilator Model 383 (respiratory rate: 50/min, respiratory volume: 2.5 mL). A rectal thermocouple was used to continuously monitor body temperature, which maintained at 37 °C using a heating pad. A left thoracotomy in the third intercostal space was performed to expose the heart. The location of the left descending coronary artery was identified and then occluded with a 6–0 silk suture. Occlusion was confirmed by monitoring the pallor of the region at risk, and an electrocardiogram was used to observe changes such as widening of QRS and ST-T segment elevation. After 30 min, the occlusion was removed, the thorax was closed and rats were returned to their cages at the local animal house. No death occurred in response to LAD occlusion, or drug injection.

2.5. FTS preparation for injections

FTS was stored in chloroform, which was evaporated under a stream of nitrogen immediately before use. The powder was dissolved in absolute ethanol and diluted to the desired concentra-

tion in sterile PBS made basic with NaOH. Carrier solution (1000 μl) containing 1.35 mg of FTS (5 mg/kg) was injected intraperitoneally (I.P.) into each rat. A control solution was prepared at the same time starting with PBS and absolute ethanol.

2.6. FTS injections

LAD ligation was performed in two groups of rats. Group 1 received 5 mg/kg FTS and group 2 received PBS according to two protocols: (A) FTS or PBS were administered daily for 7 days before LAD ligation, right before opening the LAD occlusion and continued for 14 days, every other day (pre- and post-injections); (B) FTS or PBS were administered every other day for 14 days after LAD ligation. On day 14 rats were studied by echocardiography as previously described [22]. The rats were sacrificed on day 15. Hearts were taken for histological and immunological analyses. These concentrations and regimens were chosen according to our previous work on the myocarditis model [23].

2.7. Echocardiography

Animals were lightly anesthetized by the inhalation of isoflurane. Two-dimensional (2D) guided M-mode echocardiography was performed using an echocardiogram (Philips, Sonos 5500, USA) equipped with a S12 MHz phased-array transducer. The parasternal long-axis of the hearts was viewed in a 2D image. The M-mode cursor was then positioned perpendicular to the interventricular septum and posterior wall of the LV at the level of the papillary muscles. An M-mode image was obtained at a sweep speed of 100 mm/s. Diastolic and systolic LV wall thickness,

Table 1
Baseline cardiac function and coronary flow in Langendorff-perfused isovolumically contracting rat hearts.

	Control $n = 7$	FTS $n = 7$	P value
Total weight (g)	360 ± 25.5	349 ± 38.6	0.26
Heart weight (g)	1.57 ± 0.17	1.53 ± 0.17	0.35
CF total (mL/min)	15 ± 2	14 ± 4	0.41
CF/heart weight	9.2 ± 2.45	9.3 ± 2.95	0.46
LVP (mmHg)	196 ± 14	200 ± 9	0.42
dP/dt max (mmHg/s)	4544 ± 1284	4126 ± 475	0.22
ndP/dt max (mmHg/s)	3832 ± 1177	3641 ± 587	0.35
HR (b/min)	263 ± 43	265 ± 37	0.45

Values are expressed as mean ± S.E. The baseline values of the rats and cardiac function prior to ischemia in FTS (1 μM) and control groups. No significant differences were found in any of the measurements taken from the rats in the FTS vs. the control group.

