Protective effects of amlodipine on mitochondrial injury in ischemic reperfused rat heart

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Abstract

The most significant finding of the present study was the release of nitric oxide (NO). The effect of amlodipine on NO production associated with ischemic reperfused (IR) injury was investigated in rat heart model. Cardiac tissues from animal groups were processed for biochemical, histopathological and electron microscopic studies. There was a significant increase in myocardial catalase (CAT), superoxide dismutase (SOD) and glutathione (GSH) enzymes in amlodipine treated group (1.37, 10.27, 6.39) when compared to IR injured group (0.81, 6.87, 4.53). Histopathology studies showed amlodipine reduce cardiocyte damage in cardiac injury during the cardiac IR. Transmission electron microscopic (TEM) study confirmed the cardioprotective role of amlodipine against IR induced cardiac injury. On the basis of findings, it is hypothesized that a portion of the beneficial actions of amlodipine may involve the release or action of NO and probably by its antioxidant properties.

Introduction

Mitochondria are the principal targets in the development of ischemia-reperfusion (IR) induced injury (Elimadi *et al*., 2001; Detmers *et al*., 1999) and involved both in the production of reactive oxygen species (ROS) and as targets for the damaging action of both ROS and calcium (Halestrap *et al*., 2004; 2006; Solaini, 2005). It might be due to increase in cellular calcium and ROS, initiated in ischaemia and then amplified upon reperfusion, are thought to be the main causes of reperfusion injury which is associated with a burst of ROS production (Bolli, 1998). It has been reported that IR injury lead to generation of free redical like superoxide anion, hydrogen peroxide and hydroxyl radical that causes myocardial infarction undergoing thrombolysis, coronary angioplasty or open

heart surgery (Ferrari *et al*., 1991). Upon reperfusion, molecular oxygen undergoes sequential reduction to form reactive oxygen species. The interaction of oxygen-derived free radicals with cell membrane lipids and essential proteins contribute to myocardial cell damage, leading to depressed cardiac function and irreversible tissue injury with concomitant depletion of certain key endogenous antioxidant compounds, *e.g.*, superoxide dismutase (SOD), catalase activity (CAT), reduced glutathione (GSH) and glutathione peroxidase (GPx) (Moncada and Higgs, 1993). Nitric oxide (NO) is an prototypic endothelium-derived relaxing factor which synthesized from the amino acid L-arginine by a family of enzymes, the NO synthases (NOSs) and it is involved in the control of vascular tone and plays an important role in the regulation of blood pressure

(Wang *et al*., 1993, Shen *et al*., 1994, Zhao *et al*., 1995; 1996; Koller *et al*., 1994). Nitric oxide is released from the endothelial lining of blood vessels in response shear stress stimulus (Ursell and Mayes, 1995). Studies have reported that NO produced by all of the vessels of the coronary circulation (Andries *et al*., 1998; Zhang *et al*., 1997). Studies suggested that a portion of the beneficial effects of angiotension converting enzyme (ACE) inhibitors in the treatment of heart failure may be due to the release of NO (Kichuk *et al*., 1997) secondary to the generation of kinins locally (Kichuk *et al*., 1997, Seyedi *et al*., 1995; Vanhoutte *et al*., 1993). These data support a large number of other studies and it is now widely believed that NO contributes importantly to the mechanism of action of ACE inhibitors used in the treatment of all disease states. The release of NO by a long acting, more vascular selective, second generation calcium channel blocker like amlodipine or other calcium channel blockers would not be expected because there are no known receptors for calcium channel blockers in endothelial cells and because calcium is a cofactor for NO synthase and required for activation of NO synthase. However, both ACE inhibitors and organic nitrates release NO and are useful in the treatment of heart failure. It is reported that amlodipine releases NO from blood vessels which is mediated through the kinins (Kichuk *et al*., 1997; Seyedi *et al*., 1995; Vanhoutte *et al*., 1993). The present study was designed to investigate the effect of calcium antagonist amlodipine on NO vasodilatation in coronary artery, cardiac mitochondrial enzyme and nuclear changes under ischemia and reperfusion.

