CHAPTER 2

Materials and Methods

2.1 MATERIALS

All the chemicals were high quality analytical grade reagents. MCDB 131-medium, gentamycin, antibiotic-antimycotic solution, gelatin, acrylamide, bisacrylamide, SDS, DTT, heparin, acetyl salicylic acid, ethylene diamine tetra acetic acid, ortho-dianisidine, diethyl pyro carbonate, NAD^+, curcumin (1,7-bis(4-hydroxyl-3-methoxyphenyl)-1,6 heptadiene-3,5-dione), triton X-100, ursolic acid (3β-hydroxy-urs-12-en-28-oic acid), coomassie brilliant blue R-250, DMSO, MTT, diamino benzidine, o-phenylenediamine hydrochloride, Tris, glycine, bovine serum albumin, protein A sepharose beads, heparin sepharose, monoclonal antibodies to E-selectin, Akt, phospho p38, phospho Akt (P-Theronine), von Willebrand factor/ Factor VIII antigen, β-catenin, VE-cadherin, CD 31, CD 14, CD 71, VEGF, HRP and FITC conjugated anti-rabbit and anti mouse secondary antibodies, antibodies to c-Fos, c-Jun, MMP-9, MMP-2, TIMP-1, TIMP-2, the reagents for electrophoresis and other biochemicals were purchased from M/s Sigma Aldrich Co., St. Louis, MO. Monoclonal anti-PAR antibody was purchased from Trevigen, Inc., Gaithersburg, MD. Polyclonal anti-angiopoietin-1 (Ang-1) antibody was from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. and anti-goat HRP conjugated secondary antibody from Molecular Probes Inc., Eugene, Oregon. Anti-NFκB (p65) was from M/s Chemicon Temecula, CA. Kaleidoscope protein marker and NC membranes were

from BioRad laboratories, Hercules, CA. ELISA and tissue culture plates were from NUNC A/S, Roskilde, Denmark. Perfect RNA mini isolation kits and C-Master RT Plus PCR kits were purchased from Eppendorf AG, Hamburg, Germany. Primers to VEGF, VEGFR-1,-2, FGF-2, FGFR-1, COX-1,-2, MMP-2,-9, β-catenin, VE -cadherin were taken from NCBI nucleotide database and custom synthesized by M/s Sigma Chemicals Bangalore, India. Reagents for HPLC analysis was from MERCK (Mumbai India). All other chemicals were high quality analytical grade reagents.

2.1.1 Instruments used for the study

Carbon dioxide incubator – Sanyo, Japan; Refrigerated microcentrifuge - Eppendorf, Germany; 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-Visible 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

2.2 METHODS

2.2.1 Isolation and culture of HUVECs

Endothelial cells were isolated by collagenase perfusion of umbilical vein as described before (Jaffe et al., 1973). Briefly, the umbilical vein was cannulated with a blunt ended needle. The needle was secured by clamping the cord over the needle. The vein was perfused with 100ml sterile PBS to wash out the blood and allowed to drain. The other end of the vein was then cannulated with another blunt needle and the cord secured over the needle with a clamp. 0.1% collagenase in MCDB 131 was infused into the umbilical vein and the ends of the two needles were closed. 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 endothelial cells was flushed from the cord by infusion with 10ml sterile medium. The cells were sedimented by centrifugation and the cell pellet suspended in fresh culture medium.

The yield and viability of isolated HUVECs were determined by trypan blue staining. $1.5x10^6$ cells per well in serum free MCDB 131 medium were seeded in NUNC multi-well plates and allowed to attach for 5 hrs, unattached cells were

removed, fresh medium was added and maintained in culture overnight before starting the experiment. The purity of the cultures was assessed by checking for possible contamination with other cell population like monocyte/macrophage by monitoring expression of CD 14 and CD 71 by immunocytochemical technique. The cultures were negative for both the CD markers. Further FACS analysis for CD31 was performed and about 99.8% cells were CD31 positive.

