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Calpain Inhibitors and Modulation of Ischaemia Reperfusion Induced Apoptosis and Necrotic Myocardial Cell Injury

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Abstract: *Apoptosis is a crucial event that can initiate ischaemia-reperfusion induced inflammation and subsequent tissue injury. Myocardial ischaemia reperfusion is associated with activation of intracellular death proteases known as calpains. Myocardial ischaemia was induced in isolated rat heart which was subjected to 30 min ischaemia followed by reperfusion for 120min. The effect of calpain inhibitors SJA7019 and SJA 7029(10µM and 15µM) in induced myocardial injury assessed in terms of infract size measured macroscopically, release of LDH and CK have been investigated. Agarose gel electrophoresis was used to assess the DNA smearing. TUNEL staining was done to investigate the apoptotic index. Calpain inhibitors such as SJA7019 and SJA7029 has attenuated ischaemia reperfusion induced increase in LDH,CK, myocardial infarct size ,DNA smearing and apoptotic index. In the present study it is concluded that inhibition of intracellular death proteases prevented ischaemia reperfusion induced apoptosis induced necrotic cell death.*

Keyword: *Apoptosis, Calpain, Ischaemia-reperfusion induced myocardial injury, Necrosis*

I. Introduction

Apoptosis may lead to necrotic cell death [1, 2, 3]. The transition from reversible to irreversible injury is characterized by the development of severe membrane permeability defects that allows the unregulated influx of divalent and trivalent cations including calcium [4, 5].Reperfusion is associated with brisk drop in intracellular pH and an associated rise in calcium which leads to activation of calpain- a calcium dependent membrane protease implicated in necrotic cell death [6].Calpain expression is noted to increase during ischaemia and reperfusion[5, 7]and is involved in signal transduction of apoptosis[8, 9].

Tissue damage in ischaemic areas involves both apoptosis and necrosis and calpain participate in both the processes [10].The rise in Ca^{2+} in cardiomyocytes during ischaemia and reperfusion is considered to be a pivotal event in cell death [11, 12].An increase in Ca^{2+} influx may activate dormant Ca^{2+}dependent intracellular death proteases – calpains, causing damage to myocardial structural proteins leading to membrane breakdown and eventually cell death [5, 13].Reperfusion results in sodium ion influx followed by Ca^{2+} accumulation which may activate calpain and consequently produce Bid [6, 14, 15]and Bcl-xl cleavage. The cleaved activated fragments acts on mitochondria causing its dysfunction [16] and release of pro-apoptotic factors resulting in DNA fragmentation and cell death. On the other hand release of cytochrome –c produces depletion of ATP stores [6] and lead to necrotic cell death [17].The mode of cell death shifts from apoptotic to necrotic due to use of ATP stores by apoptosis. Therefore, apoptosis may lead to necrotic cell death [2, 12, 18, 19].

The calpain has been implicated in ischaemia-reperfusion induced neuronal [8, 20] retinal [16, 21, 22] and myocardial cell death [7, 15, 23]. During ischaemia and reperfusion, calpain activity is increased through an increase in the expression of calpains. The SJA 7019 and SJA 7029 inhibit extra cellular influx of Ca^{2+} leading to down regulation of calpain activity [2, 24].The selective inhibition of calpain activity by compounds like SJA 7019 and SJA 7029has improved function of heart which was declined as a result of ischaemia and reperfusion.

In the present study inhibition of intracellular death proteases like calpain by SJA7019 & SJA7029 attenuated adverse biochemical changes, apoptotic and necrotic indices in the ischaemia reperfusion induced isolated rat heart. The proposed mechanism shows the site of action of calpain inhibitors to attenuate ischaemia-reperfusion induced myocardial cell death.

II. Materials and Methods

2.1 Drugs

SJA 7019, SJA 7029 (Senju Pharmaceutical Co., Kobe, Japan), Proteinase K(Sigma-Aldrich, St. Louis, USA) and RNAse (Hi Media, Mumbai, India) were used to carry out the study.

2.2 Animals

Wistar albino rats of either sex were used to carry out studies. Rats were heparinized and sacrificed by stunning; heart rapidly excised and immediately mounted on Langendroff's apparatus [25].The preparation was perfused with Krebs-Heinslet (K-H) solution pH 7.4, maintained at 37^{o}C and bubbled with 95% O_2 and 5% CO_2.

Coronary flow rate was maintained 6-9 ml/min and perfusion pressure was kept constant at 70mm Hg. Global ischaemia was produced for 30 min by closing the inflow of physiological solution and it was followed by reperfusion for 120 min. Two thin electrodes fixed at ventricular apex and origin of aorta was employed to record ECG (BPL, MK 801, Bangalore, India) for monitoring heart rate.

