CARDIOPROTECTIVE ACTIVITY OF ETHANOLIC EXTRACT OF *ASTRAEUS HYGROMETRICUS* (PERS.) MORG.

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Summary

Incubation of platelet rich plasma with ethanolic extract resulted in the inhibition of both secondary phase of ADP induced platelet aggregation and prostaglandin synthesis. It also stimulated the production of NO in platelets simultaneously in the absence of added ADP. Incubation of washed platelets with the extract stimulated NO synthesis too indicating that the effect of the extract was not mediated through plasma proteins. In addition, the *in vivo* effect of the extract on the increase of NO in the platelets could be demonstrated by *in vivo* exposure of platelets to the extract. It may be correlated that the inhibition of prostaglandin synthesis resulted in the synthesis of NO in resting platelets. The inhibition of platelet aggregation by the extract may not be the consequence of prostaglandin synthesis alone, and can be partly related to the stimulated synthesis of NO. The extract on being tested upon cardiomyocytes *in vitro* showed regression of hypertrophy and potent antiapototic effects. This hypertrophy was associated with increased expression of proto-oncogenes, hypertrophy marker genes, growth factors, and cytokines, with symptoms that mimicked those of human cardiomyopathy, functionally and morphologically.

Key Words: Cardiac hypertrophy, Nitric oxide, Platelet aggregation, Prostaglandin.

Introduction

Platelet aggregation by agonists such as ADP, 1-epinephrine, collagen, and thrombin has been established to be a vital pathological event leading to several thrombotic conditions including coronary artery disease [1]. On the contrary, the inhibition of platelet aggregation by agents like prostacyclin or nitric oxide (NO) has been reported to produce favorable effects in various cardiovascular conditions through their ability to inhibit platelet aggregation both *in vitro* and *in vivo* [2, 3]. Nitric oxide, a physiologic messenger molecule, is known for its diverse effects in various physiologic and pathologic events [4, 5, 6]. It activates guanylate cyclase in a variety of cells including the platelets [7]. The inhibition of platelet aggregation by prostacyclin has been determined to be mediated through the increase of cyclic AMP level [8]. The effect of NO is reported to be the result of both cyclic AMP and GMP level increase in cells [9]. It has also been reported that NO helps platelet production and may have a role in thrombopoeisis and development of megakaryocyte [10]. The inhibition of monocyte activity by NO further results in increased sensitivity to chemotactic agents, which may provide surveillance to rectify immunological problems [11].

Cardiac hypertrophy and ensuing heart failure are one of the most familiar causes of death in the whole world. Many observers have proposed that apoptosis or programmed cell death (PCD) is a chief contributor to heart failure. Apoptosis in the myocardium is a multifaceted process and complicated to make out. Volume overload and elevated end-diastolic ventricular pressure may set off the events of myocyte apoptosis [12]. Myocyte apoptosis has been exhibited after injury due to ischemia, reperfusion, myocardial infarction, ventricular pacing, cardiac aging and coronary embolization [13, 14, 15]. Besides, Olivetti *et al.* [16] demonstrated that in humans, cell death accompanies congestive heart failure.

Astraeus hygrometricus is an ectomycorrhizal wild edible mushroom commonly known as false earthstar belonging to the Family Astraeceae. It is eaten by the villagers and local people as a healthy food having a belief that consumption of this mushroom could prevent several age related disorders. Earlier works regarding *A. hygrometricus* revealed that a water-soluble glucan, Fraction I, isolated from the aqueous extract of the fruit bodies of *A. hygrometricus*, showed strong splenocyte activation [17] and ethanolic extract showed potent *in vitro* free radical scavenging, anti-inflammatory and hepatoprotective activities [18, 19]. In the present study, we examined cardioprotective effect of the ethanolic extract of *A. hygrometricus*.