2.2.2 Isolation of endothelial cells from rat aorta

The endothelial cells from rat aorta were isolated as described by Nicosia et al., (1994). Briefly, thoracic aortas excised from 3 weeks old Sprague Dawley rats were cut to obtain 1mm long rings after removing the surrounding fibro adipose tissue and thoroughly rinsing with MCDB 131 medium. The pieces were maintained in MCDB 131 medium in a Sanyo humidified CO_2 incubator at 95%air/5% CO_2 at 37° C. Under these conditions, the rings underwent endothelial sprouting. The aortic pieces were removed and the endothelial cells were cultured in MCDB 131 medium. The cells were trypsinised and used for subsequent experiments.

2.2.3 Aortic ring assay

Thoracic aortas excised from 3 weeks old Sprague Dawley rats were cut to obtain 1mm long rings after removing the surrounding fibro adipose tissue and thoroughly rinsing with MCDB 131 medium (Nicosia and Ottinetti, 1990). The proximal and distal two millimeter aorta, which were used to hold the explants with forceps during dissection were discarded. The aortic rings were cultured in MCDB 131 medium supplemented with and without test compounds in a humidified $C\theta_2$ incubator at 95%air/5% CO_2 at 37° C. Test compounds dissolved in absolute alcohol were added such that the final concentration of alcohol was less than 0.1%. Controls included cultures treated with vehicle alone. Media were replaced every day and the sprouting of aortic rings was monitored at regular intervals. The sprout area and length were quantitated using QWIN software (Leica) and expressed in area/mm^2.

2.2.4 Chorioallantoic membrane (CAM) assay

The CAM assay was performed as described by Brooks et al., (1999). Briefly, fertilized white leghorn eggs were incubated in a humidified incubator at 37°C. On the tenth day, eggs were candled to check prominent blood vessels and the development of the embryo. The CAM was separated from the shell by making a shallow hole at the

blunt end on the egg and another hole made perpendicular to the previously identified blood vessels in the center of the egg. The eggs were kept aside to allow the CAM to drop. Fine forceps were used to pick away the shell over the false air sac, to make a window and the CAM identified. 10 µl of 10mM test compound in alcohol or vehicle (10 µl alcohol) was pipetted onto a hydrocortisone dried filter disk and the disk was then placed on the CAM in an avascular area. The window was sealed with sterile parafilm and the egg returned to the incubator. The window was opened on the 12th day and analysed for changes in the microvessel density in the area where the filter paper was placed. The CAM was isolated and homogenized in Drabkins reagent and the amount of haemoglobin was determined as a measure of vascular density. An aliquot of the homogenate was precipitated with equal volume of 10% TCA for estimation of protein (Lowry et al., 1951).

2.2.5 Enzyme Linked Immunosorbent Assay

Indirect ELISA was performed to quantitate the amount of different antigens using specific antibody (Engvall and Perlman, 1971). Different concentrations of antigens precoated onto ELISA plates served as the antigen. After incubating for 3 hrs at room temperature, the wells were washed with PBS. Free binding sites were blocked with 0.2% gelatin in PBS (0.025M, pH 7.4, 0.15 M NaCl) containing 0.05% Tween 20. After washing the wells thrice with PBS-Tween, primary antibody diluted in the ratio 1:500 in PBS-Tween was added to the wells and incubated for 3hrs at room temperature. After extensive washing with PBS-Tween the wells were treated with secondary antibody (1:1000) for 1.5 hrs at room temperature. o-dianisidine (1ml of o-di anisidine in methanol (1%) + 210 ml of citrate phosphate buffer (0.1M, pH 5.0) + 24µl 30% H_2O_2) was used as substrate and the absorbance of the coloured HRP product was measured spectrophotometrically at 405nm by an automated microplate reader (Thermo Multiskan Sprectrum).

