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Cardioprotective Action of BN50739 in Myocardial Ischaemia-Reperfusion Injury in Rats

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The present study is designed to investigate the effect of BN50739, a platelet activating factor receptor antagonist, on ischaemia-reperfusion induced myocardial injury in rats. In the anaesthetized rat, the heart was subjected to ischaemia by left coronary artery ligation for 45 min followed by reperfusion for 90 min. Infarct size, electrocardiographic parameters and serum creatine phosphokinase concentration were estimated to determine the extent of ischaemia-reperfusion induced injury. Serum malondialdehyde concentration was estimated to assess the extent of lipid peroxidation. BN 50739 (5 mg/kg, i.v.) administered after 45 min of ischaemia markedly reduced the infarct size, prevented the loss of R wave, attenuated ST elevation, reduced serum creatine phospho kinase and malondialdehyde concentration. The cardioprotective effect of BN50739 may be due to inhibition of platelet activating factor-induced activation of neutrophils and consequent lipid peroxidation.

Platelet-activating factor (PAF) is released during ischaemia and reperfusion of the myocardium[1-4]. An increase in myocardial lyso-phospholipids has been observed in ischaemic myocardium[5-6]. PAF has also been demonstrated to extend the necrotic area[7] and it is known to be a potent chemotactic agent for neutrophils[1,8]. Moreover PAF receptor antagonists such as CV-6209[9], SDZ63-675[10], RP-59227[3,11] WEB 2170[12], BN50726 and BN50739[13], L-659, 989[14], TCV 309[15-16], BN52021[17] and BN50739[18] (fig. 1) have been demonstrated to protect myocardium and limit myocardial infarct size. BN52030 and BN52039 (PAF antagonists) have been noted to decrease iron initiated lipid peroxidation in murine ventricular membranes[19]. Reactive oxygen intermediates have been implicated in ischaemia-reperfusion induced myocardial injury[1,20]. Therefore, the present study is designed to investigate the effect of BN 50739, a PAF receptor antagonist on ischaemia-reperfusion induced myocardial injury and to assess its effect on reactive oxygen species.

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MATERIAL AND METHODS

Porton female rats (obtained from CCS Haryana Agricultural University, Hisar) weighing about 100-150 g maintained on rat feed (Lipton India Ltd., Mumbai, India) and having free access to tap water were employed in this study. All experimental protocols were approved by institutional animals ethics committee (IAEC) and conducted according to CPCSEA guidelines (The CPCSEA registration no. CPCSEA/0436.)

Ischaemia-reperfusion induced myocardial injury:

Rats were anaesthetized with a combination of pentobarbitone sodium (45 mg/kg, i.p.) and ether. The trachea was exposed and cannulated with a polythene cannula, which was connected to a rodent ventillator (UGO Basile, Italy). Animals were ventilated throughout the experiment with 3-4 ml. of respiratory volume and at a rate of 50-60/min. Left thoracotomy was performed at fifth intercostal space, the heart was exteriorized and the left coronary artery along

