



HISTOCHEMICAL LOCALIZATION OF NOS ENZYME IN HEART OF MICE AFTER FENOTEROL ADMINISTRATION

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ABSTRACT

Fenoterol is a short-acting β_2 agonist, which also stimulates β_1 receptors at doses above the recommended therapeutic doses. It was widely used in New Zealand in the early 1990s but withdrawn from that market because of its association with an excess number of deaths. It is thought that the association of increased risk of death was because it was typically used in excessively large doses for severe acute asthma attacks in the absence of medical assistance. The side effects of fenoterol are typical for β_2 -adrenoceptor agonists, e.g., hypokalemia, cardiac acceleration, hypotension, and tremor. Histochemistry is a combination of chemistry and histology, in which reactions are carried out on tissue sections or similar preparations and the results examined under a microscope, with the object of combining the advantages of chemical or biochemical specificity and histological localization. Histochemistry is complementary to biochemical analysis of tissue homogenates, since histochemical techniques can give simultaneously biochemical and morphological information. Nitric oxide (NO), first identified as an endothelium-derived relaxation factor, is now recognized to be an intra- and extracellular mediator of cell function. NO produced by the constitutive isoform of nitric oxide synthase (NOS) is a key regulator of homeostasis, whereas the generation of NO by inducible NOS plays an important role in inflammation, host-defense responses, and tissue repair. NO formation is increased during inflammation (rheumatoid arthritis, and ulcerative colitis, Crohn disease), and several classic inflammatory symptoms are reversed by NOS inhibitors. The correlation of structure and biochemical function made possible by histochemical techniques is of unique value in diagnostic and experimental pathology. The present investigation is an attempt to examine short term effects of fenoterol on the major non contractile apparatus which mainly comprises of collagen in left ventricle. The status of antioxidant enzyme nitric oxide synthase has been studied in heart tissue to find out their reserves during fenoterol induced myocardial necrosis. Myonecrosis is a pathophysiological state and during such stage status of antioxidant enzymes is a subject to change.

KEYWORDS: Fenoterol, NOS, Histochemistry, Left ventricle, Paraformaldehyde.

INTRODUCTION

Nitric oxide synthases are a family of enzymes catalyzing the production of nitric oxide from L-arginine. NO is an important cellular signaling molecule. It helps modulate vascular tone, insulin secretion, airway tone, and peristalsis, and is involved in angiogenesis and neural development. It may function as a retrograde neurotransmitter. Nitric oxide is mediated in mammals by the calcium-calmodulin controlled isoenzymes. The inducible isoform, NOS, is involved in immune response, binds calmodulin at physiologically relevant concentrations, and produces NO as an immune defense mechanism, as NO is a free radical with an unpaired electron. NOS isoforms catalyze other leak and side reactions, such as superoxide production at the expense of NADPH. As such, this stoichiometry is not generally observed, and reflects the three electrons supplied per NO by NADPH. In mammals, the endothelial isoform is

the primary signal generator in the control of vascular tone, insulin secretion and airway tone, is involved in regulation of cardiac function and angiogenesis. NO produced by NOS has been shown to be a vasodilator identical to the endothelium-derived relaxing factor produced in response to shear from increased blood flow in arteries. This dilates blood vessels by relaxing smooth muscle in their linings. NO activates guanylate cyclase, which induces smooth muscle relaxation. NOS signaling is involved in development and in fertilization in vertebrates. NO produced by bacterial NOS is protective against oxidative damage. It is well established fact that NO exert significant and biologically relevant effects on cardiac function. In heart, it is synthesized by NOS and is very important. Due to its antioxidant properties, it increases the life of membranes by inhibiting lipid peroxidation and free radical attack. It also protect antioxidant tocopherol from oxidation through similar

mechanisms. Nitric oxide stimulates soluble guanylate cyclase and thus reduces Ca^{2+} partly through activation of cAMP dependent protein kinase and hence causing vasodilation. Because NO is an antioxidant, it may inhibit redox sensitive MMP gene activation (Tyagi, 1996).

