The present study was designed to investigate the modulatory effects of rottlerin on ischemia-reperfusion induced myocardial injury. Isolated rat hearts were exposed to 30 min of global ischemia followed by 120 min of reperfusion using Langendorff apparatus. Myocardial injury was assessed in the terms of infarct size, release of lactate dehydrogenase (LDH), creatine kinase (CK) enzymes. Rottlerin, a selective PKCδ inhibitor, did not modulate ischemia-reperfusion (I/R) induced myocardial injury at low dose (3 μM). However, at moderate dose (6 μM) it significantly produced cardioprotective effects. On the contrary, rottlerin at high dose (12 μM) significantly enhanced I/R induced myocardial injury. However, administration of FR-167653 (1.1 μM, 2.2 μM), a selective p-38 mitogen activated protein kinase (p-38 MAPK) inhibitor, attenuated rottlerin (12 μM) mediated enhancement in I/R induced myocardial injury in a dose dependent manner. Per se administration of FR-167653 (1.1 μM, 2.2 μM) also attenuated I/R induced myocardial injury in a dose dependent manner. Pretreatment with rottlerin (6 μM) did not enhance the cardioprotective effects of FR-167653 (2.2 μM). It may be concluded that rottlerin mediated cardioprotective effects at moderate dose, possible due to inhibition of PKCδ; while at high dose it enhanced I/R induced myocardial injury which may be attributed to activation of p-38 MAPK.

**Key words** rottlerin; ischemia reperfusion injury; FR-167653

Coronary artery diseases represent a global burden on health care resources and poised to become the leading cause of morbidity and mortality in the world by 2020. Early reperfusion is essential to salvage ischemic myocardium; however reperfusion of ischemic region is not devoid of deleterious effects. Reperfusion of ischemic myocardium is noted to produce detrimental morphological and functional changes in the form of reperfusion injury manifested as reperfusion arrhythmias, myocardial stunning, micro-vascular and endothelial dysfunction leading to impairment of reflow.1,2

Myocardial ischemia and reperfusion lead to activation of two novel PKC iso-forms (PKCδ and PKCε) which are reported to play opposing roles during myocardial ischemia and reperfusion (I/R).3,4 PKCδ has been reported to mediate I/R induced myocardial injury through activation of mitochondrial pathway,5,6 whereas PKCε has been reported to provide cardioprotection through preconditioning.7 PKCδ has been implicated in I/R induced myocardial injury by producing apoptotic and necrotic cell death.4,5 A selective peptide inhibitor of PKCδ i.e. δVI-1, has been reported to reduce I/R induced myocardial injury by inhibiting apoptotic as well as necrotic cell death.6,9

Ischemia and reperfusion has also been reported to activate protein kinases such as p-38 mitogen activated protein kinase (p-38 MAPK); which further increases transcription of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α).8,9 Administration of SB 203580, a selective p-38 MAPK inhibitor, has been reported to attenuate the apoptotic and necrotic cell death of cardiomyocytes.10 Rottlerin is a specific non-peptide inhibitor of PKCδ11,12 which is also reported to activate p-38 MAPK.13 Therefore, the present study was designed to investigate the modulatory effects of rottlerin, an inhibitor of PKCδ and activator of p-38 MAPK on ischemia-reperfusion induced myocardial injury.

**MATERIAL AND METHODS**

Wistar albino, rats of either sex weighing 150—200 g maintained at standard animal diet (Kisan Feeds Ltd., Chandigarh, India) and tap water ad libitum were employed in the present study. The animals were procured from ‘disease free small animal house’ Chaudary Charn Singh, Haryana Agriculture University, Hisar (Haryana), India. They were exposed to 12 h cycle of light and dark. The protocol of the study was duly approved by Institutional Animal Ethics Committee and care of the animals was taken as per guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Govt. of India (Reg. No. 107/CPCSEA 1999).

**Drugs and Chemicals** Rottlerin (Sigma Aldrich, St. Louis, U.S.A.) and FR-167653 (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) were dissolved in 1 : 5 mixtures of ethanol with normal saline and saline respectively. All other reagents used in the study were of analytic grade.

**Isolated Rat Heart Preparation** Rats were heparinised (500 I.U., i.p.) about 20 min before sacrificing the animal by cervical dislocation. After sacrificing the rat, heart was rapidly excised and mounted immediately on Langendorff apparatus.10 Isolated heart was perfused retrogradely at constant pressure of 70 mmHg with Kreb’s Henseleit (KH) solution of pH 7.4 bubbled with 95% O2 and 5% CO2. Flow rate was maintained at 6—9 ml/min. The heart was enclosed in a double wall jacket, the temperature of which was maintained at 37 °C by circulating warm water. After 10 min stabilization, global ischemia was produced for 30 min by blocking the inflow of KH solution and it was followed by reperfusion for 120 min. Coronary effluent was collected at different time intervals i.e., basal (immediately after stabilization) and 0 min, 5 min, and 30 min after reperfusion for biochemical estimations. Coronary flow rate was measured at different time intervals to assess the degree of injury to coronary vasculature.
Two thin silver electrodes fixed at ventricular apex and origin of aorta, were employed to record ECG (BPL, MK 801, Bangalore, India) for monitoring heart rate.

