Protective effects of sinapic acid on lysosomal dysfunction in isoproterenol induced myocardial infarcted rats

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1. Introduction

Myocardial infarction (MI) is the condition of cellular injury that results in damage and ultimately necrosis of the myocardium, which occurs as a result of imbalance between coronary blood supply and myocardial demand. The animal model of MI plays an important role in the prevention, diagnosis and therapy of human MI (Wang et al., 2006). Experimental induction of MI by isoproterenol (ISO) in animals is a well established model to study the protective role of different cardioprotective agents (Stanley Mainzen Prince et al., 2009). Pharmacological induction of MI by subcutaneous administration of ISO in animals like rats has been found to be convenient because of relatively smaller size of coronary arteries (Rona et al., 1959). The advantages of ISO induced MI that occur as a result of intense inotropic and chronotropic actions of ISO compared to physical occlusion of the coronary artery as a less invasion accomplished without the complicating factors of general anesthesia and lack of foreign body remaining in the heart. Reperfusion is possible after ISO, since there is no permanent overt. Occlusion and the survival rates with ISO are consistent and reproducible after vessel occlusion (Saeed and Ahmed, 2006).

ISO, a synthetic catecholamine, in large dose, causes severe stress in the myocardium, resulting in infarct like necrosis of the heart muscle (Sushamakumari et al., 1989). The generation of highly cytotoxic free radicals through the auto-oxidation of catecholamines and a disturbance in the physiological balance between production of free radicals and an anti-oxidant defense system (Srivastava et al., 2007), have been implicated as the important factors in the loss of integrity and function of myocardial membranes during ISO induced MI (Mari Kannan and Darlin Quine, 2011). Another study demonstrated that ISO-induced oxidative stress results in hyperstimulation of beta-adrenoceptor, which ultimately leads to cardiotoxicity (Singal et al., 1982). The pathophysiological changes that take place in rat’s heart following MI induced by ISO are comparable to those changes taking place in MI in human beings (Geng et al., 2004).

Lysosomal enzymes play an important role in the inflammatory process. ISO induced MI results in increased lysosomal hydrolases activity that may be responsible for tissue damage and infarcted heart (Ravichandran et al., 1990). Alterations in the activity of lysosomal enzymes have been noticed in patients with MI (Welman et al., 1978). It is possible that stabilization of myocardial cell membranes, particularly the lysosomal membranes, may prolong the viability of ischemic cardiac muscle and prevent MI (Stanely Mainzen Prince et al., 2009).

**Keywords:** Sinapic acid Isoproterenol Myocardial infarction Lysosomal enzymes Lipid peroxidation Creatine kinase-MB

**Abstract**

In the pathology of myocardial infarction, lysosomal lipid peroxidation and resulting enzyme release play an important role. We evaluated the protective effects of sinapic acid on lysosomal dysfunction in isoproterenol induced myocardial infarcted rats. Male Wistar rats were treated with sinapic acid (12 mg/kg body weight) orally daily for 10 days and isoproterenol (100 mg/kg body weight) was injected twice at an interval of 24 h (9th and 10th day). Then, lysosomal lipid peroxidation, lysosomal enzymes in serum, heart homogenate, lysosomal fraction and myocardial infarct size were measured. Isoproterenol induced myocardial infarcted rats showed a significant increase in serum creatine kinase-MB and lysosomal lipid peroxidation. The activities of β-glucuronidase, β-galactosidase, cathepsin-B and D were significantly increased in serum, heart and the activities of β-glucuronidase and cathepsin-D were significantly decreased in lysosomal fraction of myocardial infarcted rats. Pre-and-co-treatment with sinapic acid normalized all the biochemical parameters and reduced myocardial infarct size in myocardial infarcted rats. In vitro studies confirmed the free radical scavenging effects of sinapic acid. The possible mechanisms for the observed effects are attributed to sinapic acid’s free radical scavenging and membrane stabilizing properties. Thus, sinapic acid has protective effects on lysosomal dysfunction in isoproterenol induced myocardial infarcted rats.

