

Cardioprotection from ischemia-reperfusion injury due to Ras-GTPase inhibition is attenuated by glibenclamide in the globally ischemic heart

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The present study was designed to see if acute local inhibition of Ras-GTPase before or after ischemia (during perfusion) would produce protection against ischemia and reperfusion (I/R)-induced cardiac dysfunction. The effect of glibenclamide, an inhibitor of cardiac mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels, on Ras-GTPase-mediated cardioprotection was also studied. A 40 min episode of global ischemia followed by a 30 min reperfusion in perfused rat hearts produced significantly impaired cardiac function, measured as left ventricular developed pressure (P_{\max}) and left ventricular end-diastolic pressure (LVEDP). Perfusion with Ras-GTPase inhibitor FPT III before I/R [FPT(pre)], significantly enhanced cardiac recovery in terms of left ventricular contractility. P_{\max} was significantly higher at the end of 30 min reperfusion in FPT(pre)-treated hearts compared to pre-conditioned hearts. However, the degree of improvement in left ventricular contractility was significantly less when FPT III was given only after ischemia during reperfusion [FPT(post)]. Combination treatment with FPT III and glibenclamide before I/R resulted in significant reduction of FPT III-mediated cardioprotection. These data suggest that activation of Ras-GTPase signaling pathways during ischemia are critical in the development of left ventricular dysfunction and that opening of mitoK_{ATP} channels, at least in part, contributes to cardioprotection produced by Ras-GTPase inhibition. Copyright © 2006 John Wiley & Sons, Ltd.

KEY WORDS — FPT III; glibenclamide; ischemia/reperfusion; pre-conditioning; signal transduction

INTRODUCTION

Several cellular processes including cellular growth, differentiation, proliferation, and apoptosis are tightly controlled by small GTPases of the Ras super family by switching between inactive GDP-bound and active GTP-bound states.^{1,2} Ras-GTPase, a signaling molecule upstream of mitogen-activated protein (MAP) kinases, is an important relay for G-protein coupled receptors (GPCR) and receptor tyrosine kinase (RTK)

signaling. Stimulation of small G-proteins such as Ras and kinases, for example, p90 ribosomal S6 kinase by reactive oxygen species (ROS) leads to cardiac dysfunction.^{3–6} It has been well documented that oxidative stress activates Ras which is necessary for transmission of signals by several cytokines, such as TGF-beta, involved in ischemic insult to its downstream effectors, such as phosphoinositide-3-kinase.⁷ Previously we have shown in vascular smooth muscle cells, that stimulation of GPCR by angiotensin II or norepinephrine and activation of EGFR by epidermal growth factor leads to formation of 20-hydroxyeicosatetraenoic acid, which activates Ras-GTPase-mediated MAP kinase signaling.⁸ Subsequently we reported that Ras-GTPase activity in the heart is increased during development of hypertension and normalization of Ras-GTPase activity by the selective

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inhibitor H-Cys-Val-2-Nal-Met-OH (Nal = 2-naphthylalanine)FPT III] attenuates elevation of blood pressure and hypertension-related renal and cardiac end organ damage.^{9–11}

Recent reports have shown that chronic systemic inhibition of Ras-GTPase before ischemia and reperfusion (I/R) improves recovery of cardiac function in a perfused rat heart model of global ischemia.^{12,13} However, FPT III-mediated protection against I/R-induced injuries is not well characterized. The present study was designed to evaluate if acute local inhibition of Ras-GTPase by FPT III before or after ischemia would also produce protection of ventricular global contractile functions during I/R in a model of cardiac global ischemia. The effect of glibenclamide, an inhibitor of cardiac mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels, on FPT III-mediated cardioprotection was also studied.

METHODS

Hearts were rapidly removed from male Wistar rats after intravenous heparinization (1000 U kg⁻¹ body weight). The excised hearts were immediately mounted on the Langendorff perfusion assembly (Hugo Sachs Electronics, Freiburg, Germany), and were perfused initially with a constant pressure perfusion of 50 mm Hg with oxygenated (95% O₂ + 5% CO₂) Krebs–Henseleit buffer (37°C) of the following composition (in mM): NaCl 117; KCl 3.75; CaCl₂ 2.5; NaHCO₃ 20.0; KH₂PO₄ 1.21; MgCl₂ · 6H₂O 1.2; glucose 12.0; pH 7.35. A water-filled balloon was introduced into the left ventricle and connected to a Statham pressure transducer (P23Db) and balloon volume was adjusted to give the baseline end-diastolic pressure of 5 mm Hg. Left ventricular developed pressure (P_{\max}), left ventricular end-diastolic pressure (LVEDP) and changes in left ventricular pressure ($+dP/dt$ and $-dP/dt$) were continuously monitored. Perfusion pressure was measured immediately downstream from the flow probe in a branch of the aortic cannula using a Statham pressure transducer and was electronically maintained constant at 50 mm Hg by means of a perfusion pressure control module (HSE). This system permits accurate adjustment of perfusion pressure between 5 and 300 mm Hg to an accuracy of ± 1 mm Hg.