2.2.6 Modified Enzyme Linked Immunosorbent Assay

Expression of surface antigens of endothelial cell was quantified as described before (Gupta and Ghosh, 1999). HUVECs were plated in 96 well plates and subjected to different culture conditions for different time intervals. At regular intervals of 24 hrs the cells were fixed withl% glutaraldehyde and non specific binding was blocked using bovine serum albumin (3%) in PBS. The cells were incubated overnight at 4°C with primary antibody (1:500 in PBS Tween 0.05%) followed by washing with PBS

Tween and incubation with peroxidase conjugated secondary antibody (1:1000 in PBS Tween 0.05%). After repeated washing with PBS Tween, cells were exposed to peroxidase substrate (o-phenylenediamine dihydrochloride, 40 mg/100ml in citrate phosphate buffer pH 4.5). Reaction was stopped by the addition of 2 N sulfuric acid. Absorbance was measured at 490 nm by an automated microplate reader (Thermo Multiskan Sprectrum).

2.2.7 Separation of Curcuminoids

Curcumin mixture was separated into its components by dry column flash chromatography using silica gel (TLC grade) with chloroform as eluant; a separation of 500mg of commercial curcumin afforded 350mg of curcumin 1, 75mg of curcumin II and 8mg curcumin III (Babu and Rajasekharan, 1994).

2.2.8 RT-PCR analysis

Total RNA was isolated from HUVECs using Perfect RNA Mini isolation kits procured from Eppendorf according to the manufacturer's instruction. The primer pairs used for RT-PCR analysis are given below (Table 2.1).

The primer sequences were taken from NCBI nucleotide database and custom synthesized by Sigma Chemicals Bangalore, India. RT-PCR was performed in an Eppendorf thermocycler, using the One step RT-PCR kit. 20µl (2µg) of the isolated RNA was used as template for reverse transcription and amplification. The reaction mixture was incubated at 53 °C for 60 min for reverse transcription, 94 °C for 2 min for initial denaturation and cycled 30 times each at 94 °C for 15 sec (template denaturation), 58 °C for 30 sec (primer annealing) and 68 °C for 45 sec (primer elongation). Appropriate negative controls were taken without reverse transcriptase. PCR products were resolved in a 1.75% agarose gel containing ethidium bromide and visualized by UV transillumination. The band intensity was determined by Quantity One 4.5.0 Image acquisition and Analysis software (BioRad).

Table 2.1 The primer pairs used for RT-PCR analysis

Primers	Sequence
VEGF (105bp)	Forward 5'ACGATCGATACAGAAACCACG3 ' Reverse 5'CTCTGCGCAGAGTCTCCTCT3'
VEGFR-1 (370bp)	Forward 5'GTAAAAGTAAGGAGTCGGG3 ' Reverse 5'TCTCTATCTGGAGTTACATTCT3 '

Table 2.1 Cont'd

VEGFR-2 (216bp)	Forward 5'TGCACTGCAGACAGATCTAC3 ' Reverse 5'GCAGACATAGTCTCCTTGGT3 '
FGF-2 (100 bp)	Forward 5'AGCAGCATCTGTAAGGTTCTTC3 ' Reverse 5'TGAAACATTGGGAGGGAAAC3'
FGFR-1 (218 bp)	Forward 5'CCTGAACAGGTGGTGGTATC3 ' Reverse 5'TTTGAACTTCACTGTCTTGGC3'
COX-1 (299 bp)	Forward 5'CAAACGCTCCCATTTTTACACTC3 ' Reverse 5'TGGCATGTAGTAGTCTCTTGGCA3 '
COX-2 (452 bp)	Forward 5'AATTCCTCATCCAACTATGTTCC3 ' Reverse 5'ATACTGTTCTCCGTACCTTCACC3 '
VE-cadherin (149 bp)	Forward 5'GCACCAGTTTGGCCAATATA3 ' Reverse 5'GGGTTTTTGCATAATAAGCAGG3 '
β-catenin (291 bp)	Forward 5'TTTTTAAAGGCAAGAATGCCTCA3 ' Reverse 5'CATTAATGAAGGCAAGTAGCCCA3 '
MMP-2 (195 bp)	Forward 5'ATGGGGAATCGGTTGAAGG3 ' Reverse 5'AATTGCATTTCCTGACAGAAGG3 '
MMP-9 (147 bp)	Forward 5'GACTTGGCAGTGGAGACTGCGGGCA3' Reverse 5'GACCCCACCCCTCCTTGACAGGCAA3'
GAPDH (680bp)	Forward 5'CGGAGTCAACGGATTTGGTCGTAT -3' Reverse 5'GCAGGTCAGGTCCACCACTGAC-3'