Materials and methods

Sample collection and preparation

Basidiocarp of *A. hygrometricus* was collected from local market and from the sal (*Shorea robusta* G.f.) forests of Bankura and West Midnapore, West Bengal, India. Fresh mushrooms were randomly selected into three samples of 150 g each and air-dried in an oven at 40°C for 48 h. Dried powdered mushroom sample was extracted by stirring with 200 ml of ethanol at 30°C for 24 h at 150 rpm and filtering through Whatman No. 4 filter paper. The residue was then extracted twice with another 200 ml of ethanol as described above. The total extract was then

rotary evaporated to dryness at 40°C and redissolved in ethanol to a concentration of 10 mg/ml and stored at -20°C for further use [18].

Collection of blood and preparation of platelet rich plasma

Blood was collected from normal healthy volunteers (n = 3) between the ages of 25-35 years by venipuncture using 19 gauge siliconized needles and anticoagulated by mixing 9.0 vol of blood with 1.0 vol of 130 mM sodium citrate (13 mM final). They were mixed in plastic tubes by gentle inversion. None of the volunteers had taken any medication at least for 14 days prior to the donation of blood and none of them had systemic hypertension or diabetes mellitus. Blood was centrifuged at 200 g in an Eppendorf centrifuge (5415 C tabletop centrifuge) for 15 min at 23°C to obtain platelet rich plasma (PRP) [20].

Preparation of platelet suspension

Platelet rich plasma was centrifuged at 2000 g for 20 min at 23°C. The platelet pellet thus obtained was washed thrice by resuspension and centrifugation in Kreb's buffer pH 7.4 (without Ca^{2+}) containing 1 mM EDTA [8]. After the final wash, the pellet was resuspended and washed three more times in the same buffer without EDTA containing 2 mM $CaCl_2$.

Determination of platelet aggregation

The amount of light transmitted through platelet-rich plasma or through a suspension of platelet in isotonic saline solution increases when a platelet-aggregating agent is added. Changes in light transmission, which, within limits are directly proportional to the concentration of aggregation agents, are recorded photometrically. Temperature and collision forces between platelets are controlled by constant stirring [21]. 0.5 ml of platelet-rich plasma was warmed to 37°C for 5 mins in a siliconized cuvette, which was then placed into the cell compartment of the aggregometer; the compartment temperature was maintained at 37°C. The platelet-rich plasma was stirred by small rod shaped magnet covered with polyethylene, which was coupled magnetically to a large magnet rotating below the cell compartment at 1200 rpm. The light transmittance at 600 nm through the sample tube was recorded continuously on a Coagulation Analyser Clot 2S Aggregation Recorder, Seac Radium Company. The recorded chart was calibrated by adjusting the output sensitivity of the aggregometer and the input sensitivity of the pen recorder such that the platelet-poor plasma prepared from the same sample of blood was set at 100% transmission. ADP (4.5 ± 0.5 µM final) was used to study the aggregation [22]. Maximal aggregation of the platelets induced by ADP in the absence of any inhibitor was defined as 100%.

Determination of prostaglandin synthesis in platelets

Prostaglandin synthesis in platelets was assayed by determining the synthesis of malondialdehyde by thiobarbituric acid method [23, 24, 25]. Briefly, platelet rich plasma was treated with or without extract having inhibitory effect at 37°C and the aggregation of platelets was initiated by adding 4 µM ADP. After the aggregation was completed, formation of malondialdehyde was determined.

Determination of NO

Nitric oxide was determined by conversion of oxyhemoglobin to methemoglobin [3, 26]. Typically, either platelet rich plasma or washed platelet suspension in Kreb's buffer, pH 7.4 containing 2 mM $CaCl_2$, was incubated with extract for different periods of time at 37°C. After incubation, 15 µM oxyhemoglobin was added to the incubation mixture and the conversion of oxyhemoglobin to methemoglobin due to the formation of NO in the reaction mixture was continuously monitored by determining the absorption maxima at 401, 421, 575 nm in an Elico BL 198 Biospectrophotometer.