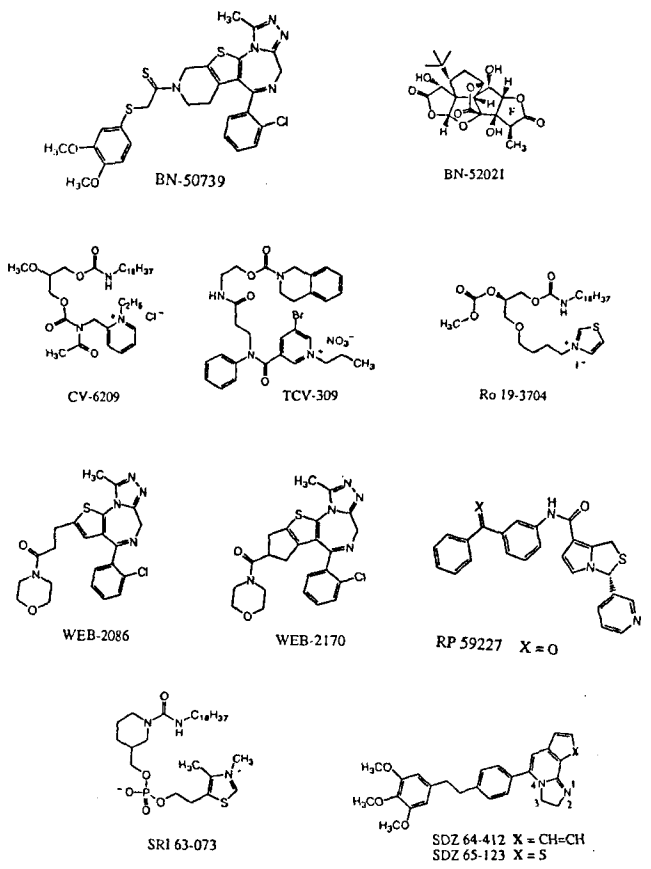

Fig. 1: PAF Receptor Antagonists.

with a thin polythene tube was ligated by a silken thread 1-2 mm away from its origin. The heart was then repositioned, chest was compressed to remove air and skin was sutured in layers. After 45 min of ischaemia, the chest was opened, heart was exteriorised and polythene tube was removed to terminate occlusion and to ensure reperfusion. Heart was repositioned and chest was closed. The reperfusion was continued for 90 min.

Estimation of myocardial infarct size:

Rats were sacrificed after 45 min of ischaemia or 45 min of ischaemia followed by 90 min of reperfusion. Heart was excised and atria, roots of great vessels and right ventricular wall were removed. The remaining left ventricle was cut into slices of 2-3 mm thickness. They were incubated in 1% triphenyl tetrazolium chloride (TTC) solution in 0.2 M Tris buffer, pH 7.8 for 3 min at 37°[21-22]. It stained the normal myocardium brick red and infarcted portion remained unstained. Infarct size was measured by macroscopic method i.e. volume and weight method[23].

Recording of electrocardiogram:

Electrocardiogram was recorded using limb lead II (Encardiorite, BPL, Bangalore.) before coronary artery ligation and immediately, 15 and 45 min after coronary artery ligation and immediately, 15, 45 and 90 min after reperfusion to monitor heart rate, R wave and ST segment elevation.

Collection of blood sample and estimation of serum creatine phosphokinase (CPK):

The blood samples were collected from carotid artery either after 45 min of ischaemia or after 90 min of reperfusion. The blood was allowed to clot, stored in refrigerator for 4-5 hours and serum was separated. The serum was stored at -80° for 8-10 d. Creatine phosphokinase was measured in the serum using CPK Kit (Code No. 25938, Span diagnostics Pvt. Ltd., Udhna, Surat) by modified method of Hughes[24].

Estimation of serum malondialdehyde (MDA):

MDA levels were measured spectrophotometrically using the thiobarbituric acid reaction[25]. To 20 μl of serum, 4.0 ml of N/12 H_2SO_4 and 0.5 ml of 10% trichloroacetic acid (TCA) was added and the mixture was centrifuged at 3000 rpm for 10 min. To the sediment portion, 2 ml of N/12 H_2SO_4 and 0.3 ml of TCA was added and the mixture was again centrifuged at 3000 rpm for 10 min. The supernatant was discarded and sediment was suspended in 4 ml of distilled water and 1 ml of thiobarbituric acid (TBA) reagent. TBA reagent is a mixture of equal volumes of 0.67% TBA aqueous solution and glacial acetic acid. The reaction mixture was heated for 60 min at 95° using a water bath. After cooling with tap water, 5 ml of n-butanol was added in each tube. It was centrifuged at 3000 rpm for 15 min and n-butanol layer was taken for spectrophotometric measurement at 532 nm. The absorbance was read against a blank prepared identically without addition of serum and results were expressed as nm of MDA per ml of serum.

