CHAPTER 2

MATERIALS AND METHODS
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2.1. Materials

MCDB131 medium, antibiotic-antimycotic solution, o-phenylene diamine dihydrochloride, diethyl pyro carbamate, diamino benzidine, Tris, glycine, protease inhibitor cocktail, calphostin C, caspase-3 assay kit, annexin-V- Cy3 apoptosis detection kit, 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, bovine serum albumin, HEPES, collagenase, snake venom phosohodiesterase, PGE2, PGD2, ADP agarose, collagen type I, polylysine, human plasma fibronectin, protein A sepharose, ethidium bromide, acridine orange, SB202190, PD98059, wortmannin, monoclonal antibodies against VEGF, FGF, E- selectin, von Willebrand factor, CD31, eNOS, Bcl-2, Bax, P-Thr, P-Ser, P-Tyr, COX-2, c-Fos, c-Jun, polyclonal antibody of phospho p38 MAPK, FITC conjugated and horse radish peroxidase- conjugated secondary antibodies were purchased from M/s Sigma Aldrich Co USA. Antibody against α5β1 integrin was a product of GIBCO BRL products, USA. Monoclonal anti- PAR antibody was purchased from Trevigian, USA. Monoclonal antibody against NF-kB (p65) was from Chemicon, CA, USA. Monoclonal antibodies against FAK and Src were kindly provided by Dr. Anil Kumar, London, UK. Perfect RNA Mini isolation kits and C- Master RT Plus PCR kits were purchased from Eppendorf, Germany. Gelatin-sepharose was purchased from Amersham, USA and Heparin-sepharose beads were from Pharmacia. FURA 2AM was purchased from Calbiochem, USA. NC membrane was from BIORAD, USA. Tissue culture plastic wares were purchased from NUNC, Denmark. Chick embryos were obtained from Regional poultry farm, Thiruvananthapuram. All other chemials used were of extra pure analytical grade from Merck, India. 14C L-arginine and 3[H]-thymidine were purchased from BARC, Mumbai, India.

2.2. Instruments Used for the Study

Carbon dioxide incubator – Sanyo, Japan; Refrigerated microcentrifuge, Thermocycler and Bio-photometer – Eppendorf, Germany; Fluorescence microscope – Leica, Germany; Inverted phase contrast microscope – Olympus, Japan; Luminescence spectrophotometer and TriLux micro beta scintillation counter – Perkin Elmer, U.S.A.; UV-Vis Spectrophotometer and HPLC system – Shimadzu, Japan; Gel documentation system and Mini double gel electrophoretic apparatus – BioRad, U.S.A.; Semidry Western blotting apparatus – Biometra, Germany ; Multiskan
spectrum multiwell plate reader – Thermo, USA.

2.3. Methods

2.3.1. Isolation and Culture of HUVECs

Endothelial cells were isolated by collagenase perfusion of the umbilical vein according to the procedure of Jaffe et al with certain modifications (Jaffe et al., 1973). A sterile technique was used in all manipulations of the cord. The cord was severed from the placenta soon after birth, clamped at both ends, placed in a sterile container filled with minimum essential medium containing antibiotics and held at 4°C until processing. The umbilical vein was cannulated with a blunt needle, about 4 cms long with polyethylene covering and the needle was secured by clamping the cord over the needle. The vein was perfused with PBS to wash out the blood and allowed to drain. The other end of the vein was also clamped. 0.1% collagenase in MCDB 131 was then infused into the umbilical vein. The cord, suspended by its ends, was placed in a sterile container containing PBS and incubated at 37°C for 30 minutes. After incubation, the collagenase solution containing the endothelial cells was flushed from the cord. The cells were sedimented by centrifugation and the cell pellet was resuspended in fresh culture medium (MCDB 131). The cell suspension was plated in plastic petri dishes. The viability of isolated HUVECs, was determined by Trypan blue exclusion test, was found to be more than 85%. The purity of the cell preparation was estimated by FACS analysis using monoclonal antibody against CD 31.