Generation of cardiac hypertrophy *in vitro*

Hearts from 2-3 day old Wistar rat pups were used for isolation of neonatal cardiomyocytes, following collagenase dispersion method [27]. They were maintained at a temperature of 25°±3°C and relative humidity of 45% to 55% under 12-h light:12-h dark cycle. The animals were maintained according to the guidelines recommended by Animal Welfare Board and approved by our Institutional Animal Ethical Committee (IAEC) constituted under the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Government of India, New Delhi. All procedures complied with the Declaration of Helsinki, as revised in 1996. To induce hypertrophy *in vitro*, isolated cardiomyocytes were treated with 10^{-8} M [Sar^1] Angiotensin II (AngII) for 24 hours. During the treatment period, AngII was replenished every 6 hours [28]. Myocyte area was determined by visualization with an inverted microscope and was confirmed by α-actinin antibody staining. Images were captured to determine cell width, length, and area of the cells [27].

Regression of hypertrophy by ethanolic extract

During the 24-hour period of hypertrophy induction by AngII on neonatal cardiomyocytes, treatment with ethanolic extract (20 ppm) was done once on the 15^{th} hour of experimentation. Total RNA was isolated and 2 µg was used for cDNA synthesis by reverse transcriptase PCR. PCR was performed using gene specific oligonucleotide primers for hypertrophy marker genes (*ANF* and *β-MHC*) and proto-oncogenes (*c-fos*, *c-jun* and *c-myc*), to analyze the cardioprotective effect of the extract [28]. *GAPDH* was used as an internal control for these experiments.

Results

Effect of ethanolic extract on the inhibition of platelet aggregation in PRP

Minimum amount of ADP capable of complete platelet aggregation was 4.5 ± 0.5 µM (mean ± SD, n = 3). Human PRP was prepared [20] and platelets in PRP were aggregated as described [21]. Ten µg of the extract in saline at 37°C was added just after addition of the aggregating agent ADP. Ethanolic extract exhibited inhibition of platelet aggregation by about 70% compared to control [Figure 1].

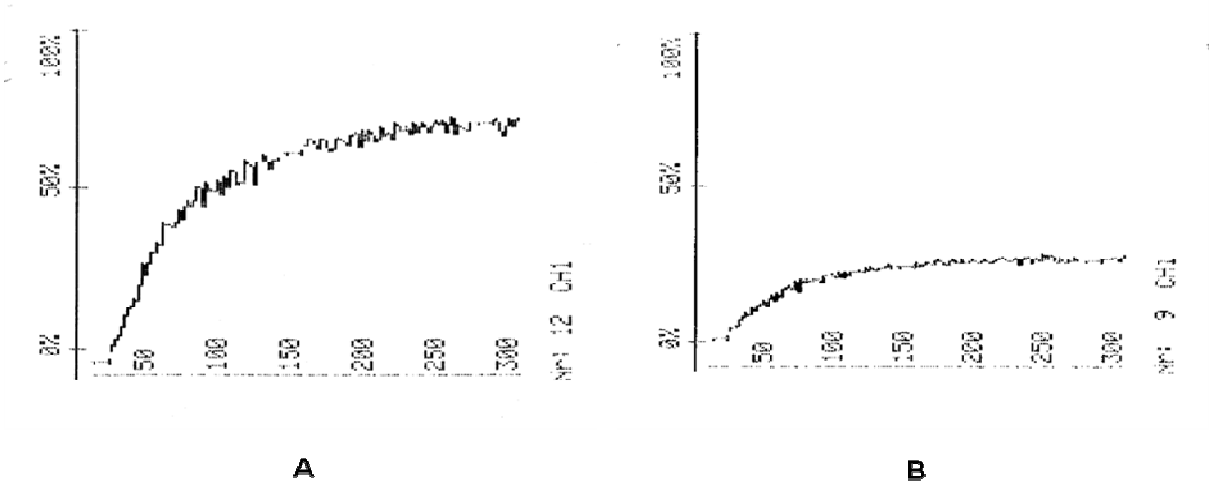

Figure 1 Aggregation of human platelets by ADP (A), and inhibition of platelet aggregation by ethanolic (B) extract.

