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Original Article

Role of aspirin activated nitric oxide synthase in controlling DOCA-salt-induced hypertension in rats through the stimulation of renal r-cortexin in kidney cortex cells

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ABSTRACT

Objectives: Because the damage of kidney tissue is associated with hypertension and impaired nitric oxide (NO) synthesis, and as aspirin is reported to stimulate the synthesis of renal r-cortexin, an anti-hypertensive protein, we investigated the role of aspirin as bolus dose on elevated blood pressure induced by deoxycorticosterone acetate (DOCA)-salt in animal model.

Methods: The chronic antihypertensive effect of aspirin on DOCA treated with ASA group of rats ($n = 6$) was evaluated after ingestion of 0.35 μM aspirin as a bolus dose in every 24 h using tail cuff methods. The plasma aspirin, NO, and r-cortexin levels were determined by spectrophotometric, methemoglobin, and ELISA methods, respectively. Synthesis of r-cortexin mRNA was determined. Aspirin activated nitric oxide synthase (AANOS) was purified by chromatographic methods.

Results: Our results showed after 3 h of administration of aspirin (0.35 μM) to the DOCA treated with ASA group of rats decreased the systolic blood pressure from 139.39 ± 7.36 mm of Hg to 116.57 ± 6.89 mm of Hg and diastolic blood pressure from 110.4 ± 7 mm of Hg to 86.4 ± 2.76 mm of Hg. The reduction of BPs was found to be related to the increased plasma aspirin from 0.00 μM to 0.042 μM, plasma NO from 0.4 ± 0.19 nM to 1.9 ± 0.5 nM, and cortexin levels from 64.36 ± 12.6 nM to 216.7 ± 21.3 nM. The molecular weight of purified AANOS is 18 kDa.

Conclusion: It can be concluded that aspirin possesses antihypertensive effect on blood pressure in chronic administration. Aspirin can stimulate NO synthesis through the activation of AANOS, which stimulated the production of r-cortexin in kidney cortex cells and thereby reducing elevated BP in hypertensive rats.

Keywords: Hypertension, aspirin, r-cortexin, nitric oxide, DOCA

Introduction

Hypertension or high blood pressure is a condition in which the force of the blood against the artery or vain wall is too high because of the constriction and dilation of the blood vessels which ultimately leads to coronary artery disease (CAD) and cerebral stroke.[1] According to the literature, approximately 40% of the world population has been affected by this deadly disease which would cause 10.4 million deaths worldwide per year.[2,3] This life-threatening condition is not a single disorder; rather it represents a heterogeneous group of diseases. Various cardiovascular risk associated with hypertension which ultimately leads to atherosclerosis, despite the two being different pathophysiological process.[4] It has been reported that uncontrolled hypertension altered renal function through dysregulation of sodium ion concentration and impairment of renin-angiotensin-aldosterone activity which ultimately leads to chronic kidney disease.[5] Recent studies showed that the association between kidney tissue damage and hypertension has been mediated by the impairment of nitric oxide (NO) synthesis.[6] It has also been reported that elevated blood pressure is the most essential factor to develop neurological disorders which is also called neurogenic hypertension.[7] However, sleep disturbance, insomnia is proposed as a possible pathophysiological factor of hypertension.[8] Since most of the hypertensive patients (>90%) is reported to have primary or essential hypertension (EH) for which no definable cause not including any gene or its product (protein) has yet been identified, the exact mechanism in the development of this condition is still remain unknown. Only 5–10% of the victims

are reported to have "secondary hypertension" due to renal or endocrine hypertension.[9]

Although hypertension is a major risk factor for death worldwide, there is an increased drive towards its prevention.[10] It should be mentioned here that uncontrolled elevated BP can cause severe harmful effects in different organs of the patients.[11] In contrast, report suggested that control of hypertension can revert back many symptoms which is caused by the condition.[12] To control hypertension, there is no specific therapy which is currently known. Although some non-specific anti-hypertensive drugs cause various unpleasant side effects.[13,14]

In the above context, it should be mentioned that endothelial cells possess a constitutive form of nitric oxide synthase (eNOS) enzyme which does not have any basal activity[6] but under proper stimulation, it produces NO, a vasodilator[15] can control elevated blood pressure.[16] It has been reported that an antihypertensive protein of molecular weight (M.W. 43kDa) was synthesized in the cortex of kidney called r-cortexin which appeared in the circulation[6] and stimulate the cNOS[17] that ultimately synthesized NO and reduce the elevated blood pressure.[6] The activation of cNOS by r-cortexin is mediated by the activation of r-cortexin gene. It was also reported that exogenous NO was capable of stimulating r-cortexin synthesis in the kidney cortex cells through the activation of r-cortexin mRNA probably in the promoter region.[6] Injection of this antihypertensive protein can reduce both systolic and diastolic BP in catecholamine induced hypertension in animal model but its effect was found to be neutralized using anti-r-cortexin antibody.[6] Some researchers also reported that cortexin protein is also expressed in brain but the exact mechanism is still remains obscure.[18] It was also investigated that person suffering from EH has reduced production of r-cortexin in their circulation.[19]

In the context of activation of r-cortexin synthesis in controlling hypertension, acetyl salicylic acid (ASA) also called aspirin has been reported to stimulate systemic NO synthesis through the activation of NOS in various cells.[20,21] Increased synthesis of systemic NO by the ingestion of ASA might have some stimulatory effect to increased synthesis of r-cortexin which, in turn, can control the elevated BP in rats. Hence, it is possible that sustained hypertension caused by different etiologic mechanisms can be controlled by the systemic increase of (r)-cortexin stimulated by systemic NO synthesis by aspirin. In the present study, we used rats as an experimental animal model to sustained elevated BP by administering deoxycorticosterone acetate (DOCA)-salt[22] because rats are ideal animal model for hypertension research that has human like cardiovascular anatomy and physiology.[23] It has 99% sequence homology of genome to humans.[24] Second, the pathogenesis of hypertension in rats and human is largely similar in terms of arterial pressure development, hemodynamic factors (including vascular resistance), mechanisms regulating arteriolar and venous constriction, renal vascular dynamics (including perfusion parameter), as well as humoral influences by nitric oxide synthase NOS.[25]