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Dietary factors play an important role in the prevention of cardiovascular diseases. Epidemiological studies have shown that diets rich in fruits, herbs and spices are associated with a low risk of cardiovascular diseases (Banerjee and Maulik, 2002). The use of antioxidants has been recognized as an important counter measure against condition in which oxidative stress is implicated. Thus, the identification of antioxidants, which can retard the process of lipid peroxidation by blocking the generation of free radical chain reaction, has gained importance in recent years (Kotamballi et al., 2002). Among such classes of compounds, naturally occurring phenolic acids have received much attention because of their role in the prevention of many human diseases, particularly atherosclerosis and cancer due to their antioxidant properties (Mattila and Kumpulainen, 2002). Sinapic acid, a phenolic acid is a cinnamic acid derivative, which possesses 3,5-dimethoxyl and 4-hydroxyl substitutions in the phenyl group of cinnamic acid. It is widely distributed in the plant kingdom and is obtained from various sources such as rye, fruits and vegetables (Andreasen et al., 2001). Scientific studies have revealed that sinapic acid exhibits anti-inflammatory (Yun et al., 2008), peroxynitrite scavenging (Zou et al., 2002) and neuroprotective effects (Kim et al., 2010). Previous studies have shown that antioxidants such as vitamin-C and vitamin-E prevents lysosomal damage and restored the altered activities of lysosomal enzymes in ISO induced myocardial damage (Yogeeta et al., 2006a; Punithavathi and Stanely Mainzen Prince, 2010). Also in our laboratory, we have previously reported an antioxidant, gallic acid, a phenolic acid decreased lysosomal damage in ISO induced myocardial damage (Stanely Mainzen Prince et al., 2009). Hence, we thought that an antioxidant, sinapic acid, a phenolic acid could prevent lysosomal dysfunction and myocardial infarct in MI. In view of the above facts, the investigation was performed to understand whether sinapic acid can aid in preventing the lysosomal damage due to ISO induced myocardial damage. In this context, we evaluated the protective effects of sinapic acid on lysosomal lipid peroxidation and lysosomal enzymes in ISO induced myocardial infarcted rats. The myocardial infarct size was determined by 2,3,5-triphenyl tetrazolium chloride (TTC) test. In addition to this, we evaluated the possible scavenging effects of sinapic acid on superoxide anion (O2•−) and hydroxyl radicals (OH•) in vitro.

2. Materials and methods

2.1. Experimental animals and diet

The experiments were performed with healthy male albino Wistar rats weighing 170–200 g. The animals were obtained from the Central Animal House, Rajah Muthiah Institute of Health Sciences, Annamalai University, Tamil Nadu, India and were housed in polypropylene cages (47 × 34 × 20 cm) lined with husk, renewed every 24 h under a 12:12 h light/dark cycle at around 22°C. The rats had free access to food and tap water. The rats were fed on a standard pellet diet (Pranav Agro Industries Ltd., Maharashtra, India). The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India and approved by the Animal Ethical Committee of Annamalai University.

2.2. Chemicals

Sinapic acid, isoprotorenol hydrochloride, N-phenyl-p-phenylene diamine, and p-nitrophenyl-p-β-glucuronide were purchased from Sigma Chemical Co., St. Louis, MO, USA. Dimethyl sulfoxide and ethylene diamine teta acetic acid were purchased from Himedia Laboratories, Mumbai, India. All other chemicals and reagents used in the study were of analytical grade.

2.3. Induction of experimental MI and experimental design

ISO (100 mg/kg body weight) was dissolved in 2 ml of saline and subcutaneously injected into male albino Wistar rats twice at an interval of 24 h (Stanely Mainzen Prince, 2011). Sinapic acid dissolved in 2 ml of 0.5% dimethyl sulfoxide (DMSO) solvent with three different doses (3 mg/kg, 6 mg/kg and 12 mg/kg body weight) was used to determine the dose dependent effect of sinapic acid in ISO (100 mg/kg body weight) induced myocardial infarcted rats. Pre- and co-treatment with sinapic acid at the doses 3, 6, and 12 mg/kg body weight daily for a period of 10 days significantly (p < 0.05) lowered the elevated levels of serum creatine kinase-MB in ISO induced myocardial infarcted rats. The dose 12 mg/kg body weight of sinapic acid showed the highest significant effect and normalized the activity of serum CK-MB with respect to the other two doses (3 mg and 6 mg/kg body weight). Hence, we have chosen 12 mg/kg body weight of sinapic acid for our further study.

