Protective role of the calcium channel blocker amlodipine against mitochondrial injury in ischemia and reperfusion injury of rat liver

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Ca^{2+} accumulation and Ca^{2+} overloading in mitochondria are responsible for the cell abnormality associated with ischemia and reperfusion injury. The present study was aimed at evaluating the efficacy of the Ca^{2+} channel blocker amlodipine on the mitochondrial Ca^{2+} accumulation, mitochondrial antioxidant status and mitochondrial respiratory enzymes in ischemia and reperfusion (I/R) induced liver injury. I/R injury induced mitochondrial damage in rats was assessed in terms of the decrease in activities ($p < 0.05$) of respiratory marker enzymes (malate dehydrogenase, succinate dehydrogenase and NADH dehydrogenase), mitochondrial antioxidant enzymes (glutathione, superoxide dismutase, catalase), and significant increase ($p < 0.05$) in the level of lipid peroxidation (LPO) and Ca^{2+} content.

Mitochondrial damage was confirmed by transmission electron microscopic (TEM) examination. Pretreatment with amlodipine effectively counteracted the alteration in mitochondrial enzymes induced by ischemia-reperfusion liver damage. TEM study confirms the restoration of cellular normalcy and the cytoprotective role of amlodipine against I/R induced hepatic injury. On the basis of our findings it may be concluded that amlodipine not only possesses Ca^{2+} channel antagonist properties but it may also reduce the extent of mitochondrial damage by its antioxidant activity.

Keywords: calcium channel blocker, amlodipine, ischemia and reperfusion injury, mitochondrial injury

Mitochondria are the principal targets in the development of ischemia-reperfusion (I/R) induced hepatic injury (1, 2). Increased production of superoxide radicals at the level of the mitochondrial respiratory chain is allegedly a major participant in I/R injury (2). Oxidant stress induced cell killing involves oxidation of pyridine nucleotides, accu-

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mulation of calcium ions in mitochondria, and superoxide formation by mitochondria, ultimately leading to formation of membrane permeability transition pores and breakdown of the mitochondrial membrane potential (3), alteration of mitochondrial enzymes activity, intracellular Ca^{2+} accumulation and Ca^{2+} overloading, contributing to irreversible cell damage (4). Ischemia is associated with impaired calcium homeostasis. Ischemic tissue injury causes an increase in free intracellular calcium that leads to diminished recovery of the dilation function after ischemia (vasoconstriction), compromised membrane integrity and decreasing reserves of cellular adenosine triphosphate. Amlodipine is a second-generation dihydropyridine calcium channel blocker shown to have a gradual onset and sustained duration of action because of the selectivity of dihydropyridines for the vascular smooth muscle (5). The pharmacokinetic profile such as that of amlodipine may prove to be valuable in protection from the ischemica and reperfused injuries. A single intravenous dose of 10 mg amlodipine resulted in absolute bioavailability of 64% and a calculated half-life of 34 hours (6). The present study evaluates the potential effect of the calcium antagonist amlodipine on hepatic mitochondrial marker enzymes, antioxidant and mitochondrial structure in I/R induced hepatotoxicity.

EXPERIMENTAL

Collagenase and bovine serum albumin (BSA) were purchased from Hymedia (India). Other chemicals were obtained from Sigma (USA).