Experimental design:

The animals were randomly divided into four groups and each group was comprised of five animals. Group I was control group subjected to 45-min ischaemia only and no reperfusion. Blood sample was collected and infarct size was measured after 45 min of ischaemia. Group II was also a control group subjected to 45 min ischaemia followed by 90 min reperfusion. Blood sample was collected and infarct size was measured after 90 min of reperfusion. Group III was vehicle treated control group. In this group, animals were subjected to 45 min ischaemia followed by 90 min reperfusion. 0.2 ml of vehicle was administered after 45 min

of ischaemia. Rest of protocol is same as in group II. Group IV was BN 50739 (5 mg/kg, i.v.) treated group. Volume of injection of drug was 0.5 ml/100 g of rat. The drug was administered after 45 min of ischaemia. Rest of protocol is same as in group II.

Statistical analysis:

All results were expressed as mean±S.E. Statistical analysis was performed using ANOVA followed by Dunnet's t-test. $p<0.05$ was considered statistically significant.

RESULTS

Modulation of myocardial infarct size:

Reperfusion instituted after 45 min of ischaemia and continued for 90 min significantly increased the infarct size as compared to extent of infraction measured after 45 min of ischaemia. BN 50739 (5 mg/kg, i.v.) administered after 45 min of ischaemia and just prior to reperfusion significantly reduced the infarct size measured by volume (fig. 2) and weight method (fig. 3)

Serum creatine phosphokinase (CPK):

Coronary artery ligation produced significant elevation of serum CPK concentration and it increased further after 90 min of reperfusion. BN 50739 (5 mg/kg, i.v.) significantly reduced reperfusion induced elevated serum CPK concentration (fig. 4).

Electrocardiographic changes:

Heart rate markedly decreased with the progression of ischaemia and it was not improved during reperfusion phase. BN 50739 (5 mg/kg, i.v.) treatment did not affect the heart rate. The voltage of R wave, recorded using limb lead II reduced markedly after coronary artery ligation (CAL) and it continued to decrease throughout the period of 45 min of ischaemia and 90 min of reperfusion. BN 50739 (5 mg/kg, i.v.) administered after 45 min of ischaemia significantly prevented the decline of R wave as compared to vehicle treated group. The ST segment (taking PR as isoelectric) elevated markedly after coronary artery ligation and it remained elevated throughout the period of ischaemia and reperfusion. BN 50739 (5 mg/kg, i.v.) administered after 45 min of ischaemia significantly attenuated ST elevation during reperfusion period (Table 1).

Serum malondialdehyde (MDA) concentration:

Coronary artery ligation produced marked elevation of serum MDA concentration and reperfusion further increased it significantly. BN 50739 (5 mg/kg i.v.) significantly reduced the elevated MDA concentration (fig. 5).

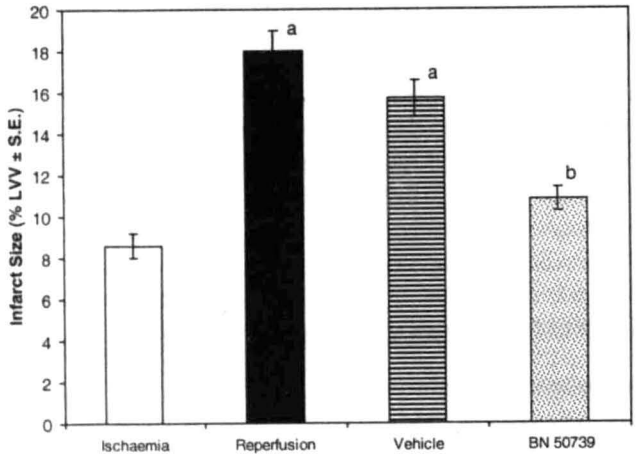

Fig. 2: Effect of BN 50739 on myocardial infarct size measured by volume method.

□ represents control subjected to ischaemia, ■ represents control subjected to ischaemia-reperfusion, ▤ represents vehicle treated group. ▩ represents BN 50739 (5 mg/kg i.v.) administered after 45 min of ischaemia. a $P<0.05$ as compared to control subjected to ischaemia, b $P<0.05$ as compared to vehicle treated control.