The effect of aspirin in lowering the elevated blood pressure was time variable. Although the effect of aspirin in lowering elevated blood pressure has been shown previously, the reason behind the variability of time to reduce the elevated blood pressure and the duration of effect of aspirin on elevated blood pressure has not been studied. Thus, in this study, we have determined the plasma aspirin level at different time intervals after ingestion of the compound and the effects of chronic administration of aspirin on blood pressures of deoxycorticosterone acetate (DOCA)-salt-induced hypertensive rats were investigated. It has been also reported that the *r*-cortexin level of DOCA-salt-induced hypertensive rats was severely reduced as compared with control (normotensive) group of rats as a consequence of decreased synthesis of nitric oxide (NO).[26] It has been reported that aspirin can stimulate NO synthesis in various cells and it was also reported that exogenous NO was capable of stimulating r-cortexin synthesis in the kidney cortex cells of goat because in the evolutionary scale, both animals (goat and rat) are placed in the same rank. Therefore, the synthesis of *r*-cortexin through the aspirin-induced NO synthesis in kidney cortex cells was studied through the *in vitro* translation of mRNA of *r*-cortexin using plant ribosome which is responsible for synthesizing proteins by translating the genetic code transcribed in mRNA into an amino acid sequence and quantitated by enzyme-linked immunosorbent assay (ELISA). This study helps us to confirm that the renal (r)-cortexin was actually synthesized by the aspirin-induced NO synthesis and not merely by the release of preexisting (r)-cortexin in the kidney cortex cells due to the vasodilatory effect of aspirin-induced NO synthesis.

We also report the purification and mechanism of action of aspirin activated nitric oxide synthase (AANOS) in arterial endothelial cells. The molecular weight (Mr.) of the purified AANOS was determined by running marker protein through SDS-PAGE.[27] The effect of ASA on this eNOS in stimulating NO synthesis was investigated. This NO could stimulate r-cortexin synthesis in kidney cortex cells, an antihypertensive protein, and thereby controlling elevated blood pressure induced by DOCA salt in animal model. Herein, we have tested that aspirin could be a unique antihypertensive compound for EH through the synthesis of *r*-cortexin. It should be mentioned that although the effect of aspirin in the reduction of hypertension in general and not in EH has been reported before, neither the process of aspirin initiated lowering of hypertension nor the involvement of any mediators for the managing of raised blood pressure in these studies was even mentioned.

Materials and Methods

Ethical clearance

Protocol used in these studies was only commencing after appropriate permission from the Institutional Ethics Committee, IPGME&R. The protocol was also approved by the Institutional Animal Ethics Committee (IAEC) no.: 544/PO/C02/CPCSEA. This study used Wister albino rats and kidney from goat obtained from the slaughter house. The permission to use those was also approved by IAEC.

Chemicals

Goat anti-rat IgG-alkaline phosphatase, N^G-nitro-L-arginine methyl ester hydrochloride (l-NAME), mercuric chloride ($HgCl_2$), ferric nitrate [Fe $(NO_3)_3$], hydrochloric acid (HCl), sodium oxalate, DOCA-salt, phenyl methyl sulfonyl fluoride (PMSF); TritonX-100; and dithiothreitol (DTT) were obtained from Sigma-Aldrich (St. Louis, MO). Enzyme-linked immunosorbent assay (ELISA) MaxiSorp plates were from Nunc, Roskilde, Denmark. Aspirin (Trade name – Ecosprin by USV Ltd., India). Non-invasive small animal tail blood pressure system was obtained from BIOPAC Systems Inc., Goleta, USA. Centrifuge, homogenizer, and spectrophotometer ELICO all other chemicals used were of analytical grade.

Selection of animal

Wistar albino male rats (120–140 gm each) of 3 months age were housed for 2–3 months at our animal facility before used for the study.[22] These inbred rats were fed standard laboratory chow, vegetables and sterilized water was given *ad libitum*. The rats were kept under 12 h cycles of light and dark at 23°C, temperature was also maintained to avoid fluctuation of blood pressure.[28]

All animal-related experiments were strictly performed in the presence of the Institutional Animal Ethics Committee (IAEC) and under the supervision of the veterinarian, and special care was taken to ensure that no rats were unnecessarily harmed or were subjected to pain during the study and all rats related to experiment are free of disease and in good health. Those rats which were used for other experiments and infected rats were also excluded from the study. A total of 24 rats were used and were divided primarily into four groups:

1. Rats administered with DOCA (20 mg/kg s.c. in olive oil) [n = 12]
   The 12 rats were divided into two groups, six rats were treated with ASA (n = 6) and the other six rats were not treated with ASA (n = 6').
2. Rats administered with only vehicle (s.c. in olive oil) [n = 6].
3. Control rats (did not inject either DOCA or vehicle) [n = 6].

Induction of hypertension in animal model

Two weeks before the preparation of hypertensive animal model, blood pressure estimations were started twice in a week. To induce hypertension, we administered DOCA-salt (20 mg/kg s.c. in olive oil) to 12 rats (n = 12) twice weekly for 4.5 weeks and 1% NaCl in their drinking water for obtaining the sustained hypertension.[22] Among these 12 hypertensive rats, six rats were treated with ASA (n = 6) and six rats were not treated with ASA (n = 6). Four groups of rats were used in this present study. The blood pressures both systolic and diastolic (approx. value) were measured by BP tail cuff sensor (RXTCUFSENSOR-9.5) (Biopac Systems Inc., Goleta, USA). The reading was confirmed using amplifier (NIBP-200A) those were connected with Data Acquisition Unit (Busy Power-MP 36) through adapter cable.

Collection of blood sample

Blood sample was collected from the tail vein of the rats by 32G needle and was anticoagulated by adding 9:1 volume of sodium citrate (0.13 nM at final concentration) to the blood sample. Cell-free plasma from the citrated blood was prepared by centrifugation at 5000 g for 10 min at 4°C.

Preparation and oral administration of acetyl salicylic acid (aspirin) solution to the test rats

Acetyl salicylic acid (ASA) solution was freshly prepared by dissolving the compound in 0.1 M $NaHCO_3$ and immediately neutralized to pH 7.0 at 0°C. The solution was administered to the DOCA-treated hypertensive rats (n = 6) and normotensive (control) rats (n = 6).

Determination of plasma aspirin level

Preparation of reagents

To prepare ferric-mercuric solution, 5 gm of mercuric chloride ($HgCl_2$) was dissolved in a small amount of distilled water in beaker A and 5 gm of ferric nitrate [Fe $(NO_3)_3$] was dissolved in 15 ml of 1(N) hydrochloric acids (HCl) in beaker B. Next, the contents of both beakers (A&B) were mixed in a 125 mL volumetric flax and made up the volume by distilled water.[29]

Preparation of standard aspirin solution

At first, stock solution of 1 µM of aspirin was prepared by distilled water with moderate heating. Next, this stock solution was further diluted up to 0.05 µM, 0.10 µM, 0.15 µM, 0.20 µM, and 0.25 µM standard solution of the drug.