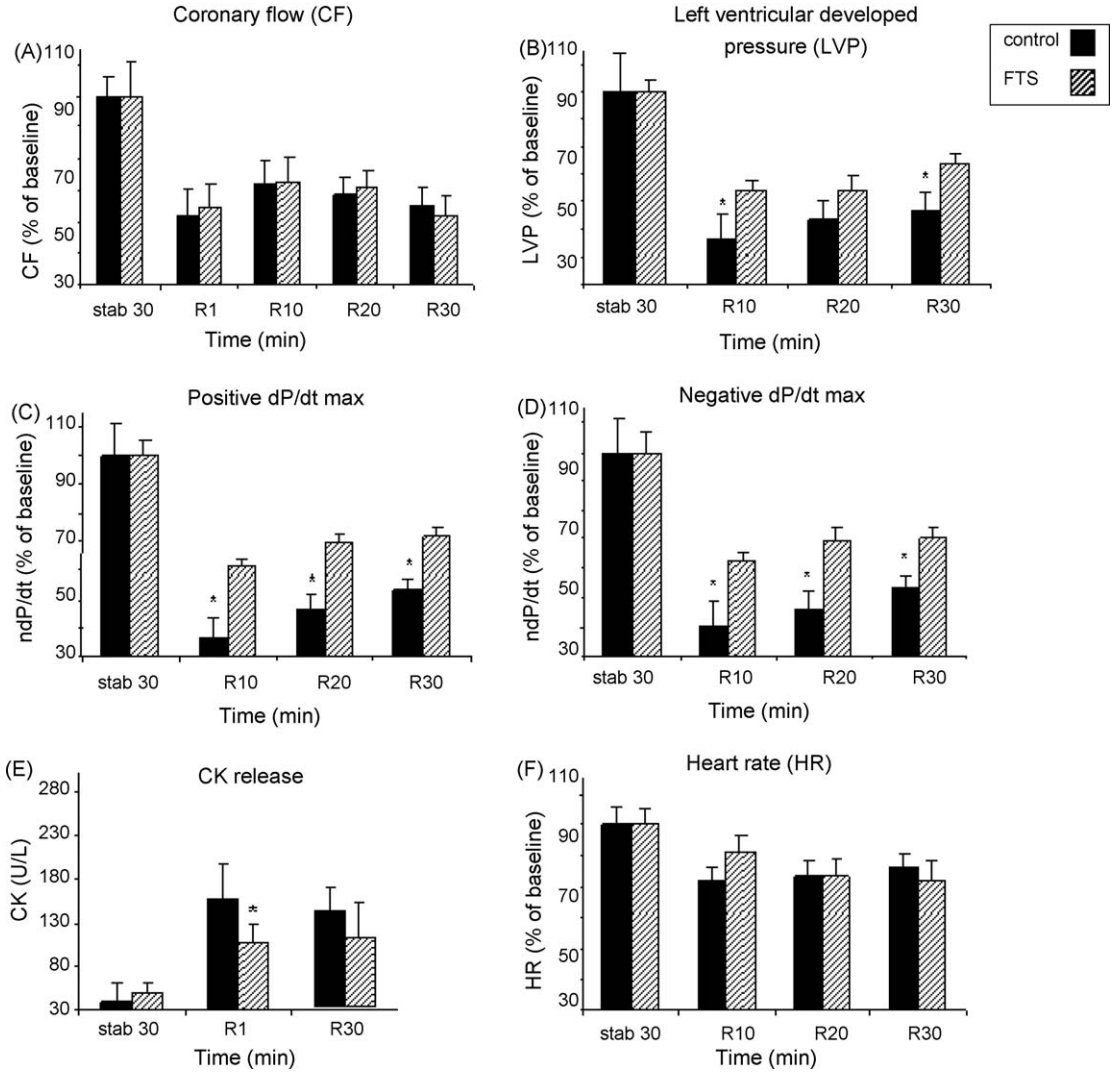

Fig. 1. The effect of FTS heart perfusion on cardiac function, coronary flow and CK release, Langendorff-perfused hearts were subjected to a 20 min stabilization period, then to 30 min ischemia and 30 min of reperfusion. In that period FTS hearts were perfused with 1 μM of FTS. Measurements were taken at the stabilization period and every 10 min in the reperfusion period. The measurements are presented as a percentage of baseline. Refer to Table 1 for baseline values for both groups. CF (A), LVP (B), dP/dt max (C), ndP/dt max (D), CK release (E), and HR (F). Columns mean ± S.E. $^*p < 0.05$ compared with control. $n = 7$ in each group.

LV end-diastolic dimensions (LVEDA), and LV end-systolic chamber dimensions (LVESA) were measured. The percentage of LV fractional shortening (LV%FS) was calculated as [(LVEDA-LVESA)/LVEDA] × 100. Echocardiography was performed 1 day before and 24 h post-LAD ligation. The technician who performed the test was blinded to treatment allocation.

2.8. Histopathology

Hearts were removed and fixed in 4% formalin, then embedded in paraffin. Several transverse sections were cut from the paraffin-embedded samples and stained with Hematoxylin and Eosin. Sections from each heart were also stained with Masson trichrome for collagen deposition. Slides were then assessed in a blinded fashion by a pathologist using light microscopy and scored for the percentage of infarcted scar (blue area) to total LV ring area [24].