2.3.2. The Rat Aortic Ring Assay

Aortic ring assay was carried out by the method of Nicosia (Nicosia and Ottinetti, 1990). Thoracic aortas excised from 4-6 week old Albino rats (Sprague-Dawley strain) were freed of the surrounding fibroadipose tissue using a fine micro dissecting forceps under sterile conditions. They were then cross sectioned to obtain 1-mm long aortic rings with a sterile sharp surgical blade. These rings were rinsed with MCDB 131 and placed individually on the bottom of the culture dishes. Medium was added and the cultures were maintained in a CO₂ incubator at 37°C in 95% air/ 5% CO₂ humid atmosphere. The morphological changes were examined microphotographically. All experiments were carried out in accordance with the guidelines laid down by the Institutional Ethical Committee.
2.3.3. Chorio Allantoic Membrane (CAM) Assay

CAM assay was performed as described earlier (Ribatti et al., 1997). Briefly, fertilized chick eggs (White Leghorn) were incubated for 4 days at 37°C and at a relative humidity of 80%. The eggs were positioned with pointed end down during this period and rotated several times. The eggs were opened on the air sac side after the incubation by carefully removing the shell with forceps and the samples soaked in filter discs were applied on to the CAM. After covering the cavity with parafilm, the eggs were incubated at 37°C at a relative humidity of 80% for further 8 days. At the end of the incubation period, the CAMs were photographed, isolated and the level of hemoglobin in the CAM, as a measure of vessel density, was estimated using Drabkin's reagent. The neovasculature formed was homogenized in Drabkin's reagent, centrifuged and the absorbance of the clear supernatant was recorded at 546 nm. CAMs treated with filter discs soaked in vehicle control served as the control.

2.3.4. Preparation of Substratum, Cell Culture and Attachment Assay

NUNC tissue culture dishes were coated passively with matrix proteins like Col I, FN, HBD of FN (20 µg/ml in phosphate buffered saline, 0.015M phosphate buffer/0.15M NaCl, pH 7.0) or poly lysine at room temperature. The coated plates were washed with 1M NaCl followed by repeated washing with PBS before seeding the cells.

Freshly isolated endothelial cells (1.5 x 10⁶ cells/ml) were seeded in tissue culture plates passively coated with Col I, FN, HBD of FN or polylysine. The cells were allowed to attach overnight, unattached cells were removed and attached cells were maintained in culture in serum-free MCDB131 medium in a Sanyo carbon dioxide incubator at 37°C and 95% air/5% CO₂, humidified atmosphere. Morphological changes were examined microphotographically. The cultures were immunostained for the expression of endothelial cell specific markers such as CD31 and vWF.

The cells were maintained in culture for specific time intervals and the medium was collected, centrifuged and the unattached cells were taken. The attached cell layer was harvested. Both attached and unattached cells were lysed and the protein was estimated. The results were expressed as percentage attachment. The effect of glycosaminoglycans (GAGs) on attachment of cells on FN and HBD of FN was assessed by incubating the cells with 100µg/ml of GAGs such as heparin, chondroitin.
sulphate A or hyaluronic acid for 2 hours (Kumar and Sudhakaran, 1992) and performing attachment assay as mentioned above.

2.3.5. FACS Analysis

Freshly isolated HUVECs suspended in sheath fluid was washed twice with 1% BSA in sheath fluid and about 25,000-50,000 cells were suspended in 1% BSA. The cells were then incubated for 15 minutes at room temperature with 5µl of primary antibody. Cells were then washed thrice with 1% BSA in sheath fluid and incubated at room temperature for further 15 minutes with 5µl of FITC conjugated secondary antibody. Cells were again washed thrice with 1% BSA in sheath fluid and analysed using a flow cytometer (BD FACS CALIBUR) at Regional Cancer Centre, Thiruvananthapuram.

2.3.6. Immunocytochemistry

HUVECs maintained in culture on coverslips were fixed with 3% paraformaldehyde in PBS for 40 minutes at room temperature. After washing twice with PBS, the coverslips were incubated with 50 mM ammonium chloride in PBS for 10 minutes and washed again with PBS. Free binding sites were blocked using 2% gelatin in PBS for 30 minutes followed by washing and incubation with primary antibody (1:50) at 4°C overnight. After washing thrice with gelatin-PBS, the coverslips were incubated with FITC conjugated secondary antibody for 30 minutes at room temperature and washed thoroughly with gelatin-PBS and finally with PBS. The fluorescent staining was visualized using Leica fluorescent microscope and photographed.