Materials and Methods

Experimental design: Wistar rats (200-250g) of either sex were maintained under standard laboratory conditions at animal house of IFTM University, Moradabad. The temperature $25\pm2^{\circ}C$, relative humidity $50\pm15\%$ and normal photo period (12 h dark 12 hr^{-1} light) was maintained in animal room throughout. Eighteen rats were divided into three groups, each having 6 animals. Group I: sham-operated control group, group II: ischemic reperfusion group and group III: Amlodipine pretreated group (1 mg kg^{-1} body weight daily by oral route for 7 days) followed by ischemia reperfusion. The cardiac IR protocol were performed as described by Kato *et al*., 2004. The study was approved by Institute Animal Ethics Committee, Department of Pharmacology and Clinical Research, College of Pharmacy, IFTM University, Moradabad. All animal care and experimental protocols were in compliance with the National Institute of Health (NIH) guidelines for the care and use of the laboratory animals (NIH, 1985).

Estimation of nitric oxide production and biochemical parameters: A single cell suspension (cardiocytes) was obtained from the heart tissue samples immediately after removal by mechanical disaggregation with a 50 μm filtered medimachine system (BD Bioscience, CA, USA). Cardiocytes were washed with cold phosphate buffer saline (PBS) to exclude cell debris and re-suspended in culture medium (RPMI 1640) phenol red free medium supplemented with 10% fetal calf serum in petri dishes and incubated at $37^{\circ}C$ in 5% CO_2 for 4 hr. At the end of 48 hr, 5 ml of Griess reagent [mixture of 1:1 of napthlethlenediamine dihydrochloride (0.1% in water) and sulphanilamide (1% in phosphoric acid)] was added and incubated in the dark at $30^{\circ}C$. Finally, the absorbance was measured at 546 nm and a standard curve using sodium nitrite was used to calculate the concentration of nitrite (Hibbs *et al*., 1998). Cardiac tissue (200 mg) was weighed and homogenized with 0.35M sucrose buffer at $4^{\circ}C$ and centrifuged at 10,000 rpm for 5min. The resultant mitochondrial pellate was then resuspended in 0.25M sucrose solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA and made up to a final volume of 2 ml with the same (Johnson and Lardy, 1947). Myocardial antioxidant such as reduced glutathione (GSH), (Elman, 1969), Catalase activity (CAT) (Aebi, 1954) and Superoxide dismutase (SOD) (Beauchamp *et al*., 1969) were estimated.

Histopathological examination: Cardiac tissue was fixed in 10% formalin, routinely processed and embedded in paraffin. Paraffin sections (5 μm) were cut, and stained with hematoxylin and eosin (H&E) and examined under a microscope as described by Shamatsu *et al*. (1997). Cardiac tissues was fixed in Karnovsky's solution pH 7.4 for 4 h at $4^{\circ}C$ for electron microscopic studies. After washing overnight in sodium cacodylate buffer at $4^{\circ}C$, the specimens were postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4), dehydrated in ethanol and then embedded in araldite resin. Ultra thin sections (40-60 nm thick) were placed on copper mesh grids (200 mesh) and doubly stained with uranyl acetate and lead citrate. Stained sections were examined under transmission electron microscope (TEM) (Moragagni 268D Netherlands).

Statistical analysis: All the results were statistically interpreted using one-way analysis of variance (ANOVA) followed by Bonferroni test. A value of $P < 0.05$ was considered statistically significant.

