Differential mechanisms of angiotensin II and PDGF-BB on migration and proliferation of coronary artery smooth muscle cells

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Abstract

Angiotensin II (Ang II) and platelet-derived growth factor-BB (PDGF-BB) are associated with excessive cell migration, proliferation and many growth-related diseases. However, whether these agents utilise similar mechanisms to trigger vascular pathologies remain to be explored. The effects of Ang II and PDGF-BB on coronary artery smooth muscle cell (CASMC) migration and proliferation were investigated via Dunn chemotaxis assay and the measurement of $[^3H]$thymidine incorporation rates, respectively. Both atherogens produced similar degrees of cell migration which were dramatically inhibited by mevastatin (10 nM). However, the inhibitory effects of losartan (10 nM) and MnTBAP (a free radical scavenger; 50 μM) were found to be unique to Ang II-mediated chemotaxis. In contrast, MnTBAP, apocynin (an antioxidant and phagocytic NADPH oxidase inhibitor; 500 μM), mevastatin and pravastatin (100 nM) equally suppressed both Ang II and PDGF-BB-induced cellular growth. Although atherogens produced similar changes in NADPH oxidase, NOS and superoxide dismutase activities, they differentially regulated antioxidant glutathione peroxidase activity which was diminished by Ang II and unaffected by PDGF-BB. Studies with signal transduction pathway inhibitors revealed the involvement of multiple pathways i.e. protein kinase C, tyrosine kinase and MAPK in Ang II- and/or PDGF-BB-induced aforementioned enzyme activity changes. In conclusion, Ang II and PDGF-BB may induce coronary atherosclerotic disease formation by stimulating CASMC migration and proliferation through agent-specific regulation of oxidative status and utilisation of different signal transduction pathways.

Keywords: Atherosclerosis, Arteries, Superoxide anion, Nitric oxide, Antioxidants

1. Introduction

Coronary atherosclerosis, associated with enhanced oxidative stress, continues to be the leading cause of morbidity and mortality in the Western World. Atherosclerosis is a progressive inflammatory disease that ultimately leads to formation of advanced or complicated focal lesions which develop subsequent to a series of specific cellular and molecular responses including enhanced vascular smooth muscle cell (VSMC) proliferation and migration [1]. Several agents including platelet-derived growth factor (PDGF) exacerbate atherogenesis through inducing VSMC proliferation and plaque neovascularisation. The HMG-CoA reductase inhibitors (statins) e.g. simvastatin have been implicated in the suppression of PDGF-induced DNA synthesis in human glomerular mesangial cells in addition to reducing the risk of primary and secondary events [2-4]. Another non-lipid-lowering effect of statins is their ability to prevent migration of VSMC to PDGF by blocking the production of isoprenoids which are required for prenylation of small GTP-binding proteins, such as Ras and Rho, involved in cell proliferation and migration control [5]. Hence, the suppression of PDGF-induced mitogenesis and migration should dramatically limit its atherogenic effects.

Similar to PDGF, angiotensin II (Ang II), the active component of the renin-angiotensin system, stimulates VSMC proliferation and migration and is therefore implicated in atherogenesis [6]. Previous data have concluded that Ang II type 1 receptor (AT_1R) blockers (ARB) exert direct anti-atherosclerotic effects [7,8]. Ang II displays its proatherogenic effects through AT_1R stimulation and consequent NADPH oxidase enzyme activation to generate excess levels of superoxide anion (O_2^-), a reactive oxygen species (ROS) [9]. The effects of ROS range from eliciting vasoconstriction to scavenging nitric oxide (NO), an endogenous vasodilator produced from L-arginine by NO synthase (NOS) [10,11]. Under physiological conditions, O_2^- is converted to H_2O_2 by superoxide dismutases (SOD) which is then further

metabolised to H_2O by catalase and glutathione peroxidase (GPx) [12]. However, in pathological conditions, O_2^- may lead to atherosclerotic plaque formation via diminished concentrations and/or aberrant regulations of antioxidant enzymes.

