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Exploring The Electrophysiological Consequences Of Reactive Oxygen Species On Rat Papillary Muscle: Insights Into Calcium Channels And High-Energy Phosphates

Manju Lekshmy[1], Renuka Nair [2], Vishnu Sasidharan Lathakumari[3], Jisha Sarassamma[4] Rejiya Chellappan Sobitham[5]*,

[1]St.Xavier's College, Thiruvananthapuram – 695 586, India, E-mail – drlmanjuakathalam@gmail.com
[2]Division of Cellular and Molecular Cardiology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram – 695 011, India, E-mail – renukanairsctimst@gmail.com
[3]Sree Narayana College for Women, Kollam -691001, India, E-mail – vishnusncw@gmail.com
[4]Bishop Moore College, Mavelikara-690110, Kerala, India,Email- sjishabmc@gmail.com
*[5]*Sree Ayyappa College, Eramallikkara, Chengannoor, Alapuzha-689109, Kerala, India, Email-rejiyas@gmail.com*

***Corresponding author:** Rejiya Chellappan Sobitham*
**Sree Ayyappa College, Eramallikkara, Chengannoor, Alapuzha-689109, Kerala, India, Email-rejiyas@gmail.com*

Reviewers

1. ***Dr. Madanan M G***
Scientist G Division of Biochemistry Regional Medical Research Centre, (ICM R) Post Bag # 13 Port Blair - 744 101 A&N Islands Mob: +919434284576Tel: +91 3192 251158 Email: madanan.mg@icmr.gov.in
2. ***Dr. Mini S***
Professor Department of Biochemistry University of Kerala Kariavattom ,Thiruvananthapuram 695 581, India Mob: +91 9495276631 E-mail – minisaraswathy@gmail.com
3. ***Rajesh K Harijan***
Albert Einstein College of Medicine 1300 Morris Park Ave. Bronx, NY 10461 Tel: 718.430.2777 Fax;718.430.8565 E-mail – rajesh.harijan@einsteinmed.org

***Abstract*:**

Purpose: Oxidative stress can affect myocardial contractility. The mechanism mediating the functional change at pathophysiological concentrations of reactive oxygen species (ROS) is not well defined. Two major factors influencing myocardial contractility are – calcium (Ca) transients and availability of high-energy phosphates. Inotropic response to physiologically relevant concentrations of ROS and the role of Ca channels and availability of energy metabolites in the mediation of ROS induced inotropic changes were studied in rat heart preparations.

Procedures: Reactive oxygen species were enzymatically generated using hypoxanthine (0.5mM) and xanthine oxidase (0.02 U/ml). Changes in isometric contraction in response to ROS and the role of ion channels were studied in left ventricular papillary muscle. Effect of oxidative stress on tissue injury and energy metabolites were assessed in Langendorff perfused heart.

Findings: A negative inotropic response was seen on exposure to the free radical generator. Superoxide anions appear to modify voltage gated Ca channels and sarcoplasmic reticular Ca uptake and release. Creatine phosphate and ATP levels were significantly reduced.
Conclusion: The negative inotropic response to reactive oxygen species can be the co-ordinated response to decrease in Ca transients associated with a decrease in the energy available for contraction.

Keywords: Reactive oxygen species, myocardial contractility, papillary muscle, energy metabolites, Ca channels

Introduction

Oxidative stress may cause numerous cardiac disorders. Studies show that oxygen-derived free radicals and oxidants may induce myocardial reperfusion damage [1]. Excessive myocardial ROS production can lead to ischemia reperfusion-related cardiomyopathy and abnormal Ca^{2+} homeostasis and ventricular arrhythmias [2,3]. Reactive oxygen species (ROS) generation in the endoplasmic reticulum (ER) is triggered by changes in the oxidative environment of ER and intra-ER Ca^{2+} levels [4]. Oxidative damage to myosin can cause intrinsic dysfunction in coronary heart failure [5]. Posttranslational modifications of myofilament proteins induced by ROS, myofilament protein phosphorylation and myofilament protein cleavage has been analysed by Steinberg et al [6]. Hydrogen peroxide was also found to reduce Na^+ channel availability in cultured atrial (HL-1) myocytes[7]. Inhibition of glycolytic and oxidative metabolism by H_2O_2 in patch-clamped guinea pig ventricular myocytes, causing cell shortening and limiting functional recovery from an ischemic insult were also studied [8,9]. We have also observed that complete functional recovery was not attained following treatment with varying concentrations of H_2O_2, implicating irreversible damage to the cardiac tissue[10]. Most of the earlier studies were carried out using high levels of free radical generators. The purpose of present study was to investigate, the mechanical response of the myocardium to pathophysiologically relevant concentrations of enzymatically generated reactive oxygen species (ROS) and to investigate, the variables that can possibly influence cardiac mechanics, such as the levels of high-energy phosphates and ion channels that modulate Ca transients.