Effect of ethanolic extract on the synthesis of malondialdehyde in PRP

The time course of the inhibition of prostaglandin synthesis, as determined by malondialdehyde synthesis, showed that the maximal inhibition of malondialdehyde formation was achieved after 30 min of incubation [Figure 2]. Malondialdehyde formation was of intermediate level after 60 min of incubation. It increased slightly at 75 minutes and remained constant after 90 minutes of incubation.

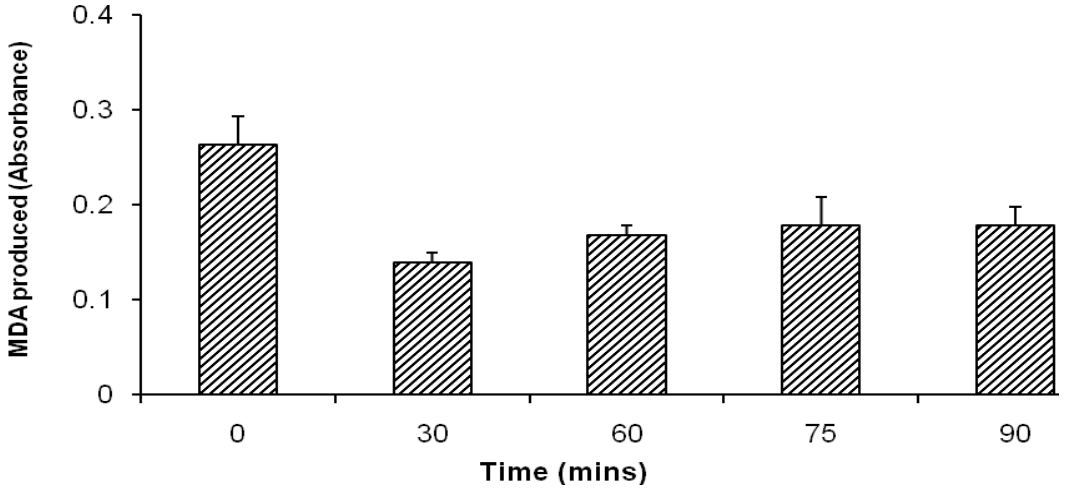

Figure 2 Effect of ethanolic extract on the synthesis of malondialdehyde (MDA) indicating prostaglandin synthesis inhibition in platelet rich plasma (PRP). Results are presented as mean ± SD of three separate experiments, each in triplicate.

Stimulation of NO synthesis in PRP and washed platelets by ethanolic extract

To determine whether the production of NO by extract in PRP was mediated through the plasma proteins, washed platelets free of plasma proteins were suspended in Kreb's buffer and analysed. The platelet suspension was treated with the ethanolic extract and time course of NO synthesis was determined. It was found that the time course of stimulation of NO in washed platelets in the presence of the extract was similar to that of PRP. The exposure of washed platelets to the extract for increasing period of time resulted in the enhanced synthesis of NO, and the synthesis of NO reached its maximum after 30 minutes of incubation [Figure 3].