MATERIALS AND METHODS

Adult Swiss albino female mice weighing 22-25g were procured from the Central Research Institute (CRI), Kasauli (H.P) and were maintained in the animal house of Department of Biosciences, HP University under suitable hygienic conditions with 16 hrs day light and temperature of $24 \pm 2^{\circ}\text{C}$. The mice were provided feed Hindustan lever Ltd. And water *ad libitum*. In order to study the short term effects of fenoterol on cardiac muscles, the animals were divided in to two main groups-

- 1) Animals of first group served as control and received equal volume of saline water.
- 2) Animals in group two received equal volume of single oral dose of fenoterol (2.5 mg/ kg of body wt). Mice were sacrificed after 2 hrs, 4,10,20 and 72 hrs. For each experiment, left ventricular tissue of heart was taken for histochemical studies.

HISTOCHEMICAL STUDIES

Nitric oxide synthase (NOS) activity was determined by using a histochemical approach (Prabhaker *et al.*, 1993). After washing in phosphate buffer saline (PBS), left ventricular tissue was frozen at -25°C and 15 μm thick sections of the tissue were cut followed by fixation in 4% paraformaldehyde solution. These were washed again in phosphate buffer saline and then incubated for one hour in 0.3% triton, 1mM β - NADPH and 0.2mM NBT. After incubation the sections were washed in 100% alcohol and counter stained with eosin. After clearing in xylene these were mounted in glycerol jelly.

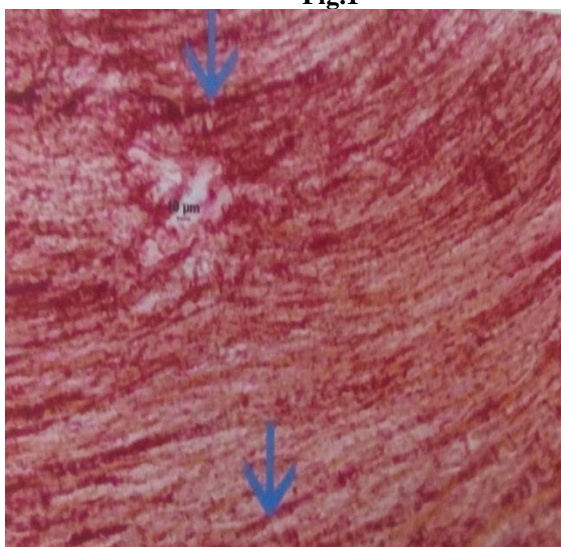
RESULTS

a) Normal cardiac muscle: Cardiac muscles of left ventricular tissue are analysed for nitric oxide synthase activity by employing NADPH defarose assay. Normal ventricle shows sparse NOS activity in the myocardial region. NOS activity can be visualized as a measure of colour intensity in which fine light blue strips are demonstrating NOS activity. (Fig. 1).

b) Drug treated: A negligible rise in NOS activity is observed after 2nd and 4 hrs of drug administration. Here staining characteristic revealing NOS activity are showing slight change in comparison to normal (Fig. 2 and Fig. 3).

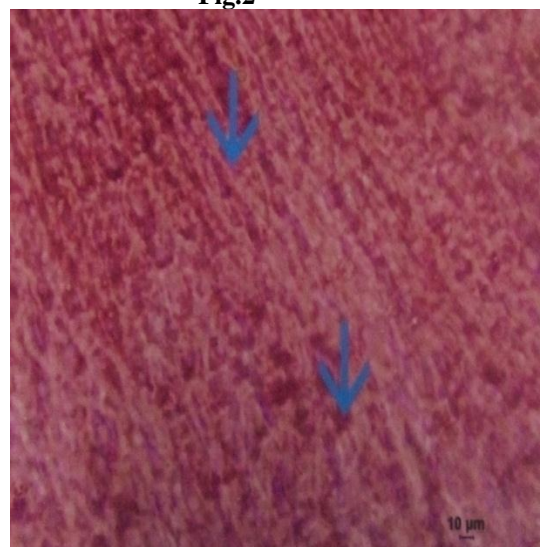
A significant rise in the NOS activity is exhibited after 10 hrs of drug administration. Here a prominent rise in color intensity indicating increase in NOS activity is observed (Fig. 4). A rise in nitric oxide synthase activity is followed by decline through 20 and 72 hrs stages as shown by staining characteristics where color intensity falls. After 72 hrs of drug administration enzyme activity falls equivalent to normal (Fig.5). It seems that this antioxidant enzyme (NOS) is stimulated by β -agonist fenoterol after 4hrs of administration and maximum rise in its activity is observed after 10 hrs of drug administration, thereafter a decline in NOS activity is noticed. The increase in activity of antioxidant enzyme is thought to be a preventive measure against the deleterious effects caused by β - agonist fenoterol. As after certain time period (20hrs), a decline in NOS activity is witnessed which indicate that tissue is recovering from the deleterious effects of drug and at 72 hrs left ventricular myocardium exhibiting NOS activity almost equivalent to normal (Fig.6).