Results and Discussion

Biochemical parameters and nitric oxide (NO) production: Production of NO in ischemic rat heart increased significantly ($P < 0.01$) in amlodipine pretreated group as compared to IR group. (Tables 1) There was a significant decrease in myocardial NO, GSH, SOD and CAT activity in IR group (9.07, 4.53, 6.87 and 0.81 units mg^{-1} protein; $p < 0.05$) as compared to sham operated group (13.34, 7.15, 12.38 and 1.54 units mg^{-1} protein). Activities of these enzymes in mitochondria were maintained to near normal ($p < 0.05$) level in amlodipine pretreated groups with significant changes (11.45, 6.39, 10.27 and 1.37 units mg^{-1} protein) when compared to IR group.

Production of NO in ischemic rat heart, activity of myocardial antioxidant enzymes increased significantly that accredits the inhibition of injury in histopathology and electron microscopic studies by amlodipine pretreated group as compared to IR group. The most widely used drugs in the treatment of heart failure are organic nitrates and angiotensin converting enzyme (ACE) inhibitors (Murdoch and Heel, 1991). Both of these compounds release NO either chemically, as in the nitrates, or due to inhibition of kinin breakdown, by kininase-II inhibition, as in the case of ACE inhibitors.

Table - 1: Values of reduced glutathione (GSH), super oxide dismutase (SOD), catalase (CAT) and nitric oxide (NO) of cardiac tissue of rat exposed to amlodipine for 7 days

Groups	Treatment	GSH (nmol min^{-1} mg^{-1} of protein; nmol 100 mg^{-1} protein)	SOD (unit mg^{-1} 100 mg^{-1} protein)	CAT (nmol H$_2$O$_2$ min^{-1} mg^{-1} protein	NO production
I	Sham operated	7.15 ± 0.73	12.38 ± 1.32	1.54 ± 0.49	13.34 ± 5.64
II	Ischemic reperfusion	4.53 ± 0.60*	6.87 ± 0.97*	0.81 ± 0.20*	9.07 ± 2.54*
III	Amlodipine treatment followed by ischemic reperfusion	6.39 ± 0.76#	10.27 ± 1.26#	1.37 ± 0.96	11.45 ± 4.13##

Values are mean of six replicates ± SD. Significant different (*$P<0.05$), from sham operated rats. Significant different (#$P<0.05$), vehicle treated ischemia and reperfusion
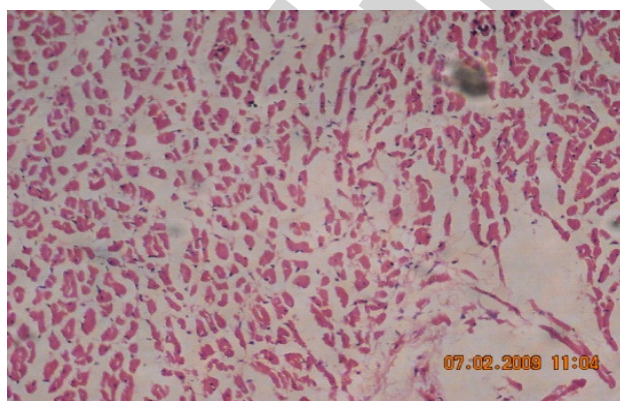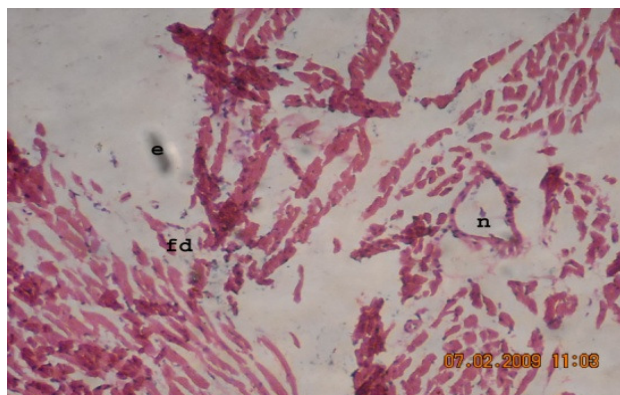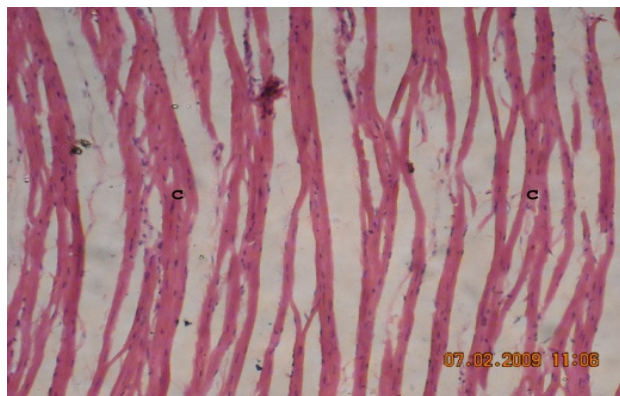