The experiment was performed with four groups of rats, each group consisting of six rats. Group I: normal untreated rats. Group II: rats were orally treated with 2 ml of sinapic acid dissolved in 0.5% DMSO using an intragastic tube (12 mg/kg body weight/day, for 10 days). Group III: rats were subcutaneously injected with 2 ml of ISO (100 mg/kg body weight) dissolved in saline twice at an interval of 24 h (on 9th and 10th day). Group IV: rats were pre- and co-treated with 2 ml of sinapic acid dissolved in 0.5% DMSO (12 mg/kg body weight/day, for 10 days) orally using an intragastic tube and injected with 2 ml of ISO (100 mg/kg body weight) dissolved in saline twice at an interval of 24 h (on 9th and 10th day). Sinapic acid was dissolved in 0.5% DMSO and administered to rat’s 2 ml orally using an intragas trig-acetate (P. Stanely Mainzen Prince, 2011). Sinapic acid dissolved in 0.5% DMSO was administered alone to normal control (Group-I) and ISO control rats (Group-III) orally using an intragastic tube daily for a period of 10 days. Twelve hours following the second dose of ISO injection, all the rats were anesthetized with pentobarbital sodium (60 mg/kg body weight) and then sacrificed by cervical decapitation. Blood was collected without anticoagulant for serum. Serum was separated by centrifugation. Heart tissues were excised immediately, rinsed in ice-chilled saline and stored at –80°C till further use for the biochemical estimations. All enzyme assays were done immediately.

2.4. Separation of heart lysosomal fraction

Heart tissue samples were cut open and placed in isotonic saline to remove the blood and then the tissues were homogenized in 0.25 M ice-cold sucrose solution at 4°C. One portion of the prepared homogenate was used to determine the total activity of lysosomal enzymes whereas the other portion of the prepared homogenate was subjected to centrifugation and lysosomal fraction was separated according to the following procedure: structural proteins, nucleus, and cell debris at 600 g for 10 min; mitochondria at 5000 g for 10 min; lysosomes at 15,000 g for 10 min. Myocardial lysosomal fraction was treated with Triton X-100 (final concentration 0.2% w/v) in ice for 15 min prior for the determination of enzyme activities (Sathish et al., 2003).

2.5. Estimation of lipid peroxidation products in lysosomal fraction of the heart

Thiobarbituric acid reactive substances (TBARS) in the heart lysosomal fraction were estimated by the method of Fraga et al. (1988).

2.6. Assays of serum cardiac marker enzyme, lysosomal enzymes in the serum, total heart, lysosomal fraction and protein content in heart tissue

Activity of serum creatine kinase-MB (CK-MB) was measured by using standard commercial kit (Product No. 11405001) according to the manufacturer’s instructions (Agappe Diagnostics Ltd., Ernakulam, Kerala, India). The activity of β-glucuronidase in the serum, total heart tissue homogenate and lysosomal fraction was assayed by the method of Kawai and Anno (1971) and the activity of β-galactosidase in the serum and total heart tissue homogenate was assayed by the method of Conchie et al. (1967). Also, the activity of cathepsin B in the serum and total heart tissue homogenate was assayed by the method of Barrett (1972). Cathepsin-D activity in the serum, total heart tissue homogenate and lysosomal fraction was assayed by the method of Sapolsky et al. (1973). Protein content in the heart tissue homogenate was determined by Lowry et al. (1951).