Experimental design

Male Wistar rats weighing 200 to 250 g were purchased from Laboratory Animal Resources, Indian Veterinary Research Institute, Izzatnagar, UP, India. Animals were kept in temperature-controlled rooms at the animal house, College of Pharmacy, IFTM, Moradabad, UP India, with 12 h alternating light and dark cycles and were given adequate nutrition and water *ad libitum*. All experimental protocols using animals were performed according to the »Principles of Laboratory Animal Care« (NIH publication 85–23, revised 1985). Experimental protocols were reviewed and approved by the institutional Ethical Committee. Eighteen Wistar rats were divided into a control group (I) (*n* = 6), ischemia and reperfusion group (II) (*n* = 6), and amlodipine pre-treated group (1 mg kg^{-1} body mass daily by oral route for 7 days before induced ischemia-reperfusion maneuver) (III) (*n* = 6). Ischemia and reperfusion injury was produced as per the procedure described by Hayashi *et al.* (7). In short, after the induction of anesthesia (urethane 10 mg kg^{-1} body mass daily by *i.p.*), the liver of each animal was exposed through a midline laparotomy. Clamping the left branches of the portal vein and the hepatic artery for 60 min produced complete ischemia of the median and left hepatic lobes. The right hepatic lobe was perfused to prevent intestinal congestion. After the period of ischemia, the ligatures around the left branches of the portal vein and hepatic artery were removed. The wound was closed with 3–0 silk. Sham-operated animals were similarly prepared except that no ligature was placed to obstruct the blood flow to the left and median hepatic lobes. Instead, the blood flow to the right lobe of the liver was occluded. After 3 h reperfusion, rats were euthanized by diethyl ether (S.D. Fine Chemicals Ltd., India) and blood and tissue samples were collected, as described below.

Peripheral tissue procurement

A portion of ischemic and non-ischemic liver lobes were fixed in buffered 10% formalin and Karnovsky's solution (2.5% glutaraldehyde and 2% formaldehyde in 0.2 mol L^{-1} phosphate buffer) for histopathology and TEM studies. Another portion of ischemic and non-ischemic liver lobes were snap frozen in liquid nitrogen and stored for 3 days at –70 °C for mitochondria isolation.

Isolation of mitochondria

The liver mitochondria were isolated by the method of Johnson and Lardy (8). A portion of liver tissue was weighted and homogenized with 0.35 mol L^{-1} sucrose buffer at 4 °C and centrifuged at 10,000×*g* for 5 minutes. The resultant mitochondrial pellet was then resuspended in 0.25 mol L^{-1} sucrose solution containing 10 mmol L^{-1} Tris-HCl (pH 7.4) and 1 mmol L^{-1} EDTA up to a final volume of 2 mL. Mitochondrial integrity of the hepatocytes was measured by reduction of mitochondrial enzyme succinate dehydrogenase using the 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Barltrop *et al.* (9).

Assay of enzymes

The mitochondrial respiratory marker enzymes such as malate dehydrogenase (MDH), succinate dehydrogenase (SDH), NADH dehydrogenase and mitochondrial antioxidants such as glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT) and mitochondrial lipid peroxides (LPO) were determined as described previously by Mehler *et al.* (10), Slater and Bonne (11), Minakami *et al.* (12), Habig *et al.* (13), Hodgson and Fridovich (14), Takahara *et al.* (15) and Ohkawa *et al.* (16), respectively. Mitochondrial calcium was estimated by using the diagnostic kit (Quiligens Diagnostics, India) according to manufacturer's guidelines.

Transmission electron microscopy (TEM) of liver tissue

Liver tissue was fixed in Karnovsky's solution pH 7.4 for 4 h at 4 °C. After fixation and an overnight wash in sodium cacodylate buffer at 4 °C, the specimens were postfixed with 1 % osmium tetroxide in 0.1 mol L^{-1} phosphate buffer (pH 7.4), dehydrated in ethanol and then embedded in Araldite resin (Ducupan, Fluka BioChemika, USA). Semi--thin sections (1 µm) were cut for optical microscopy. Ultra-thin sections (40–60 nm) were placed on copper mesh grids (200 nm) and double-stained with uranyl acetate and lead citrate. Sections were examined using a transmission electron microscope (Moragagni 268 D, The Netherlands) and photomicrographs were taken.

Statistical analysis

All values were expressed as mean ± SD. Differences in mean values were compared by one-way ANOVA and Student-Newman-Keul (SNK) test.