Fig. 1: T.S of cardiac tissue of (A) sham operated, (B) IR group and (C) IR and amlodipine treatment. N=necrosis, FD=focal destruction of myocardial in fibers

These actions result in vasodilation, increased coronary blood flow, decreased peripheral vascular resistance, and other actions, such as inhibition of platelet aggregation (Taylor, 1991). In addition, the organic nitrates cause venous dilation to reduce preload and myocardial oxygen consumption (Stadtman, 1992; Kevin *et al*., 2003) Because amlodipine releases NO and this may be mediated by a kinin mechanism, a portion of its cardiovascular actions should be similar to that of ACE inhibitors and organic nitrates. Indeed, amlodipine is a long-acting vasodilator, increasing blood flow in the coronary, renal, and mesenteric vascular beds (Taylor, 1991; Stadtman, 1992). In addition, amlodipine reduces myocardial oxygen consumption (Taylor, 1991). The mechanism responsible for the release of NO by amlodipine is similar to that of ACE inhibitors that is, modulation of the actions or formation of kinins. Cardiac myocytes are the likely targets of ROS attack in the failing heart. It is conceivable that free radicals cause damage at or near the site of their formation. Therefore, as a major source of ROS production, mitochondria could also be the major targets susceptible to ROS attack (Kevin *et al*., 2003). The defects in mitochondrial architecture would lead to the alteration of the mitochondrial metabolism, resulting in decreased activities of mitochondrial enzymes, in the heart, injury induced by IR, thus become a key contributor to intrinsic cell dysfunction (DiLisa and Bernardi, 2006) and it is also reported that preconditioning reduces ROS production both at the end of ischemia and during reperfusion (VandenHoek *et al*., 2000, Ozcan *et al*., 2002; Ylitalo *et al*., 2000) and decreases mitochondrial calcium overload (Schulz *et al*., 2001). In the present study, the profile of oxidative/antioxidative status in heart after acute injury induced by ischemic and reperfusion revealed marked alterations in antioxidant enzyme activities. Low activities of antioxidant enzymes such as SOD, CAT, and GSH might be due to the overwhelming effects of free radicals, cellular antioxidant enzymes such as SOD, CAT, and free radical scavengers like GSH protect cells and tissues against noxious radicals. An imbalance between cellular pro-oxidant and antioxidant levels results in the oxidative stress that leads to tissue damage. The antioxidant enzymes react directly with ROS to yield non-radical products. SOD, a mitochondrial as well as cytosolic enzyme, O_2^- is converted to H_2O_2 by dismutation, which is decomposed by CAT to H_2O (Meister and Anderson, 1983). A number of studies have reported that the activity of SOD, CAT and GSH were decreased significantly in lung, kidney and liver after
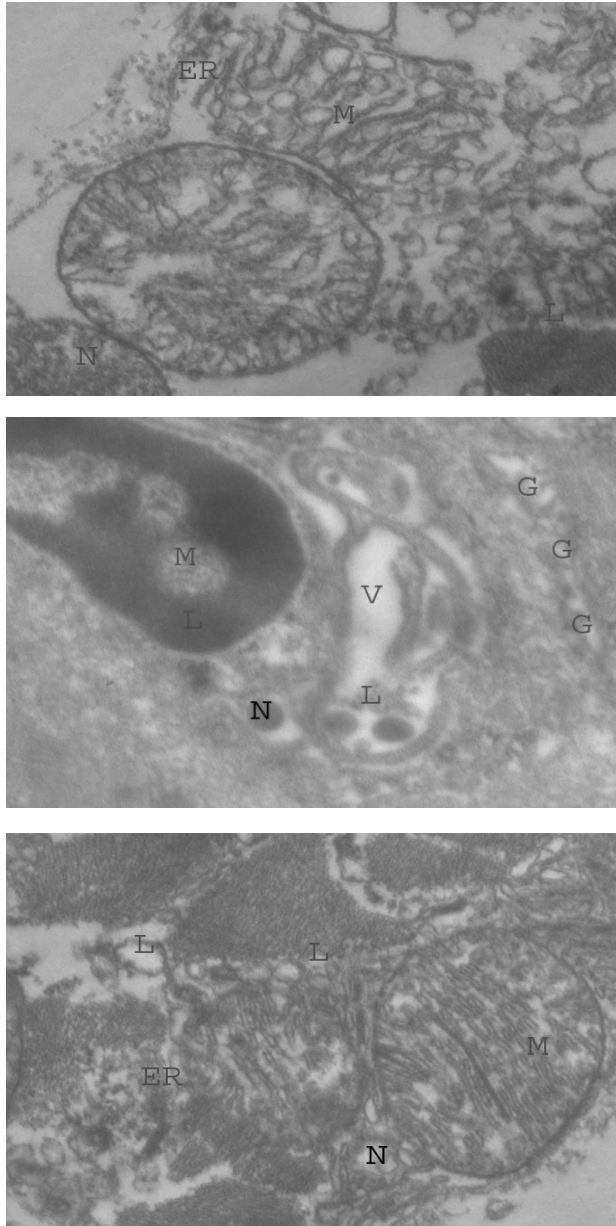
Fig. 2: Electron microscopic observations of (A) Normal morphological finding seen in cardiomyocytes of sham operated group. (B) Showing mitochondria were swollen, cristae disappear, matrix clear out and (C) Showing prominent nucleus with an extended smooth endoplasmic reticulum. ER=Endoplasmic reticulumn, M=Mitochondria, N=Nucleus, L=Lysosome, V=Vacuole, G=Golgi complex