2.3.7. Fragmentation of Fibronectin and Isolation of Heparin Binding Domain (HBD)

Human plasma fibronectin (3mg/ml) was subjected to digestion at 30°C by trypsin (final concentration of 0.2%) in Tris/HCl buffer (50mM, pH 7.0/CaCl₂, 1mM/NaCl, 30mM) for 30 minutes as described before (Garcia-Pardo et al., 1987; Kumar and Sudhakaran, 1992; McCarthy et al., 1988). The tryptic digest was dialyzed, subjected to prechromatography over gelatin-sepharose and the HBD of FN was isolated by affinity chromatography over heparin-sepharose as described before (Kumar and Sudhakaran, 1992). The purity of the fragment was tested by SDS-PAGE.
2.3.8. Estimation of Protein

Protein was measured by Lowry's method (Lowry et al., 1951). Different volumes of sample and standard BSA (1 mg/ml) were taken and made up to 100 µl with 0.1N NaOH. To this 1 ml of alkaline copper reagent [A+B in 1:50 ratio; A-1% copper sulphate + 2% sodium potassium tartarate (1:1) B- Sodium carbonate (2% in 0.1 N NaOH)] was added and kept for 10 minutes. After adding 100 µl of Folin phenol reagent (1:1 with water), the solution was allowed to stand for 30 minutes before measuring the optical density at 650 nm.

2.3.9. Enzyme Linked Immunosorbent Assay (ELISA)

Amount of VEGF, PAR modified VEGF, FGF2, COX-2, E-selectin, Bcl-2, Bax, CD 31, eNOS and phosphorylated eNOS at Thr and Ser residues were quantitated by ELISA by the method of Engvall (Engvall and Perlman, 1971). Different concentrations of antigen were coated on the wells of multiwell plate. After incubating overnight at 37°C, the wells were washed with PBS. Free binding sites were blocked for 1 hour using 0.2% gelatin in PBS containing 0.05% Tween 20. After the incubation period, the wells were washed with PBS-Tween and the primary antibody diluted in PBS-Tween was added to the wells and incubated for 3 hours at room temperature. After extensive washing in PBS-Tween, the wells were treated with HRP conjugated secondary antibody for 1.5 hours at room temperature. This was followed by the addition of the chromogen solution (containing o-phenylene diamine dihydrochloride as substrate in citrate-phosphate buffer (0.1 M, pH 5.0) and 30% H₂O₂). The reaction was stopped by the addition of 3N HCl. Color intensity at 495 nm was read in a multiwell microplate reader (Thermo Multiskan Spectrum).

2.3.10. Isolation of VEGF and Assay of Angiogenic Activity

HUVECs in MCDB131 medium were seeded in culture dishes, passively coated with various matrix substrata, allowed to attach overnight at 37°C in a 95% air and 5% CO₂ atmosphere. The medium was collected, centrifuged to remove cell debris and protease inhibitor cocktail was added. The medium was incubated with heparin-Sepharose beads at 4°C overnight with gentle agitation. The beads were then washed thrice with Tris-HCl buffer (20 mM, pH 7.8/NaCl, 0.4 mM). VEGF was eluted from the beads using 1.5 M NaCl in the above buffer. Recovery of VEGF was determined by ELISA. Assay of angiogenic activity of the heparin binding fraction was carried out in
chick CAM using aliquots of the eluted samples containing equivalent amounts of VEGF as determined by ELISA.

2.3.11. Analysis of VEGF Bound PAR

The extent of PAR modification of VEGF was analyzed by digesting equivalent amounts of VEGF secreted by cells with pronase, followed by phosphodiesterase. Affinity purified VEGF samples were subjected to pronase digestion (1 mg/ml pronase in Tris HCl buffer, pH 7.5) for an hour (Shima et al., 1970), and stopped by adding 15% perchloric acid. The supernatant was neutralized with 1.8 N KOH and the purity of PAR was confirmed by measuring the ratio of A$_{230}$ to A$_{260}$ (0.2389) (Kanai et al., 1980). The supernatant was further subjected to phosphodiesterase digestion (100 µg snake venom phosphodiesterase/ml of Tris HCl buffer pH 8 containing 1 mM MgCl$_2$) for an hour and the reaction was stopped by the addition of 15% perchloric acid and the supernatant neutralized with KOH. Phosphoribosyl AMP (PR-AMP), the major product of phosphodiesterase digestion, was quantitated by HPLC with ADP ribose as the standard (PR-AMP corresponds to ϕADP ribose) (Fujimura et al., 1967). The amount of PR-AMP liberated was quantitated at 260 nm by isocratic HPLC on a C18 column with a solvent system consisting of potassium phosphate buffer (pH 6): methanol (75:25), in a Shimadzu LC-10AT instrument.