RESULTS AND DISCUSSION

The activities of mitochondrial respiratory, *viz.* malate dehydrogenase (MDH), succinate dehydrogenase (SDH) and NADH dehydrogenase (NADH), marker enzymes were significantly decreased ($p < 0.05$) in the I/R liver injured group of rats compared to the control group (Table I). Pretreatment with amlodipine (group III) reverted the altered activities of these enzymes to near control levels when compared to the sham-operated control group (Table I).

The activity of mitochondrial antioxidant enzymes, *viz.* glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), was significantly ($p < 0.05$) decreased while Ca^{2+} content and lipid peroxides (LPO) activity was increased significantly ($p < 0.05$) in the I/R injured group of rats compared to the control group (Table II). Activities of these enzymes in mitochondria were almost completely reversed in the amlodipine pretreated group of rats (Table II). Pretreatment with amlodipine also prevented lipid peroxidation compared to the I/R liver injured group of animals.

Table. I. Activities of mitochondrial and respiratory enzymes in the liver of control and experimental groups of rats

Group	MDH[a]	SDH[b]	NADH dehydrogenase[c]
Control	293.12 ± 25.34	201.40 ± 17.65	124.36 ± 19.65
I/R Injury	187.25 ± 33.51[d]	112.87 ± 13.46[d]	85.63 ± 5.61[f]
Amlodipine + I/R injury	249.70 ± 27.18[e]	182.32 ± 24.50[e]	108.44 ± 17.49[g]

Results are expressed as mean ± *SD* (*n* = 6).
[a] Expressed as nmol of MDH oxidized per min per mg protein.
[b] Expressed as nmol of succinate oxidized per min per mg protein.
[c] Expressed as nmol NADH oxidized per min per mg protein.
Significantly different from control group ([d] $p < 0.05$, [f] $p < 0.01$).
Significantly different from I/R injury group ([e] $p < 0.05$, [g] $p < 0.01$).

Table II. Activities of mitochondrial antioxidant enzymes, Ca^{+2} content and LPO in the liver of control and experimental groups of rats

Group	GST[a]	SOD[b]	CAT[c]	LPO[d]	Ca^{2+e}
Control	64.37 ± 5.90	15.41 ± 1.34	1.32 ± 0.19	4.10 ± 0.60	7.11 ± 0.39
I/R Injury	40.29 ± 4.87[f]	4.98 ± 0.91[h]	0.94 ± 0.08[h]	7.25 ± 0.33[f]	12.36 ± 1.54[f]
Amlodipine + I/R injury	60.87 ± 5.19[g]	13.22 ± 1.47[i]	1.30 ± 0.46[g]	3.49 ± 0.47[i]	8.70 ± 1.22[i]

Results are expressed as mean ± SD (*n* = 6).
[a] Expressed as nmol of 1-chloro 2,4-dinitrobenzene (CDNB) oxidized per min per 100 mg protein.
[b] Expressed as units per min per 100 mg protein.
[c] Expressed as nmol of H_2O_2 decomposed per min per mg protein.
[d] Expressed as nmol of MDA per mg of protein.
[e] Expressed as nmol per mg of protein.
Significantly different ([f] $p < 0.05$, [h] $p < 0.01$) from control group.
Significantly different ([g] $p < 0.05$, [i] $p < 0.01$) from I/R injury group.

Transmission electron microscopy (TEM) studies showed that the I/R injured rats mitochondria of hepatocytes were severely swollen with less quantity and cristae. Pretreatment with amlodipine showed improvement in cytoplasmic changes as well as the fine ultra structure of the mitochondria similarly to the control liver section (Fig. 1).

In the present study, ischemia and reperfusion injury caused severe mitochondrial degeneration leading to the tricarboxyl acid (TCA) cycles marker enzymes (MDH, SDH) which were observed in I/R injured rats. The impaired mitochondrial function, which may contribute to the pathogenesis of I/R induced hepatotoxicity, might be a reason for activation of Kupfer cells leading to generation of reactive oxygen species (ROS).