2.2.9 Immunoprecipitation of VEGF

After exposure to different culture conditions, media from HUVECs were collected and centrifuged. Medium was concentrated after adding protease inhibitors and dialyzed extensively in double distilled water. Cells were harvested and lysed in buffer (RIPA buffer containing 1% sodium deoxy cholate, 0.1% SDS, 0.01M Tris pH 8.0, 0.14 M NaCl) containing proteinase inhibitor cocktail (Sigma Aldrich) for 30 min at 4°C. The cell lysates were centrifuged at 10,000 rpm for 15 min and supernatant fractions were collected. Media and cell lysates were immunoprecipitated for determining VEGF production (Kim et al., 2002). Briefly, 5µl of monoclonal anti human VEGF antibody was added to medium and cell lysates (protein normalized amount) and kept overnight at 4°C on a rocker in a total volume of 500µl, 25µl of protein A sepharose beads was then added and incubated at 4 °C for another 4 h. The beads were collected by centrifugation, washed two times with RIPA buffer and once in 0.05M Tris pH 6.8. The beads were boiled for 3 min in SDS gel loading buffer and the

supernatant were collected, divided into two equal portions before subjecting to electrophoresis. Separated proteins were blotted on to a NC membrane; one portion was probed with anti VEGF and the other portion, with monoclonal anti PAR antibody to analyze the amount of PAR modification on VEGF. Alternately, total cell lysates in Laemmli sample buffer were electrophoresed and blotted on to NC membrane and probed with anti VEGF antibody. The extent of PAR modification was expressed as the percent of total which was taken as the intensity corresponding to that probed with anti VEGF.

2.2.10 Akt Phosphorylation

Cell layer was washed twice in cold phosphate-buffered saline and lysed in ice with 900 µl of lysis buffer containing 1% Triton X-100, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate and 1 mM sodium ortho vanadate as previously described (Deregibus et al., 2003). Equivalent amounts of cell lysates normalized with respect to total Akt as determined by ELISA were pre-treated with protein A-protein G (1:1) agarose slurry and Akt was immunoprecipitated from clear cell lysate using anti-Akt mAb followed by adsorption on protein A beads at 4°C. Protein A beads were washed, extracted with electrophoresis sample buffer and subjected to immunoblot analysis using anti-phospho Akt.

To examine the intracellular signaling pathways that are responsive to curcumin and ursolic acid the cells in culture were treated with inhibitors of PI3K-Akt (LY294002), p38 MAPK (SB202190) and ERK (PD98059) at a concentration of 20µM for 48hrs and the expression of angiogenic markers were analysed by ELISA as described above.

2.2.11 Assay of NAD^+

The concentration of NAD^+ was estimated fluorimetrically (Estabrook et al., 1967). 300µl aliquots of cell suspension was added to 0.5ml of perchloric acid solution and mixed vigorously in ice. Denatured proteins and debris were removed by centrifugation and the supernatant was neutralized by slow addition of 0.5 ml of 0.4 M triethanolamine containing 1.8 N KOH. 0.5ml of the neutralized acid extract was diluted to 2.5ml with a buffer containing 0.2 M glycine and 0.15 M ethanol, pH 9 (adjusted with KOH). After compensating for the background fluorescence of the samples, the increase in fluorescence occurring on addition of alcohol dehydrogenase

(1500 units) was determined by excitation at 340 nm and emission at 470 nm in a Perkin Elmer LS 45 luminescence spectrometer. The system was calibrated using appropriate freshly prepared NAD^+ standard.