2.7. Determination of myocardial infarct size

The infarcted myocardium was determined by the TTC test according to the method of Lie et al. (1975). A freshly prepared 1% TTC solution in phosphate buffer solution was prewarmed at 37–40°C for 30 min in a darkened glass. The excess blood was removed from the heart tissues by washing rapidly in cold water. The heart was transversely cut across the left ventricles to obtain slices not more than 0.1–0.2 mm in thickness. The slices were then kept in a covered darkened glass dish containing prewarmed TTC solution and the dish was kept in an incubator and heated to 37–40°C for 45 min. At the end of the incubation period, the heart slices were kept in fixing solution to fix the tissue. Colour photographs of the heart tissue slices were obtained by a camera with macro lenses.

2.8. In vitro studies

The free radical scavenging effects of sinapic acid on O2•− and OH• in vitro were determined by the standard procedures of Halliwell et al. (1987) and Nishimiki et al. (1972) respectively.
2.9. Statistical analysis

Statistical analysis was performed by One-way Analysis of Variance (ANOVA) followed by Duncan’s Multiple Range Test (DMRT) using Statistical Package for the Social Science (SPSS) software package version 12.00. Results were expressed as mean ± S.D. for six rats in each group. P values <0.05 were considered significant.

3. Results

A pilot study with 6 rats in each group was performed to understand the dose dependent effect of sinapic acid with three different doses, (3, 6 and 12 mg/kg body weight) in ISO (100 mg/kg body weight) induced myocardial infarcted rats. The activity of cardiac marker enzyme, serum CK-MB in rats pretreated with sinapic acid and injected with ISO was examined. We observed normalized activity of serum CK-MB compared to normal control rats on the 10th day of pre-and co-treatment with sinapic acid. The effect exerted by 12 mg/kg body weight of sinapic acid showed the highest significant effect and normalized the activity of serum CK-MB (Fig. 1). Hence, we have chosen 12 mg/kg body weight of sinapic acid for our further study.

Fig. 2 shows the levels of TBARS in heart lysosomal fraction in normal and experimental rats. ISO induced myocardial infarcted rats revealed a significant ($P < 0.05$) increase in the levels of TBARS in the lysosomal fraction of the heart compared to normal control rats. Pre-and co-treatment with sinapic acid (12 mg/kg body weight) daily for a period of 10 days normalized ($P < 0.05$) the levels of lipid peroxidation products in the lysosomal fraction of the heart compared to ISO alone induced myocardial infarcted rats (Fig. 2).

ISO induced myocardial infarcted rats showed a significant ($P < 0.05$) increase in the activities of $\beta$-glucuronidase, $\beta$-galactosidase, cathepsin-B and D in the serum compared to normal control rats. Pre-and co-treatment with sinapic acid (12 mg/kg body weight) normalized ($P < 0.05$) the activities of these enzymes in the serum of ISO induced myocardial infarcted rats compared to ISO alone induced myocardial infarcted rats (Fig. 3).

ISO induced myocardial infarcted rats showed a significant ($P < 0.05$) increase in the activities of $\beta$-glucuronidase, $\beta$-galactosidase, cathepsin-B and D in the total heart homogenate compared to normal control rats. Pre- and co-treatment with sinapic acid (12 mg/kg body weight) normalized ($P < 0.05$) the activities of these enzymes in the total heart homogenate of ISO induced myocardial infarcted rats compared to ISO alone induced myocardial infarcted rats (Fig. 4).

Fig. 5 reveals the activities of $\beta$-glucuronidase and cathepsin-D in the heart lysosomal fraction of normal and experimental rats. ISO induced myocardial infarcted rats showed a significant
(P < 0.05) decrease in the activities of β-glucuronidase and cathepsin-D in the heart lysosomal fraction compared to normal control rats. Pre-and co-treatment with sinapic acid (12 mg/kg body weight) normalized (P < 0.05) the activities of β-glucuronidase and cathepsin-D in the heart lysosomal fraction of ISO induced myocardial infarcted rats compared to ISO alone induced myocardial infarcted rats.