Construction of standard graph

A 1 mL of each of the diluted standard solution was taken in separate test tubes and heated in a water bath for 3–5 min at 45 ± 2°C, and after cooling in running water, 5 mL of ferric-mercuric reagent was added to each of the standard test tubes and mixed well. For blank determination, 1 mL of distilled water was taken in another test tubes and the same procedure

was followed. All the test tubes were again heated for 1–2 min and then cooled.

The absorbance at 540 nm against blank was determined for all of the standard solutions. When absorbance values were plotted against concentrations, a more or less straight line was obtained which represented a 5-point calibration curve.

Plasma aspirin analysis

After ingestion of 0.35 μM of aspirin solution of each of these six (n = 6) rats of both groups (DOCA-treated with ASA and control, that is, normotensive with ASA), 0.5 mL of blood sample was withdrawn from each group of rats into separate Eppendorf in every hour from 0 to 4 h containing 25 mg of sodium oxalate. After centrifuged at 4500 r.p.m for 5 min, 200 μL of plasma of each Eppendorf was transferred to other Eppendorf and 1 mL of ferric-mercuric reagent was added to each of them and mixes well. The mixture was again centrifuged at 4500 r.p.m (for complete precipitation of plasma protein by forming complex with the reagent). Then, the supernatant solution was collected and absorbance was measured and compared with the standard curve.

Assay of NO

The amount of NO was determined using the conversion rate of oxyhemoglobin to methemoglobin through NO using a scanning spectrophotometer (Beckman spectrophotometer). The NO content was quantified by recording the spectral changes in the reaction mixture due to the conversion of oxyhemoglobin to methemoglobin, that is, a decrease in the absorbance at 575 and 630 nm as described.[30] The quantification of NO was independently verified by chemiluminescence method.[31]

Preparation of the goat kidney cortex cell suspension

The organ was obtained from freshly killed goat supplied by the local slaughterhouse and was stored in ice-cold Krebs buffer pH 7.4 as described before.[6] The organ was next freed from all adhering fats and debris and cut in half on its longitudinal axis. The cortex portion was then removed chopped into 5 mm of slices and homogenized as described.[6]

Separation of RNA

The kidney cortex cell homogenate in Krebs buffer (pH 7.4) was incubated with different concentrations (0–80 μM) of aspirin (ASA). After incubation, the nucleic acids which contained r-cortexin mRNA from the aspirin-treated kidney cortex cell were extracted using Trizol-chloroform method.[32] To 1 ml of each cell suspension, 0.5 ml of Trizol was added to lyse the cells, later 0.5 ml of chloroform per 1 ml of Trizol was added. The samples were then centrifuged at 12,000 rpm for 15 min at 4°C. Following centrifugation, the mixture separated into a lower red phenol-chloroform phase, an interface, and a colorless upper aqueous phase. Total RNA remained exclusively in the aqueous phase, the aqueous phase was transferred into an Eppendorf, to precipitate RNA, and 1 ml isopropanol per 1 ml of Trizol was added. It was then centrifuged at 12,000 rpm for 10 min at 4°C. RNA pellet was then washed with 75% ethanol and then centrifuged at 7500 rpm for 5 min at 4°C. RNA was dissolve in RNAse-free water.

Separation of plant ribosomes

Bael leaf (*Aegle marmelos*) around 10 g was taken and washed to remove debris twice and rinsed in distilled water and homogenized and later centrifuged at 5000 rpm to remove the debris, the supernatant was taken, and centrifuged at 13,000× *g*. The supernatant was layered on top of a 1 mM sucrose cushion and centrifuged at 200,000× *g* to pellet ribosome as described.[33]

In vitro translation of *r*-cortexin mRNA

RNA was isolated by Trizol-chloroform method from different concentrations of aspirin-treated kidney cortex cell described above.[32] Briefly the mRNAs were incubated with ribosomal preparation, mixture of all 20 amino acids (0.1 μmol/ml, each) and 2 mM ATP as described.[34] After 6 h, the reaction mixture was centrifuged at 10,000 g at 0°C for 10 min. The supernatant was used for the synthesis of r-cortexin by ELISA as described.[35]

Isolation and purification of aspirin activated nitric oxide from goat arterial endothelial cell

Preparation of particulate fraction from arterial endothelial cell suspension

Endothelial cells were isolated from the arterial lumen using a soft nylon bottle brush and were dispersed in Tyrode's buffer, pH 7.4 and centrifuged for 10 min at 500 g at 4°C. The endothelial cells were washed 2 more times appropriately and suspended in the same buffer. Next, this suspension was disrupted by repeated freezing and thawing in liquid N_2 and centrifuged at 30,000 g at 0°C for 30 min. The obtained cytosolic supernatant was discarded while the remaining pellets were mixed and incubated with 0.05% Triton X-100. The Triton X-100 treated pellets were again centrifuged at 30,000 g for 60 min at 4°C and the supernatant was used as a membrane protein source[36] for further purification of the enzyme.

DEAE cellulose chromatography

All chromatographic procedures described below were performed at 4°C. The clarified endothelial cell suspension containing Triton X-100 solubilized aspirin activated NO generating protein (9 mL containing 54 mg protein) was immediately applied to DEAE cellulose column (2.5 cm × 29 cm) as described.[20] The fractions showing highest NO activity were collected, pooled, and concentrated to 5 mL using polyethylene glycol as described.[37]

Assay of aspirin activated NO generating protein from the chromatographic column

Typically, 0.25 mL of the elute containing the enzyme was incubated in Tyrode's buffer pH 7.4 containing 10 µM arginine, 2.0 mM $CaCl_2$, and 80 µM aspirin in a total volume of 2.5 mL for 60 min at 37°C. The formation of NO in the assay mixture was determined by the oxyhemoglobin method as described above.[30]

Gel electrophoresis and identification of protein band of highest NOS activity

About 10% SDS-polyacrylamide gel electrophoresis was carried out for the protein mixture from the DEAE cellulose chromatographic fraction. The gel was stained using Coomassie brilliant blue as described.[33] Similarly, in a parallel experiment, an identical gel that was not stained with Coomassie brilliant blue was prepared to avoid denaturation and it was done repeatedly for the determination of maximal NOS activity.[20]

The protein bands that were not stained as described above were excised into slices (1.5 mm) from the gel and triturated separately in 0.9% NaCl. The aspirin activated NOS activity of the clarified supernatant of each of the slices was determined as described.[20,38] The protein band with highest NO activity was further processed for the determination of homogeneity activity.