2.2.12 MTT Assay

The viability of HUVECs in culture maintained under different experimental conditions was assessed by performing MTT assay (Sladowski et al., 1993). Briefly HUVECs were maintained in MCDB 131 medium containing different test materials. Cells supplemented with MCDB 131 medium alone served as control. At intervals of 24 hrs the medium was removed and the cells were washed with Krebs Hanseleit buffer. The cells were then incubated with 0.5mg/ml MTT in Krebs Hanseleit buffer for 4 hrs at 37° C in a CO_2 incubator after which the solution was removed and the formazan dye formed in the cell was solubilized in DMSO. The absorbance at 570nm was measured using a multi well scanning spectrophotometer and the viability of the test groups expressed as percentage of the untreated control.

2.2.13 SDS-PAGE

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

Reagents

1. ABA- Acrylamide- Bis acrylamide- 30:0.8 w/w in 100ml
2. SDS- 10%
3. Electrode buffer- Tris/Glycine (0.05M, pH 8.3/0.1%SDS)
4. Spacer buffer- Tris/HCl (0.05M, pH 6.8)
5. Discontinous buffer- Tris/HCl (3M, pH 8.9)
6. Ammonium per sulphate (freshly prepared)- 10% (w/v)
7. Sample buffer (reducing gel)- Tris, pH-6.8-2.5ml, SDS-4ml, β-Mercaptoethanol-1ml, Glycerol- 2ml, Water-10.5ml
8. Coomassie blue- 0.2%w/v in 45% methanol and 7% acetic acid.
9. Destaining solvent- 10% methanol and 7.5% acetic acid

Composition of resolving gel	**7.5%(ml)**	**10%(ml)**
ABA	10	13
Tris, pH8.9	10	10
Water	19	16
SDS	0.4	0.4
APS	0.6	0.6

TEMED	0.06	0.06
Composition of spacer gel (ml)		
ABA	1.22	
Tris, pH8.9	1.25	
Water	7.28	
SDS	0.1	
APS	0.15	

Samples for analysis were dissolved in Laemmli sample buffer and boiled for 3 minutes and centrifuged to remove any insoluble material. The supernatant was loaded on the gel. Electrophoresis was performed in a Hoeffer electrophoresis unit with 10% gel giving a current of 30mA for 5-6h. Gels were stained with coomassie blue or silver stained.

2.2.14 Western Blotting

Western blotting was done using a semidry apparatus according to the procedure of Mastroiani et al., (Mastroiani et al., 1991; Towbin et al., 1979).

Reagents

1. Transfer buffer: 0.025M Tris, 0.15M glycine, pH 8.3/ 10% methanol

2. Phosphate buffered Tween- PBS (0.025M, pH 7.4, 0.15 M NaCl, 0.05% Tween)

3. Substrate buffer- di aminobenzidine in citrate phosphate buffer pH 5.0 + 0.5% H_2O_2

After electrophoresis, the gel was taken out and washed. The nitrocellulose and filter paper were cut to the same size of the gel and soaked in the transfer buffer. The filter paper, nitrocellulose membrane, gel and filter paper were arranged in that order on the anode of semidry apparatus in the form of a sandwich, carefully avoiding air bubbles. The cathode was placed on to the top. A constant current of 1mA per cm^2 was given for 1 hr. After blotting, the nitrocellulose was blocked for 2hrs in 2.5% BSA in 0.05% Tween 20, PBS at room temperature. The nitrocellulose membrane was then incubated overnight at 4°C with primary antibody. After washing thoroughly, it was incubated with secondary antibody (IgG-HRP) for 2hrs at room temperature. The membrane was again washed in PBS-Tween and immersed in substrate buffer in dark till brown bands appeared. The band intensity was determined by Quantity One 4.5.0

Image acquisition and Analysis software (BioRad).