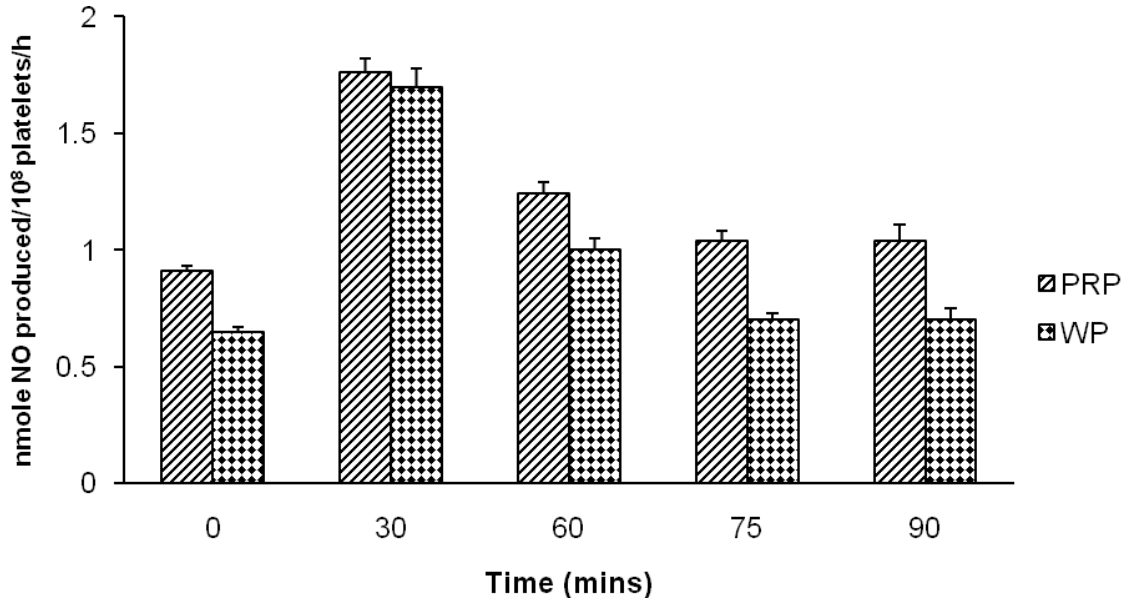

Figure 3 Effect of ethanolic extract on the synthesis of nitric oxide (NO) in PRP and washed platelets (WP) after different periods of incubation. Results are presented as mean ± SD of three separate experiments, each in triplicate.

Hypertrophy generation in neonatal cardiomyocytes

10^{-8} M AngII treatment resulted in considerable hypertrophy in isolated neonatal cardiomyocytes. Myocyte cross-sectional area was increased from 1609 ± 89.9 μm^2 in control to 2034 ± 293.1 μm^2 in AngII treated myocytes [Figure 4], which was approximately >20% increase as compared to untreated controls ($p<0.05$).

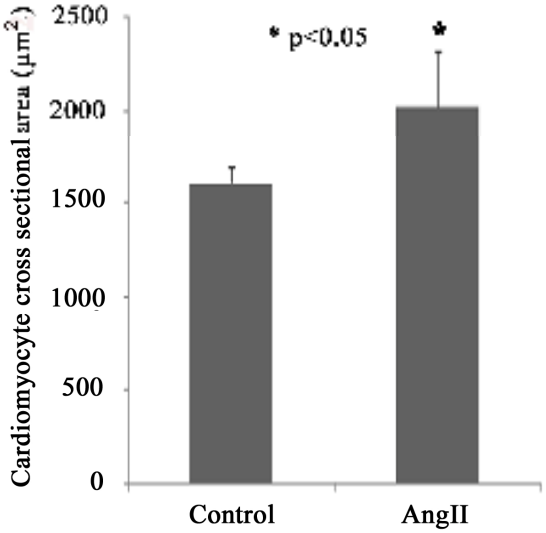

Figure 4 Increase in myocyte cross-sectional area by Ang II treatment as compared to control.

Effect of ethanolic extract on expression of hypertrophy marker genes

As shown in Figure 5, ethanolic extract treated hypertrophied cardiomyocytes showed downregulation of expression of hypertrophy marker genes and proto-oncogenes, compared to the AngII treated cardiomyocytes as evident by RT-PCR analysis. Significant downregulation in expression of hypertrophy marker genes ANF (< 2.5 fold; $p<0.05$), β-MHC (< 2.0 fold; $p<0.05$) and proto-oncogenes like c-fos (< 1.8 fold; $p<0.01$), c-jun (< 3.8 fold; $p<0.05$), c-myc (< 3.5 fold; $p<0.05$) were observed in the ethanolic extract treated hypertrophied cardiomyocytes, compared to hypertrophic cardiomyocytes.

