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Studies on the signal cascade mechanism mediating the cardioprotective actions of bradykinin

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The cardioprotective involvement of bradykinin was evaluated using the ACE inhibitor, lisinopril, and the APP inhibitor, 2-mercaptoethanol alone and in combination in rats with experimental myocardial infarction. The signal cascade mechanism mediating the cardioprotective actions of bradykinin was evaluated by administering aspirin and methylene blue prior to lisinopril and 2-mercaptoethanol combined treatment. Myocardial infarction was produced by occlusion of the left anterior descending coronary artery for 30 min followed by 4 h of reperfusion. Infarct size was measured by the TTC stain method. Serum free radical levels were estimated by the method developed by Yagi. A lead II electrocardiogram was monitored throughout the experiment. With the combined inhibition of both the enzymes ACE and APP, a better cardioprotection was observed. The observed cardioprotection was decreased with the prior administration of aspirin and methylene blue. The results suggest the cardioprotective role of bradykinin during experimental myocardial infarction. The results are further suggesting the involvement of both prostaglandins and nitric oxide pathways in the cardioprotective actions of bradykinin.

1. Introduction

Myocardial ischemia can occur due to abnormal narrowing of the coronary arteries (coronary atherosclerosis) prior to obstruction [1], cardiac hypertrophy [2], circulatory shock [3] or diabetes [1]. The reduction in blood flow will result in an imbalance between the demand of the heart tissue for nutrients (such as glucose and O_2) and the blood supply to the heart [4]. Prolonged ischemia will lead to poor force generation, contracture, arrhythmia, calcium overload, a decrease in tissue pH, release of cytosolic enzymes from cells and cellular necrosis [5]. Ischemic injury depends upon the length of ischemia [6]. The heart can recover gradually from a short duration of ischemia (reversible ischemia), but if ischemia persists for a longer period of time, the chance for recovery will diminish (irreversible ischemia) [6]. Reperfusion of the previously ischemic myocardium, although essential for tissue survival, results in increased necrosis and reduced tissue validity [7]. Reperfusing the ischemic heart after a period of time results in a paradoxical loss of contractile force, contracture formation, rise in resting potential, structural damage, release of creatine phosphokinase (CPK) into the coronary effluent, arrhythmia and necrosis [8]. Numerous mechanisms for the increase in tissue injury after reperfusion have been identified including the generation of oxygen derived free radicals, complement activation and the infiltration of neutrophils into the ischemic zone [9–11]. Myocardial necrosis is a dynamic process that develops after occlusion-reperfusion of the coronary artery. Most studies have concluded that the ultimate infarct size is attained when the tissue is processed after several hours of reperfusion [12, 13]. There is a consensus that oxygen derived free radicals are generated during myocardial ischemia [14, 15] and particularly upon reperfusion [14, 16, 17]. These oxygen-derived free radicals are of paramount importance for the pathogenesis of myocardial stunning [6, 18, 19].

The attenuation of myocardial necrosis by several angiotensin converting enzyme (ACE) inhibitors has been demonstrated in a number of experimental studies *in vitro* [20–23] and *in vivo* [24–26] . The mechanism underlying the cardioprotective action of ACE inhibitors, however, is not fully clear [27]. One mechanism might be the prevention of bradykinin (BK) degradation during myocardial ischemia-reperfusion, since ACE is responsible not only for the conversion of angiotensin I to angiotensin II but also for the catabolism of bradykinin [28]. In fact, in isolated, buffer-perfused rat hearts, the reduction of ventricular arrhythmias and the improvement of ventricular function during reperfusion by the ACE inhibitor ramipril were similar to those achieved by bradykinin [22, 29]. These cardioprotective effects of ramipril and bradykinin were completely abolished by a bradykinin B_2 receptor antagonist [23, 29]. Thus these *in vitro* studies suggested a relation of the attenuation of myocardial stunning by ramipril to bradykinin. Bradykinin potently induces vasodilation through stimulation of vasodilator release from endothelium [30]. Endothelial mediators released by bradykinin include prostacyclin (PGI_2), nitric oxide (NO) and as yet unidentified endothelium-derived hyperpolarizing factors, depending on the size and origin of the blood vessels investigated [31, 32].