2.9. Determination of Ras, Erk, p-Erk, Jnk, p-Jnk, P38, p-P38, Mst-1, p-Mst1

At the end of the *in vivo* and *in vitro* ischemia and reperfusion experiments ($n = 5$ from each group) left ventricular tissue (100 mg) was homogenized in cold homogenization buffer containing protease inhibitors. The composition of the homogenization buffer was: 150 mM NaCl, 20 mM $MgCl_2$, 50 mM Tris–HCl pH 7.6, 1 mM DTT, and 5 mM NP40. Benzamidine 2 mM, aprotinin 0.4 M, leupeptin 105 μM, pepstatin 0.4 mM, 3.33 mM PMSF, and vanadate 0.01 mM. Protein concentration was measured by the Bradford assay. Samples containing 100 μg protein were used for the determination of protein levels by Western immunoblotting using: pan-anti-Ras Ab (Ab03; Santa Cruz, CA, USA) anti JNK Ab (Cell Signaling), anti p-JNK Ab (Cell Signaling) anti P38 Ab (Cell Signaling), anti p-P38 Ab (Cell Signaling), anti Mst-1 Ab (Cell Signaling) and anti p-Mst1 Ab (Cell Signaling). Enhanced chemiluminescence (ECL) and densitometry analysis were performed as detailed previously [25].

3. Determination of Ras-GTP

The hearts were prepared as mentioned above. Samples containing 0.5 mg protein were used for assessing the levels of active GTP-bound Ras by the glutathione S-transferase-RBD pull-down assay, followed by Western immunoblotting with pan-anti-Ras Ab, as detailed previously [26].

4. Statistical analysis

Results are expressed as means ± standard error (S.E.) of the mean. Values during stabilization period were defined as 100%. A statistical difference between the groups was assessed by ANOVA with repeated measurements using the multiple comparison option of Duncan. If differences were established, values were compared using Student's *t*-test: $p < 0.05$ was considered significant.

5. Results

5.1. In vitro study using the isolated heart

5.1.1. Baseline data

Table 1 summarizes the baseline values of the rats and cardiac function prior to ischemia in FTS (1 μM) and control groups. No

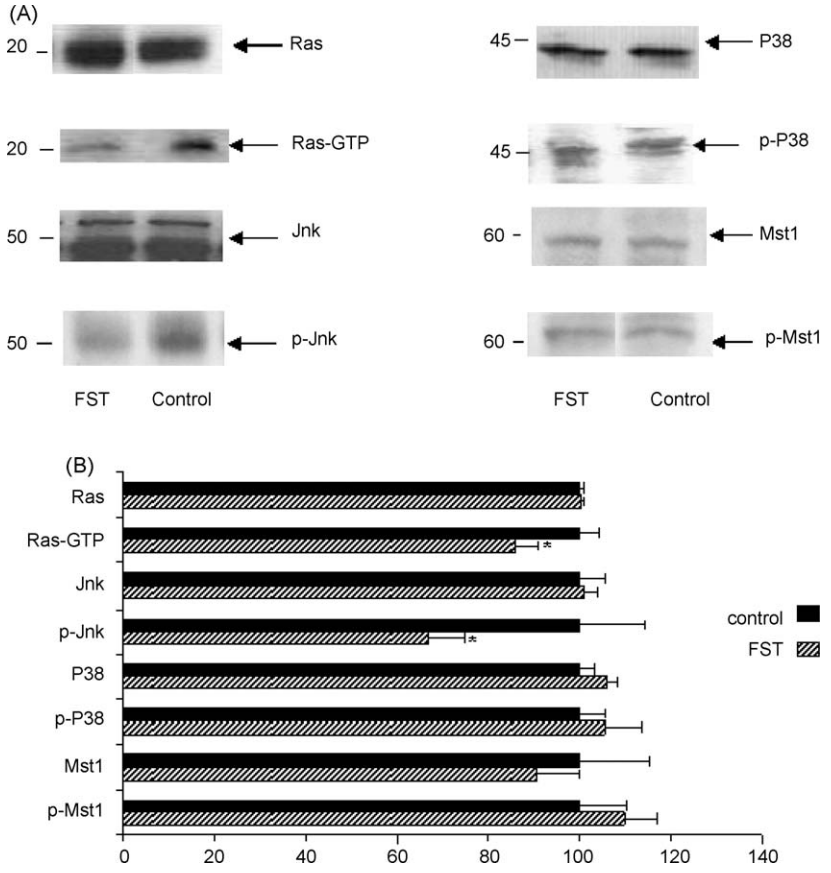

Fig. 2. Western immunoblot analysis on the isolated hearts. (A) In order to examine the effect of FTS on Ras signaling, we performed Western immunoblotting on control and FTS perfused hearts. (B) The results of densitometric analysis of the immunoblots in both groups. Columns mean ± S.E., $n = 5$ from each group. $^*p < 0.05$ compared with control.