Methodology

Isolated hearts from 200 to 250 g Sprague–Dawley rats were used in the experiments. The Institutional Animal Ethics Committee gave their stamp of approval to the project (SCTIMST No.98/1999/CPCSEA of 28/4/99).Variation in left ventricular papillary muscle isometric tension measured contractile response to oxidant stress. Using channel-specific antagonists the role of channels and pumps play significant role in inducing inotropic changes were measured. Langendorff perfused rat heart preparations were used to measure biochemical variables that may influence contractile variation. These included high-energy phosphate compounds and markers of tissue injury.

Isolation of left ventricular papillary muscle and measurement of myocardial contraction: Oxygenated Krebs Ringer Henseleit (KRH) and HEPES-3 was used to separate papillary muscles. Using a force transducer and two platinum electrodes electrical pulses of 0.5 hertz at 5 milliseconds duration were created and the intensity of the contraction was measured using a physiograph. The viability of muscle preparations was 6 hours[11].

Measurement of inotropic response of papillary muscle to superoxide anions:
The inotropic response of papillary muscle was studied using pathophysiologically relevant oxygen radical generator concentrations. Hypoxanthine (HX) and xanthine oxidase (XO) generate free radicals in vivo and in vitro. Three different concentrations of the superoxide anion generators used were: 0.5mM HX + 0.01 U/ml XO; 0.5mM HX + 0.02 U/ml XO and 1mM HX + 0.04 U/ml XO. It has been found that 0.02 U/ml of xanthine oxidase generate as much superoxide anion as activated neutrophils in vivo [12].
After 1h stabilization in modified KRH buffer, the baseline contraction was observed. Superoxide anion generators were introduced, and steady-state contraction was measured which was obtained in 15-20 min. The

treatment response was the percentage change in developed force from baseline. This method eliminated disparities in initial contractility for each preparation.

Role of ion channels influencing mechanical variation due to superoxide anions:
The contraction force of the myocardium is related to the Ca^{2+} available for contraction. Ca^{2+} flow through the sarcolemma (SL) and sarcoplasmic reticulum (SR) is necessary for this to occur. Primary Ca^{2+} influx channels are the voltage gated sarcolemmal L-type and T-type Ca^{2+} channels. The sarcoplasmic reticulum Ca^{2+} channel and Ca^{2+} pump are the intracellular regulators of Ca^{2+}. To examine the influence of a particular channel, it was inhibited using the channel specific antagonist and the inotropic response to ROS was determined. Participation of channel in the modulation of contractile variation is suggested by the attenuation of responsiveness to ROS in the presence of the antagonist. Sarcolemmal (SL) L-type Ca channel was inhibited using 1 μM verapamil [13] and the T-type channel with 40 μM $NiCl_2$ [14]. The sarcoplasmic reticular (SR) Ca pump was inhibited with 10mM caffeine[15] and the Ca release channel with 1 μM ryanodine[16]. The inotropic response to HX + XO was then recorded as the change in force of contraction compared to the baseline obtained after inhibiting the channel.

Isolated heart preparation for measurement of tissue metabolites:
Heart was perfused with oxygenated KRH buffer through the aorta at 37^0C. Hearts were perfused for 30 minutes with KRH buffer and then with 0.5mM HX + 0.02 U/ml XO. Lactate dehydrogenase (LDH) was determined in coronary effluent. Left ventricular myocardium was freeze-clamped in liquid nitrogen and kept at –80 37^0C until analysis.

Lactate dehydrogenase assay: LDH was measured in coronary effluent immediately after collection by measuring the rate of fall in NADH absorbance at 340 nm when lactate is generated from pyruvate under non-limiting substrate conditions [17].