2.3.12. Dot Blot Assay

FN and HBD (1 µg of each) were spotted on NC membrane, allowed to dry and blocked with 2.5% BSA in binding buffer (Tris, 20 mM, pH 7.5/NaCl, 0.15 mM/0.1% BSA). The NC membrane was then incubated with the affinity isolated VEGF from medium for 30 minutes at 37°C, washed with binding buffer, incubated with monoclonal anti-human VEGF/monoclonal anti-PAR antibody at a dilution of 1:1000, followed by incubation with the secondary anti-mouse IgG conjugated to horseradish peroxidase (dilution of 1:2,000). The bands were detected by staining with DAB and quantitated the relative intensity of the spots using BIORAD Quantity One 4.5.0 Software.

2.3.13. Solid-Phase VEGF Binding Assay

Microtiter plates were coated with FN and HBD (50 µg/ml each) and allowed to dry at 37°C. The wells were blocked with PBS containing 2% BSA and incubated with
the affinity isolated VEGF from media for 6 hours at 37°C. After washing, the plates were either used for ELISA using monoclonal anti-human VEGF/monoclonal anti-PAR antibodies or the bound VEGF was eluted with Tris-HCl containing 1.5 M NaCl and used for chick CAM assay.

2.3.14. Semi Quantitative Reverse Transcription-PCR (RT-PCR)

Total RNA from HUVECs maintained in culture on different substrata was isolated using Perfect RNA Mini isolation kit (Eppendorf, Germany) according to manufacturer's instruction.

<table>
<thead>
<tr>
<th>Table 2.1.: Primer sequences for RT-PCR</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Forward Primer (5’-3’)</td>
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<tr>
<td>------------------------</td>
</tr>
<tr>
<td>VEGF-A (105bp)</td>
</tr>
<tr>
<td>VEGFR2 (216bp)</td>
</tr>
<tr>
<td>VEGFR1 (370bp)</td>
</tr>
<tr>
<td>FGF (100 bp)</td>
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<td>COX-1 (299 bp)</td>
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<tr>
<td>COX-2 (452 bp)</td>
</tr>
<tr>
<td>eNOS (280 bp)</td>
</tr>
<tr>
<td>Bax (338 bp)</td>
</tr>
<tr>
<td>Bcl-2 (447bp)</td>
</tr>
<tr>
<td>GAPDH (680bp)</td>
</tr>
</tbody>
</table>

The primer sequences were selected from NCBI nucleotide database (Table: 2.1.) and custom synthesized by Sigma Aldrich Chemicals, Bangalore, India. RT-PCR was performed in an Eppendorf thermocycler, in a single step. 20 µl (2 µg) of the isolated RNA was used as template for reverse transcription and amplification. The
reaction mixture was incubated at 53°C for 60 minutes for reverse transcription and 94°C for 2 minutes for initial denaturation and cycled 30 times each at 94°C for 15 seconds (template denaturation), 58°C for 30 seconds (primer annealing) and 68°C for 45 seconds (primer elongation). Appropriate negative controls were used without reverse transcriptase. PCR products were resolved in a 1.75% agarose gel containing ethidium bromide and the bands were visualized by UV transillumination in a BioRad gel doc. The relative intensity of the bands was quantitated using BIORAD Quantity One version 4.5 software.

2.3.15. SDS-Poly Acrylamide Gel Electrophoresis

SDS-PAGE was done by the method of Laemmli (Laemmli, 1970).

Reagents:
1. ABA: - Acrylamide: Bis acrylamide – 30:0.8 w/w in 100 ml
2. SDS: - 10%
3. Electrode Buffer: - Tris/Gly (0.05M, pH 8.3/0.1% SDS)
4. Spacer Buffer: - Tris/HCl (0.05M, pH 6.7)
5. Discontinuous Buffer: - Tris/HCl (3M, pH 8.9)
6. Ammonium per sulphate (fresh): - 10% (w/v)
7. Sample Buffer (reducing gel): - Tris, pH 6.8 – 2.5 ml, SDS-4 ml, β-mercaptoethanol – 1 ml, Glycerol – 2 ml, Water – 10.5 ml
8. Coomassie blue: - 0.2% w/v in 45% methanol and 7% acetic acid

<table>
<thead>
<tr>
<th>Composition</th>
<th>Resolving gel</th>
<th>Spacer gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.5% (ml)</td>
<td>10% (ml)</td>
</tr>
<tr>
<td>ABA</td>
<td>2.5</td>
<td>3.375</td>
</tr>
<tr>
<td>Tris/HCl 8.9</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Tris/HCl 6.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>4.75</td>
<td>3.88</td>
</tr>
<tr>
<td>SDS (for reducing gels only)</td>
<td>0.100</td>
<td>0.100</td>
</tr>
<tr>
<td>APS</td>
<td>0.150</td>
<td>0.150</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.015</td>
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</tr>
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</table>