significant differences were found in any of the measurements taken from the rats in the FTS as opposed to the control group. Hemodynamic measurements also showed no difference between groups, including LVP, $\pm dP/dt$ max and HR.

5.1.2. The effect of FTS perfusion on the heart recovery

We tested the effect of 1 μM FTS perfusion to rat hearts in the Langendorff system after 30 min of ischemia (Fig. 1). FTS had no effect on the coronary flow recovery as compared to the PBS control group (Fig. 1A) during 30 min of reperfusion. LVP and $\pm dP/dt$ max recovery was significantly better in the FTS perfused hearts compared to controls ($p < 0.05$) (Fig. 1B–D). CK leakage to the coronary effluent increased in both groups compared with the baseline measurements. CK release in the coronary effluent during reperfusion was lower in the FTS group compared with control group (Fig. 1E). There was no significant difference in the recovery of HR between both groups (Fig. 1F).

5.1.3. FTS reduced irreversible ischemic injury

TTC staining revealed that FTS perfusion to the isolated heart subjected to 30 min ischemia and 30 min of reperfusion resulted in a reduced area of irreversible ischemic injury compared to the control group (12.7 ± 2% vs. 23.7 ± 4% respectively, $p < 0.05$).

5.1.4. The effect of FTS on Ras signaling in the perfused hearts

In order to examine the effect of FTS on Ras and its prominent downstream effectors, we performed Western immunoblotting using homogenates of the perfused hearts. Quantitative analysis of the immunoblots disclosed a reduction in levels of active Ras (Ras-GTP) and p-Jnk in hearts of the FTS group ($p < 0.05$) after 30 min of reperfusion. No differences were observed in total Ras, Jnk, P38, p-P38, Mst-1 and p-Mst1 levels in both groups (Fig. 2).

5.2. In vivo experiments

5.2.1. FTS pre-treatment improves LV function post-LAD ligation

In order to further investigate the effect of systemic FTS treatment on the heart we conducted *in vivo* experiments. FTS given 7 days prior to LAD ligation at a dose of 5 mg/kg and then for a period of 14 days (protocol A, pre and post-treatment) resulted in a significant improvement in most hemodynamic parameters of cardiac function compared with control I/R group (Fig. 4). Left ventricular end-systolic and end-diastolic area (LVESA and LVEDA) were significantly better in the FTS protocol (A) group compared with the control I/R group, $p < 0.05$ (Fig. 3A and B). The FTS-treated rats in protocol A demonstrated significantly improved fractional shortening of the area compared with the control I/R rats (55 ± 6%