The myocardial infarct size was measured by TTC test. Fig. 6A–D reveals the images of heart slices of rats after TTC staining. Fig. 6A is the TTC-stained heart slice of normal rat showing completely viable tissue without any infarction. Fig. 6B is the TTC-stained heart slice of sinapic acid (12 mg/kg body weight) treated group rats showing completely viable tissue without any infarction. Fig. 6C is the TTC-stained heart slice of ISO (100 mg/kg body weight) induced myocardial infarcted rat showing infarcted region. Fig. 6D is the TTC-stained heart slice of rat pre-co-treated with sinapic acid (12 mg/kg body weight) and induced with ISO (100 mg/kg body weight) revealing much reduced infarct size compared to C.

Fig. 7 reveals the percentage in vitro free radical scavenging effects of sinapic acid on O− and OH·. As shown in Fig. 7, sinapic acid scavenged the O− and OH· in vitro in a concentration-dependent manner (15, 30, 45 and 60 μM). The percentage scavenging effect of sinapic acid increased with increasing concentration. At 60 μM concentration, sinapic acid showed higher percentage O− scavenging effect (86.4%) than OH· scavenging effect (66%).
Treatment with sinapic acid (12 mg/kg body weight) alone to normal rats (Group-II) did not show any significant effect in all the biochemical parameters and myocardial infarct size examined.

4. Discussion

In this study, we observed that pre-and co-treatment with sinapic acid (12 mg/kg body weight) maintained the membrane integrity, restored the levels of lysosomal lipid peroxidation and the activities of lysosomal enzymes in ISO induced myocardial infarcted rats, by virtue of its free radical scavenging and membrane stabilizing effects. A control group with DMSO alone (Group-I) was used as a comparator and that sinapic acid performed better than DMSO in the experimental trials. We observed an increase in the activity of cardiac marker enzyme, CK-MB in the serum of myocardial infarcted rats. The observed increase in serum CK-MB is due to the damage caused to the sarcolemma by the ISO, which has rendered it leaky (Devika and Stanely Mainzen Prince, 2008). Sinapic acid pre-and co-treatment normalized the activity of CK-MB, which may explain its ability to protect the myocardium from damage by preventing the leakage of CK-MB from the heart.

ISO metabolism produces quinines, which react with oxygen to produce superoxide anion and hydroxyl peroxide, leading to oxidative stress thereby damaging the myocardial cells. The excessive formation of free radicals as well as accumulation of lipid peroxides has been recognized as one of the possible mechanisms for the myocardial damage caused by ISO (Sathish et al., 2003). Lipid peroxidation is one of the main manifestations of oxidative damage initiated by reactive oxygen species (ROS) and has been linked to the altered membrane structure and enzyme inactivation (Yogeea et al., 2006b). Increased levels of lysosomal lipid peroxidation is indicated by increased levels of lysosomal TBARS, which might be due to increased production of free radicals in ISO induced MI. Increased free radicals react with the lipid bilayer of intracellular organelles including lysosomes and destabilizes lysosomal membranes resulting in the rupture of lysosomes. Also, exposure of mammalian cells to oxidant stress causes early lysosomal rupture followed by apoptosis or necrosis of the cell (Zhao et al., 2003). Sinapic acid pre-and co-treatment normalized the levels of lipid peroxidation products, thereby inhibiting oxidative stress and stabilized lysosomal membrane in ISO induced rats. This effect revealed the antilipid peroxidation and membrane stabilizing properties of sinapic acid. In this context, gallic acid, a phenolic acid also prevents lipid peroxidation by virtue of its antilipid peroxidation and membrane stabilizing properties in ISO induced myocardial infarcted rats (Stanely Mainzen Prince et al., 2009).