Measurement of tissue lipidperoxidation: Lipid peroxidation was quantified with malonedialdehyde (MDA) assay, and thiobarbituric acid reactive substance was measured[18].

Assay of adenosine tri phosphate: Utilizing commercially available kits, adenosine triphosphate (ATP) was measured enzymatically (Sigma Chemical Co)

Assay of creatine phosphate:creatine phosphate was assayed enzymatically by method of Heinz and Weiber[19].

Assay of protein: Protein content was assayed by Lowry's method[20].

Statistical analysis: Each sample has at least six independent analyses performed on it. All data are shown as mean ± SE. Student's t test was used to determine statistical significance between the treatment and control groups, and a p value of 0.05 was considered statistically significant.

RESULTS

Inotropic response of papillary muscle to superoxide anions:
Papillary muscle exposed to superoxide ion generators exhibited a negative inotropy. There was a concentration-dependent reduction in force of contraction when XO concentration increased from 0.01 U/ml to 0.04 U/ml. (Figure 1). Pre-treatment of the papillary muscle with the free radical scavenger superoxide dismutase attenuated the contractile change induced by HX + XO, thereby confirming that the inotropic variation was induced by superoxide anions (Figure 2). Inclusion of catalase along with SOD did not have an additive effect. The negative inotropic response produced by 0.01 and 0.02 U/ml XO was completely reversed in fresh medium. Incomplete recovery was seen in fresh medium when exposed to a higher concentration of XO (0.04 U/ml), and pre-treatment with SOD also afforded partial protection. (Figure 2).

Role of ion channels and pumps in mediating the inotropic changes:
Contractile force is mostly regulated by calcium flux through the SL and SR. Channels and pumps that modulate Ca transients were therefore studied. Attenuation of the inotropic response to HX+XO consequent to inhibition of a channel suggests the involvement of the channel in the free radical induced contractile

variation. Following inhibition of the SL L-type Ca channel, the negative inotropic response to HX+XO was significantly lower (10.9%) compared to that in the absence of the antagonist (17.3%) Inhibitors of the sarcoplasmic reticular Ca channel and Ca pump attenuated the negative inotropic effect, suggesting their involvement in contractile variation (Table 1).

Alteration in myocardial tissue metabolites in the presence of reactive oxygen species: The biochemical indicators of tissue injury and levels of high energy phosphates that can possibly influence cardiac mechanics were examined in Langendorff perfused heart preparations. Tissue lipid peroxidation and release of lactic dehydrogenase into the coronary effluent were used as markers of tissue injury. MDA levels, quantified as thiobarbituric acid reactive compounds, were used to evaluate the degree of lipid peroxidation. The level of MDA in the myocardial tissue increased by 12% on perfusion with HX (0.5mM) + XO (0.02 U/ml). Release of LDH increased by 45% in the presence of the free radical generators (Table 2).
High-energy phosphate compounds are the source of energy for myocardial contraction. Hence the levels of ATP and CP were assayed for delineating the possible involvement of these compounds in the induction of contractile variation. Adenosine tri phosphate was assayed in the left ventricular tissue of hearts perfused with HX+XO. Myocardial ATP content decreased by 27% on exposure to the free radical generator (Table 2). Creatine phosphate is the storage form of energy, which is mobilized for the generation of ATP. The reduction of CP (45%) in the presence of superoxide anion generator was highly significant (Table 2).