Slab gel was prepared between two perfectly clean glass plates held together with two spacers between them along the sides, placed vertically and held together by clamps. After sealing the glass plates with agar, the running gel (7.5% or 10%) was poured rapidly after mixing, between glass plates upto 2.5 cm below the top. Gel was
overlaid with butanol saturated with water to achieve a flat surface. After polymerization of the resolving gel, stacking gel solution was added on top of it and the comb was inserted gently avoiding air bubbles. After polymerization, the comb was gently removed and samples were applied in each slot using a micropipette. The wells were filled to the top using electrode buffer and the assembly was placed inside the chamber. The top and bottom chambers of the apparatus were filled with electrode buffer and the electrodes were connected to the power supply. The electrophoresis was run at a current of 7mA till the sample entered the resolving gel and 14mA thereafter till the marker dye reached the bottom of the gel. At the end of the run, glass plates were removed from the chamber and after removing the spacers, the gel was taken out gently, washed and stained with coomassie blue. The dye was removed after overnight staining and the gel was destained using destaining solvent till the bands become visible.

2.3.16. Western Blotting

Western blotting was done using a semidry apparatus according to the procedure of Towbin et al (Towbin et al., 1979). After electrophoresis with equivalent amounts of protein, the polyacrylamide gel (10%) was taken out quickly and the required portion was cut out. The gel was soaked and incubated in transfer buffer (0.025M Tris, 0.15M glycine, pH 8.3, 20% methanol) for an hour. The nitrocellulose membrane and filter paper were cut to the same size of the gel and the NC membrane was also soaked in the transfer buffer. The filter paper, NC membrane, gel and filter paper were arranged in the order stated on the anode of semidry apparatus in the form of a sandwich, carefully avoiding air bubbles and the lid (cathode) was placed on top. A constant current of 1mA/cm² was given for 1 hour. After blotting, the NC membrane was treated with blocking solution (2.5% BSA in PBS-Tween) for 2 hours, washed in PBS-Tween and incubated with primary antibody (1:1000) overnight at 4°C. After washing thoroughly with PBS-Tween, the membrane was incubated with the secondary antibody conjugated to horseradish peroxidase (dilution of 1:2,000) for 2 hours. The membrane was again washed in PBS-Tween and the bands were detected by staining with DAB and the relative intensity of bands was quantitated using BIORAD Quantity One version 4.5 software in a BioRad gel doc.

2.3.17. Silver Staining

After SDS PAGE, the gel was washed in washing solution (1% formalin, 40%
ethanol) for 10 minutes with slow shaking followed by rinsing with distilled water for 2 minutes (Shevchenko et al., 1996). The gel was then soaked in sodium thiosulphate (0.02%) for 1-2 minutes, washed twice with distilled water (1-2 minutes each) and soaked in silver nitrate (0.1%) with gentle shaking. This was followed by washing twice with distilled water (1-2 minutes each) and developing using developer (3 g sodium carbonate in 100ml distilled water containing 1 ml formalin and 1 ml 0.02% sodium thiosulphate). After 5 minutes, the reaction was stopped by adding 5% acetic acid.

2.3.18. Immunoprecipitation

Media were collected and cells harvested after exposure to different culture conditions. Media were centrifuged at 12,000 g for 5 minutes to remove cell debris and mixed with protease inhibitor cocktail whereas the cells were lysed in lysis buffer [RIPA buffer containing 1% sodium deoxycholate, 0.1% SDS, 0.01 M Tris, pH 8.0, 0.14 M NaCl (1mM sodium ortho vanadate and 20mM sodium fluoride were added in experiments where phosphorylation was detected)] containing protease inhibitor cocktail. To the media or cell lysate, equivalent to same amount of protein of interest, 4 µl of the respective antibody was added and kept overnight at 4°C with occasional shaking. 25 µl of protein A sepharose beads were then added and incubated for another 6 hours at 4°C. The sepharose beads were collected by centrifugation, washed twice in RIPA buffer and once with TSA buffer (0.01 M Tris, pH 8.0/ 0.14 M NaCl) and finally with 0.05 M Tris, pH 6.8. After boiling for 5 minutes in SDS gel loading buffer, supernatants were collected and used for further analysis.