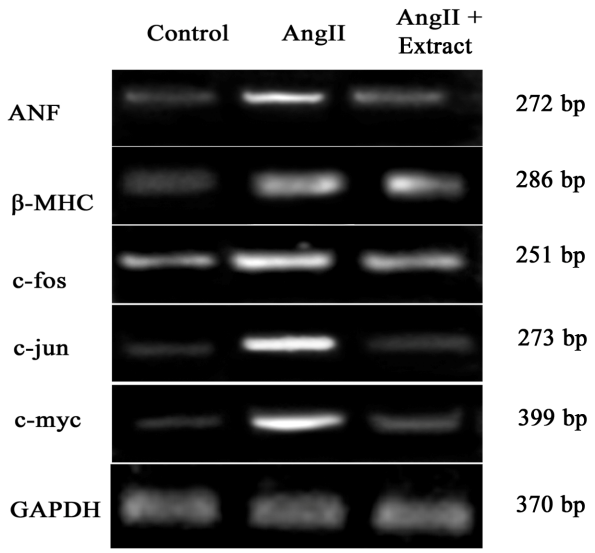

Figure 5 Expression profile of transcripts of hypertrophy marker genes (*ANF*, *β-MHC*) and proto-oncogenes (*c-fos*, *c-jun*, *c-myc*) by RT-PCR. Significant downregulation of hypertrophy marker genes and proto-oncogenes was observed during ethanolic extract treatment, compared to respective Ang II treated cardiomyocytes ($p<0.05$). *GAPDH* was used to normalize the expression.

Discussion

The results suggested that the ethanolic extract was a potent inhibitor of prostaglandin synthesis and also stimulated NO synthesis in human blood platelets. This effect in platelets was not mediated through plasma proteins in the incubation mixture because the incubation of either platelet rich plasma or washed platelets in Kreb's buffer with the extract resulted in the stimulation of NO synthesis. It was found that not only the treatment of platelets with ethanolic extract resulted in the synthesis of NO in the suspension; the time course of NO synthesis in platelet rich plasma was found inversely related to the inhibition of malondialdehyde production. Our results indicated that the inhibition of platelet aggregation occurred via two ways. One was by inhibiting prostaglandin synthesis and the other by the stimulation of synthesis of NO.

Platelets participate in fatal and non-fatal myocardial infarction due to coronary thrombosis. They also contribute to the development and progression of coronary artery atherosclerosis. Inhibition of platelet aggregation by agents like prostaglandin or NO has been reported to produce beneficial effects in various cardiovascular conditions [3, 8, 20]. Incubation of PRP and washed platelets with ethanolic extract resulted in the inhibition of ADP induced platelet aggregation and prostaglandin synthesis with simultaneous stimulation of the production of NO. Inhibition of platelet aggregation, prostaglandin synthesis and stimulation of NO synthesis by the

extract significantly reduces the incidence of cardiac hypertrophy, first myocardial infarction, recurrent infraction and vascular death among patients with cardiovascular disease [29].

Our results also suggest that the ethanolic extract may have a role in preventing the development and progression of hypertrophy. AngII known to be involved in hypertrophic changes induced by renal vascular constriction, showed neonatal cardiomyocyte cross sectional area to be significantly increased mimicking the hypertrophic state. Treatment of cardiomyocytes with AngII, a potent vasoconstrictor showed expression of hypertrophy marker genes like *ANF* and *β-MHC* as well as proto-oncogenes (*c-fos*, *c-myc* and *c-jun*) [28]. Ethanolic extract after treatment on hypertrophied cardiomyocytes showed decreased expression of these genes, that corroborates protection from hypertrophy by the extract of *A. hygrometricus*. We believe the extract of *A. hygrometricus* may act as a novel therapeutic strategy against hypertrophy of cardiomyocytes. Future application of this compound as a therapeutic agent for treating hypertrophy would be an interesting avenue to explore.

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