Bradykinin degrades to [1–5] BK, [2–9] BK and [1–7] BK by ACE, aminopeptidase P (APP) and neutral endopeptidase (NEP) respectively. The proportions of various kininases present were determined using specific enzyme inhibitors [33]. The two predominant kininases found in the rat myocardium are ACE and APP [33]. Hence in rat myocardium bradykinin is not only metabolized by ACE but also by APP. A combination of inhibitors may provide protection superior to that given by a single agent [34]. In the present study, the involvement of bradykinins was investigated by giving lisinopril (ACE inhibitor) and 2-mercaptoethanol (APP inhibitor) alone and in combination. To evaluate the signal cascade mechanism mediating the cardioprotective actions of bradykinin, the effects of prior administration of a prostaglandin synthesis inhibitor, aspirin, and a nitric oxide pathway inhibitor, methylene blue [35] on the cardioprotective actions offered by lisinopril and 2-mercaptoethanol were studied.

2. Investigations and results

2.1. Quantification of infarct size

In all the groups after sacrificing the animal, the heart was excised from the thorax rapidly and the greater vessels

were removed. The left ventricle was separated from the heart and was weighed. It was sliced parallel to the atrioventricular groove to 0.1 cm thick sections and the slices were incubated in 0.5% TTC solution prepared in pH 7.4 phosphate buffer for 30 min at 37 °C. In viable myocardium TTC is converted by dehydrogenase enzymes to a red formazan pigment that stains tissue dark red [36]. The infarcted myocardium that does not take TTC stain where the dehydrogenase enzymes are drained off, remains pale in colour [37]. The pale necrotic myocardial tissue was separated from the stained portions and weighed on an electronic balance (Dhona 200D). Myocardial infarct size was expressed quantitatively in terms of percent left ventricle necrosis (PLVN).

2.2. Biochemical estimations

In all the groups after sacrificing the animal at the end of 4 h of reperfusion, 2 ml of blood sample was collected from the left ventricle for the estimation of malondialdehyde (MDA) in blood serum. Serum free radical levels were estimated by the method developed by Yagi [38].

2.3. Protocol 1: Role of bradykinin in mediating the effects of lisinopril and 2-mercaptoethanol

PLVN was found to be 51.79 ± 1.68 in control group animals. It was decreased with lisinopril and 2-mecaptoethanol individual treatments (Table 1) and the difference was statistically significant ($P < 0.05$). PLVN was further reduced with the combined treatment of lisinopril and 2-mercaptoethanol when compared to individual treatments (Table 1). Serum MDA concentration was found to be 2.08 ± 0.69 nmol ml^{-1} in control group animals. Statistically there is no significant difference in serum MDA concentration in lisinopril and 2-mercaptoethanol individual and combined treatments compared to control (Table 1). The heart rate for all the above groups at various stages of occlusion and reperfusion is given in Table 2.

Table 1: Percent left ventricle necrosis (PLVN), and malondialdehyde (MDA) concentration in the serum of all groups of animals at the end of reperfusion

Experimental group	PLVN	MDA (nmol ml^{-1})
Group 1	51.79 ± 1.68	2.08 ± 0.69
Group 2	$24.37 \pm 0.87^{*}$	1.78 ± 0.41
Group 3	$44.19 \pm 1.25^{*}$	2.12 ± 0.78
Group 4	$14.33 \pm 1.39^{*}$	1.24 ± 0.74
Group 5	$31.79 \pm 1.33^{**}$	0.48 ± 0.22
Group 6	$22.39 \pm 0.48^{**}$	$2.98 \pm 0.35^{**}$

Values represent means ± SD from 5 rats. Group 1, saline. Group 2, lisinopril. Group 3, 2-mercaptoethanol. Group 4, lisinopril + 2-mercaptoethanol. Group 5, aspirin + lisinopril + 2-mercaptoethanol. Group 6, methylene blue + lisinopril + 2-mercaptoethanol.
* $P < 0.05$ compared to Group 1. ** $P < 0.05$ compared to Group 4

