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Rat Model of DOCA-Salt-Induced Hypertension

[1]Department of Pharmacology, Abhilashi University, Mandi,India
[2]Department of Pharmacology, School of Pharmaceutical Sciences, CT University, Ludhiana, Punjab, India

Nitric oxide (No) is the most powerful vasodilator .Caveolin has been documented to inhibit eNOS and decrease the activity of NO. Hypertension is associated with an impaired endothelial function that includes reduced production and/or NO release. Caveolin directly binds to eNOS, thereby regulating the NO production. Daidzein- A caveolin inhibitor by decrease caveolin activity, increase eNOS and NO production. In present study we fouced on NO pathway to further

various stimuli such as VEGF and shear stress, eNOS binds to HSP90 which facilitates calmodulin-induced displacement of caveolin-1 from eNOS (Gratton et al., 2000). However, the interrelationship of caveolin-1 and eNOS is a dynamic one. For example, caveolin-1 is crucial for agonist-induced eNOS activation. us, the aim of this study was to demonstrate daidzein a caveolin inhibitor may have bene cial actions on NO activity and perhaps have the potential to selectively improve hypertension and its associated vascular complications.

Materials and Method

Experimental animals

Wistar albino rats weighing 125–175 g (8–10 week old) purchased from animal house, I.S.F College of Pharmacy, Moga, Punjab, were employed in the present study. ey were housed in an air-conditioned room and were kept in standard laboratory conditions under natural light and dark cycles (approximately 14 h light/10 h dark) and humidity 60 ± 5% and temperature of 25 ± 3^{0}C in the departmental animal house. e animals were allowed free access of standard diet (24% protein, 5% fat, 4% ber, 55% carbohydrates, 0.6% calcium, 0.4% phosphorous, 7% moisture and 4% ash w/w) and tap water ad libitum and allowed to acclimatize for 2 weeks before starting the experiments. e experimental protocol was approved by the Institutional Animal Ethics (IAEC) and conformed to the Indian National Science Academy (INSA) Guidelines for the use and care of experimental animals in research.

Drugs and chemicals

Daidzein was purchased from Medchem Expess LLC chemicals and DOCA from Sigma-Aldrich. Other chemicals and biochemical reagents of analytical grade were used freshly.

DOCA- salt induced hypertension

DOCA- Salt-induced hypertension is due to the sodium-retaining properties of the steroid causing increases in plasma and extracellular volume. e hypertensive e ect is increased by salt loading and unilateral nephrectomy in rats. Wistar Albino rat (125-175g) were anesthetized with Ketamine (70 mg/kg, i.p.) and xylazine (5mg/kg, i.p.) through a ank incision, the le kidney was removed. e rats were injected twice weekly with 40 mg/kg, s.c. deoxycorticosterone-acetate in olive oil for 6 weeks. Drinking water is replaced with a 1% NaCl solution. Blood pressure starts to rise a er one week and reaches Mean arterial blood pressure value between 160 and 190 mm Hg a er 6 Week.

Experimental protocol: (Each group n=6)

Group I Normal control: Rats were maintained on normal chow diet with drinking water.

Group 11 Hypertensive control: Rats were uninephrectomized and DOCA Salt was administered twice weekly for 6 weeks and drinking water replaced with 1% NaCl solution.

Group III Hypertensive rats were treated with Daidzein (0.2mg/kg/day, s.c) for the 1st week started from the 5th week of DOCA Salt administration.

Group IV Hypertensive rats were treated with Daidzein (0.4mg/kg/day, s.c) for lst week started from the 5th week of DOCA Salt administration.

Group V Hypertensive rats were treated with Lisinopril (1mg/kg/day, p.o) for the 1st week started from the 5th week of DOCA Salt administration.

Assessment of Hypertension

Assessment of mean arterial blood pressure

e rat blood pressure was measured by Tail cu method (NIBP-BIOPACK MP 100, USA). Rat tail was heated with the exact time of 3 minutes a er that tail was inserted into the cu , the pressure was applied with cut o pressure of 250 mmHg and mean arterial blood pressure was recorded by Data Acquisition System (BIOPAC).

Assessment of oxidative stress

Reduced glutathione (GSH)

To measure reduced glutathione level, aortic tissue homogenate (in 0.1 M phosphate bu er Ph 7.4) was taken. e procedure was followed initially as described by Ellman, 1959. e homogenated was added with an equal volume of 20% trichloroacetic acid (TCA) containing 1mm EDTA to precipitate the tissue proteins. e mixture was followed to stand for 5 min prior to centrifugation for 10 min at 200 rpm. e supernatant (200µl) was then transferred to a new set of the test tube and added 1.8 ml of the Ellman reagent (5,5`-dithio bis-2-nitrobenzoic acid) (0.1mm) was prepared in 0.3M phosphate bu er with 1%of sodium citrate solution. Volume was made up to 2ml of all the test tubes. A er the completion of the total reaction, the solution was measured at 412 nm against blank. Absorbance values were compared with a standard curve generated from a standard curve from known GSH. e level of GSH was expressed as µmol/mg of Aortic wt [3-8].