2.3 Infarct size Measurement

Infarct size was measured and expressed as percentage of total left ventricular volume (%LVV) and left ventricular weight (%LVW) respectively by Volume and Weight method [26, 27].

2.4 DNA extraction and Gel electrophoresis

DNA extraction and gel electrophoresis was carried out for measuring the extent of necrosis of myocardium [28]. The concentration of DNA was determined spectrophotometrically at 260nm. Protein contamination of DNA was accessed by determining the ratio of absorbance at 260nm and 280nm which should not be more than 1.75. To detect the internucleosomal cleavage, 10-12 µg of extracted DNA was added to equal volume of loading dye and it was loaded in the well. Electrophoresis was carried out using 1.8% agarose gel in 1X TBE buffer for 1.5 hrs. at 400mA and 3W in submarine electrophoresis unit (Pharmacia Biotech, Freibury, Germany). Ethidium bromide was added to gel for DNA detection.

2.5 TUNEL Staining

TUNEL positive cardiomyocytes were counted and apoptotic index was calculated using the formula(1) [29].

Apoptotic Index = (Number of TUNEL positive cell nuclei / Number of total cell) x 100 (1)

2.6 Estimation of LDH

LDH was estimated in coronary effluent [10] by 2, 4-DNPH method [30] spectrophotometrically at 440nm.Optical density of Test ($O.D._T$) and control ($O.D._C$) was measured (2) against distilled water.

Net optical density of test ($O.D._{Tn}$) = $O.D._T - O.D._C$ (2)

Enzyme activity was calculated from standard plot by making $O.D._{Tn}$on Y-axis and extrapolating it to corresponding enzyme activity on X-axis (Fig. 1).

2.7 Estimation of CK

CK was estimated in coronary effluent and optical density of test ($O.D._T$), standard ($O.D._S$) and blank ($O.D._B$) was measured spectrophotometrically (3) against distilled water at 520nm [31].

$CK = (O.D._T - O.D._B / O.D._S - O.D._B) \times (10^3 \textbf{ x } \text{Creatinine taken } (\mu M))/\text{Incubation time } \textbf{x} \text{ volume of coronary effluent}).$ (3)

III. Results and Discussion

The release of LDH is a biochemical index of myocardial injury. Maximum increase in the release of LDH is noted either immediately or 30 min after reperfusion. It may be tentatively suggested that noted release of LDH immediately after reperfusion may be due to sustained ischaemia and late spurt in the release of LDH noted after 30 min of reperfusion may be as a result of reperfusion. Release of CK during reperfusion is used as a measure of lethal cardiomyocyte injury.

Apoptosis generate high molecular weight fragments. TUNEL staining uses the divalent cations Ca^{2+} and enzyme terminal deoxynucleotidyl transferase (Tdt) to add fluoresceinisothiocyanate (FITC) labeled deoxyuridinetriphosphate (dUTP) to 3'OH ends of DNA which are detected by fluorescent microscope. TUNEL assay labels apoptosis in tissues sections on a single cell level making it much more sensitive than agarose gel electrophoresis. TUNEL technique has been employed in the present study to determine the apoptotic cell death.Fig.3indicates that global ischaemia for 30 min followed by reperfusion for 120 min has significantly increased apoptotic index estimated by TUNEL staining. Calpain is specifically inhibited by SJA 7019 and SJA 7029 .The SJA 7019 and SJA 7029 inhibit extracellular influx of Ca^{2+} leading to down regulation of calpain activity and responsible for the noted decrease in apoptotic index (Fig.4). The mechanism underlying the reperfusion induced necrotic cell death involves onset of mitochondrial permeability transition (MPT). Opening of permeability transition pores in mitochondria causes release of cytochrome c along with depletion of ATP and ultimately leads to necrotic (energetic) cell death. Thus the present study showed that the global ischaemia for 30 min followed by reperfusion for 120 min has significantly increased necrotic cell death measured in terms of infarct size and release of LDH and CK. The specific inhibition of calpain activity by SJA7019 and SJA 7029 may be responsible for the noted decrease in release of LDH (Fig.5), CK (Fig.6) and infarct size (Fig.7).