The activity of the respiratory marker NADH dehydrogenase was lowered in I/R rats. This could be due to enhanced phospholipids degradation and participation of ROS in the mitochondrial electron transfer system. Pretreatment with amlodipine reduced the extent of mitochondrial damage and reversed the activities of TCA cycle and respiratory marker enzymes towards normalcy. The enhanced oxidative stress and mitochondrial calcium overload have been taken to be responsible for mitochondrial dysfunction (17). We observed an increase in the level of calcium in the mitochondria in I/R induced hepatocytes. Pretreatment with amlodipine prevented the increase in mitochondrial Ca^{2+} concentration, thereby restoring the mitochondrial function.

In the cellular defence system, GSH (reduced glutathione) plays an important protective role, as an intracellular free radical scavenger, by conjugation with metabolites, and also protects the mitochondrial membrane from the damaging action of LPO (18). Low level of GSH was observed during increased oxidative stress caused by I/R. Cellular GSH depletion is closely related to the lipid peroxidation and disturbance of Ca^{2+} influx induced by oxidative injury (19). This observation supports our findings where we observed a decline in GST levels in I/R injured rats. Pretreatment with amlodipine in
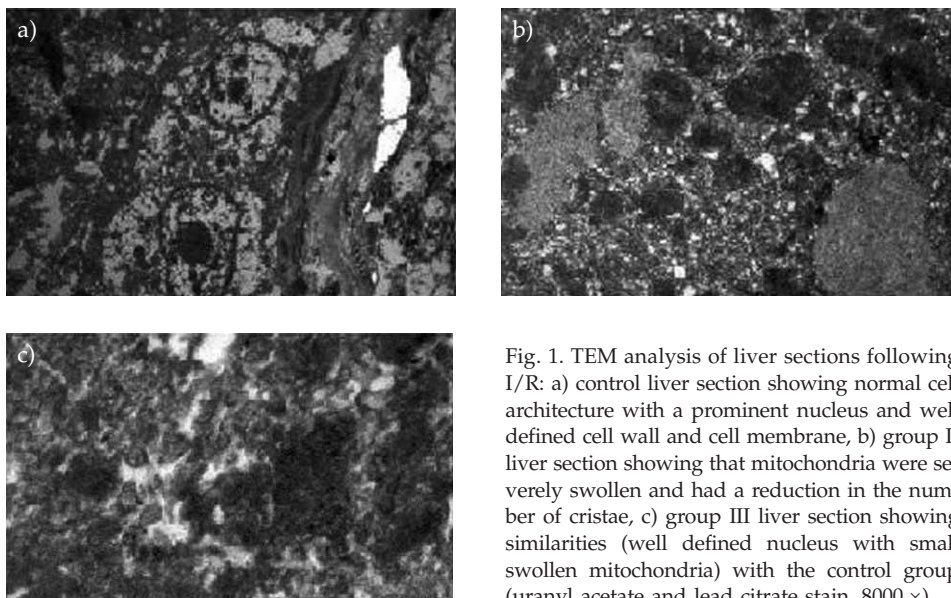

Fig. 1. TEM analysis of liver sections following I/R: a) control liver section showing normal cell architecture with a prominent nucleus and well defined cell wall and cell membrane, b) group II liver section showing that mitochondria were severely swollen and had a reduction in the number of cristae, c) group III liver section showing similarities (well defined nucleus with small swollen mitochondria) with the control group (uranyl acetate and lead citrate stain, 8000 ×).

I/R rats maintained the concentration of GSH at near normal levels, which prevented cell disruption, probably by decreasing the Ca^{2+} influx.