In addition to their respective roles in SMC proliferation, migration and oxidative stress, Ang II and PDGF share somewhat similar signal transduction characteristics such as activation of tyrosine kinases and mitogen-activated protein kinases (MAPK) [13,14]. Furthermore, recent data have implicated PDGF-BB in Ang II-induced chemotaxis and revealed the ability of Ang II to activate PDGF-β receptor [15,16]. Taken these close functional interactions into account, the present study examined whether mechanistic dis/similarities exist between these atherogens to trigger vascular pathologies. To this end the putative differences in: i- oxidative status as assessed by pro- and anti-oxidant enzyme activities; ii- proliferative and migratory responses; iii- signal transduction pathways involved in atherogen-mediated oxidative stress regulation, and iv- the correlation of ARBs, an antioxidant, a free radical scavenger or statins to cell growth and enzyme activities were investigated using coronary artery smooth muscle cells (CASMC) exposed to physio-pathological concentrations of Ang II or PDGF-BB.

2. Materials and Methods

2.1. Human CASMC culture and characterisation

Human CASMC (n=3 donors) were purchased from Cambrex (UK) and cultured in SmGM-2 according to manufacturer's instructions. Cells grown on coverslips were rinsed with ice-cold PBS and fixed in 4% formaldehyde for 20 min at room temperature before permeabilisation with 0.1% Triton X-100. To characterise the cells as SMC they were stained with a monoclonal antibody raised against SM α-actin for 1 h in the dark at 4^oC prior to examination by confocal microscopy (Micro Radiance, Bio-Rad, UK). Moreover, the cells

were stained positive for SM myosin, calponin and caldesmin by the commercial company. CASMC between passages 4 and 8 were used in the current study.

2.2. Manipulation of cell growth

To exclude potential effects produced by differences in cell density, an identical number of cells was seeded into flasks (0.5×10^6). Cell passages were performed using a 1:3 ratio and culture medium was replaced every 24 h. This ensured that the number of mitoses was unaltered amongst experiments.

2.3. [^{3}H]Thymidine incorporation

[^{3}H]Thymidine (37 kBq/ml) was directly added to the culture medium with the test compounds and incubated with CASMC for 18 h. Incubations were stopped by removing the medium, washing the cells with ice-cold 5% trichloroacetic acid (TCA) and incubating them in 5% TCA on ice for 20 min. After two additional washings with cold 95% ethanol, precipitates were solubilised with buffer (0.1 M NaOH, 2% Na_2CO_3, 1% sodium dodecyl sulphate) and their radioactivity was analysed by liquid scintillation counting. Data were expressed as counts per minute (cpm) [^{3}H]thymidine incorporated per flask.

2.4. Migration Assays

Cell migration was assessed by direct observation and recording of cell behaviour in concentrations of recombinant human PDGF-BB (5-50 ng/ml) or Ang II (5-50 nM) using the Dunn chemotaxis chamber (Weber Scientific International, Teddington, UK). This apparatus permits the direction of movement of individual cells to be measured in relation to the direction of the gradient, as well as the time course to be followed. The apparatus was set up, filmed by time-lapse computer photography and data analysed as previously described [17,18]. The rationale behind all chemotaxis experiments was the same; the potential chemoattractant was placed in the outer well of the Dunn chamber in addition to the components that were available in the inner well to rule out the contribution of those other components. Between the 2 wells cells on an inverted coverslip are exposed to the

chemoattractant gradient and can be directly filmed for up to 24 h when the gradient will start to break down. For example, for Ang II experiments SmGM-2 was placed in the inner well and SmGM-2 with varying concentrations (5-50 nM) of Ang II in the outer well.

All cells were serum starved for 18 h prior to migration assays to exclude effects attributable to factors present in serum other than the expected chemoattractant. Experiments were run for 18 h with a time-lapse interval of 10 min. Minimum of 30 cells from three independent experiments were analysed for each study and the proportion of cells migrating towards the atherogenic agent are given as a percentage of total cells in that field.

2.5. Measurement of NADPH oxidase activity and detection of O_2^- levels

O_2^- levels were measured by cytochrome C reduction assay. Briefly, CASMC were collected in Hanks' balanced salt solution at a density of 20×10^6 cells/ml. Aliquots (250 μl) containing 50 μM cytochrome C were then incubated for 60 min at 37°C. O_2^- generation was measured as the superoxide dismutase (10 μg/ml)-inhibitable reduction of cytochrome C and monitored as the change in absorbance at 550 nm using a GENios plate reader (TECAN). Absorbances were recorded for 8 min with 60 sec intervals and the activity was calculated as pmoles O_2^- per mg protein following subtraction of background levels at 550 nm.