**Assessment of Infarct Size** After 120 min reperfusion, heart was removed from Langendorff apparatus. The atria, the root of aorta were excised and heart was kept overnight at 0°C. Frozen heart was sliced into uniform sections of 2—3 mm thickness. The slices were incubated in 1% triphenyl tetrazolium chloride (TTC) at 37°C in 0.2 M Tris buffer (pH 7.4) for 20 min. The extent of myocardial infarct size was estimated macroscopically by volume and weight method. The infarct size was expressed as percentage of total left ventricular volume (LVV) and total left ventricular weight (LVW) respectively.15—17)

**Estimation of Lactate Dehydrogenase (LDH)** LDH was estimated in samples of coronary effluent collected after stabilization (basal), immediately and 30 min after reperfusion using 2,4-DNPH method as described by King.18)

**Estimation of Creatine Phosphokinase (CK)** The levels of CK were estimated in coronary effluent samples after stabilization (basal) and 5 min after reperfusion using method of Hughes.19)

**Experimental Protocol** Ten groups, each comprising six albino rats, were employed in the present study.

- **Group I (Sham group):** Isolated rat heart was perfused with K-H solution for 160 min, after stabilization for 10 min.
- **Group II (Control group):** After stabilization, isolated rat heart perfused with K-H solution 10 min and then subjected to 30 min global ischemia followed by reperfusion for 120 min.
- **Group III (Rottlerin 3 μM Treated Group):** After stabilization, isolated rat heart perfused with K-H solution containing rottlerin (3 μM) for 10 min and then subjected to 30 min global ischemia followed by reperfusion for 120 min.
- **Group IV (Rottlerin 6 μM Treated Group):** After stabilization, isolated rat heart perfused with K-H solution containing rottlerin (6 μM) for 10 min and then subjected to 30 min global ischemia followed by reperfusion for 120 min.
- **Group V (Rottlerin 12 μM Treated Group):** After stabilization, isolated rat heart perfused with K-H solution containing rottlerin (12 μM) for 10 min and then subjected to 30 min global ischemia followed by reperfusion for 120 min.
- **Group VI (FR-167653 1.1 μM Treated Group):** After stabilization, isolated rat heart perfused with K-H solution containing FR-167653 (1.1 μM) for 10 min and then subjected to 30 min global ischemia followed by reperfusion for 120 min.
- **Group VII (FR-167653 2.2 μM Treated Group):** After stabilization, isolated rat heart perfused with K-H solution containing FR-167653 (2.2 μM) for 10 min and then subjected to 30 min global ischemia followed by reperfusion for 120 min.
- **Group VIII (Rottlerin 12 μM and FR-167653 1.1 μM Treated Group):** After stabilization, isolated rat heart perfused with K-H solution containing rottlerin (12 μM) and FR-167653 (1.1 μM) for 10 min and then subjected to 30 min global ischemia followed by reperfusion for 120 min.
- **Group IX (Rottlerin 12 μM and FR-167653 2.2 μM Treated Group):** After stabilization, isolated rat heart perfused with K-H solution containing rottlerin (12 μM) and FR-167653 (2.2 μM) for 10 min and then subjected to 30 min global ischemia followed by reperfusion for 120 min.
- **Group X (Rottlerin 6 μM and FR-167653 2.2 μM Treated Group):** After stabilization, isolated rat heart perfused with K-H solution containing rottlerin (6 μM) and FR-167653 (2.2 μM) for 10 min and then subjected to 30 min global ischemia followed by reperfusion for 120 min.

**Effect of Rottlerin and FR-167653 on Myocardial Infarct Size** Global ischemia for 30 min followed by reperfusion for 120 min produced significant increase in myocardial infarct size assessed by both volume and weight method. Rottlerin (3 μM), a PKCδ inhibitor, treatment before global ischemia produced no marked change in ischemia reperfusion (I/R) induced infarct size. However, rottlerin (6 μM) treatment before global ischemia significantly attenuated I/R myocardial infarct size. On the contrary, rottlerin (12 μM) treatment before global ischemia produced significant increase in I/R induced infarct size. However, administration of FR-167653 (1.1 μM, 2.2 μM) significantly attenuated rottlerin (12 μM) mediated enhancement in I/R induced increase in infarct size. Per se treatment with FR-167653 (1.1 μM, 2.2 μM) also attenuated I/R induced myocardial infarct size significantly in a dose dependent manner (Fig. 1). However, administration of combination of rottlerin (6 μM) and FR-167653 (2.2 μM) did not attenuate infarct size significantly as compared to FR-167653 (2.2 μM) alone.