Homogeneity and subunit composition of the aspirin activated purified NOS

The homogeneity and the subunit composition of the preparation obtained from the gel slice with the highest NOS activity after 10% SDS-polyacrylamide gel electrophoresis which was determined as demonstrated.[39] The molecular weight of aspirin activated nitric oxide synthase (AANOS) enzyme was determined by alkaline SDS-polyacrylamide gel electrophoresis under reducing and non-reducing condition using marker proteins of known molecular weights.[39]

Construction of Lineweaver-Burk plot of the purified aspirin activated NO generating protein

Typically, the reaction mixture containing 0.1 mg of the purified protein was incubated with 2 mM $CaCl_2$ and different concentrations of *l-arginine* and 80 µM of aspirin at 37°C for 30 min, the amounts of NO formed in the reaction mixture were determined as described above. In a parallel experiment, identical reaction mixtures containing *l-arginine* but without the added aspirin and the formation of NO was determined and Lineweaver-Burk plot was similarly constructed.[40] Addition of 0.1 mM L-NAME, an inhibitor of nitric oxide synthase[41] to the identical reaction mixtures for the inhibition of NO synthesis, was also investigated.

Statistical analysis

Results are expressed as mean±SD of at least three independent experiments. The comparisons were performed using the Student's t-test or one way ANOVA. $P < 0.05$ was defined as statistically significant. The coefficient of correlation (r) was determined by Pearson test.

Results

Determination of plasma aspirin level after ingestion of the compound (0.35 µM) at different time interval of both groups (DOCA treated and control group) of rats

It was previously reported that ingestion of 0.35 µM aspirin was capable of reducing the blood pressure in DOCA-induced hypertensive rats but the reduction of the elevated BPs by the ingestion of aspirin after 2 and 3 h was found to be variable.[26] To find out the reason behind it, studies were conducted to determine the plasma aspirin level of different groups of rats at different time interval after ingestion of 0.35 µM aspirin solution.

It was found that before the administration of 0.35 µM of aspirin, the plasma aspirin level of DOCA treated rats was 0.00 µM which increased to 0.020 µM after 2 h of administration of aspirin and maximally increased to 0.042 µM after 3 h of administration of 0.35 µM aspirin [Figure 1]. Similarly, in control groups of rats (without DOCA treated), the plasma aspirin level increased from 0.00 µM to 0.043 µM after 3 h of administration of aspirin [Figure 1]. Hence, it can be concluded that after 3 h of administration of aspirin, the plasma aspirin level reached the optimal level.

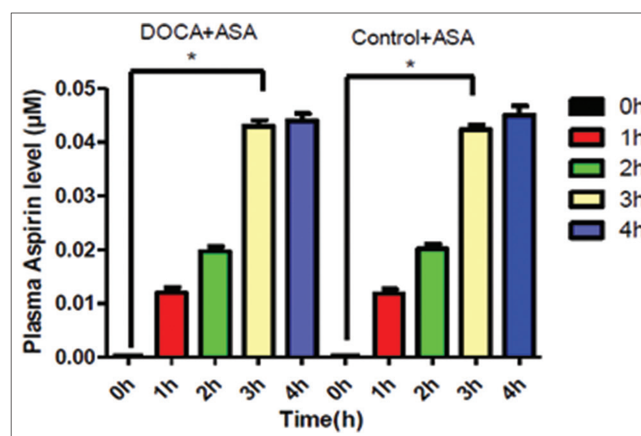

Figure 1: Plasma aspirin level after ingestion of the compound (0.35 µM) at different time intervals of both groups (DOCA treated and control group) of rats: Plasma aspirin levels at different intervals (0–4 h) after ingestion of 0.35 µM of aspirin solution of DOCA + ASA and Control + ASA-treated groups of rats where black (▬), red (▬), green (▬), yellow (▬), and blue (▬) colored bars indicate the plasma aspirin level at 0 h, and after1 h, 2 h, 3 h, and 4 h intervals respectively. $n = 6$; $P < 0.05$. (*plasma aspirin level between 0 h and 3 h of DOCA +ASA and Control+ ASA-treated groups of rats)

Effect of 0.35 μM aspirin on the determination of NO and r-Cortexin in the CFP of different groups (DOCA treated, DOCA treated with ASA, and control with ASA group) of rats

Since aspirin at 0.35 μM was capable of reducing the blood pressure in DOCA-induced hypertensive rats[26] and it was previously reported that aspirin is a stimulator of r-cortexin (an anti-hypertensive protein) synthesis, investigation was carried out to determine the effect of the administration of 0.35 μM of aspirin on the production of NO and r-cortexin in the cell-free plasma (CFP) of different groups of rats.

It was found that the amount of NO present in the CFP of DOCA-treated rats was 0.4 ± 0.19 nmol/h/mg protein, and in the case of DOCA + ASA-treated rats, the amount of NO increased to 1.9 ± 0.5 nmol/h/mg protein, and in control+ASA-treated rats, the amount of NO was 3.4 ± 1.5 nmol/h/mg protein which was 8 times higher compared to DOCA-treated rats [Figure 2a]. Similarly, the amount of r-cortexin present in the CFP of DOCA-treated rats was 64.36 ± 12.6 nM which increased to 216.7 ± 21.3 nM in DOCA + ASA-treated rats. In control + ASA groups of rats, the amount of r-cortexin present in the CFP was 251.32 ± 16.31 nM [Figure 2b].

Hence, aspirin can stimulate plasma NO synthesis which, in turn, stimulate the production of anti-hypertensive protein r-cortexin. There could be some factor which can activate by aspirin to reduce the elevated blood pressure in hypertensive rats through the increased production of systemic NO and r-cortexin.

Kinetics of aspirin induced NO production in *in vitro* system

The dose-response curve of the aspirin-induced NO production in the kidney cortex cell suspensions in Krebs buffer, pH 7.4, showed that the addition of different concentrations of the aspirin (20 μM–200 μM) to the incubation mixtures resulted in the increased production of NO from basal 0.044 nmol/h/mg protein to 1.41 nmol/h/mg protein, and at 80 μM aspirin, the production of NO was maximally achieved. Further increase of aspirin (200 μM) in the incubation mixture, however, resulted in decreased production of NO by almost 75% [Figure 3].

Effect of aspirin in the synthesis of r-cortexin in the kidney cortex cell suspension

As described above, oral administration of aspirin in the hypertensive rat model resulted in the reduction of both SBP and DBP (approximate)[26] that were related to the increase of the plasma NO level [Figure 2a]. Aspirin has been reported to stimulate NO synthesis in various cells through the activation of NOS,[20] and it has been also reported that the NO is the activator of *r*-cortexin synthesis in kidney cortex cell preparations *in vitro* and *in vivo*.[6] In an effort to determine the effect of different concentrations of aspirin in the kidney cortex, the cell suspensions were studied *in vitro* as described in the Methods section.