2.4. Protocol 2: Role of prostaglandins and nitric oxide in mediating the cardioprotective effects of bradykinin

PLVN was increased significantly with the prior administration of aspirin to lisinopril and 2-mercaptoethanol combined treatment compared to group 4 (Table 1). There was also a significant increase with the prior administration of methylene blue to lisinopril and 2-mercaptoethanol combined treatment compared to group 4 (Table 1). Serum MDA concentration was significantly increased with the prior administration of methylene blue to lisinopril and 2-mercaptoethanol combined treatment compared to group 4 (Table 1). The heart rate for all the above groups at various stages of occlusion and reperfusion is given Table 2.

2.5. Statistical analysis

The results are expressed as mean ± SD. Differences in PLVN, serum MDA levels were determined by factorial one-way analysis of variance. Individual groups were compared using Dunnet's 't' test. Differences with $P < 0.05$ were considered statistically significant.

3. Discussion

The present study using a protocol of 30 min of coronary occlusion followed by 4 h of reperfusion was performed in a model of fully reversible ischemia reperfusion damage. Coronary artery occlusion results in the acute activation of renin angiotensin system and production of angiotensin II, a potent vasoconstrictor and positive inotropic agent [39]. This has raised the possibility that ACE inhibitors might be cardioprotective, i.e., they may attenuate myocardial injury, dysfunction and necrosis in the setting of acute ischemia and infarction. ACE inhibitors inhibit the generation of endogenous angiotensin II and are shown to increase coronary flow *in vivo* [40]. In the present study, the ACE inhibitor lisinopril when administered intravenously before reperfusion attenuated the myocardial necrosis caused by ischemia – reperfusion. Previous studies on cardioprotection by ramiprilat in isolated rabbit hearts concluded that ramipril does possess direct cardioprotective properties that are independent of the inhibition of angiotensin II generation but that may be related to protentiation of the effects of bradykinin [41]. The cardioprotection of ramipril was mimicked by bradykinin and abolished by co-administration of a bradykinin antagonist

Table 2. Heart rate (beats min^{-1}) recorded at various stages of occlusion and reperfusion

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
BO	402.34 ± 22.46	396.43 ± 29.34	385.71 ± 23.96	413.73 ± 37.83	407.14 ± 29.34	352.38 ± 42.59
MOP	368.74 ± 19.42	401.43 ± 26.79	289.09 ± 14.94	385.71 ± 23.96	369.04 ± 39.26	333.33 ± 24.86
IAR	352.31 ± 16.48	375.64 ± 19.77	313.33 ± 18.25	353.92 ± 20.84	330.59 ± 19.09	310.47 ± 21.66
1 h AR	348.06 ± 24.26	375.64 ± 19.77	321.67 ± 33.12	353.43 ± 14.74	314.54 ± 27.47	330.59 ± 19.09
2 h AR	342.84 ± 17.06	385.71 ± 23.96	338.16 ± 21.95	327.08 ± 20.05	330.59 ± 19.09	333.33 ± 18.54
3 h AR	361.23 ± 15.77	375.64 ± 19.77	341.66 ± 18.63	306.67 ± 14.90	337.25 ± 8.77	333.33 ± 18.54
4 h AR	361.23 ± 15.77	375.64 ± 19.77	341.66 ± 18.63	272.72 ± 18.76	333.33 ± 22.34	333.33 ± 18.54

Values represent means ± SD from 5 rats. Group 1, saline. Group 2, lisinopril. Group 3, 2-mercaptoethanol. Group 4, lisinopril + 2-mercaptoethanol. Group 5, aspirin + lisinopril + 2-mercaptoethanol. Group 6, methylene blue + lisinopril + 2-mercaptoethanol. BO, before occlusion. MOP, middle of LAD occlusion period. IAR, immediately after reperfusion. AR, after reperfusion.

