CALCIUM ANTAGONIST PREVENTS CALCIUM FLUX INDUCED NECROSIS AND APOPTOSIS IN ISCHEMIC REPERFUSION OF RAT LIVER

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ABSTRACT

Ca²⁺ accumulation in mitochondria is responsible for the cell abnormality associated with ischemia and reperfusion injury. The present study was aimed to evaluate the efficacy of Ca²⁺ channel blocker-amlodipine on the mitochondrial Ca²⁺ accumulation in ischemic and reperfusion (I/R) induced liver injury. Eighteen wistar rats were divided in sham-operated control group-I (n = 6), ischemia and reperfusion group-II (n = 6) and amlodipine treated group-III (1 mg/kg body weight /daily by oral route for 7 days before induced ischemia reperfusion manouver) (n = 6). Rats were subjected to 1h of hepatic ischemia followed by 3 h reperfusion. Mitochondrial Ca²⁺ content was measured and damage of mitochondria was confirmed by transmission electron microscopic examination. Bcl-2 gene expression was measured by reverse transcriptase polymerase chain reaction method. Pretreatment with Amlodipine effectively counteracted the alternation in mitochondrial Ca²⁺ content. TEM and expression of Bcl-2 protein confirms the restoration of cellular normalcy and accredits the cytoprotective role of Amlodipine against I/R induced hepatic injury. These findings showed that the mechanism of regulation of Bcl-2 gene expression by amlodipine may be the inhibitory action of Ca²⁺ entry into mitochondria and prevent apoptosis and necrosis.

KEY WORDS

Calcium channel blocker, Amlodipine, Ischemic and reperfusion injury.

INTRODUCTION

Ischemic reperfusion (I/R) injury is a phenomenon whereby cellular damage in a hypoxic organ is accentuated following the restoration of oxygen delivery. In the liver, this form of injury was recognized as a clinically important pathological disorder during studies of experimental liver transplantation (1). Mitochondria are the principal targets in the development of I/R induced hepatic injury (2-3). Alteration of mitochondrial functions viz., mitochondrial enzyme activity, intracellular Ca²⁺ accumulation and Ca²⁺ overloading, contribute to irreversible cell damage (4). Calcium overload induced cell killing involves oxidation of pyridine nucleotides, accumulation of calcium in

mitochondria, and superoxide formation by mitochondria, which ultimately leads to formation of membrane permeability transition pores and breakdown of the mitochondrial membrane potential (5). In support of this intracellular signaling mechanism, the mitochondrial membrane permeability transition was observed during hepatic ischemia-reperfusion (6). Bcl-2 family of genes play a major role in determining the ultimate sensitivity or resistance of cells to myriad stimulus and insults that induce apoptosis in mitochondria (7). However, studies dealing with the effects of amlodipine on hepatic I/R injury is not reported. Thus it was decided to study the effect of amlodipine on hepatic I/R injury in rats and its possible mechanism.

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MATERIALS AND METHODS

Chemicals: If not mentioned otherwise all reagents were obtained from Sigma (Sigma, St Louis, Mo, USA).

Experimental Design: Male Wistar rats weighing 200 to 250g purchased from Laboratory Animal Resources, Indian Veterinary Research Institute, Izatnagar, UP, India, were

maintained in temperature-controlled room to the animal house with 12h alternating light and dark cycles, and were given adequate nutrition and water *ad libitum*. All animals received human care in compliance as per Guide for the care and use of Laboratory Animals. Experimental protocols were reviewed and approved by Institutional ethical committee. Ischemia and reperfusion injury was produced as per the procedure described by Chattopadhyay et al (8). Eighteen Wistar rats were divided into sham-operated control group-I ($n = 6$), ischemia and reperfusion, group-II given 0.9 % saline (5ml/kg, p.o) for 7 days ($n = 6$) and Amlodipine treated group-III was given 1 mg/kg body weight /daily by oral route for 7 days before inducing ischemia reperfusion manouver ($n = 6$). In all the rats were sacrificed after 1 h ischemia and 3 h reperfusion. Ischemic lobe was snap frozen in liquid nitrogen and stored at -70°C for isolation of mitochondria, Bcl-2 gene expression and TEM studies.