Hearts were treated as follows ($n = 7$ /group): Group 1 [ischemia (Isch)]: Hearts were perfused for 30 min and then subjected to 40 min ischemia followed by 30 min reperfusion. Group 2 (pre-conditioning

(PC) + Isch): Hearts underwent a PC step involving 2 cycles of 5 min ischemia followed by 10 min reperfusion prior to 40 min ischemia followed by 30 min reperfusion. Group 3 [(FPT(pre) + Isch)]: Hearts were perfused with FPT III for 30 min then subjected to 40 min ischemia followed by 30 min reperfusion. Group 4: [FPT(pre) + PC + Isch]: Hearts were perfused with FPT III for 30 min and underwent a PC step prior to 40 min ischemia followed by 30 min reperfusion. Group 5: [(FPT(post) + Isch)]: Hearts were perfused for 30 min then subjected to 40 min ischemia followed by a period of 30 min reperfusion with FPT III. Group 6: [glibenclamide + (FPT(pre) + Isch)]: Hearts were perfused with FPT III and glibenclamide (1 μ M) for 30 min then subjected to 40 min ischemia followed by 30 min reperfusion. The present study conformed to the guidelines for the care and use of laboratory animals published by the U.S. National Institute of Health (NIH publication No 85–23, revised 1985).

All drugs and reagents used in the study were purchased from Sigma-Aldrich Co., unless otherwise indicated. FPT III was obtained from Calbiochem (USA). One milligram FPT III was dissolved in 300 ml Krebs–Henseleit buffer and perfused with a flow rate of 10 ml min⁻¹ to deliver 3.33 μ g ml⁻¹ kg FPT III. Glibenclamide (1 μ M) was dissolved in Krebs–Henseleit buffer. The doses of drugs used in these studies were determined on the basis of previous observations.^{8–15}

The results are expressed as mean \pm SEM. Mean values were compared using analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparisons test (GraphPad InStat). Comparison of the time-course of changes in reperfusion recovery of P_{\max} , $+dP/dt$, $-dP/dt$, and LVEDP was done by a two tailed unpaired t -test for each time period. Computerized statistical analysis was accomplished with SPSS for Windows (V.6.0.1; SPSS Inc. Evanston, Illinois, USA). A p value of less than 0.05 was considered to be statistically significant.

RESULTS

Actual values of left ventricular developed pressure (P_{\max}), LVEDP and changes in left ventricular pressure ($+dP/dt$ and $-dP/dt$) are given in Table 1. Figure 1 shows the time-course of reperfusion recovery during the 30 min reperfusion step in P_{\max} and LVEDP after ischemia (Isch), PC followed by ischemia (PC + Isch), pre-treatment with FPT III

Table 1. Effect of FPT III on left ventricular contractility of perfused hearts exposed to global ischemia

	P_{\max} (mm Hg)			$+dP/dt$ (mm Hg)			$-dP/dt$ (mm Hg)			LVEDP (mm Hg)		
	Con	Rep	%R	Con	Rep	%R	Con	Rep	%R	Con	Rep	%R
Isch	110 ± 4	44 ± 5	40 ± 4	2051 ± 110	761 ± 36	37 ± 2	1527 ± 99	557 ± 30	37 ± 2	5.2 ± 0.1	43.3 ± 3.8	832 ± 84
PC + Isch	116 ± 5	75 ± 6	65 ± 5*	2182 ± 133	1192 ± 68	55 ± 3*	1543 ± 86	939 ± 44	62 ± 4*	5.1 ± 0.2	18.0 ± 1.9	347 ± 27*
FPT(pre) + Isch	115 ± 4	93 ± 6	81 ± 2 [†]	2052 ± 88	1505 ± 78	74 ± 4 [†]	1609 ± 91	1133 ± 45	70 ± 4*	5.5 ± 0.3	20.5 ± 1.9	374 ± 34*
FPT(pre) + PC + Isch	109 ± 5	88 ± 6	81 ± 4	1954 ± 34	1411 ± 37	72 ± 1	1615 ± 31	1271 ± 38	79 ± 2	5.0 ± 0.1	13.5 ± 1.2	277 ± 31
FPT(post) + Isch	105 ± 3	52 ± 2	49 ± 2*	2793 ± 115	1383 ± 69	50 ± 4*	2039 ± 56	934 ± 35	46 ± 2*	5.4 ± 0.2	39.8 ± 4.3	752 ± 97
Glib + FPT(pre) + Isch	101 ± 5	70 ± 2	70 ± 3 [#]	3022 ± 104	1750 ± 128	59 ± 5 [#]	2213 ± 89	1329 ± 49	61 ± 4 [#]	5.2 ± 0.1	28.2 ± 3.6	540 ± 73 [#]