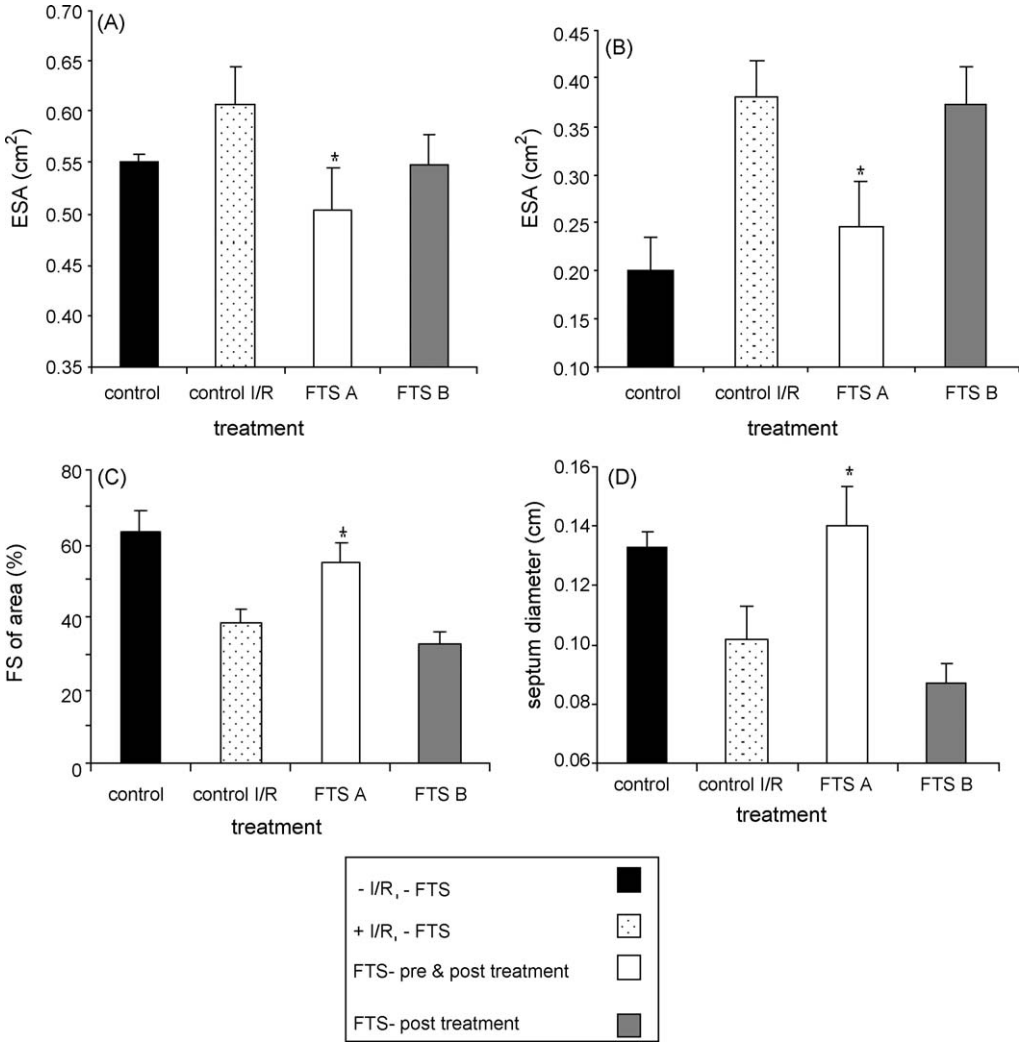

Fig. 3. Echocardiographic studies in FTS vs. PBS-treated rats, LAD ligation was performed on two groups of rats. The rats were injected with 5 mg/kg FTS or PBS according to the two protocols. Fourteen days after ligation, rats were taken to echocardiography, and then sacrificed. These studies demonstrated LVESA and LVEDA of the rats (A and B). In addition, fractional shortening of area (FS of area) is also presented (C), and changes in the septum thickness (D) were measured. Columns mean ± S.E., $n = 5$ from each group. $^{*}p < 0.05$ compared with control I/R.

vs. 38 ± 4% respectively, $p < 0.05$) (Fig. 3C). The left ventricular septal diameter was significantly higher in the rats from the FTS group treated according to protocol (A) as compared with the control I/R rats (0.14 ± 0.01 vs. 0.1 ± 0.01 respectively, $p < 0.05$; Fig. 3D). Heart rates were similar in both groups (287 ± 20 vs. 283 ± 28); posterior wall thickness was similar (0.16 ± 0.02 vs. 0.159 ± 0.02).

When FTS was given 14 days post-LAD ligation (protocol B, post-treatment) no improvement in cardiac hemodynamic function was observed compared to control I/R. (Fig. 3A–D).

5.2.2. FTS attenuates ischemia/reperfusion damage

To determine the morphological damage caused to the heart tissue, heart sections were stained with H&E and Masson's Trichrome. Typical sections from FTS- and PBS-treated rats are shown in Fig. 4B. The area of irreversible damage presented as a percentage of LV wall is illustrated in Fig. 4A. Rats that received FTS according to protocol (A) showed a significant reduction in the area of irreversible damage (17.3 ± 2.5%) compared with control I/R rats (36 ± 7%). Rats that received FTS according to protocol (B) did not show a significant difference compared with the control I/R group (Fig. 4A, $p < 0.05$).