2.3.19. Assay of NAD⁺

The concentration of NAD⁺ was estimated fluorimetrically (Estabrook et al., 1968). 1 ml aliquot of cell suspension was added to 0.5 ml of 15% perchloric acid solution and mixed vigorously in ice. Denatured protein and debris were removed by centrifugation and the supernatant was neutralized by slow addition of 0.5 ml of 1.8 N KOH. 0.4 ml of neutralized acid extract was diluted to 2 ml with a buffer containing 0.2 M glycine, 0.4 M hydrazine hydrate and 0.15 M ethanol pH 9.0. After compensating for the background fluorescence of the samples, the increase in fluorescence occurring on addition of Alcohol dehydrogenase (1500 units) was determined with an excitation wavelength of 340 nm and an emission wavelength of 470 nm in a Perkin Elmer LS45 luminescence spectrometer. Freshly prepared NAD⁺ solution was used as the standard.
2.3.20. MTT Assay

The viability of HUVECs was assessed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay by the procedure of Tada et al. with certain modifications (Tada et al., 1986). Briefly, MTT solution (5 mg/ml PBS) was added to the well to a final concentration of 0.5 mg/ml. After incubation for 6 hours, the mixed media and MTT solution were carefully discarded, washed the cell layer with PBS and then the crystallized dye was solubilized with DMSO by incubating for 2 hours. The amount of purple blue dye formed was determined by measuring the absorbance at 570 nm.

2.3.21. Cell Proliferation Assay - $^3$H-Thymidine Incorporation

HUVECs in culture were incubated with $^3$H-thymidine (15µCi/ml medium) on each day for 24 hours. After the incubation period, the medium was removed, the cell layer was washed with PBS, harvested and incubated with 0.5M NaOH for 1 hour at 37°C. After thoroughly mixing with 1% BSA, TCA was added to a final concentration of 10%. The precipitate obtained on centrifugation was washed with 10% TCA and heated in a boiling water bath with 0.5M perchloric acid. The supernatant after centrifugation was used for radioactive counting.

2.3.22. Acridine Orange/Ethidium Bromide Staining

HUVECs were stained with 2 µl of acridine orange (100 µg/ml) and 2 µl of ethidium bromide (100 µg/ml) and then examined by Leica fluorescent microscope.

2.3.23. Annexin V-Cy3 Staining

Annexin staining was done using the kit from Sigma Chemicals Co. as per the manufacturer’s instructions. Cells were incubated with double label staining solution (binding buffer containing AnnCy3 and 6-CFDA) for 10 minutes, washed thrice with binding buffer and immediately observed under a Leica fluorescent microscope and photographed. Live cells were labeled only with 6-CFDA (green) while necrotic cells were labeled only with AnnCy3 (red). Cells in the early stage of apoptosis however get labeled with both 6-CFDA and AnnCy3.

2.3.24. DNA Fragmentation - Colorimetric Assay

Fragmented and intact DNA were evaluated as described earlier (Colotta et al., 1992). Briefly, the cell pellet was lysed with hypotonic buffer (10mM Tris pH 7.5, 1mM EDTA & 0.2% Triton X-100) by incubating on ice for 15 minutes. The low and
high molecular weight DNAs were separated by centrifugation at 13,000g at 4°C for 20 minutes. The supernatant containing the low molecular weight DNA was precipitated with 12.5% TCA for 18 hours and the pellets were treated with cold 12.5% TCA. Both the samples were then centrifuged at 13,000g at 4°C for 5 minutes, and DNAs in the precipitates were extracted with 5mM NaOH and 1M perchloric acid at 70°C for 20 minutes. Then diphenyl amine reagent was added and samples were incubated at 37°C for 18 hours. The absorbance was measured at 600 nm in an automated plate reader (Thermo MultiSkAn Spectrum).

2.3.25. Caspase-3 Assay (EC. 3.4.22.56)

Caspase-3 was assayed colorimetrically using caspase assay kit from Sigma Chemicals Co. USA as per manufacturer's instructions. This assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitro anilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of the pNA. The concentration of pNA released from the substrate was calculated from the absorbance values at 405 nm and from a calibration curve prepared with pNA solutions of known concentrations.


COX was assayed by oxygraphic method (Fritsche et al., 2001). The enzyme extract was incubated in 0.1M Tris-HCl (pH 8.0) containing 5 mM EDTA, 2 mM phenol and 1 mM hematin at 37°C. The reaction was started by the addition of 100 µM arachidonic acid and monitored by measuring the amount of oxygen used, using an Oxygraph (Hansa Tech, England). The rate of the reaction (linear slope) in nanomoles of oxygen used/minute was recorded.