DISCUSSION

Different forms of cardiac damage have been linked to oxygen-derived free radicals and their metabolites. Reports on mechanical response of the myocardium to ROS at pathophysiological concentrations are relatively few. When papillary muscles were exposed to enzymatic superoxide producers, a concentration-dependent negative inotropic response was found. The contractile changes were negated in the presence of the scavenging enzyme, SOD. Neutralization of the inotropic changes by SOD confirms that the effects are due to oxygen radicals. Blaustein et al, showed that a combination of purine and xanthine oxidase reduced active tension in rat papillary muscle by 38% without affecting rest tension[21]. Even in our investigation, we found that at higher levels of the free radical generator (0.04 U/ml XO), the decrease in contraction was about 32%. Recovery of contraction to baseline values on wash out of the free radical generators shows that the contractile variation is reversible at lower concentrations. Earlier studies using 100µM H_2O_2 showed that the force of contraction decreased by about 32% and the recovery on wash out was only partial, indicating irreversible injury[10]. This shows that at low levels of oxidative stress, the tissue changes are reversible leading to total recovery of contractile function.
Since ion transients often mediate mechanical changes, using competitive channel blockers, the functional contribution of different channels was evaluated. When the SL L-type Ca channel and the SR Ca uptake and release channels were inhibited, the negative inotropic response generated by HX+XO was greatly attenuated, suggesting the modulation of these channels by ROS. According to research conducted on the hearts of rats that were exposed to xanthine and xanthine oxidase, Kaneko et al suggested that Oxygen free radicals may lower the voltage-dependent Ca^{2+} influx in cardiac cells by lowering the number of Ca^{2+}channels in the cell membrane[22, 23]. Whole cell voltage clamping revealed a decrease of the peak of the L-type Ca^{2+} current with 0.04 U/ml XO [24]. H_2O_2 significantly reduced L-type calcium current in single guinea pig ventricular myocytes[9]. Ca^{2+}absorption and release in the sarcoplasmic reticulum is regulated by endogenous chemicals like Mg^{2+} and is a complex interplay of multiple regulatory systems that are being unraveled. [25]. Arrhythmia due to ROS is caused by the activation of Ca^{2+}/CaM-dependent kinase II, c-Src tyrosine kinase, protein kinase C, and the aberrant splicing of cardiac sodium channels[26]. Structural damage within the microtubule lattice of oxidized tubulin as well as myocyte microtubule network remodeling and cellular contractile failure were reported by Rebecca et al.[27]. Role of oxidative stress induced Ca^{2+}handling defects as well as protease activation in progression of heart failure has also been reported[28]. Evidence of oxidative damage to DNA and lipid peroxidation in the myocardium within the first few hours following a cardiac arrest, as well as ROS-based therapeutics in the failing heart and mitochondria-targeted antioxidant therapies, are also available.[29,30].
Oxidativestress causes mitochondrial damage and reduces high-energy phosphates. Elevated LDH release is a common biomarker of tissue injury because it indicates SL damage. The treatment considerably enhanced the release of LDH into the coronary effluent. Sarcolemmal damage as indicated by increased LDH release can be the consequence of membrane lipid peroxidation. When oxygen radicals combine with polyunsaturated fatty acids, lipid peroxides and hydro peroxides are produced. In rat heart and cultured heart cells, free radical

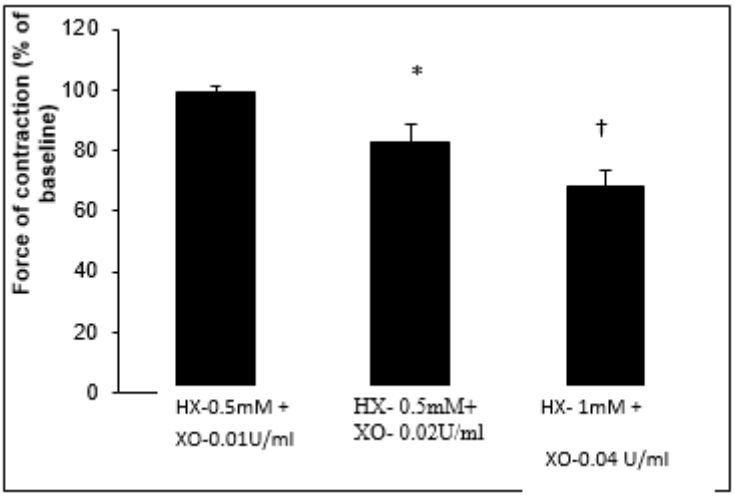
induced increased TBARS release and LDH leakage has been reported[31].In the present study significant decrease in ATP and CP was seen in hearts perfused with HX+XO. Cain and Davis provided the clearest evidence yet that ATP is directly involved in the process of contraction [32].ATP-dependent and independent mechanisms of cardiac contraction regulated by mitochondria has also been reported [33].

CONCLUSION

The findings point to the fact that at pathophysiological levels, the force of isometric contraction can be reduced by reactive oxygen species. The oxidative stressinduced negative inotropic change appears to be the coordinated response to membrane lipid peroxidation and decrease in ATP, which can affect calcium ion transients and also limit the energy available for contraction.