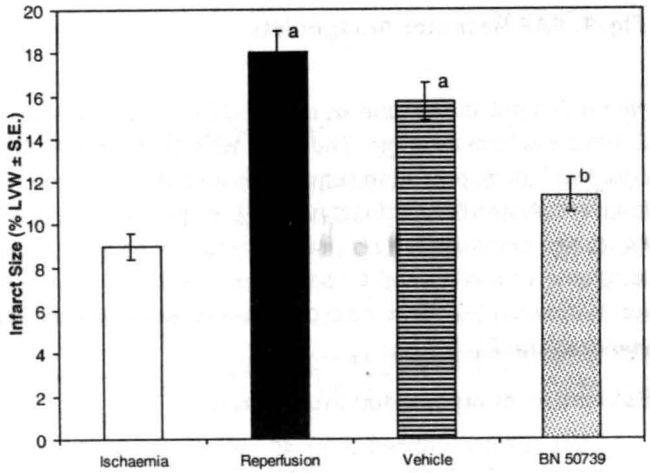

Fig. 3: Effect of BN 50739 on myocardial infarct size measured by weight method.

□ represents control subjected to ischaemia, ■ represents control subjected to ischaemia-reperfusion, ▤ represents vehicle treated group. ▩ represents BN 50739 (5 mg/kg i.v.) administered after 45 min of ischaemia. a $P<0.05$ as compared to control subjected to ischaemia, b $P<0.05$ as compared to vehicle treated control.

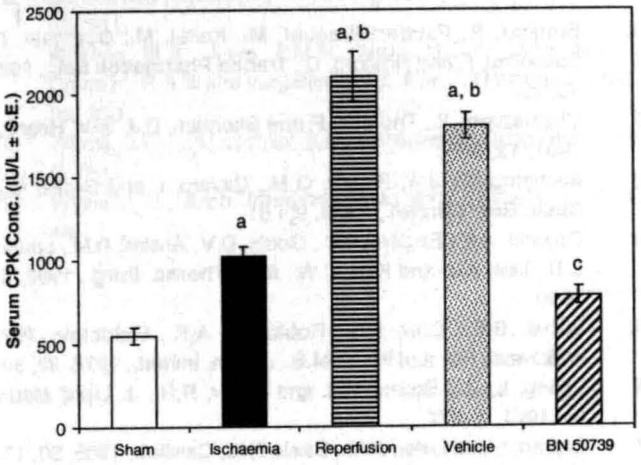

Fig. 4: Effect of BN 50739 on CPK concentration in serum.

□ represents sham operated control, ■ represents control subjected to ischaemia, ▤ represents control subjected to ischaemia-reperfusion. ▩ represents vehicle treated group. ▨ represents BN 50739 (5mg/kg i.v.) administered after 45 min of ischaemia. a P<0.05 as compared to sham operated control, b P<0.05 as compared to control subjected to ischaemia, c P<0.05 as compared to vehicle treated control.

DISCUSSION

PAF has been implicated in ischaemia-reperfusion injury[1-4]. In the present study, BN 50739 (PAF receptor antagonist) significantly protected myocardium from ischaemia-reperfusion injury because it reduced myocardial infarct size, attenuated loss of R wave-an electrophysiological measure of ischaemic injury, prevented ST elevation and reduced plasma CPK activity, elevated as a result of ischaemia-reperfusion. This ameliorative effect of BN 50739 appears to be independent of hemodynamic changes because heart rate noted in the present study remained unchanged with BN 50739[18]. Inhibition of platelet aggregation may also not be involved in the observed protective effects of BN 50739 because rat platelets are not sensitive to PAF[26-27].

PAF is known to be chemotactic agent for neutrophils, which are crucial cell type with regard to free radical generation and a major component of ischaemia-reperfusion injury[28-29]. PAF activates neutrophils and stimulates them to produce various substances including leukotrienes and superoxide anions[1, 20]. A transient alteration of neutrophil function in coronary circulation may augment neutrophil infilteration to the ischaemic myocardium after reperfusion and generation of oxygen free radicals by these cells may lead to progression of myocardial ischaemia-reperfusion injury[30]. PAF is reported to be involved in the recruitment of

TABLE 1: EFFECT OF BN 50739 ON R-WAVE VOLTAGE AND ST ELEVATION (LIMB LEAD II).