2.3.27. Estimation of Prostaglandins, PG E₂ and PG D₂

The level of prostaglandins in the culture supernatants were determined by HPLC by the method of Holtmann et al (Holtmann et al., 1990). Briefly, the culture medium was subjected to solid-phase extraction on 1ml Sep Pak C18 columns (Waters, Massachusetts). The eluates containing the eicosanoids were concentrated under a stream of nitrogen. Initial calibration of the C18 column was done using PG E₂ and PG D₂ standards by isocratic reversed-phase high-pressure liquid chromatography at a flow rate of 2ml/min in a Shimadzu LC-AT HPLC and the mobile phase consisted of acetonitrile (31.9% v/v), acetic acid (0.1% v/v) and water (68% v/v) adjusted to pH 4.5 with sodium acetate. Each sample was analysed for the separation and determination of
PG E₂ and PG D₂ in a single run. Peak areas corresponding to different concentrations of PG E₂ and PG D₂ were determined and a calibration curve was constructed from which the concentrations of PG E₂ and PG D₂ in the samples were calculated.

2.3.28. Assay of NOS (EC. 1.14.13.39)

The whole cell assay of NOS was carried out according to the procedure of Demura et al. (Demura et al., 1998) using [¹⁴C]-α-arginine as substrate. Briefly, sub confluent HUVECs pre-equilibrated with Kreb's Hansleit buffer were incubated for 10 minutes with 1 µCi of [¹⁴C]-α-arginine and 10 mM L-αarginine in the same buffer. To assess the calcium dependence of NOS activation, experiments were performed under calcium free conditions. The reaction was stopped using cold HEPES buffer containing 4 mM EDTA and supernatant was removed. Cell protein was precipitated by adding absolute ethanol, the soluble amino acids and derivatives were extracted using HEPES buffer (pH 5) and mixed with Dowex G50 WX-8 Na⁺ resin and vigorously shaken. Radioactivity in the unbound fraction was measured by liquid scintillation counting using Perkin Elmer microbeta counter. The NOS activity was expressed as picomol of citrulline released/mg protein. The activity in the absence of calcium gives iNOS activity.

2.3.29. Isolation of eNOS

eNOS was isolated from cultured cells by the procedure of Garvey et al (Garvey et al., 1994). Briefly, cells were harvested and lysed in RIPA buffer containing 1 mM sodium ortho vanadate and 20 mM sodium fluoride. The supernatant was applied to 2'5'-ADP agarose, pre equilibrated with HEDS buffer (20 mM HEPES pH 7.8, 0.1 mM EDTA, 5 mM DTT and 0.2 M sucrose). eNOS was eluted from the column by 2 mM NADPH in HEDS buffer. The recovery of eNOS was confirmed by ELISA using monoclonal anti-eNOS antibody.

2.3.30. Assay of Intracellular Calcium Ions

Ca²⁺ levels in HUVECs were analysed fluorimetrically according to the procedure of Simpson, 2006. Freshly harvested HUVECs were incubated in Kreb's-HEPES buffer containing Fura-2-AM (5µM) for 30 minutes at 37°C followed by 20 minutes at room temperature to allow for complete dye deesterification. Loaded cells were washed twice, resuspended in Kreb's-HEPES buffer and maintained at room temperature in dark. Cell suspension was taken in a quartz cuvette and equilibrated at
22°C for 3 minutes and fluorescence was measured in a Perkin Elmer-LS45 fluorimeter, where excitation wavelengths were changed every 2 seconds between 340 and 380 nm and emission light was detected at 510 nm. [Ca\textsuperscript{2+}]i was calculated from the 340/380 ratio according to Grynkiewicz et al., 1985, where R\textsubscript{max} and R\textsubscript{min} were determined using triton X 100 (0.1% v/v) and EGTA (4.5 mM). K\textsubscript{D} of Ca\textsuperscript{2+} for FURA-2 was 145 nM at 22°C. The intracellular free Ca\textsuperscript{2+} was calculated according to the equation:

\[
[\text{Ca}^{2+}]_i = \beta K_D (R - R_{\text{min}})/(R_{\text{max}} - R)
\]

where R is E\textsubscript{340}/E\textsubscript{380}; R\textsubscript{min} is E\textsubscript{340}/E\textsubscript{380} in zero calcium; R\textsubscript{max} is E\textsubscript{340}/E\textsubscript{380} in Ca\textsuperscript{2+} saturated solution and K\textsubscript{D} is the dissociation constant of dye. \beta is E\textsubscript{380} in zero Ca\textsuperscript{2+}/E\textsubscript{380} in Ca\textsuperscript{2+} in Ca\textsuperscript{2+} saturated solution.