Fig.1



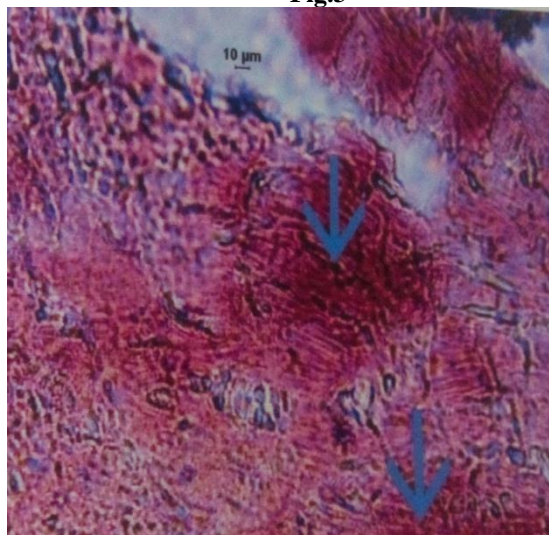
L.S of normal myocardium associated with Ventricular myocytes (↓)

Fig.2



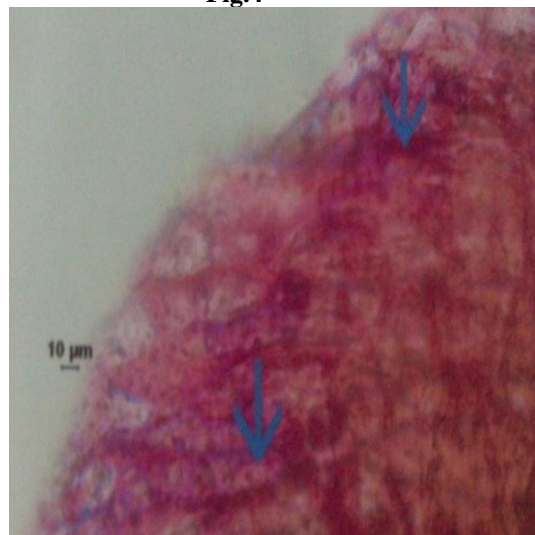
L.S of left ventricular tissue showing no change in NOS activity after 2hrs of drug treatment (↓)

Fig.3



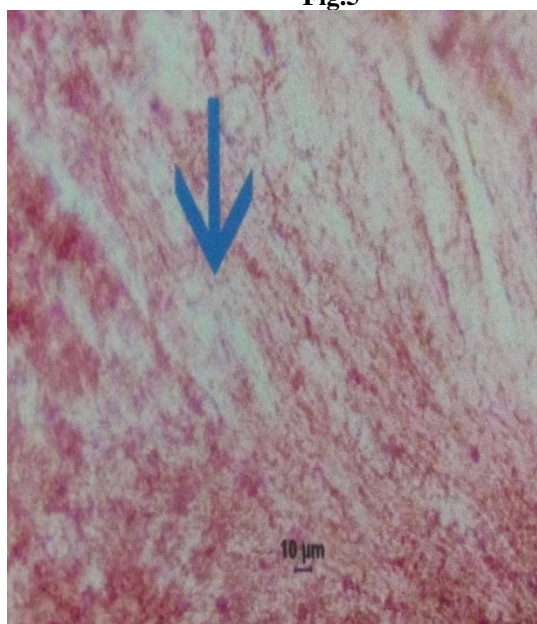
After 4hrs of drug treatment NOS activity (↓)