in dogs suggesting the role of bradykinin in the cardioprotective action of ramipril [42]. Bradykinin is metabolized mainly by ACE, APP and NEP. ACE and APP are the two predominant kininases found in the rat myocardium. ACE and APP form a metabolic barrier which effectively reduces kinin concentrations in the interstitium. APP activity is responsible for most of the remaining metabolism of bradykinin and this was confirmed by the inhibitory action of 2-mercaptoethanol and apstatin [43, 44]. In the present study, the APP inhibitor 2-mercaptoethanol when administered intravenously before reperfusion attenuated myocardial necrosis caused by ischemia-reperfusion. The degree of cardioprotection was higher with lisinopril than with 2-mercaptoethanol. Recent studies proposed that ACE inhibitors potentiate bradykinin beyond blocking its hydrolysis by inhibiting desensitization of its B_2 receptors [45]. This may be the reason for the enhanced cardioprotective action observed with lisinopril. A combination of inhibitors may provide superior protection to that given by a single agent [34]. Inhibition of both ACE and APP and potentiating the bradykinin effect could be an interesting approach in the treatment of ischemia reperfusion induced myocardial infarction. With the combined treatment of lisinopril and 2-mercaptoethanol more cardioprotection was observed compared to individual treatments. With the combined inhibition of both the enzymes ACE and APP, degradation of bradykinin may be further inhibited resulting in elevated levels of bradykinin which may account for the enhanced cardioprotection confirming the role of bradykinin in mediating the beneficial cardiac effects.

Activation of bradykinin B_2 receptors in cultured bovine [23, 46] and human [46] endothelial cells has been demonstrated to stimulate the formation of prostacyclin as well as nitric oxide, as measured by an enhanced formation of 6-keto prostaglandin $F_{1\alpha}$ and cGMP. In the same experimental model, a similar increase in the formation of prostacyclin and nitric oxide was induced by ramiprilat [23, 46] and prevented by the bradykinin B_2 receptor antagonist HOE 140. Thus stimulation of both the prostaglandin and nitric oxide pathways could potentially mediate the beneficial effect of ACE inhibitors on myocardial function during reperfusion. In a previous study using isolated, buffer-perfused rat hearts, the beneficial effects of captopril on ventricular function during reperfusion were completely abolished by the cyclooxygenase inhibitor indomethacin [20]. A beneficial effect of nitric oxide in the setting of ischemia reperfusion is expected from results of a previous study performed in isolated, buffer perfused rat hearts, in which the administration of a stable nitric oxide radical scavenged oxygen derived free radicals and protected against reperfusion induced arrhythmias [47]. Indeed, in two previous studies in isolated, buffer perfused rat [23] and guinea pig [48] hearts, the beneficial effect of ramiprilat and bradykinin on ventricular function during reperfusion was completely abolished by the nitric oxide synthase inhibitor L-NNA. In the present study both a prostaglandin synthesis inhibitor, aspirin, and a nitric oxide pathway inhibitor, methylene blue, were shown to inhibit the cardioprotective action offered by the combined treatment of lisinopril and 2-mercaptoethanol, suggesting the involvement of both prostaglandins and nitric oxide in the cardioprotective actions of lisinopril and 2-mercaptoethanol. Methylene blue is known to antagonize both arterial relaxation and associated increase in cGMP levels elicited by nitro vasodilator drugs [35]. Methylene blue enters cells and its inhibition of relaxation is believed to be mediated intracellularly by the oxidation of a component of guanylate cyclase [49]. Nitric oxide induced vasorelaxation may occur independent of guanylate cyclase. Bradykinin is known to activate Ca^{2+} dependent K^+ channels by stimulating tyrosine kinase and there by producing hyper polarization of vascular smooth muscle [45]. Hence with the administration of methylene blue, nitric oxide induced vasorelaxation may not be completely inhibited. The oxidant property of methylene blue may be attributed to the increase in serum MDA levels.