Lysosomal membrane plays an important role in various inflammatory conditions (Ignarro, 1974) and the regulation of lysosomal enzyme secretion in pathophysiology (Pillay et al., 2002). An increase in the activities of lysosomal enzymes such as β-glucuronidase, β-galactosidase, cathepsin-B and D were observed in the serum and total heart of ISO induced myocardial infarcted rats. Increased lipid peroxidation observed in ISO induced myocardial infarcted rats was the reason for the leakage of myocardial lysosomal enzymes from the lysosomes due to lysosomal membrane damage. Pre-and co-treatment with sinapic acid normalized the activities of lysosomal enzymes both in the serum and myocardium by its inhibitory effect on lipid peroxidation, thereby preventing lysosomal damage induced by ISO in myocardial infarcted rats. Gallic acid also decreased the activities of lysosomal enzymes both in the serum and myocardium by its inhibitory effect on lipid peroxidation in ISO induced myocardial infarcted rats (Stanely Mainzen Prince et al., 2009).

Myocardial infarct can be detected by using TTC dye test, which forms a red formazan precipitate with lactate dehydrogenase (LDH) of the viable myocardial tissue, but the infarcted myocardium fails to stain. ISO induced myocardial infarcted rat's heart revealed larger myocardial infarct size with less dye absorbing capacity, which reveals considerable leakage of LDH enzyme. Pre-and co-treatment with sinapic acid much reduced the infarct size demonstrating a mild leakage of LDH. Thus, sinapic acid much reduced the infarct size and protected the heart from ISO induced MI.

ISO metabolism produces quinines, which react with oxygen to produce ROS such as O$_2^-$ and hydrogen peroxides, thereby damaging the myocardial cells. Hence, in vitro free radical scavenging effects of (−) epicatechin on O$_2^-$ and OH$^-$ was evaluated. O$_2^-$ directly initiate lipid peroxidation (Wickens, 2001). It is a precursor to active free radicals that have the potential to react with biological macromolecules thereby inducing tissue damage. OH$^-$ is chiefly responsible for lipid peroxidation, which impairs the normal function of cell membranes. In this study, sinapic acid in vitro exhibits 86.4% of O$_2^-$ and 66% of OH$^-$ scavenging activities at the concentration of 60 μM. The free radical scavenging activity of sinapic acid appears to reduce myocardial infarct size, inhibit lipid peroxidation levels, maintain lysosomal enzymes activity, thereby stabilizing lysosomal membranes and protect lysosomes, resulting in the normalization of lysosomal lipid peroxidation, lysosomal enzymes and prevented lysosomal dysfunction in ISO induced myocardial infarcted rats.

In our earlier study, we used 15 mg/kg body weight of gallic acid and decreased the extent of lysosomal damage in ISO (100 mg/kg body weight) induced myocardial infarcted rats (Stanely Mainzen Prince et al., 2009). But in this study, we used only 12 mg/kg body weight of sinapic acid and observed better effects and restored the lysosomal damage when compared to the effects of gallic acid in ISO (100 mg/kg body weight) induced myocardial infarcted rats. Thus, the protective effects of sinapic acid on lysosomal dysfunction in ISO (100 mg/kg body weight) induced myocardial infarcted rats were better than the effects of gallic acid.
It has been reported that the content of sinapic acid is found to be 70–200 mg/kg in whole grain of rye and wheat (Mattila et al., 2005). An intake of 20 g of wheat bran would provide about 2 mg of sinapic acid (Kern et al., 2003). In a human study, the concentration of sinapic acid in plasma was about 40 nM after consumption of high-bran cereals (about 20 mg of sinapic acid) (Kern et al., 2003). According to our findings, a 70 kg person requires 840 mg of sinapic acid daily. As reported earlier (Mattila et al., 2005; Kern et al., 2003), 840 mg of sinapic acid can be obtained from 10 kg of whole grain of rye and wheat or 120 g of wheat bran. Daily intake of 10 kg of whole grain of rye and wheat is high. But one can take 120 g of wheat bran daily. Therefore, further studies are needed to find out the exact dosage of sinapic acid in human MI.

5. Conclusion

Our study revealed that sinapic acid (12 mg/kg body weight) could prevent the lysosomes and prevented lysosomal dysfunction in ISO induced myocardial infarcted rats. Administration of sinapic acid (10 mg/kg body weight) to normal control rats (Group-II) had no effects on the measured biochemical parameters and myocardial infarct size. This study may be beneficial to prevent the occurrence of MI.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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References