**Effect of Rottlerin and FR-167653 on Ischemia-Reperfusion Induced LDH and CK Release** Global ischemia for 30 min followed by reperfusion for 120 min significantly increased the release of LDH in coronary effluent noted immediately, 30 min and 120 min after reperfusion. Similarly, significant increase in release of CK was noted at 5 min after reperfusion. Rottlerin (3 μM) treatment before global ischemia did not produce any marked change in I/R induced release of LDH and CK. However, rottlerin (6 μM) treatment significantly attenuated I/R induced release of LDH and CK.

**Statistical Analysis** The results were expressed as mean±standard error of means (S.E.M.). Statistical analysis for LDH, CK, and coronary flow rate was done using two way repeated ANOVA while the results of infarct size were analyzed by one-way ANOVA followed by Tukey’s multiple range tests as post-hoc analysis. A value of p<0.05 was considered to be statistically significant.

**RESULTS**

![Fig. 1. Effect of Pharmacological Interventions on Infarct Size in Isolated Perfused Rat Heart Subjected to 30 min Global Ischemia Followed by 120 min Reperfusion](image-url)

Infarct size was measured macroscopically by volume and weight method. Values are mean±S.E.M of six animals. *p<0.05 vs. sham; †p<0.05 vs. control; ‡p<0.05 vs. rottlerin 12 μM; ‡p<0.05 vs. FR-167653 1.1 μM.
Isolated perfused heart preparation has been employed in the present study because it permits the use of pharmacological interventions without any interference due to changes in systemic circulation. Electrical pacing has not been used in this study because it is reported to release norepinephrine.20 Infarct size was assessed macroscopically because a good correlation has been demonstrated between macroscopic and microscopic measurement of infarct size.21 Lactate dehydrogenase (LDH) and creatine kinase (CK), localized in myocytes, are released during ischemia-reperfusion induced irreversible myocardial injury. Therefore, release of these enzymes in coronary effluent is documented as an index of myocardial injury. Earlier report from our laboratory had suggested the peak release of LDH immediately and 30 min after reperfusion, whilst peak release of CK after 5 min of reperfusion during myocardial injury.16,22,23 Therefore, samples of coronary effluent were collected at these time intervals to estimate the release of LDH and CK.

Rottlerin is a specific non-peptide inhibitor of PKCδ.11,12 In the present study, administration of rottlerin (3 μM) at low dose did not modulate ischemia-reperfusion (I/R) induced increase in myocardial injury measured in terms of infarct size and, release of LDH and CK. Some studies have revealed that rottlerin at concentrations, 1 μM and 3 μM, is ineffective in vitro as PKCδ inhibitor.24,25 However, administration of rottlerin (6 μM) at moderate dose attenuated I/R induced increase in myocardial injury assessed in terms of reduction in infarct size and release of LDH, CK in coronary effluent. The noted cardioprotective effects of rottlerin (6 μM) may be attributed to its PKCδ inhibiting property. The activation of PKCδ has been reported to play an important role in producing I/R induced myocardial injury26 and inhibition of this selective isoform of PKC has been demonstrated to exhibit cardioprotection.27

On the contrary, administration of rottlerin (12 μM) at higher dose significantly enhanced I/R induced myocardial injury. Several studies have demonstrated that activation of p-38 MAPK in cardiomyocytes plays a key role in I/R induced myocardial injury.28,29 The noted per se cardioprotective effects of FR-167653 a selective p-38 MAPK inhibitor in our study further supports these reports. Studies have also documented activation of p-38 MAPK in cardiomyocytes by rottlerin.13 To explore the contradictory effect of rottlerin in high dose on I/R induced myocardial injury, a selective p-38 MAPK inhibitor was employed. Administration of FR-167653, a potent inhibitor of p-38 MAPK, attenuated rottlerin (12 μM) induced enhancement in I/R induced myocardial injury, suggesting the critical role of p-38 MAPK activation in mediating the effects of rottlerin in high dose (12 μM). Therefore, activation of p-38 MAPK induced due to high dose of rottlerin may be responsible for its deleterious effects in I/R induced myocardial injury. With the aim that perhaps simultaneous inhibition of p-38 MAPK and PKCδ might produce stronger cardioprotective effects, hearts were pretreated with rottlerin (6 μM) and FR-167653 (2.2 μM). However, contrary to our assumption, pretreatment with rottlerin (6 μM) did not enhance the cardioprotective effects of FR-167653 (2.2 μM).

Nevertheless, further studies are required to unfold the
role of other MAP kinases in modulating the effects of rottlerin on heart during ischemia and reperfusion.

CONCLUSION

On the basis of these results, this may be concluded that rottlerin produces differential effect on I/R induced myocardial injury depending on the dose. At moderate dose, it exhibits cardioprotective effects through its inhibitory action on PKCδ. In contrast to this rottlerin induces enhancement of myocardial injury at high dose, which may be possibly attributed to its p-38 MAPK activating property. The cardioprotective effects of PKCδ and p-38 MAPK modulators suggest that these target sites may further be exploited clinically for attenuating I/R induced myocardial injury.

REFERENCES