5.2.3. The effect of FTS on Ras signaling in the hearts from in vivo treatment

Similar to the *in vitro* experiment, in this experiment we also examined the effect of FTS on Ras and its downstream effectors, by using Western immunoblotting. Quantitative analysis demonstrated a reduction in levels of total Ras, active Ras (Ras-GTP) and p-Mst1 in hearts of rats from FTS group ($p < 0.05$). Levels of Mst, P38, p-P38, Jnk and p-Jnk did not differ in both groups (Fig. 5).

6. Discussion

I/R injury induces a series of events that culminate in cardiomyocyte death [27,28]. FTS is a Ras protein inhibitor, which has shown efficacy in various immune mediated disorders [29,30]. FTS perfusion to the isolated heart resulted in the improvement in

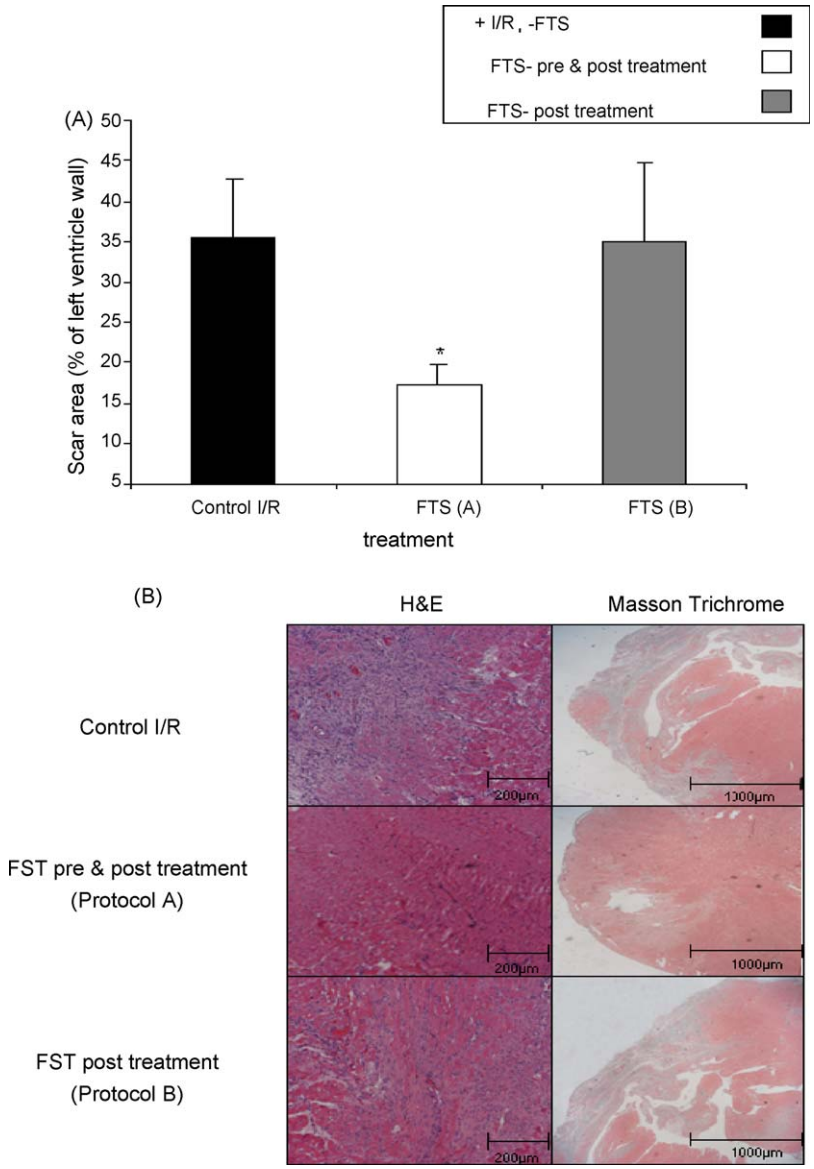

Fig. 4. Histological evaluation of irreversible damage area in FTS vs. PBS-treated rats. Hearts from rats-treated pre and post with FTS (protocol A) or 14 days post-LAD ligation (protocol B) were sectioned and stained with H&E and Masson trichrome. Scar area was determined as described in Section 2. (A) Columns mean ± S.E.*$p < 0.05$ compared with control I/R. (B) Microscopic examination of the hearts from all experimental groups. $n = 5$ from each group.