Assay of mitochondrial Ca^{2+} effluxes: The mitochondria of liver were isolated by the method of Johnson and Lardy (9). Mitochondrial calcium was estimated using diagnostic kit (Quilgens, diagnostics, India) as per manufacturer's guidelines. The increase in absorbance was measured at 570 nm using beam spectrophotometer (ECL, Hyderabad India) and the results were expressed as nmol calcium per mg protein in all the samples.

Reversal transcription polymerase chain reaction Total RNAs were isolated from samples of frozen liver (1g) using by the Trizol method (Life Technologies, Rockville, MD, USA). RNA quality and integrity was assured by spectrophotometric analysis ($\text{OD}_{260\text{nm}}$). Total RNAs (5 μg) were first reverse-transcribed into cDNA using oligo (dT) 12-18 as primer and AMV reverse transcriptase (Boehringer, Mannheim, Germany). Reverse transcripts (equivalent to 125 ng of total RNA) were used directly for each amplification reaction. Experiments were performed using a light cycle rapid thermal cycle (Eppendorf, Germany). Polymerase chain reaction primer were against rat Bcl-2 sequences obtained from gene bank (Fastaf). The sense primer was a 21 –mer with a sequences of CGT-CAT-AAC-TAA-AGA-CAC-CCC and the reverse primer was also a 21 mer with a sequences of TTC-ATC-TCC-AGT-ATC-CGA-CTC and purchased from Integrated DNA technologies, Inc. Milpitas, CA 95035, USA. The product length was 234 and the PCR profile was set for denaturation 1 min at 94°C , annealing 90 s at 56°C and extension 2 min at 72°C and semi- quantization was optimized to 35 cycles. GAPDH (glyceraldehyde- 3 – phosphate dehydrogenase) transcript abundance was used as an endogenous control. The cDNA was amplified by PCR

amplification with Ampli Taq Polymerase (Bangalore Genni, India). Amplified product was resolved by electrophoresis on 1.5% agarose gels (sigma, St Louis USA), stained with ethidium bromide and visualized under ultraviolet light. A 1 kbp DNA ladder molecular weight marker (Life Technologies, Rockville, MD, USA) was run on every gel to confirm expected molecular weight of the amplification product. Bands were quantitatively measured by densitometry analysis system (Molecular Analyst/PC, Windows software for Bio-Rad's (Hercules CA). Image Analysis System Version 1.5, and the data are expressed in relative optical density (OD) units.

Transmission electron microscopy assay: Liver tissues were 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 0.1M phosphate buffer (pH 7.4), dehydrated in ethanol and then embedded in Araldite resin and semi thin sections (1 μm) were removed for optical microscopy. Ultra thin sections (40-60nm thick) were placed on copper mesh grids (200 mesh) and doubly stained with uranyl acetate and lead citrate. Sections were examined using a transmission electron microscope (Moragagni 268D by Netherlands) and photomicrographs were taken.

Fluorescence Microscopy: Viable hepatocytes were prepared by the collagenase perfusion modified method (10). Cell morphology of I/R induced apoptosis was investigated by staining the cells acridine orange and ethidium bromide. Cells were harvested and washed with PBS after isolation of hepatocytes from different group. After staining with mixture of 100 $\mu\text{g}/\text{ml}$ acridine orange and ethidium bromide for 5 min and the cells were observed under a fluorescence microscope (Olympus).

Flow cytometry analysis: Flow cytometry was used to determine the sub – $G_1 - G_0$ fraction in fixed cells stained with propidium iodide (PI) as described previously (11). Hepatocytes ($1 \times 10^9/\text{L}$) washed with PBS, exposed to propidium iodide (PI) 50 mg/L, 0.1% Triton X-100, 0.01 mmol/L EDTA (Na_2) and RNase 50 mg/L at normal temperature in darkness for 12-24 h. Specimen were then presented to the FACS-420 Flow Cytometry Analyzer (New York, Becton Dickinson and Company, USA) to evaluate apoptosis levels. The apoptotic and necrotic cells were finally analyzed with the Modfit 3.0 DNA software on the basis of percentage of hepatocytes staining with PI.