The data were computed at 30 min reperfusion and expressed as mean ± SEM. Con, control; Rep, reperfusion; P_{\max} , Left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; %R = % recovery = (reperfusion/control × 100).

*indicates significantly different compared to ischemia, $p < 0.05$.

[†]indicates significantly different compared to PC + ischemia, $p < 0.05$.

[#]indicates significantly different compared to FPT(pre) + ischemia, $p < 0.05$.

before ischemia [(FPT(pre) + Isch)]; treatment with FPT III after ischemia during perfusion [(FPT(post) + Isch)]. Figure 2 shows the time-course of reperfusion recovery during the 30 min reperfusion step in P_{\max} and LVEDP after ischemia (Isch), pre-treatment with FPT III before ischemia (FPT(pre) + Isch); pre-treatment with glibenclamide and FPT III combination followed by ischemia (GLIB + FPT(pre) + Isch).

The P_{\max} values indicate that pre-treatment with PC (75 ± 6 mm Hg) resulted in significant improvement in recovery after I/R as compared to ischemia alone (44 ± 6 mm Hg; $p < 0.05$). Similarly, LVEDP values were significantly lower with PC (18 ± 1.9 mm Hg) compared to ischemia alone (43.3 ± 3.8 mm Hg; $p < 0.05$). Changes in left ventricular pressure were significantly higher in the PC group (+ dP/dt : 1192 ± 68, - dP/dt : 939 ± 44) compared to ischemia alone (+ dP/dt : 761 ± 36, - dP/dt : 557 ± 30; Table 1).

Pre-treatment with FPT III before ischemia [(FPT(pre) + Isch)] produced beneficial effects significantly better than PC (Table 1, Figure 1). The P_{\max} values indicate that FPT(pre) + Isch showed even better improvement than PC (93 ± 6 mm Hg vs. 75 ± 6 mm Hg) where the percentage recovery was doubled in FPT(pre) + Isch compared to ischemia alone (81 ± 2 vs. 40 ± 5). Similar trends were observed in LVEDP, + dP/dt and - dP/dt (Table 1, Figure 1). Interestingly, treatment with FPT III after ischemia [FPT(post) + Isch] produced significantly less protection compared to FPT(pre). When hearts were perfused with FPT III for 30 min, followed by a PC step prior to 40 min ischemia followed by 30 min continuous reperfusion [FPT(pre) + PC + Isch], recovery was not significantly different from FPT(pre) + Isch (Table 1).

When treatment with FPT III was combined with glibenclamide before I/R, it resulted in significant reduction of FPT III-mediated cardio-protection (Table 1, Figure 2). The P_{\max} values indicate that FPT III and glibenclamide combination treatment resulted in significant reduction (70 ± 2 mm Hg) in recovery after I/R as compared to FPT(pre) alone (93 ± 6 mm Hg; $p < 0.05$). An increase in LVEDP was observed with combination of FPT III and glibenclamide treatment (28.2 ± 3.6 mm Hg) compared to FPT(pre) treatment alone (20.5 ± 1.9 mm Hg; Table 1, Figure 2). Similar trends were observed in + dP/dt and - dP/dt indicating significant attenuation of FPT III-mediated cardioprotection due to inhibition of cardiac mitochondrial ATP-sensitive potassium (mito- K_{ATP}) channels by glibenclamide (Table 1). Glibenclamide treatment also attenuated PC-mediated