Major antioxidants like SOD, CAT and GST are important for cellular protection due to their ability to detoxify free radicals such as ROS. A number of studies have reported diverse results for the changes of these antioxidant enzyme activities in I/R induced hepatic injury (20). Accentuated mitochondrial lipid peroxidation, accompanied by a deteriorating mitochondrial antioxidant status is evident in I/R injured rats. The rise in the activities of mitochondrial antioxidant enzymes in amlodipine pretreated group highlights the protective role of amlodipine in combating the oxidative insult. Lipid peroxidation is one of the major outcomes of free radical-mediated tissue injury and is an indicator of oxidative damage by a series of chain reactions. Increased level of MDA, the end product of LPO, was observed in I/R injured rats. Mitochondrial lipid peroxidation was significantly ($p < 0.05$) lowered in the amlodipine pretreated group compared to the I/R injured group. Our results are in concord with the earlier work done by Wang *et al.* (21), where it was reported that amlodipine regulates membrane fluidity and cholesterol depositions, acts as an antioxidant and regulates matrix deposition.

Ultra structural changes were demonstrated microscopically in I/R rats. Our study shows that, after 1 h ischemia and 3 h reperfusion, there is accumulation of Ca^{2+} which leads to autophagosomes, reduction in the number of mitochondria and nuclear condensation. Pretreatment with amlodipine showed considerable prevention in the ultra structural alteration, including disruption of mitochondrial and nuclear fine structures. Both Ca^{2+} accumulation and oxidative stress increase the probability of changes in mitochondrial permeability and together with opening the transition pore in the mitochondria carry the signals for cell death (22). Amlodipine prevents cellular damage in the membrane probably by inhibiting the calcium channel of hepatocytes. A previous study reported that amlodipine reduced hepatic I/R injury by cytoprotective effects on parenchymal and non-parenchymal hepatocytes during both preservation and reperfusion (23). Other Ca^{2+} channel antagonists, verampril (24) and nimodipine, are also reported to have a protective role in hepatic I/R induced injury. Thus, the present study, consistent with our previous study, provides evidence that amlodipine protects I/R rats from mitochondrial injury, giving an insight into the underlying mechanisms of the effects of amlodipine.

CONCLUSIONS

The present investigation suggests that the protective action of amlodipine, a calcium channel blocker, against the I/R induced liver injury in rats is due to its inhibitory action on Ca^{2+} influx into the mitochondria and probably to its antioxidant property. Additional studies are needed to determine whether there is a link between reductions in the I/R induced injury liver markers as achieved with calcium antagonists and that obtained with oxygen radical scavengers.

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S A Ž E T A K

Zaštitna uloga amlodipina, blokatora kalcijevih kanala, protiv oštećenja mitohondrija kod ishemičnih i reperfuzijskih ozljeda jetre štakora

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Akumulacija Ca^{2+} iona i njihovo nakupljanje u mitohondrijima uzrok je abnormalnosti u stanicama nakon ishemičnih i reperfuzijskih ozljeda. Cilj ovog rada bio je ispitati učinak amlodipina, blokatora kalcijevih kanala, na nakupljanje iona kalcija u mitohondrijima, antioksidativni status mitohondrija i na aktivnost enzima u dišnom lancu kod ishemičnih i reperfuzijskih (I/R) ozljeda jetre. Kod tih ozljeda smanjeno je djelovanje ($p < 0,05$) enzima dišnog lanca (malat dehidrogenaze, sukcinat dehidrogenaze i NADH-dehidrogenaze), antioksidativnih enzima mitohondrija (glutation, superoksid dismutaze, katalaze), stupanj lipidne peroksidacije (LPO) i nakupljanje iona Ca^{2+}.

Oštećenje mitohondrija potvrđeno je transmisijskom elektronskom mikroskopijom (TEM). Prethodna obrada s amlodipinom učinkovito sprječava promjenu aktivnosti enzima mitohondrija uzrokovanih I/R oštećenjima jetre. TEM potvrđuje uspostavljanje normalnih uvjeta u stanici i citoprotektivno djelovanje amlodipina. Na temelju rezultata naših istraživanja može se zaključiti da amlodipin zbog antioksidativnog djelovanja reducira oštećenje mitohondrija.

Ključne riječi: blokator kalcijevih kanala, amlodipin, ishemična i reperfuzijska ozljeda, oštećenje mitohondrija

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