Statistical analysis: All values were expressed as mean $\pm$ SD.

Differences in mean values were compared using SPSS 11.0 by one-way ANOVA and Student-Newman-Keul (SNK) test. $P < 0.05$ was considered as statistically significant.

RESULTS

The Ca^{2+} level in the sham operated control rats was 7.11 ± 0.39 and increased to 12.36 ± 1.54 after 1 h ischemia followed by 3 h reperfusion. The significant increase in Ca^{2+} level in the I/R group was significantly suppressed by administration of 1 mg/kg amlodipine (Table 1).

Table 1: Effects of amlodipine on % apoptosis, %necrosis and Ca^{2+} efflux after 1h ischemia followed by 3h reperfusion injury in rat liver

Group	% of Necrotic cells	% of Apoptotic cells	Ca^{2+} effluxes
Sham-operated control (I)	1.02 ± 0.32	0.70 ± 0.08	7.11 ± 0.39
Ischemia and reperfusion (II)	$21.54 \pm 7.1^*$	$26.44 \pm 6.01^*$	$12.36 \pm 1.54^{**}$
Amlodipine treated (III)	$8.21 \pm 0.22^{*##}$	$14.65 \pm 2.36^{*##}$	$8.70 \pm 1.22^{*##}$

Results are expressed as mean $\pm$ SD (n=6).

Significantly different (* $P < 0.05$, ** $P < 0.01$) from sham operated group.

Significantly different (# $P < 0.05$, ## $P < 0.01$) from I/R injury group.

The necrotic and apoptotic cells were 1.02 ± 0.32 and 0.70 ± 0.08 , respectively in the sham operated control rats and increased to 21.54 ± 7.1 and 26.44 ± 6.0 after 1h ischemia followed by 3h reperfusion. Amlodipine (1mg/kg) was attenuated increased necrosis and apoptosis caused by I/R induced toxicity (Table 1 & Figure 1)

Figure 1: I/R -induced necrosis in hepatocytes. Flow cytometry analysis assessing apoptosis after propidium iodide staining. a: Flow-cytogram of sham operated control rats (I). b: Flow - cytogram of ischemic and reperfused rats (II). c: Flow- cytogram of amlodipine treated rats (III).

Anti-apoptotic Bcl-2 gene and house keeping glyceraldehyde-3 phosphate dehydrogenase (GPDH) gene amplified by RT-PCR were separated by electrophoresis by staining with ethidium bromide. PCR products of Bcl-2, and GPDH were expressed at 234 bp and 510 bp respectively (Figure 2). Expression of Bcl-2 gene in I/R group was negligible in comparison to sham-operated group after 1h ischemia followed by 3h reperfusion. Pre treatment with amlodipine significantly increased Bcl-2 gene expression as compared to I/R group.

Figure 2: Bcl-2 gene expression. a: Expression of Bcl-2 gene by RTPCR analysis. Lane M: Marker, Lane 1: Sham-operated control rats (I), Lane 2: I/R rats (II), Lane 3: amlodipine treated (III) rats. b: The expression of Bcl-2 gene. Data are expressed as the mean $\pm$ SD

Apoptosis was evaluated based on distinct morphological features viz., cell shrinkage, chromatin condensation, oligonucleosomal DNA fragmentation and break down of the cell into smaller units (apoptotic bodies). Reperfusion of the ischemic liver caused severe hepatocellular apoptosis (Figure 3b). In contrast in sham operated rat there were no sign of apoptosis (Figure 3a). The increase apoptosis that

occurred after 1 h ischemia followed by 3h reperfusion in the I/R group rats was significantly suppressed by administration of amlodipine acid (Figure 3c).