2.3.31. Assay of Calmodulin – HPLC

Calmodulin (CaM) activity in the cells was assayed by measuring its ability to stimulate the activator-deficient phosphodiesterase (PDE), which converts cAMP to 5'-AMP. The 5'-AMP released during the reaction was measured by HPLC according to the procedure of Watterson et al. (Watterson et al., 1980). The cells were extracted in 40 mM HEPES buffer (pH 7.0), 0.5 M NaCl, 5 mM CaCl\textsubscript{2}, and 28 mM L-ascorbic acid, centrifuged, and the supernatant was heated to 90°C for 5 minutes in a hot water bath. The heat-denatured proteins were removed by spinning at 12,500g for 5 minutes and the supernatant obtained was used for assaying calmodulin activity. The PDE assay mixture was composed of 40 mM Tris/HCl (pH 8.0), 1 mM CaCl\textsubscript{2}, 0.4 mM MnCl\textsubscript{2}, and 2 mM cAMP. The reaction was started by adding PDE to a final concentration of 0.02 units/ml, incubated at 30°C for 20 minutes and was stopped by heating in a boiling water bath for 5 minutes. 5'-AMP produced during the enzymatic reaction was quantitated using a C18 column. The mobile phase consisted of 10 mM KH\textsubscript{2}PO\textsubscript{4} (pH 2.5) delivered at the rate of 1.5 ml/minutes. The absorbance was monitored at 260 nm. The activity was expressed as nanomoles of AMP released/min/mg protein.

2.3.32. Estimation of Nitrosothiol Content

Nitrosothiol content was determined according to the procedure of Saville (Saville, 1958). Briefly, 180 µl of each sample was incubated with 30 µl of 0.5% ammonium sulphamate in water for 2 minutes. A total of 300 µl of 2.7% sulphanilamide and 0.25% HgCl\textsubscript{2} in 0.4N HCl were added followed by 240 µl of 0.1% N-(1-naphthyl) ethylenediamine in water. The controls were incubated without HgCl\textsubscript{2}. The OD of formed azo compound formed was determined after 20 minutes at 540 nm.
2.3.33. Estimation of Nitrite

The amount of nitrite formed was determined by Griess reaction, as described previously (Xiong et al., 1998). Briefly, 75 µl of culture medium was placed in a 96-well plate, followed by 75 µl of cold 350 mM ammonium chloride, pH 9.6. 150 µl of a mixture of 1 part 5 mM sulfanilic acid, 1 part 5 mM N-(1-naphthyl) ethylenediamine, and 3 parts glacial acetic acid was added. After 10 minutes of incubation in the dark at room temperature, absorbance at 570 nm was determined using a microplate reader (Thermo Multiskan Spectrum).

2.3.34. Preparation of Nuclear Fractions of HUVEC Lysates

Nuclear fraction of HUVECs was prepared as described before (Savani et al., 2001). HUVECs cultured in T-75 flasks were washed with Ca\(^{2+}\)/Mg\(^{2+}\)-free PBS. The cells were then harvested and centrifuged at 3000 rpm for 5 minutes. The pellets were washed twice with ice-cold PBS and then resuspended in 1 ml of fresh Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl\(_2\), 0.2% NP-40, 1.0 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride), incubated for 5 minutes on ice with occasional stirring and then centrifuged at 300 rpm for 5 minutes. Nuclear extracts were then released by resuspending the pellets in Buffer B (20 mM HEPES, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl\(_2\), 25% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and 1.0 µg/liter leupeptin and pepstatin) and incubating the tubes on ice for 10 minutes with intermittent gentle shaking. Nuclear extracts were stored at -80°C.

2.3.35. Measurement of Radioactivity

\(^3\)H and \(^14\)C radioactivity was measured using a Perkin Elmer 1450 LSC and Luminescence counter Microbeta Trilux. An aliquot of the sample was mixed with 3 ml of scintillant (PPO-6g, POPOP-0.2g, triton X\(_100\), 100 ml, methanol-80 ml and toluene to 1000 ml) and the activity was measured in liquid scintillation counter (Perkin Elmer).

2.3.36. Statistical Analysis

All the data were expressed as mean with standard error of mean. The statistical significance of difference was analysed by Duncan's One way Analysis of Variance (ANOVA) using SPSS 11.0 Software. A value of P<0.05 was considered significant.