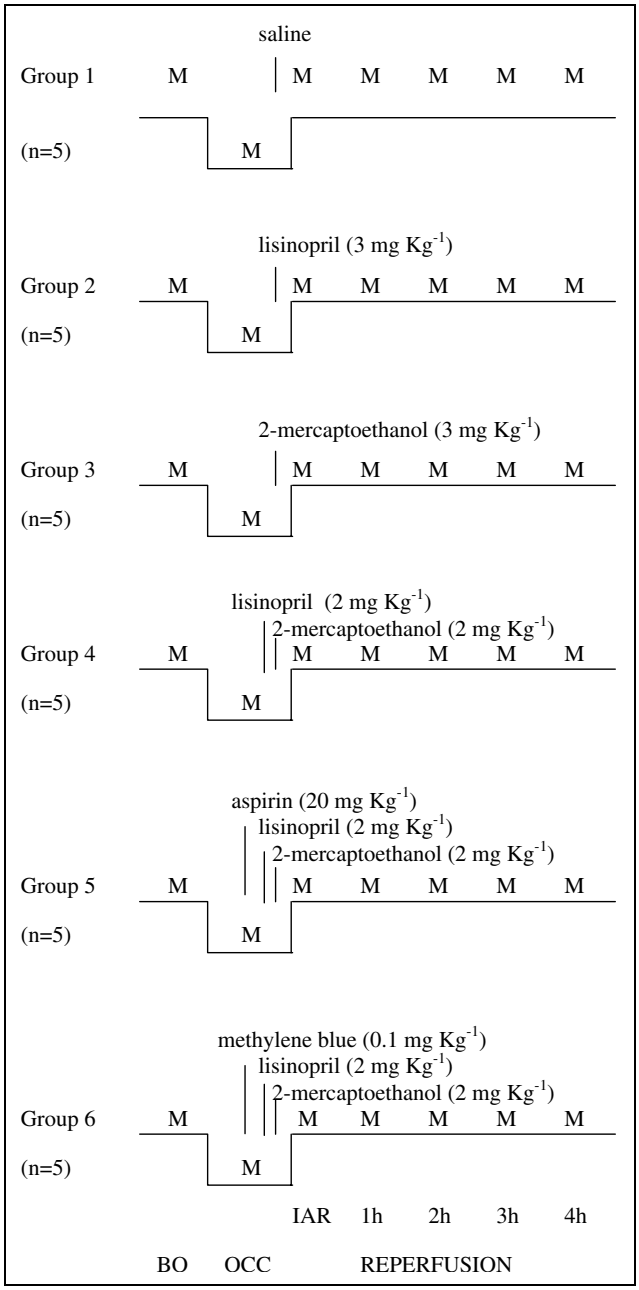

Fig.: Illustration of the experimental protocol in rats of group 1 (saline), group 2 (lisinopril), group 3 (2-mercaptoethanol), group 4 (lisinopril + 2-mercaptoethanol), group 5 (aspirin + lisinopril + 2-mercaptoethanol), group 6 (methylene blue + lisinopril + 2-mercaptoethanol). M indicates ECG measurement. BO, before occlusion. OCC, 30 min LAD occlusion. IAR, immediately after reperfusion.

The beneficial effects of captopril [50] and ramipril [51] on the long-term prognosis of patients after an acute myocardial infarction have been clearly demonstrated. The AIRE study on ramipril, in contrast to the SAVE study on captopril [50], revealed a beneficial effect even within first 30 days of treatment. In the AIRE study, there was a trend

to a greater benefit from ramipril in patients not receiving aspirin, supporting the data of the present study that prostaglandins are involved in the beneficial effects of lisinopril.

In conclusion, lisinopril in the present study attenuated myocardial necrosis through a signal cascade of bradykinin, prostaglandins and nitric oxide.

4. Experimental

4.1. Drugs

Lisinopril was a generous gift of Dr. Reddys Laboratories (Hyderabad, India), and aspirin was a generous gift of NATCO Pharma Ltd., (Hyderabad, India).