exposing for long term to chemical stimuli like smokeless tobacco (Mates *et al.*, 1999). Our results concord with the earlier work in ischemic heart, a calcium channel blocker, against the IR induced liver injury in rats was due to its inhibitory action on Ca^{2+} influx into the mitochondria and probably to its antioxidant property (Pronobesh *et al.*, 2008). Production of NO in ischemic rat heart increased significantly ($P<0.01$) by amlodipine pretreated group as compared to IR. In Fig. 1, showing severe necrosis, marked edema and focal destruction of myocardial in fibers (B). The degree of myocardial damage in amlodipine administered group showing mild

edema, cardiomyopathy with occasional loss of myofibre in amlodipine administered group (C), similar to sham operated group (A). In Fig. 2, normal morphological finding were seen with the sham operated group (A). After 30 min ischemia and 45 min reperfusion, mitochondria of cardiomyocyte were swollen, cristae disappear, metrix clear out. Increased endoplasmic reticulum (ER), dilated lysosome (L) and nucleus was not well marked (C). In amlodipine treated group on IR induced injury showing digested mitochondria (M) but normalcy of mitochondria (M) and lysosome (L) are accredits the inhibition of injury (B). Thus, the present study provides evidence that the protective effects of amlodipine against IR injury on rat heart might be due to production of NO.

The present study suggests the protective action of amlodipine, a calcium channel blocker, against the IR induced heart injury in rats is due to increased production of NO and possibly to its antioxidant property. Molecular studies may establish more on this calcium channel blocker.

Acknowledgments

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