Serum Nitrite/Nitrate level using greiss reagent

Serum nitrite/nitrate was estimated using Greiss reagent which served as an indicator of nitric oxide production. 400µl of carbonate bu er (Ph 9.0) was added to 100µl of serum sample followed by addition of small amount (˜0.15g) of the copper-cadmium alloy. e tubes were incubated at room temperature for 1 hour to reduce nitrate to nitrite. e reaction was stopped by adding 100µl of 0.35 M sodium hydroxide following this,400 µl of zinc sulfate solution (120mm) was added deproteinate the serum samples, the samples were allowed to stand for 10 min and then centrifuged at 4000g for 10 min. Greiss reagent (250µl of 1.0% sulphanilamide and 250µl of 0.1% napthaylaminediaminedihydrochloric acid in water) was added to aliquots (500µl) of clear supernatant and serum nitrite/nitrate was measured spectrophotometrically at 545 nm. e standard curve of sodium nitrite was plotted and expressed as µmol/l [9].

Estimation of tumor necrosis factor –alpha (TNF-α) levels

TNF- **α** level was estimated by using rat TNF-a kit (RayBio, Rat TNF-alpha ELISA kit protocol) which uses a microtitre plate reader read at 450nm. Concentrations of TNF-α were calculated from the plotted standard curve.

Principle

is assay employs the quantitative sandwich enzyme immunoassay technique. An a nity polyclonal antibody speci c for mouse TNF-α has been precoated onto a microplate. Standards, cotede(6(2ml)0.6(ofkmml w

a blue product that turns yellow when the stop solution is added. e intensity of the color measured is in proportion to the amount of rat TNF-a bound in the initial step. e sample values are then read o the standard curve.

Vasoreactivity

Ach and SNP induced vasorelaxation: Isolated rat aortic ring preparation: - e rats were sacri ced by cervical dislocation, followed by decapitation. oracic aorta was removed, cut into a ring of 4-5mm in length and mounted in an organ bath containing Krebs- henseleit solution (NaCl,119Mm, Kcl,4.7mm; KH_2PO_4.1.2Mm and $CaCl_2$,2.5 Mm) of PH 7.4, bubbled with carbonated oxygen (95% O_2 and 5% CO_2) and maintained at 37ºC. e preparation was allowed to equilibrate for 90min under 1.5g tension. e isometric contractions were recorded with a force transducer (Ft-2040) connected to a physiograph (INCO, Ambala, India) the aortic ring preparation was primed with 80mm KCL to check its functional integrity and to improve its contractility. e cumulative dose response of Ach (10^{-8} to 10^{-4} M) or sodium nitroprusside SNP($10^{-8}$to 10^{-4}M) were recorded in phenylephrine (3×10^{-6}M) precontracted with intact or denuded endothelium, respectively [10].

Molecular Assays

Assessment of mRNA expression of eNOS

100 mg of aortic tissue was homogenized in 1ml of triazole reagent and incubated for 5 min at 20ºC. 0.2ml of chloroform was added to the homogenate , incubated at 20ºC for 3 min. the mixture was centrifuged at 10,000 g for 15 min at 4ºC, the upper aqueous phase was isolated and 0.5ml isopropyl alcohol was added to precipitate RNA. e sample was centrifuged at 10,000g for 15 min at 4ºC to form a gel-like pellet of RNA in the tube. e supernatant was removed, RNA pellet was washed with 75% ethanol, mixed, centrifuged at 7,500g for 5 min at 4ºC and RNA pellet was brie y vacuum dried for 5-10 min. e RNA was quanti ed by ultraviolet absorbance spectrophotometry to ascertain A260/A280 ratio<1.6 and dissolved in RNase-free water. e 5µl reverse primer was added to crude RNA, 29µl reverse transcriptase bu er incubated for 10 min at 65ºC and cooled on ice. 16 U AMV transcriptase (10U/µl) and 5µl 10nM dNTP mixture were added, incubated at 42ºC for 1hr and 10mM Tris bu er (pH- 7.5) was added to synthesized single-stranded cDNA. 5µl cDNA product was mixed with 4µl dNTP mixture, 5µl forward primer, backward primer , 10 X ampli cation bu er , 0.9µl of Taq DNA polymerase enzyme (3U/µl) and 70.1µl RNase free water in PCR tube and overlaid with 100µl mineral oil . 24 PCR cycles of GAPDH (94ºC for 1 min, 62ºC for 1 min, 72ºC for 1 min) and 30 PCR cycles of eNOS (94ºC for 1min , 62ºC for 1min, 72ºC for 1 min) followed by 1 cycle at 57ºC for 2 min and 72ºC for 7min were performed using 1 half of the reverse transcription mixture (Biorad , MJ Mini ermal cycler), sense and antisense primers for eNOS: (5'-TCCAGAAACACAGACAGTGCA-3' and 5'–CAGGAAGTAAGTGAGAGC -3'resp.) and for GAPDH (5'-TCCCTCAAGATTGTCAGCAA -3' and 5'- AGATCCACAACGGATACATT -3' resp.) were used. The PCR products so obtained were analyzed on ethidium bromide-stained agarose (1.5%) gel on Gel electrophoresis apparatus (Biorad). e eNOS and GADPH products were quanti ed using the image (Gel Doc EZ image, Biorad) and the amount of eNOS was normalized with respect to the amount of GAPDH product [11].