2-mercaptoethanol was purchased from Otto – Kemi (Mumbai, India). 1,1,3,3-tetraethoxy propane was purchased from Sigma Chemicals (Banglore, India), and methylene blue from Loba Chemicals (Mumbai, India) and triphenyl tetrazolium Chloride (TTC) from BDH (England). All other reagents used were of analytical grade. All the drug solutions were prepared in saline and were administered intravenously through femoral vein before commencement of reperfusion.

4.2. In vivo studies of myocardial ischemia reperfusion surgical preparation

Wistar albino rats of either sex weighing 140–210 g each were anaesthetised with thiopental sodium (30 mg kg^{-1}, i.p) and were ventilated with room air by a Techno positive pressure respirator (Crompton Parkinson Ltd, England). The femoral vein was isolated and cannulated to administer saline and drugs. A left thoracotomy and pericardiotomy were performed, followed by identifying the marginal branch of the left anterior descending coronary artery (LAD). A silk thread was passed behind the artery and was occluded for 30 min by lifting the thread. The silk thread was removed after 30 min to allow reperfusion of the heart for succeeding 4 h. A lead II electrocardiogram was monitored throughout the study by using Cardiart 408 (BPL) with sensitivity 20 mm mv^{-1} at a paper speed of 50 mm s^{-1}.

4.3. Study protocols

After the instrumentation was completed, control measurements of ECG were taken. The rats were then subjected to a 30 min LAD occlusion. At 15 min of LAD occlusion ECG was taken again. Measurements of ECG were repeated immediately after release of LAD occlusion and at 1 h, 2 h, 3 h and 4 h intervals of reperfusion. ECG measurements were taken at the above-specified intervals for all the groups of animals.

4.3.1. Protocol 1: Role of bradykinin in mediating the effects of lisinopril and 2-mercaptoethanol

In a first set of experiments, 20 rats were randomly assigned to four groups of five in each (Fig.). Group 1 was the placebo group. In this group of rats, saline (0.2 ml) was administered before release of LAD occlusion. Group 2 received lisinopril. After control and 15 min LAD occlusion measurements lisinopril (3 mg kg^{-1}) dissolved in saline was administered intravenously 5 min before release of LAD occlusion. Group 3 received 2-mercaptoethanol. After control and 15 min LAD occlusion measurements 2-mercaptoethanol (3 mg kg^{-1}) was administered intravenously 5 min before release of LAD occlusion. Group 4 received lisinopril and 2-mercaptoethanol. After control and 15 min LAD occlusion measurements lisinopril (2 mg kg^{-1}) and 2-mercaptoethanol (2 mg kg^{-1}) were administered intravenously 10 min and 5 min before release of LAD occlusion.

4.3.2. Protocol 2: Role of prostaglandins and nitric oxide in mediating the cardioprotective effects of bradykinin

In a second set of experiments, 10 rats were randomly assigned to two groups of 5 rats each. Group 5 received aspirin. After control and 15 min LAD occlusion measurements, the cyclooxygenase inhibitor aspirin (10 mg kg^{-1}) was administered intravenously 15 min before release of LAD occlusion. After administration of aspirin, lisinopril and 2-mercaptoethanol were administered intravenously as described above (Fig.). Group 6 received methylene blue. After control and 15 min LAD occlusion measurements nitric oxide pathway inhibitor methylene blue (0.1 mg kg^{-1}) dissolved in saline was administered 15 min before release of LAD occlusion. After administration of methylene blue, lisinopril and 2-mercaptoethanol were administered intravenously as described above.

4.4. Limitations of the present study

The major limitation of the present study is the lack of measurement of bradykinin, prostaglandins and nitric oxide level which was not possible for technical reasons. Furthermore, we realize that even the determination of these mediators in regional coronary venous blood could not have adequately reflected their concentration at the site of action, which is particularly important in autacoids, and thus would not have permitted establishment of a cause-and-effect relation. The lack of a specific bradykinin B_2 receptor antagonist, HOE 140, and a nitric oxide synthase inhibitor, L-NAME, are also a major limitation of the present study. Therefore the evidence for the attenuation of myocardial necrosis by lisinopril, through a signal cascade of bradykinin, prostaglandins and nitric oxide is indirect in the present study.

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