Myocardium subjected to ischaemia and reperfusion has often shown gel electrophoresis pattern that do

not demonstrate clear cut or pure DNA laddering and appear to represent mixtures of DNA smearing. Moreover the mode of cell death shifts from apoptotic to necrotic because of use of ATP during apoptosis and consequent depletion of ATP stores. Hence global ischaemia and reperfusion showed atypical pattern of DNA fragmentation (Fig.8; L-2) which likely represent a smear formation. Therefore it is possible to suggest that DNA smearing noted in present study may reflect apoptosis induced necrotic cell death .The specific inhibition of calpain activity by SJA7019 and SJA 7029 which ultimately block apoptosis induced necrotic cell death (Fig.8; L-3, L-5; L-4, L-6) may be responsible for the noted decrease in DNA smear formation. The compound SJA 7019 is more effective and selective than compound SJA 7029 to inhibit calpains because of the presence of methoxy group in phenyl ring of compound SJA 7019. Therefore compound SJA 7019 has been demonstrated to be more effective than compound SJA 7029 to attenuate ischaemia reperfusion induced increase in apoptotic index, DNA smearing and myocardial injury.

IV. Conclusion

On the basis of results obtained in the present study, it may be concluded that isolated rat heart subjected to ischaemia of 30 min followed by reperfusion for 120 min produced significant increase in myocardial injury measured in terms of infarct size and release of LDH and CK. It produced marked increase in apoptotic index and DNA smearing. It suggests that ischaemia and reperfusion has produced apoptotic and apoptosis induced necrotic cell death in isolated rat heart.The nonpeptide (SJA-7019 and SJA-7029) inhibitors of calpain significantly decreased ischaemia-reperfusion-induced apoptotic index. It indicates that nonpeptide inhibitors of calpain have attenuated ischaemia and reperfusion induced apoptotic cell death. The nonpeptide (SJA-7019 and SJA-7029) calpain inhibitors have prevented ischaemia-reperfusion-induced increase in ventricular DNA smearing, which has occurred as a result of apoptotic-induced-necrotic cell death and significantly attenuated ischaemia-reperfusion-induced increase in myocardial infarct size and release of LDH and CK. These results suggests that ischaemia and reperfusion induced necrotic injury has been prevented by calpain inhibitors. The compound SJA-7019 is more effective than compound SJA-7029 to attenuate ischaemia-reperfusion-induced increase in apoptotic index, DNA smearing and myocardial injury. It may be due to presence of methoxy group in the phenyl ring of compound SJA-7019. On the basis of above findings the following mechanism has been proposed for the attenuation of ischaemia-reperfusion induced cardiac cell death with the use of calpain inhibitors (Fig. 9).

Acknowledgement

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Figures

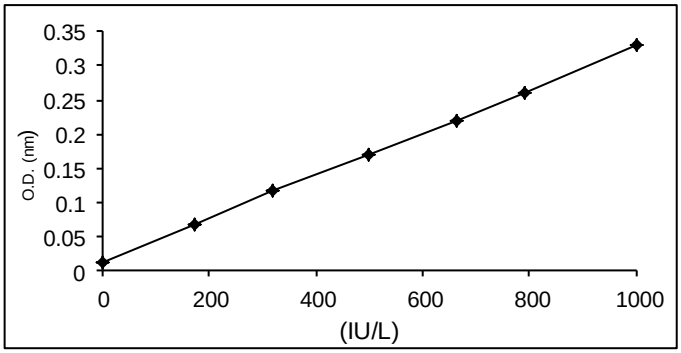

Figure 1: Standard Plot of Lactate Dehydrogenase (LDH) Activity, $r^2 = 0.998$

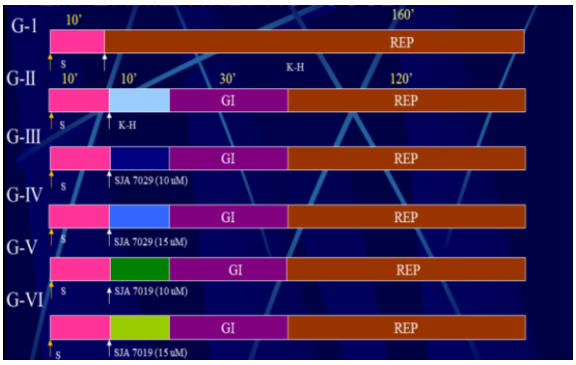

Figure 2: Diagrammatic representation of experimental protocol (S-stabilization; K-H perfusion with K-H solution; GI- global ischaemia; REP- reperfusion with K-H solution)

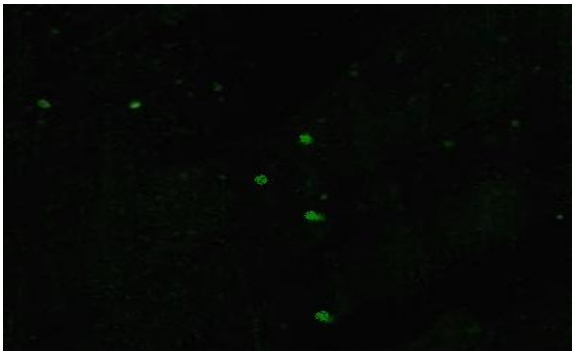

Figure 3: TUNEL +ve nuclei (control)