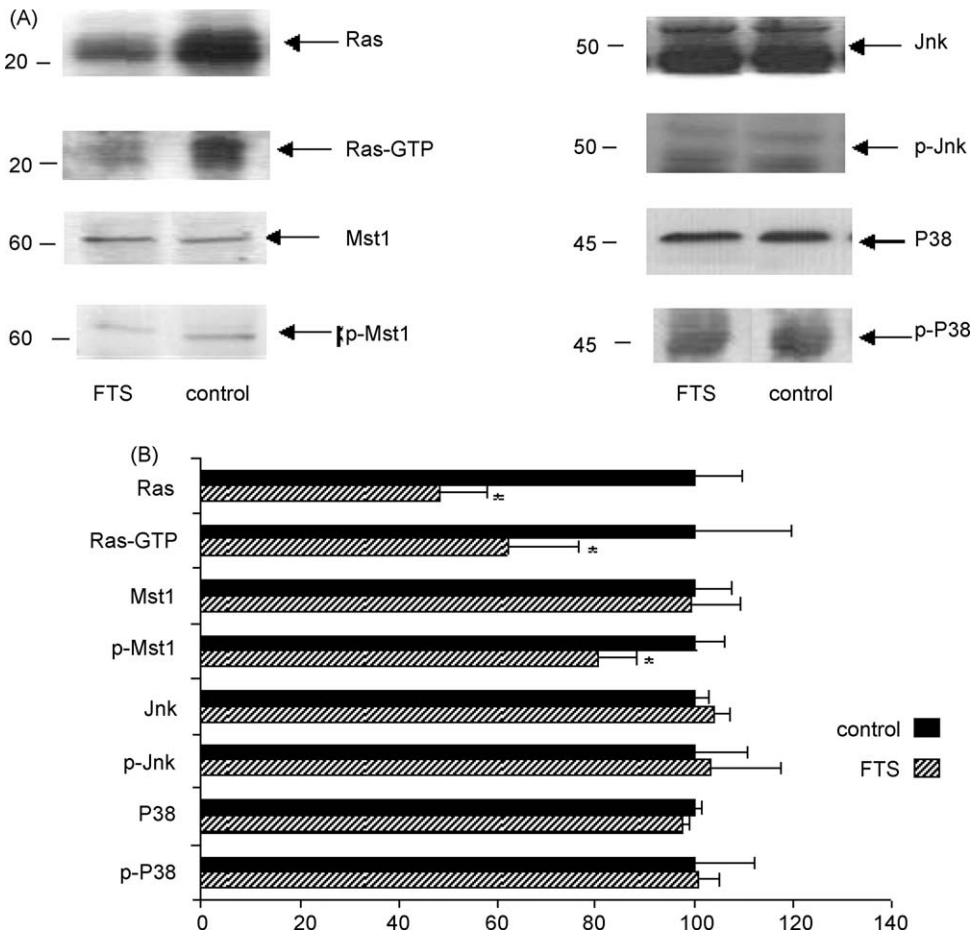

Fig. 5. Western immunoblot analysis to the hearts in LAD ligation model. In order to examine the effect of FTS on Ras signaling, we performed Western immunoblotting on control I/R and FTS-treated rats. (B) The results of densitometric analysis of the immunoblots in both groups. Columns mean ± S.E. $^{*}p < 0.05$ compared with control I/R. $n = 5$ from each group.

hemodynamic measurements and reduced the irreversible cardiac damage. Our present *in vivo* results indicate that in order to produce the delayed improvement during reperfusion, Ras inhibition must begin before ischemia, since amelioration was observed only when FTS was administered before I/R.

Chronic activation of Ras proteins is mostly recognized in proliferative disorders, the most prominent of which is cancer. In recent years, chronic activation of Ras and some of its downstream effectors were found to be associated with increased propensity of cells to undergo apoptosis [11,12]. As cardiomyocyte apoptosis is a key mediator of damage caused by I/R injury, we reasoned that Ras inhibition might result in a reduction of I/R damage and improvement in cardiac function *in vitro* and *in vivo*. Indeed, FTS perfusion to the isolated heart also resulted in a better recovery, as observed by reduced LVP and the rate of pressure development and relaxation. Correspondingly, histological analysis confirmed the reduction in the myocardial areas with irreversible injury and this finding was supported by the reduced cardiac enzyme leakage in the FTS-treated rats. Similar to our results, previous studies have shown that administration of Ras-GTP inhibitor (FPT III) prior to being subjected to I/R in the isolated heart perfusion system improved LVP function [31,32]. They conclude that ischemia induced activation of Ras-GTPase signaling produces injury that cannot be corrected by later inhibition of Ras during reperfusion. It seems that Ras signaling is only a potentially therapeutic applicable target for inhibition when administered before ischemia.