Experiments were carried out to determine the aspirin-induced NO synthesis in kidney cortex cells through the activation of NOS which were actually involved in the synthesis of r-cortexin in kidney cortex cells by *in vitro* translation of r-cortexin mRNA to facilitate the reduction of systemic elevated blood pressure induced by DOCA salt. It was found that the incubation of kidney cortex cell suspensions with different amounts of aspirin for 30 min at 37°C resulted in the increased synthesis of *r*-cortexin and at 80 μM aspirin concentration, the synthesis of r-cortexin was maximally achieved from basal level 34.2 ± 3.1 nM to 53.09 ± 1.5 nM at 30 min as quantitated by ELISA [Figure 4]. Further increase

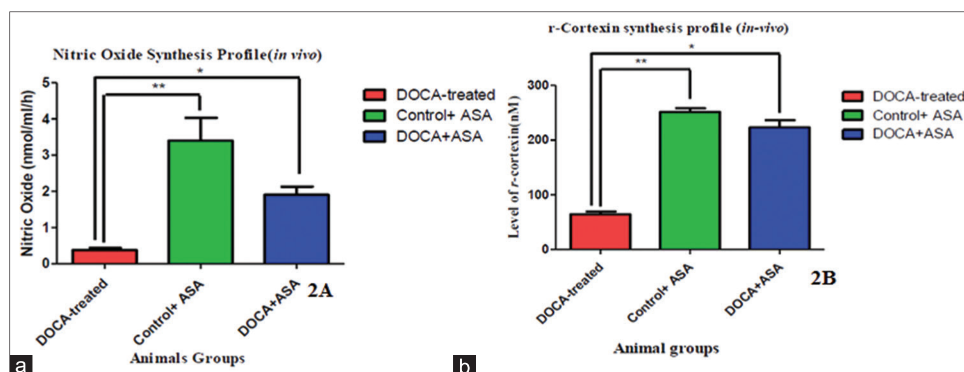

Figure 2: (a) Effect of 0.35 μM aspirin on the determination of NO in the CFP of different groups (DOCA treated, DOCA + ASA and control + ASA group) of rats: Plasma NO levels of different groups of rats where red bar (▬) indicates plasma NO level of only DOCA-treated hypertensive group, green bar (▬) represents control+ASA-treated group, and blue bar (▬) demonstrates the same in DOCA + ASA-treated groups of rats = 6. (*NO between DOCA treated and DOCA + ASA rats $P < 0.05$. (**NO between Control + ASA (normotensive) and DOCA treated rats $P < 0.001$). (b) Effect of 0.35 μM aspirin on the determination of r-cortexin in the CFP of different groups (DOCA treated, DOCA + ASA and control + ASA group) of rats: Plasma *r*-cortexin levels of different groups of rats where red bar (▬) indicates *r*-cortexin level of only DOCA-treated hypertensive group, green bar (▬) represents control+ASA-treated group, and blue bar (▬) demonstrates the same in DOCA+ASA-treated groups of rats. *n*=6. (*r-cortexin between DOCA treated and DOCA+ASA rats: $P < 0.05$. **r-cortexin between Control + ASA (normotensive) and DOCA treated rats. $P < 0.001$)

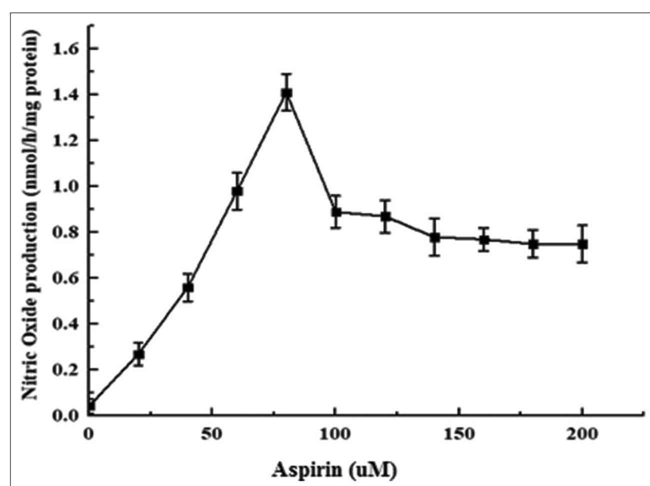

Figure 3: Dose response curve for the production of NO in kidney cortex cell suspensions in the presence of different concentrations of aspirin. The kidney cortex cell suspension in Krebs buffer was prepared from the goat kidney as described in the Methods section. The suspension (particulate fraction) was incubated with different concentrations of aspirin solutions as indicated for 30 min at 37°C. The production of NO was determined by the conversion of oxyhemoglobin to methemoglobin as described. Solid squares (■) represent the production of NO. The results are mean ± SD of six different experiments using six different kidneys (n = 6)

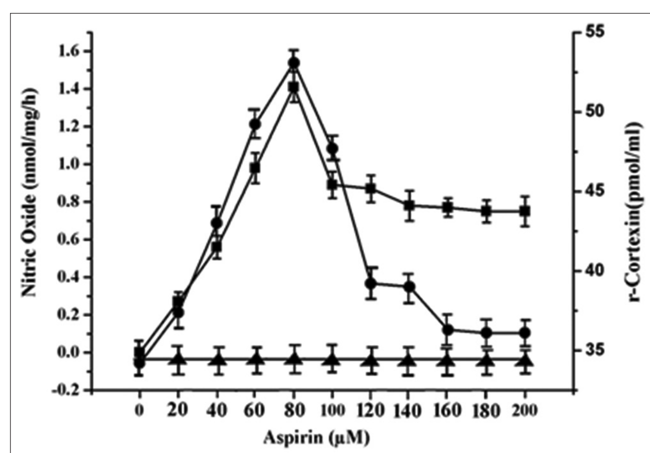

Figure 4: Aspirin-induced production of NO and (r)-cortexin in kidney cortex cells. The goat kidney cortex cells were incubated with different concentration of aspirin and it was found that at 80 µM aspirin, production of NO and cortexin was maximum. Here, solid squares (■) represent the production of NO; solid circles (●) represent the production of (r)-cortexin, and solid triangles (▲) represent the NO production in the presence of NAME. Moreover, the correlation coefficient between NO production and (r)-cortexin is +0.992 up to the 80 µM concentration of aspirin and the corresponding (r)-cortexin synthesis was maximal at 80 µM concentration of aspirin n = 5

of aspirin concentration in the reaction mixture, however, not only resulted in the decreased production of NO but was also found to result in the reduced synthesis of r-cortexin in the reaction mixture [Figure 4].