2.2.15 Zymography

The activity of MMPs secreted by HUVECs into the medium maintained in culture was determined by gelatin zymography (Ambili and Sudhakaran, 1998). Zymogram gels consisted of 7.5% polyacrylamide (native) gel polymerized together with gelatin (1mg/ml). After electrophoresis, the gels were washed twice with 2.5% Triton x 100 and incubated with substrate buffer (50mM Tris, 5mM $CaCl_2$, pH 7.5) at 37^0C for 24hr. The gels were stained with Coomassie brilliant blue R 250 and destained with water. Gelatinolytic activities appearing as clear zone were quantitated using Quantity One - 4.5.0 - Software (BioRad).

2.2.16 Reverse Zymography

In order to study the activity of TIMP proteins, reverse zymographic analysis was performed (Oliver et al., 1997). Aliquots of freeze dried medium equivalent to same amount of cell protein were applied to polyacrylamide gels containing 0.1% (w/v) SDS, 0.5 mg/ml gelatin and MMPs from HeLa cells conditioned medium. After electrophoresis, gels were washed thrice with 2.5% (v/v) Triton X-100 at intervals of 1hour, followed by incubating gels overnight at 37°C in substrate buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, pH 8.0). Subsequently, gels were stained for 30 minutes with 0.1% (w/v) Coomassie Blue R-250 and destained with a solution of 30% (v/v) methanol and 10% (v/v) glacial acetic acid. TIMPs in the samples inhibit MMP action and were visualized as dark bands on a clear background. The intensity of bands was quantitated using Quantity One - 4.5.0 - Software (BioRad).

2.2.17 Estimation of protein

Protein was estimated by Lowry's method (Lowry et al., 1951)

Reagents

1. Alkaline copper reagent: A- 1% copper sulphate + 2% sodium potassium tartarate (1:1) B- Sodium carbonate (2% in 0.1 N NaOH). Added A and B in the ratio of 1:50.

2. Folin phenol reagent: Folins reagent + Water (1:1)

Different volumes of sample and standard BSA (1mg/ml) were taken and made upto 10µl. To this added 100 µl alkaline copper reagent and kept for 10 minutes. After

adding 10 µl Folins reagent the solution was incubated at room temperature for 30 minutes before measuring optical density at 650 nm.

2.2.18 Estimation of prostaglandins, PG E_2 and PG D_2

The level of prostaglandins in the culture supernatants were estimated by HPLC by the method of Holtmann (Holtmann et al., 1990; Fritsche et al., 2001). The culture medium (pH 3.5 adjusted with HPLC grade acetic acid) was passed through 1ml Sep Pak C18 columns prewashed with 1 ml of ethanol and equilibrated with 1 ml of 0.1% EDTA for solid-phase extraction of polar materials. After columns were washed with 1 ml of water, they were eluted with 2 ml of methanol; eluates containing the eicosanoids were concentrated under a stream of nitrogen followed by analysis by isocratic reversed-phase high-pressure liquid chromatography at a flow rate of 2 ml/min and UV detection at 275 nm. The mobile phase consisted of acetonitrile (31.9%, v/v), acetic acid (0.1%, v/v) and H_2O (68%, v/v) adjusted to pH 4.5 with sodium acetate. A C18 narrow-bore column constituted the stationary phase. Appropriate standards of PGE_2 and PGD_2 (100 µg) were subjected to HPLC and the area corresponding to these eicosanoids were used to calculate the concentration of PGE_2 and PGD_2 in the samples and their concentrations expressed as µmoles/mg protein.