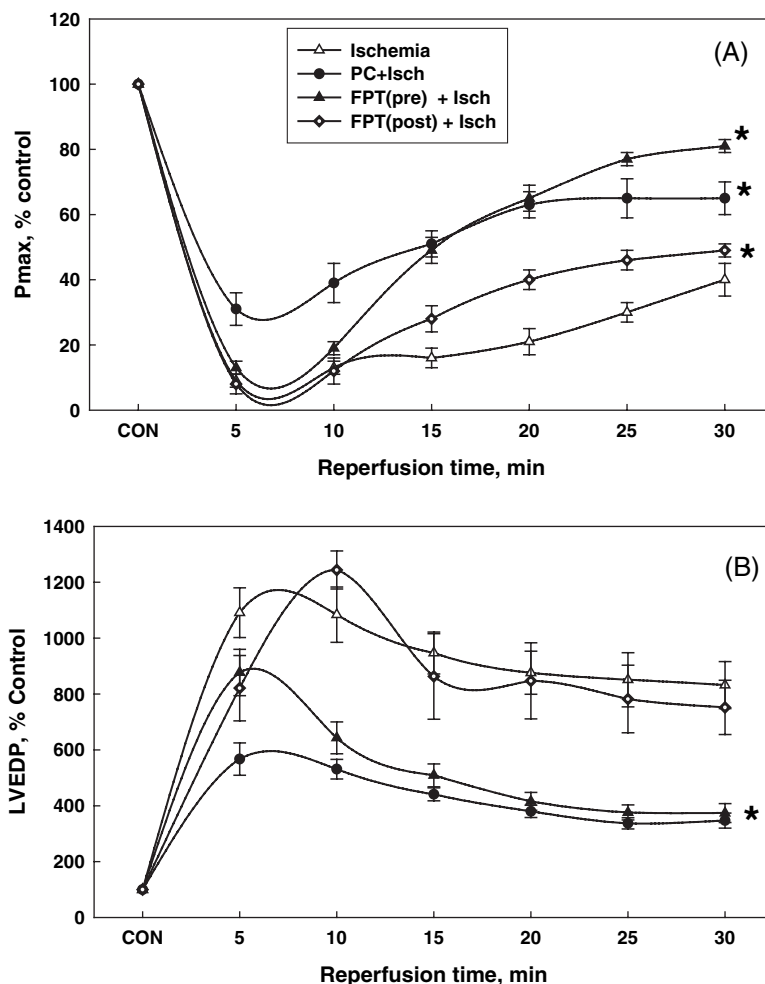


Figure 1. Time course of reperfusion recovery in P_{max} (A) and LVEDP (B) after ischemia (Isch), pre-conditioning + ischemia (PC + Isch), pre-treatment with FPT III before ischemia [FPT(pre) + Isch]; treatment with FPT III after ischemia [(FPT(post) + Isch)]. * indicates significantly different compared to ischemia, $p < 0.05$

cardioprotection (data not shown) as previously shown by others.^{23–27}

DISCUSSION

This is the first study to show that acute local inhibition of Ras-GTPase farnesylation can protect the heart against I/R-induced dysfunction better than PC. Perfusion with FPT III for 30 min before I/R significantly improved left ventricular contractility (P_{max} and LVEDP) to values similar to those observed

with PC. In contrast, improvement in left ventricular contractility was less pronounced when FPT III was given only during reperfusion. Interestingly, glibenclamide was able to significantly attenuate FPT III-mediated cardioprotection.

Ischemia/reperfusion exposes the heart to many cell stresses including increased production of ROS, ionic imbalances, metabolic deprivation, and osmotic and mechanical stresses.^{3,4,16} As a result, such ischemic triggers are known to initiate release of mediators that activate GPCR and tyrosine kinases (TKs).^{5,17} Ras has been shown to be directly involved in the regulation of the intracellular redox state and to be associated with