It was also found that addition of 0.1 mM *l*-NAME an inhibitor of NO synthesis[41] resulted in the complete inhibition of both NO and *r*-cortexin production in the kidney cortex cell suspensions [Figure 4].

Duration of the antihypertensive effect of bolus of aspirin ingestion in DOCA-induced hypertensive rats

Since it was reported that aspirin at concentration 0.35 µM capable of reducing blood pressure in DOCA-induced hypertensive rats[26] by the systemic stimulation of NO production [Figure 2a] through the activation of aspirin activated nitric oxide synthase (AANOS) which, in turn, synthesized antihypertensive protein r-cortexin in the kidney cortex cells [Figure 2b], it was necessary to investigate the effect of ingestion 0.35 µM aspirin as a bolus dose on elevated blood pressures of rats.

To determine the effect of ingestion of 0.35 µM aspirin as a bolus dose on elevated blood pressure, after eaten adequately, the DOCA treated with ASA group of rats (n = 6) was administered with 0.35 µM aspirin whereas DOCA treated without ASA group of rats did not ingest the same and used as control. Both systolic and approximate diastolic blood pressures were measured after 24 h of the ingestion of the compound. It was found that as a result of ingestion of 0.35 M of aspirin, both systolic and diastolic (approx.) pressures of the DOCA treated with ASA group of rats remained at normotensive level even after 24 h (systolic pressure = 116.57 ± 6.89 mm of Hg and approximate diastolic pressure = 82 ± 2.68 mm of Hg). Whereas, the BPs level of the DOCA treated without ASA group of rats remain unchanged (systolic pressure = 139.39 ± 7.36 mm of Hg and approximate diastolic pressure = 110.41 ± 7 mm of Hg).

In a separate study, the possibility of maintaining the elevated BPs at normotensive levels in DOCA treated with ASA group of rats, 0.35 µM of aspirin solution was orally administered to those rats (n = 6) every 24 h after adequate feeding. It was found that the ingestion of 0.35 µM aspirin to these DOCA treated with ASA group of rats decreased the systolic blood pressure from 139.39 ± 7.36 mm of Hg before the administration of aspirin to 116.57 ± 6.89 mm of Hg after 3 h of ingestion of the compound [Figure 5a and b].

Continuation of daily ingestion of 0.35 µM aspirin to these groups of hypertensive rats resulted in the maintenance of the systolic pressure at 115.41 mm of Hg at day 10. Further continuation of daily ingestion of the same amount of aspirin maintained the systolic blood pressure at 116.57 ± 6.89 and 115.41 ± 4.41 mm of Hg at day 20 and 30, respectively [Figure 5a]. The diastolic pressure which was 110.41 ± 7 mm of Hg before 0.35 µM aspirin ingestion decreased to 86.4 ± 2.76 mm of Hg after 3 h of ingestion of the compound in these rats. Continuous daily ingestion of 0.35 µM aspirin capable of maintained diastolic blood pressure at 84 ± 2.74 mm of Hg at day 10, 83 ± 2.16 mm of Hg at day 20 and at 82 ± 2.68 mm of

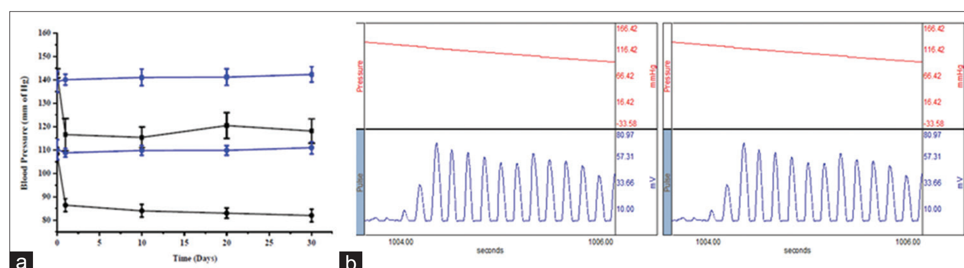

Figure 5: (a) The duration of the antihypertensive effect of aspirin after continuous ingestion of the compound in hypertensive rats: DOCA + ASA-treated group of rats ($n = 6$) was ingested 0.35 μM of aspirin daily for 1 month as described in the Materials and Methods section. Both systolic and diastolic (approx.) pressures were measured after 24 h, 10, 20, and 30 days after continuous ingestion of the compound. Solid black squares (■) indicate the systolic and solid black circles (●) represent the diastolic BPs (approx.) of DOCA + ASA-treated group of rats ($n = 6$) and solid blue squares (■) indicate the systolic and solid blue circles (●) represent the diastolic blood pressures (approx.) of control group (only DOCA treated) of rats ($n = 6$). Each point represents Mean ± SD of five different experiments. (b) Panel-A showing pressure and pulse wave forms (original tracing) exhibiting changes in systolic and diastolic (approx.) blood pressure in DOCA+ASA-treated group of rats after 24 h of administered with 0.35 μM of aspirin. Panel-B shows pressure and pulse wave forms (original tracing) exhibiting blood pressure levels of only DOCA treated, that is, control group of rats at the same time

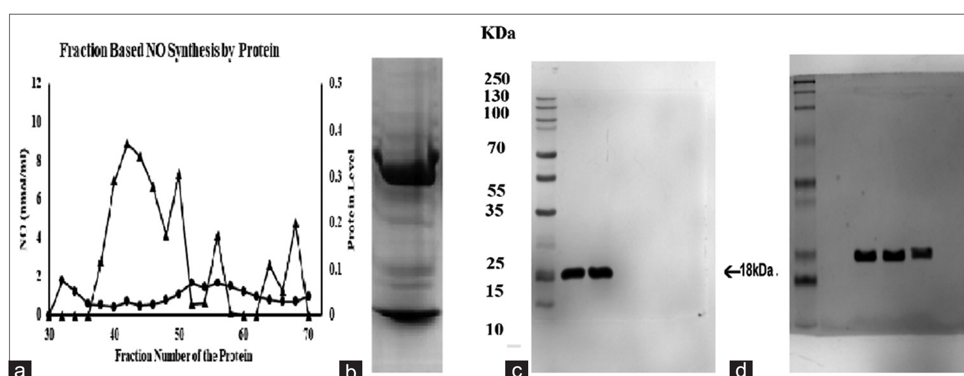

Figure 6: (a) DEAE-cellulose chromatography of Triton X-100 solubilized AANOS from endothelial cells: Isolation and purification of AANOS was described in the Methods section. Aspirin activated nitric oxide synthase was found to emerge from the column in three peaks. The fractions from the major peak were collected. Solid black circle (●) indicates protein, solid black triangles (▲) AANOS activity. (b-d) SDS-PAGE of purified AANOS protein in the presence and absence of reducing agent: The AANOS protein purified from membranous fraction of goat carotid artery endothelial cells by chromatographic methods as described Materials and Methods. In the figure, Panel-6B demonstrates the electrophoresis of DEAE-cellulose chromatographed protein after stained with Coomassie brilliant blue shows seven dark bands and repeatedly run at the same condition (non-reduced) but not stained and triturated separately. It was found that the 18 kDa triturated protein showed the maximum NO activity and no other elute from the slices in the entire gel showed any stimulation of NO synthesis in the endothelial cells. Panel 6c and 6d demonstrate the sliced, triturated, and electrophoresis of the 18 kDa band in presence (reduced) and absence of SDS (non-reduced), respectively