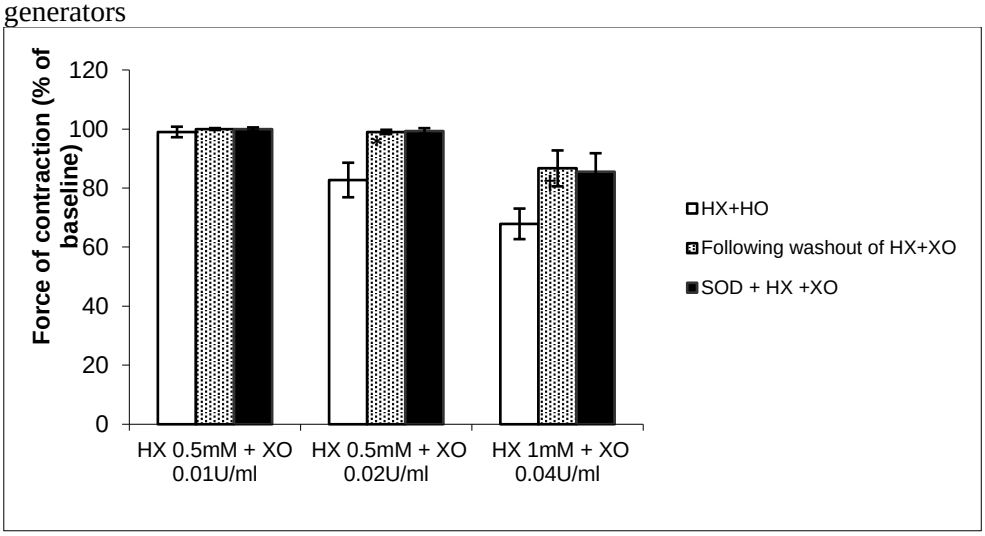
Figures:

Figure 1. Concentration dependent variation in inotropic response of rat papillary muscle to superoxide anion generators



No. of replicates- 15-20
*p<0.05 Vs baseline, † p<0.01 Vs baseline

Figure 2. Concentration dependent recovery of force of contraction following wash out and protective effect of superoxide dismutase of rat papillary muscle exposed to different concentrations of the free radical generators



HX- Hypoxanthine, XO- Xanthine oxidase, SOD- Superoxide dismutase
No. of replicates 6-9
* p< 0.05 compared to baseline, † p<0.01 compared to baseline

Table 1. Inotropic response of papillary muscle to superoxide anion generator after inhibition of membrane channels using specific antagonists

Channel/pump Inhibited	**Antagonist Used**	**HX+XO induced decrease in contraction (%)**
None (Control)	None	17.27 ± 6.05
L-type Ca channel	Verapamil - 1µM	10.93 ± 1.39 *
T-type Ca channel	$NiCl_2$ – 40 µM	12.69 ± 5.56
SR Ca channel	Ryanodine - 1µM	7.22 ± 3.39 **
SR Ca pump	Caffiene – 10mM	6.41 ± 2.41 **

HX+XO - hypoxanthine (0.05 mM) + xanthine oxidase(0.02U/ml)
SR- Sarcoplasmic reticulum
* $p<0.05$ compared to control, ** $p<0.01$ compared to control

Table2- Myocardial tissue metabolites in hearts perfused with hypoxanthine (0.5 mM) + xanthine oxidase (0.02U/ml) compared to untreated control

Variable	**Control**	**Treated**
Lactate dehydrogenase (mU/min/g)	19.62 ± 1.47	35.45 ± 3.02 *
Malondialdehyde (mmoles/100g wet wt.)	0.40 ± 0.04	0.45 ± 0.04 *
Adenosine tri phosphate (µmoles/g protein)	26.37 ± 3.19	19.29 ± 1.58 *
Creatine phosphate (µmoles/g protein)	39.80 ± 1.90	22.08 ± 3.85 *

N = 6-9, * $p<0.01$ compared to control

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Highlights

- Inotropic response of papillary muscle is affected by physiologically relevant concentrations of reactive oxygen species.
- Superoxide anions can modify voltage gated Ca channels and sarcoplasmic reticular Ca uptake and release.
- Creatine phosphate and ATP levels were significantly reduced on exposure to reactive oxygen species.
- Co-ordinated response to decrease in Ca transients associated with a decrease in the energy availability are influencing cardiac inotropy.

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