Our *in vivo* results show that FTS, given according to protocol A (prevention regimen), resulted in a reduction of ~20% in the scar area while FTS administered according to protocol B (treatment regimen) did not demonstrate a beneficial effect, compared with control animals. Indeed, animals treated according to protocol A, but not according to protocol B, showed an advantage in most of the functional parameters (observed in echocardiography), compared with controls. The reduction in cardiomyocyte death and the smaller scar area were associated with improved cardiac function. One of the potential mechanisms by which FTS could attenuate myocardial damage is by interference with leukocyte and lymphocyte migration to the myocardium. Studies have reported that improved cardiac function and lower cardiac damage in the LAD ligation model is associated with attenuated inflammation as observed in H&E staining [33,34]. FTS afforded myocardial protection against ischemic damage as seen by both scar area and septal thickness yielding a better fractional shortening in protocol A group vs. controls. The measurement of scar area as a percentage of the left ventricle cross-section area, following permanent coronary ligation, has previously been reported [35]. Our experience with FTS supports the potent immumomodulatory action of this agent that could involve several effector cells such as lymphocytes, antigen presenting cells and leukocytes [23].

We and others have shown that FTS acts via Ras. A large body of evidence has been published in many papers and reviews supporting the notion that FTS is a Ras inhibitor that

downregulates Ras. FTS was shown to act particularly well on active GTP-bound Ras [36]. In this work, FTS down regulated total Ras and Ras-GTP in the *in vivo* experiment, whereas in the *in vitro* experiment, we observed a decrease in Ras-GTP, but not in total Ras levels. These results are consistent with a short-term effect on active Ras that is being tuned down as the study is extended. The *in vitro* protocol that is sampled after 30 min is insufficient to bring down total Ras levels, whereas in the *in vivo* and longer study, levels of total Ras fall after the reduction of active GTP-Ras levels. In order to investigate the effect of FTS on Ras signaling activity, we examined several Ras downstream effectors. In the *in vivo* experiment there was a significant decrease in p-Mst1, but not in Mst-1 levels in the FTS-treated rats. P38, p-P38, JNK and p-JNK did not differ in both *in vivo* groups. These kinases are known to be affected in the immediate phase of I/R injury (37). Mst-1 is a pro-apoptotic downstream effector of Ras. It was previously shown that inhibition of endogenous Mst-1 prevents cardiac myocyte apoptosis and reduces MI size in response to I/R [13,14]. These findings support the potential role of Mst-1 as a downstream mediator of the effect provided by Ras. However, further studies are warranted to further explore these mechanisms. In the *in vitro* experiment, there was a significant decrease in p-JNK but not in JNK levels in FTS perfused hearts. JNK phosphorylation is associated with a tendency to undergo apoptosis in many cell types including cardiomyocytes [38,39]. It has recently been demonstrated that I/R triggers JNK activation [40] and suppression of JNK activation is associated with improved heart recovery from I/R in the isolated heart model [37]. Collectively, these data suggest that the JNK and Mst-1 downstream pathway may play differential roles in mediating the beneficial effects of Ras inhibition by FTS in the immediate and long-term.

In the present study we showed that FTS was effective in reducing the damage caused to the heart in the I/R model. The effect was demonstrated both *in vivo*, by LAD ligation. The result may be associated with JNK in the short-term and with Mst-1 in the long-term. If these findings are further supported, FTS may be employed as a novel therapeutic agent to attenuate I/R injury in the heart. Currently, patients with acute MI undergo either percutaneous coronary angioplasty or are treated with thrombolytic agents. In both these strategies, pre-treatment with a protective agent is possible and applicative. In view of our results the possibility of administering FTS prior to stent implantation in the context of urgent catheterization, aiming to reduce ischemic reperfusion injury, should be investigated.

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