Hg at day 30, respectively [Figure 5a]. It was also found that in control experiment, where DOCA-induced hypertensive rats did not ingest 0.35 μM aspirin, there are no significant changes which were observed at systolic and diastolic blood pressures in these rats [Figure 5a and b]. Hence, it can be suggested that continuous ingestion of aspirin is capable of controlling the elevated blood pressure in DOCA-induced hypertensive rats for long time.

Purification, properties, homogeneity, and subunit composition of aspirin activated NO generating protein (AANOS)

Since aspirin was capable of stimulating NO synthesis both *in vivo* and *in vitro* [Figures 2a and 3], indicates that oral administration of aspirin must activate an enzyme released from the endothelium resulted in the increase of plasma NO level through the activation of the enzyme. Experiments were carried out to solubilize and purify the enzyme from endothelial cells. When endothelial cell suspension in 10 mM Tris-HCl buffer pH 7.4 was treated with 0.05% TritonX-100 as described in Methods, it was found that supernatant fractions contain NO activity. The supernatant fraction was chromatographed on DEAE cellulose column as described in Methods. It was found that the active fractions (fraction no. 38–52) emerged from the column as major peak containing 0.05 M NaCl in the buffer [Figure 6a]. Only the fractions from the major peak were pooled, concentrated, and used for further studies. It was found that the supernatant fractions showed aspirin activated NOS (AANOS) activity. As described in Table 1, the combination of the above steps used in the purification of aspirin activated NOS enzyme from endothelial cells provided over 4339-fold purification of the protein over the starting material, which represents 5.4% of the total endothelial cell membrane protein.

Table 1: Summary of purification steps of aspirin activated nitric oxide synthase of arterial endothelial cells

Protein	Conc. of protein (mg/ml)	Specific activity (nmol/mg of protein/hr)	Folds of purification	Total protein yield (%)
Crude homogenized endothelial mass	20	0.014	1	
0.05% TritonX-100 treated supernatant	6	0.0871	6.22	30
DEAE cellulose chromatography	4.88	2.132	152.28	24.4
After gel electrophoresis in the presence followed by in the absence of SDS	1.08	60.75	4,339	5.4

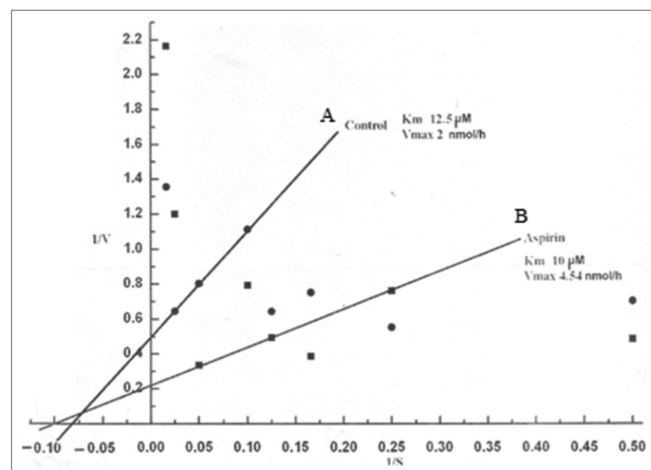

Figure 7: Lineweaver-Burk plot of the stimulation of aspirin-induced NO synthesis in purified protein (NOS): The protein in Krebs buffer, pH-7.4, was incubated in the presence and absence of 80 µM aspirin with different amounts of l-arginine and 2.0 mM $CaCl_2$ in a total volume of 1.0 ml. After incubation for 30 min. at 37°C, the synthesis of NO was determined by methemoglobin method as described in the Methods section. Line A represents the synthesis of NO in the absence of added aspirin to the reaction mixture with different concentrations of l-arginine. Line B represents the synthesis of NO in the reaction mixture in the presence of 80 µM aspirin and with different amounts of l-arginine

The concentrated protein (4.88 mg/ml) from the fractions no. 38–52 was next applied to SDS-PAGE[40] under alkaline condition for further purification. Seven dark protein bands were found [Figure 6b] which were cut and triturated separately for NO activity determination. The final product of the non-reduced protein under alkaline condition which showed highest NO activity demonstrated that the apparent molecular weight was 18 kDa [Figure 6b]. When the purified AANOS was reduced by 2% β-mercaptoethanol and subjected to SDS-PAGE, the electrophoretic mobility of the reduced protein [Figure 6c] remained unchanged [Figure 6c] when compared to the non-reduced protein [Figure 6d] under otherwise identical conditions.

Kinetics of aspirin activated endothelial nitric oxide synthase (AANOS)

It was previously reported that DOCA salt-induced significant rise in blood pressure in animal model was associated with systemic reduction of NO, reported to be an endothelial cell derived vasorelaxing factor and a global antihypertensive agent.[42] However, endothelial NOS by itself has been reported to have little or no basal enzymatic activity for the production of NO, and this enzyme in the endothelial cells has been reported to be stimulated only in the presence of appropriate activators for the synthesis of NO, leading to the control of elevated BPs.[42]

A Lineweaver-Burk plot was constructed to determine the enzymatic activity of the purified proteins (NOS) of arterial endothelial cells, in the presence of 80 µM aspirin (activator). It was found that the basal enzymatic activity (i.e., the basal activity in the absence of the added aspirin) [Km = 12.5 µM, Vmax = 2 nmol NO/mg protein/h] of the purified protein [Line-A, Figure 7] that indicated NO production by the purified protein was stimulated due to the addition of 80 µM aspirin in the reaction mixture [Km = 10 µM, Vmax = 4.54 nmol NO formed/mg protein/h] [Line-B, Figure 7]. The enzymatic activity was completely inhibited by adding 0.1 mM NAME to the reaction mixture. Hence, aspirin could be the appropriate activator to stimulate the endothelial NOS for the production of NO (i.e., AANOS) to control the systemic elevated blood pressure.