Figure 3: Fluorescence microscopy of hepatocytes stained with mixture of ethidium bromide and acridine orange (100mg/ml). Viable cells observed with green nuclear fluorescence and apoptotic cells containing an orange nucleus (marked arrow) and exhibiting chromatin condensation. Magnification x200. a: Sham operated group (I); b: Ischemia followed by reperfusion group (II). c: amlodipine treated group (III).

TEM studies showed that I/R injured the mitochondria of hepatocytes which swell, had less quality and cristae are clear out. (Figure 4b). Pre treated with amlodipine group (Figure 4c) showed that improved cytoplasmic changes as

Figure 4: Transmission electron microscope analysis of liver sections following I/R. a: Sham operated control rat (I). b: I/R induced group (II). c: amlodipine treated group (III). Endoplasmic reticulum (E); Mitochondria (M); Glycogen (G); Nucleus (N) (Uranyl acetate and lead citrate stain x 8000).

well as retained the fine ultra structure of the mitochondria (Figure 4a).

DISCUSSION

The present study shows that after 1h ischemia followed by 3h reperfusion, Ca^{2+} increases and Bcl-2 expression is inhibited. Pretreatment with Amlodipine significantly attenuated efflux of Ca^{2+} into mitochondria and up regulated Bcl-2 expression which resulted in diminished hepatic apoptosis caused by liver I/R in rats.

Earlier work showed that ischemia is associated with impaired calcium homeostasis. Ischemic tissue injury causes an increase in free intracellular calcium that leads to diminished recovery of dilatation function after ischemia (vasoconstriction), compromised membrane integrity and decrease in reserves of cellular adenosine triphosphate (12). There is no report to support the assertion that amlodipine directly affects mitochondria and Bcl-2. Amlodipine specifically blocks L-type Ca^{2+} channels which are exclusively localized to the plasma membrane. In addition to its localization to the mitochondria significant amounts of Bcl-2 is localized to the mitochondrial membrane, endoplasmic reticulum, and nuclear membrane (13), where it functions to maintain low Ca^{2+} levels. Therefore, present study support that in I/R rats after treatment with amlodipine Bcl-2 function from I/R injury by indirectly blocking of Ca^{2+} channels is restored.

It is also presumed that amlodipine restored indirectly Bcl-2 expression by inhibiting Ca^{2+} efflux into mitochondria and prevention of activation of calcineurin which reduces phosphorylation of Bad (a Bcl-2 family pro-apoptotic protein) therefore reduced apoptosis in I/R rat. Previous study showed that Bad is a regulator of apoptosis that appears to function by sequestering Bcl- X_L and by preventing Bcl- X_L from heterodimerizing with Bax or up regulation Bcl-2 expression (14). However, this study provides new insight into the mechanism of calcium channel blocker by which mediates protection from apoptosis by indirectly Bcl-2 up regulation.

Further, our observation showed that after 1 h ischemia followed by 3 h reperfusion accumulation of Ca^{2+} increases which lead to autophagosomes, the reduction in the number of mitochondria and nuclear condensation. Pre - treatment with amlodipine showed considerable prevention in the ultra structural alteration including disruption of mitochondrial and nuclear fine structure, restored numbers of E R as compared to sham operated control rats.

Data are consistent with previous study that overexpression of Bcl-2 reduced apoptosis (15) and this study showed that calcium channel blocker prevents necrosis and apoptosis by up regulation of Bcl-2 which was confirmed by flow cytometry and fluorescence microscopy.

In our study, sham operated control rats showed a very few numbers of apoptotic and necrotic hepatocytes because apoptosis is an important and continuous event in mitosis cell division and this observation is consistent with previous study that in physiological hepatocytes turnover, older differentiated hepatocytes are typically eliminated by apoptosis and replaced by the division progeny of adult stem cells (16).

The present investigation thus shows that protective action of calcium channel blocker-amlodipine against the I/R induced injury has protective effect mainly due to inhibitory action of Ca^{2+} efflux into mitochondria.

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