2.19 Analysis of AP-1 and NFκB activation

Following experimental treatment of endothelial cells, nuclear extracts were prepared as described earlier (Molitor et al., 1990) with the following modifications. Cell monolayers (3-5 x 10^6 cells) were harvested, washed in cold phosphate-buffered saline (PBS) and incubated in 80 µl of buffer A (10 mM Hepes, pH 8.0/1.5 mM $MgCl_2$/10 mM KCl/0.5 mM dithiothreitol/200 mM sucrose/0.5 mM phenylmethanesulfonyl fluoride/0.5 µg of leupeptin per ml/0.5 mg of aprotinin per ml/0.5% Nonidet P-40) for 15 min at 4°C. Nuclear pellet was collected by microcentrifugation, rinsed once in buffer A and resuspended in 80 µl of buffer B [20 mM Hepes, pH 8.0/20% (vol/vol) glycerol/0.1 M KCl/0.2 mM EDTA/0.5 mM phenylmethanesulfonyl fluoride/ 0.5 mM dithiothreitol / 0.5 µg of leupeptin per ml/0.5 mg of aprotinin per ml]. Nuclear pellet was sonicated for 10s and clarified by microcentrifugation for 30s. The protein content of resulting supernatants was assayed by Lowry's method. Protein normalized nuclear and cytosolic extracts were subjected to immunoblotting and probed with anti-NFκB. In order to study the nuclear translocation of AP-1, the nuclear extracts were immunoprecipitated with anti-c-Jun

and were probed with anti-c-Fos and c-Jun.

2.2.20 Flow Cytometric analysis

HUVECs subjected to different experimental conditions were maintained in culture for different time intervals. The cells were dislodged using 2mM EDTA at 4°C. The cells were suspended in 1 ml of PBS and washed with 2 ml 1% BSA in PBS. The cells were then pelleted down by centrifugation at 2000 rpm for 5 minutes at 4°C and processed for indirect staining. Briefly, cells ($5x10^4$) were treated with 5 µl of primary antibody for 15 minutes at room temperature and suspended in 1 ml 1% BSA in PBS by gently tapping the tubes. Centrifuged at 2000 rpm for 5 minutes at 4°C and removed the supernatant. Repeated this step twice and the cells were incubated for 15 minutes with 5µl of secondary FITC conjugated antibody at room temperature. The cells were then washed twice with 1% BSA in PBS. Finally the cells were suspended in 0.5ml of 1% BSA in PBS. Fluorescence was determined by FACS Vantage using 488 nm argon laser (Becton Dickinson, Mountainview, CA). The results are expressed as scatter plots and fluorescence histograms. Quadrant statistical analysis was done on scatter plots to derive percentage positive cells. Acquisition of data employed gated cell population.

2.2.21 Nuclear translocation of β-Catenin

Cell pellets were resuspended in 500 µl PBS ice-cold nuclear buffer (150 mM NaCl, 150 mM sucrose, 20 mM HEPES, pH 7.4, 5 mM KCl, 2mM dithiothreitol, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM PMSF, and protease inhibitors) (Herren et al., 1998). The suspension was gently mixed with a pipet on ice for 3 min and then centrifuged at 500 × *g* for 10 min to pellet the nuclei. Then the remaining cytosolic supernatant was centrifuged for 60 min at 13000 × g at 4°C. Nuclear pellet and the cytosolic fraction collected were suspended in Laemmli sample buffer. Total protein concentration was determined by Lowry's method (Lowry et al., 1951). Cytosolic and nuclear fractions were resolved on SDS polyacrylamide gels and immunoblotted to analyse the levels of β-catenin in nucleus and cytosolic fraction. For immunoprecipitation, equivalent amount of cellular proteins were brought to a volume of 1.0 ml in buffer containing 50 mM Tris-HCl, pH 7.5, 0.4 M NaCl, 5 mM EDTA and 0.5% NP-40. The clear supernatant was incubated with the anti-β-Catenin antibody for 2 hours on ice followed by Protein A/G-Sepharose immunoprecipitation. Beads were washed 3 times with the same buffer and extracted in Laemmli sample buffer; samples were then subjected to SDS PAGE, immunoblotted and probed with monoclonal antibodies against

phosphotyrosine and phosphoserine.

2.2.22 Statistical Analysis

All the data are 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 SPSS10 Software.