Discussion

The above results showed that hypertension caused by mineral corticoid could be managed in animal (rats) by oral administration of acetyl salicylic acid (ASA), a commonly used compound for the prevention of coronary artery disease (CAD) with excellent safety records.[43] The results demonstrated that the antihypertensive effect of aspirin was mediated through the synthesis of (r)-cortexin, an antihypertensive protein in the kidney cortex cells with the stimulation of nitric oxide (NO) produced by the activation of membrane bound constitutive form of NOS (cNOS) in various cells by aspirin. This activator (aspirin) was found to reduce the DOCA-induced elevated blood pressure (both systolic and diastolic) in rats.[26] It has been reported that aspirin can stimulate the synthesis of NO by the activation of cNOS in erythrocytes, platelets, leukocytes, etc.[20,21] It has also been reported that NO thus produced, stimulate the synthesis of r-cortexin, an antihypertensive protein which is also activate the eNOS of endothelial cells in the cardiovascular system.[6] Although aspirin was capable of stimulating systemic NO synthesis (20) which itself is a global vasodilator,[42] the half-life of aspirin actuated NO is extremely

small (≈10^{-8} s.) in the circulation and as such, NO may not be available to reach the vascular system to control the elevated blood pressures. At this point of view, (*r*)-cortexin, a protein hormone (43 kDa), has been reported to activate a membrane nitric oxide synthase, which has no basal NOS activity and the presence of (*r*)-cortexin activated NOS is crucial for the control of the elevated blood pressure through the synthesis of NO (6). The chronic use of aspirin (0.35 μM) for 30 days maintained both the systolic (115.41 ± 4.41 mm of Hg) and diastolic blood pressure (82 ± 2.68 mm of Hg) at normal level in DOCA-induced hypertensive rats [Figure 5] through the increase of plasma NO and r-cortexin. Hence, it could be postulated that continuous ingestion of aspirin as bolus dose controls the elevated blood pressure indefinitely. As a consequence, the increase of plasma aspirin level, the plasma NO, and r-cortexin level in both hypertensive rats and in normotensive control were found to be increased [Figure 2] which control the elevated blood pressure in DOCA-induced hypertensive rats. However, in our previous study, it was found that ingestion of aspirin by untreated control group of rats ($n = 6$) did not lead to hypotensive condition. These results might suggest that aspirin could be a more acceptable antihypertensive compound compared to other antihypertensive drugs that usually are known to produce hypotension in normotensive subjects. The failure of aspirin to cause hypotension in normotensive rats was probably related to the fact that the quantity of NO produced by the ingestion of aspirin was not high enough to cause hypotension. Other explanations including short half-life of NO in the circulation are also possible. The decrease of both systolic and diastolic blood pressure by the administration of aspirin was apparently mediated by the increase of NO level in the circulation through the activation of aspirin activated nitric oxide synthase (AANOS) which ultimately stimulates the synthesis of r-cortexin in the kidney cortex cells. At the time of determining the role of aspirin in the synthesis *r*-cortexin in *in vitro*, the addition of *l*-NAME, an inhibitor of NO synthesis to the incubation mixtures of kidney cortex cells homogenates with aspirin, impaired the production of NO in the system, simultaneously, the synthesis of *r*-cortexin was also inhibited. This study indicated that the reduction of DOCA-salt-induced high blood pressure in rats by aspirin was mediated through the systemic production of NO which has been reported to be a global vasodilator. The vasodilatory effect of aspirin through NO synthesis, in turn, was found to mediate its antihypertensive effects through the synthesis of r-cortexin in the kidney cortex cells. Our results indicated that while the addition of 80 μM of aspirin to the incubation mixture increased the synthesis of r-cortexin 2-fold over the basal level. Because the increased cellular level of either NO or *r*-cortexin has been reported to result in vasodilation,[6,26] the aspirin-induced antihypertensive effect was apparently increase of r-cortexin in kidney cortex cells. Although the mechanism of DOCA-salt-induced inhibition of systemic r-cortexin synthesis was well studied but still elevation of blood pressure is not known. However, it is well known that DOCA-induced hypertension causes an impaired endothelial function thereby developed elevated blood pressures. However, from the previous study of our enzyme kinetics, it was found that aspirin can inhibit the function of DOCA and as a result the activity of eNOS restored as normal condition. Furthermore, as aspirin decreased the DOCA-induced elevated blood pressures in rats, our results may show that the vasodilatory effect of aspirin was endothelium dependent. The injection of L-NAME, an inhibitor of NO synthesis[40] which impaired the aspirin-induced increase of NO [Figure 3] in the system, simultaneously negated the antihypertensive effect of the activator through the inhibition of r-cortexin synthesis [Figure 4], indicating that the reduction of DOCA-induced high BP by aspirin in rat was mediated through the systemic production of NO [Figure 2]. From the *in vitro* study, it was found that inhibition of NO synthesis resulted the reduction of *r*-cortexin synthesis (0–10 pmol/mL) in the kidney cortex cell suspensions. On contrary, *in vitro* translation of cortexin m-RNA in the kidney cortex cells suspension indicated that incubation of aspirin stimulates the NO synthesis that was able to stimulate the synthesis of cortexin in these cells. In this context, it could be suggested that the antihypertensive effect of aspirin, a NO generating agent in *in vivo* might also be mediated through NO induced cortexin synthesis. Moreover, it was also observed that *l*-NAME in *in vitro* translation inhibited the synthesis of both NO and the hypotensive protein (r-cortexin) in the kidney cortex cells. The NO-induced stimulation of cortexin synthesis and again cortexin actuated NO production, demonstrated a “positive” feedback stimulation of both NO and cortexin.[6] This feedback character of NO has been reported earlier.[44]

Conclusion

From the above results, it can be concluded that DOCA-induced hypertension in animal model could be neutralized by aspirin activated nitric oxide synthase (AANOS). Moreover, AANOS-induced nitric oxide can stimulate r-cortexin in kidney cortex cells, this cortexin can also induce nitric oxide again. Thus, the above-stated observation might be a vicious cycle which may actually explain how AANOS acts in controlling hypertension through NO and cortexin.

Authors’ Declaration Statements

Research involving human participants and/or rats

Research involves with rats and ethical permission has been obtained from the Institutional Ethical Committee board, which has been approved by IAEC. All applicable international, national, and/or institutional guidelines for the care and use of rats were followed.

Consent for publication

None.

Availability of data and material

The data used in this study are available and will be provided by the corresponding author on a reasonable request.

Competing interests

No conflicts of interest exist

Funding statement

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Authors' Contributions

All authors contributed equally.

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