Statistical analysis

All values will be expressed as mean ± S.D. e data for aortic ring preparations were statistically analyzed using repeated measures of ANOVA followed by Newman-Keul` test. e data for aortic glutathione level, serum nitrate/nitrite level, Mean arterial blood pressure (MABP) were statistically analyzed using one-way ANOVA followed by Tukey`s multiple comparison tests. e p-value <0.05 was considered to be statistically signi cant.

Results

E ect of pharmacological interventions on mean arterial blood

Uninephrectomy + DOCA salt-induced hypertension for six weeks has been noted to produce vascular endothelial dysfunction. Uninephrectomy + DOCA administration increases mean arterial blood pressure (MABP) in rats a er 5 weeks as compared to normal control group. Treatment with Daidzein (0.2mg/kg/day, 0.4mg/kg/day, s.c) and lisinopril for the 1st week started from the 5th week of DOCA Salt administration signi cantly decreased mean arterial blood pressure (MABP) as compared to hypertensive rats [12-14].

E ect of pharmacological interventions on serum nitrite/nitrate concentration

e serum concentration of nitrite/nitrate was noted to signi cantly reduced in Hypertensive control (DOCA, 40 mg/kg"', s.c.) as compared to normal control. Treatment with Daidzein (0.2mg/kg/day, 0.4mg/kg/day, s.c) and lisinopril for the 1st week started from the 5th week of DOCA Salt administration signi cantly increased the serum concentration of nitrite/nitrate as compared to hypertensive control.

E ect of pharmacological interventions on reduced glutathione level in thoracic aorta

Aortic GSH concentration was noted to signi cantly reduce in hypertensive control a er 6 weeks in comparison to normal control rats. Treatment with Daidzein (0.2mg/kg/day, 0.4mg/kg/day, s.c) and lisinopril for the 1st week started from the 5th week of DOCA Salt administration signi cantly increased the serum concentration of nitrite/nitrate as compared to hypertensive control.

E ect of Pharmacological Interventions on endothelium-dependent and independent relaxation: - Ach and SNP in a dose-dependent manner produced endothelium-dependent and independent relaxation in phenylephrine ($3X10^{-6}$M) pre-contracted isolated rat aortic ring preparation. DOCA (40 mg kg^{-1}, s.c.) induced hypertension markedly attenuated Acetylcholine-induced endothelium-dependent relaxation but they did not a ect SNP-induced endothelium-independent relaxation. Moreover, Daidzein (0.2mg/kg/day, 0.4mg/kg/day, s.c) and lisinopril for the 1st week started from the 5th week of DOCA Salt administration signi cantly prevented hypertension induced attenuation of Ach-induced endothelium-dependent relaxation.

E ect of pharmacological interventions on TNFα levels

Administration of DOCA-Salt (40 mg/kg, s.c.) for 6 weeks caused a signi cant increase in TNFα levels as compared to normal control rats. Treatment with Daidzein (0.2mg/kg/day, 0.4mg/kg/day, s.c) and lisinopril for the 1st week started from the 5th week of DOCA Salt administration signi cantly reduced the TNFα levels as compared to hypertensive control.

E ect of pharmacological interventions on mRNA expression of eNOS

Administration of DOCA-Salt (40 mg/kg, s.c.) for 6 weeks caused a

signi cant signi cant decrease in expression ratio of eNOS/GAPDH as compared to normal control rats. Treatment with Daidzein (0.2mg/kg/day, 0.4mg/kg/day, s.c) and lisinopril for the 1st week started from the 5th week of DOCA Salt administration signi cantly reduced the TNFα levels as compared to hypertensive control.

Discussion

e major ndings of our study are that blood pressure lowering e ects of Daidzein are associated with increased activation of eNOS and increased serum NO levels. Hypertension is the most common cardiovascular disorder worldwide, and oxidative stress is a major contributor to the elevation of blood pressure and its complication [1-3, 6]. Our ndings, as well as previous studies, demonstrated that DOCA/HS administration in rats display many characteristics of hypertension including increased SBP, DBP, MBP, oxidative stress, and decreased serum NO levels and eNOS activation. Uninephrectomy + DOCA administration increased mean arterial blood pressure (MABP) in rats a er 5 weeks as compared to normal control group [15].

Vascular endothelial dysfunction was assessed in terms of decreased serum nitrite/nitrate conc., reduced aortic glutathione levels, ACh-induced endothelium-independent vasorelaxation, and SNP-induced endothelium-independent vasorelaxation. e earlier results suggest that NO is, in fact, the most important endothelium-derived mediator

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