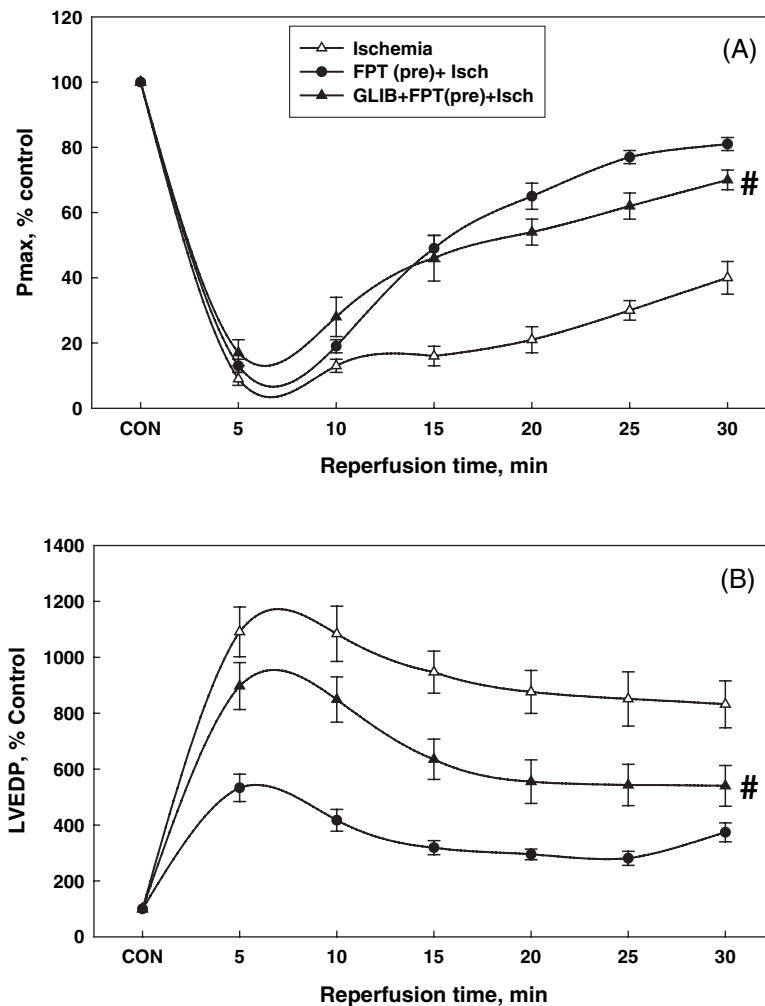


Figure 2. Time course of reperfusion recovery in P_{\max} (A) and LVEDP (B) after ischemia (ischemia), pre-treatment with FPT III before ischemia (FPT(pre) + Isch); treatment with glibenclamide and FPT III before ischemia [GLIB + (FPT(pre) + Isch)]. # indicates significantly different compared to [(FPT(pre) + ischemia)], $p < 0.05$

high levels of intracellular ROS and related oxidative injuries.¹⁸ Inhibition of Ras signaling in HUVECs was shown to increase resistance to apoptosis.¹⁸ Inflammation and apoptosis have been proposed as potential targets for limiting organ damage following ischemic insult where Ras is thought to play a crucial role in both events.^{19,20} Ha-Ras has been reported to increase apoptosis induced by oxidative stress in endothelial cells.¹⁸ NADPH oxidase is stimulated by Ha-Ras resulting in ROS production.²¹ We concluded in a previous study that the beneficial effects of Ras-

GTPase inhibition were exclusively present during reperfusion as the initial P_{\max} value after ischemia was not significantly different from that of ischemia alone whereas a rapid and marked change in P_{\max} was only observed during sustained reperfusion.¹² However, our present results indicate that in order to produce the delayed improvement during reperfusion Ras inhibition must begin before ischemia since improvement was significantly greater when FPT III was given before I/R. These results indicate that ischemia-induced activation of Ras-GTPase signaling produces

injury that cannot be corrected by later inhibition of Ras during reperfusion. This observation is in agreement with a recent report showing that inhibition of farnesylation and consequently of Ras signaling reduces acute ischemic renal injury.²⁰

It has been well documented that ATP-sensitive potassium channels (K_{ATP}) have an endogenous cardio-protective role.²² Furthermore, it has been suggested that K_{ATP} channels may be involved in triggering and maintaining cardioprotection by ischemic pre-conditioning.²³ Therefore, utilization of selective potassium channel openers to mimic pre-conditioning has been a target of several studies.²⁴ Glibenclamide, an inhibitor of cardiac mito K_{ATP} channels, has been reported to prevent ischemic pre-conditioning suggesting that PC-mediated cardioprotection is at least partly due to opening of K_{ATP} channels.^{5,25–27} The opening of mito K_{ATP} channels causes the depolarization of mitochondria and reduces Ca^{++} overload during I/R.^{5,26,27} It has also been shown that the signaling pathway between atrial muscarinic cholinergic receptors, heterotrimeric G-proteins and single muscarinic K^+ channels can be interrupted by Ras p21 or one of its guanosine triphosphatase activating proteins.^{28–30} Results from the present study show that removal of Ras-GTPase-mediated inhibition of mito K_{ATP} channels is partially responsible for cardioprotection due to FPT III.

In conclusion, the results of the present study indicate that inhibitors of Ras-GTPase could serve as pharmacological tools to reduce I/R-induced dysfunction in the heart. For example, short-term pre-treatment with an inhibitor of Ras-GTPase could protect the heart during surgical procedures without necessitating pre-conditioning.

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