	R-wave voltage (mV±SE)	ST elevation (mV±SE)
Before Coronary artery ligation (CAL)	0.57±0.02	0.073±0.013
Ischaemia		
Immediately after ischaemia	0.35±0.01[a]	0.34±0.02[a]
15 min. after ischaemia	0.22±0.02[a]	0.33±0.02[a]
45 min. after ischaemia	0.19±0.02[a]	0.29±0.02[a]
Reperfusion (control)		
Immediately after reperfusion	0.19±0.02[a]	0.3±0.02[a]
15 min. after reperfusion	0.18±0.02[a]	0.293±0.02[a]
45 min. after reperfusion	0.19±0.01[a]	0.286±0.02[a]
90 min. after reperfusion	0.24±0.01[a]	0.286±0.02[a]
Reperfusion (vehicle treated)		
Immediately after reperfusion	0.21±0.02[a]	0.3±0.02[a]
15 min. after reperfusion	0.21±0.02[a]	0.293±0.02[a]
45 min. after reperfusion	0.25±0.03[a]	0.286±0.02[a]
90 min. after reperfusion	0.24±0.03[a]	0.286±0.02[a]
Reperfusion (BN 50739, 5 mg/kg i.v.)		
Immediately after reperfusion	0.4±0.04[a,b]	0.26±0.013
15 min. after reperfusion	0.43±0.02[a,b]	0.213±0.01[b]
45 min. after reperfusion	0.46±0.02[a,b]	0.173±0.01[b]
90 min. after reperfusion	0.47±0.03[a,b]	0.173±0.01[b]

A represents p<0.05 as compared to before CAL, b represents p<0.05 as compared to R wave voltage / ST elevation at the same time intervals in vehicle treated group.

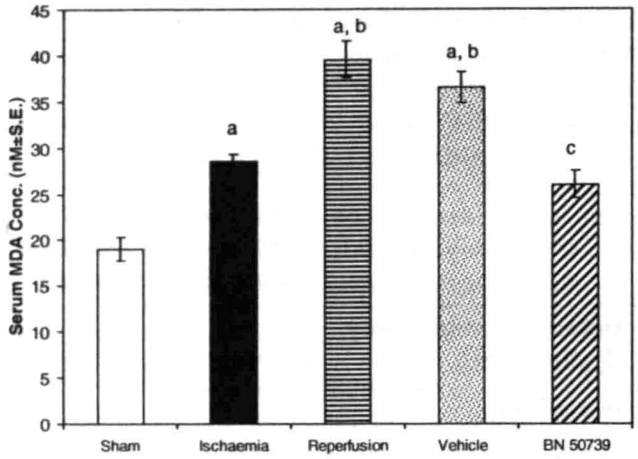

Fig. 5: Effect of BN 50739 on MDA (malondialdehyde) concentration in serum.

□ represents sham operated control, ■ represents control subjected to ischaemia, ▤ represents control subjected to ischaemia-reperfusion. ▨ represents vehicle treated group. ▨ represents BN 50739 (5mg/kg i.v.) administered after 45 min of ischaemia. a P<0.05 as compared to sham operated control, b P<0.05 as compared to control subjected to ischaemia, c P<0.05 as compared to vehicle treated control.

neutrophils, as well as in their activation to generate oxygen free radicals[31-32] and activation of arachidonic acid dependent pathways[33-34]. Therefore, BN 50739 may have protected the ischaemia-reperfused myocardium by preventing the migration of neutrophils and formation of reactive oxygen species from polymorphonuclear cells migrated to the site of ischaemia during reperfusion phase. This possibility is supported by MDA data of this study. Elevated MDA levels due to ischaemia and reperfusion have been reduced by BN 50739 treatment.

On the basis of above discussion, it may be concluded that BN 50739 provided protection against ischaemia-reperfusion-induced myocardial injury, perhaps by specific inhibition of PAF-induced activation of neutrophils and subsequent lipid peroxidation as a result of release of reactive oxygen intermediates.

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