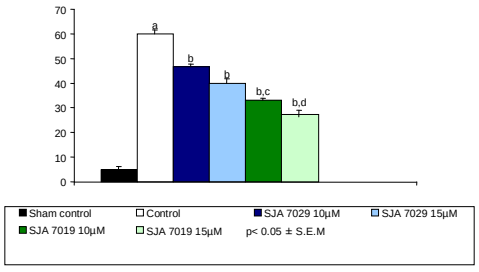

Figure 4: Effect of Calpain on inhibitors apoptotic index

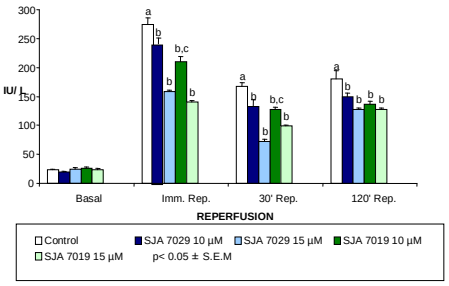

Figure 5: Effect of Calpain inhibitors on LDH release

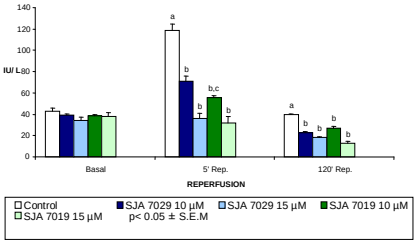

Figure 6: Effect of Calpain inhibitors on CK release

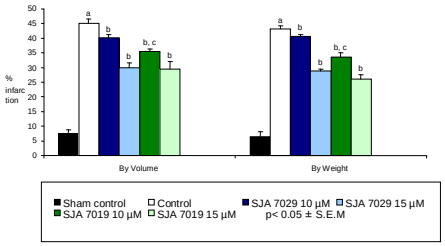

Figure7: Effect of Calpain inhibitors on infarct size

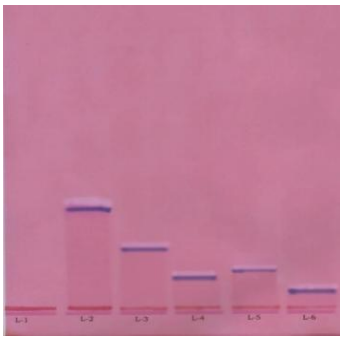

Figure 8: Electrophoretic pattern of DNA

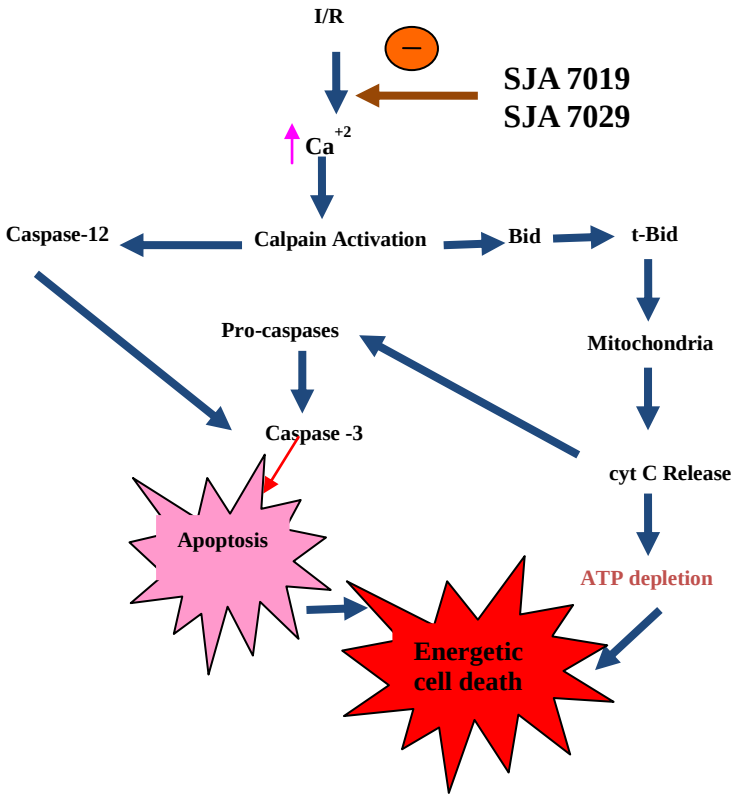

Figure 9: Proposed mechanism of calpain inhibitors to attenuate ischaemia-reperfusion-induced myocardial cell death