Fig.4



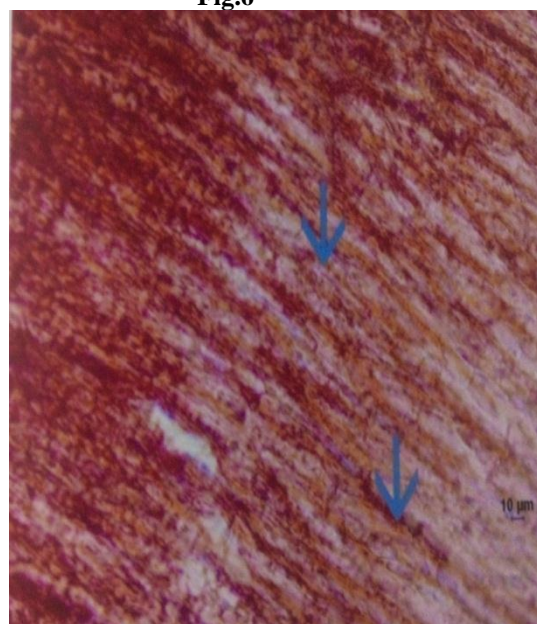
After 10 hrs of drug treatment NOS activity (↓)

Fig.5



Decline in NOS activity after 20 hrs of drug

Fig.6



NOS activity to normal after 72 hrs of drug treatment

DISCUSSION

Fenoterol is a drug that has been used for the treatment of disease like asthma for many years. Chronic beta agonist administration affects cardiac function of rat, independent of β - adrenoceptor density (Burniston *et al.*, 2002). Although, β - adrenoceptor agonist have clinical merit for attenuating the age related loss of skeletal muscle mass and strength, cardiac related side effects may limit their clinical application. Cardiac hypertrophy was associated with increase in left ventricular developed pressure, reduction in cardiac output, reduction in coronary flow per unit heart mass. Systemic administration of can stimulate cardiac hypertrophy (Ryall, 2002). B- agonist stimulate the antioxidant enzyme reserve of muscle tissue. Oxidative stress is the ability of organ or cell to protect itself against the free radicals results in oxidative damage

(Halliwell and Gutteridge, 1985). Enzymes in the mitochondrial respiratory chain in liver generate oxygen radical even in the resting state (Chance *et al.*, 1979). Adequate free radicals scavenging enzymes are available in tissue in resting state of the normal sedentary individual whereas intense physical activity would increase the oxidant reserves of their tissue resulting in oxidative stress (Ji *et al.*, 1988). Antioxidant enzymes act directly or indirectly to remove reactive oxygen species and thus an elevation of these enzymes suggest an increased need for protection against free radicals. It has been proposed that the level of antioxidant enzyme protection in muscle is related to tissue oxygen consumption (Laughlin *et al.*, 1990).

An interesting finding of present study is increase in the level of nitric oxide synthase after single small dose of

fenoterol. There is ample support for the hypothesis that an increase in generation of oxygen free radicals is involved in the pathogenesis as well in the progression of heart failure. The increased level of antioxidant enzymes are indicator of controlled oxidative stress in response to myocardial damage caused by fenoterol. Uncontrolled oxidative stress is combined with a decrease in antioxidant enzyme activity (Dhalla and Singh, 1994; Dhalla *et al.*, 1996). Nitric oxide synthase produces nitric oxide which is a protective antioxidant in the heart. Immediately after fenoterol administration, a heavy rise in NOS activity result in increased NO production, which may be responsible for the changed expression of MMPs. Therefore NO seems to have its effects indirectly on the extracellular matrix turnover. Since NO is a vasodilator, another possibility is that increased NOS activity might have resulted as an adaptive response to the tissue against the myocardial fibrosis induced by β -agonist fenoterol. It appears that an increase in the activity of antioxidant enzymes can be regarded as an effort made by heart to protect itself from an oxidative assault. Some early clinical and preclinical observations have implicated β -agonist in the development of hypertrophic myocardial pathology (Benjamin *et al.*, 1989; Burniston *et al.*, 2002; Lakatta *et al.*, 2003). Although β -agonists show considerable potential as an intervention for sarcopenia, much research is needed to test their efficacy and safety, especially the need to separate skeletal muscle and cardiac effects. Such issues need to be addressed before these agonists can be recommended for clinical applications.

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