NADPH oxidase activity was measured in similar experiments via detection of changes in O_2^- levels by cytochrome C reduction assays. In order to ascribe all cytochrome C reduction to NADPH oxidase, the inhibitors of other ROS-generating enzymes i.e. L-NAME (100 μM, NOS inhibitor), rotenone (50 μM, mitochondrial complex I inhibitor), allopurinol (100 μM, xanthine oxidase inhibitor) or indomethacin (50 μM, cyclooxygenase inhibitor) were added simultaneously to aliquots at the beginning of 60 min incubation period prior to determining O_2^- generation.

NADPH oxidase activity was also measured by lucigenin-enhanced chemiluminescent detection of O_2^-. Briefly, cells were lysed on ice in lysis buffer containing 1 mM EGTA, 20

mM monobasic potassium phosphate (pH 7.0), 0.5 μg/ml leupeptin, 10 μg/ml aprotinin, 0.7 μg/ml pepstatin and 0.5 mM phenylmethylsulfonyl fluoride. The reaction buffer contained 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin and 1 mM NADPH. The reaction was initiated by addition of 50-200 μg of protein in 100 μl of homogenate and luminescence was recorded every 60 sec for 10 min using a luminometer following subtraction of the counts obtained from a buffer blank.

2.6. Isolation of cellular membranes

Differential centrifugation was used to isolate subcellular fractions. CASMC were suspended (2×10^7/ml) in MOPS-KOH buffer (20 mM MOPS-KOH, 250 mM sucrose, pH 7.4) containing 1 mM PMSF, 0.1 mM EDTA, 2 μM leupeptin and 2 μM pepstatin. Cells were disrupted successively by snap freezing in liquid nitrogen, two cycles (15 sec each) of homogenisation and two cycles of sonication at 100 W for 20 sec on ice. The homogenate was quickly centrifuged at 800 x g for 10 min to remove nuclei and unbroken cells. The supernatant was centrifuged at 20,000 x g for 20 min at 4°C to obtain a pellet of submitochondrial particles and smaller organelles. The supernatant was centrifuged again at 100,000 x g for 60 min to collect membrane fractions to be used in the experiments. The pellets were resuspended in MOPS-KOH buffer and the quantities of the proteins were determined using a kit (Pierce) prior to detection of NADPH oxidase activities.

2.7. NOS Assay

NOS activity was determined, in cell homogenates, using the NOSdetect assay kit (Alexis Biochemicals). Briefly, cells were lysed in buffer containing 25 mM Tris, pH 7.4, 1 mM EDTA and 1 mM EGTA and centrifuged for 30 min at 4000 g. 10 μl supernatant and 40 μL reaction buffer (50 mM Tris, 6 μM tetrahydrobiopeterin, 2 μM flavin adenine mononucleotide, 2 μM flavin adenine dinucleotide, 10 mM NADPH, 6 mM CaCl$_2$) containing 100.000 dpm [^{3}H]L-arginine were combined. Following 30 min incubation at room

temperature, the enzymatic reaction was stopped by the addition of stop buffer (50 mM HEPES, 5 mM EDTA, pH 5.5). Newly formed [^{3}H]L-citrulline, neutral at pH 5.5, was then separated from the incubation mixture by cation exchange resin (Dowex AG 50 W-X8) and quantified using a liquid scintillation counter. The contribution of iNOS to overall NOS activity was assessed in similar experiments by replacing the calcium in reaction buffer with EGTA (5 mM). Data were normalised by the amount of protein and reaction time. Results were expressed as pmol L-citrulline/mg protein/min.

In parallel experiments, the cellular homogenates were incubated with N^{ω}-hydroxy-nor-L-arginine (NOHA; 100 μM) for 30 min to inactivate arginase activity before measuring the NOS activities.

2.8. Nitrite Detection

Nitrite levels were measured by Griess reaction as an index of NO generation following conversion of nitrate to nitrite by nitrate dehydrogenase [19]. An aliquot of the cellular homogenate was mixed with an equal volume of Griess reagent (sulfanilamide 1% w/v, naphthylethylenediamine dihydrochloride 0.1% w/v and orthophosphoric acid 2.5% v/v) and incubated at room temperature for 10 min prior to measurement of absorbances at 540 nm. The amount of nitrite formed was compared to those of known concentrations of sodium nitrite and normalised to the protein content of the respective flask.

2.9. GPx assay

The GPx activity was measured using a specific assay kit (Merck Biosciences) based on the reduction of oxidised glutathione, produced by GPx-mediated reduction of hydroperoxide, by glutathione reductase and NADPH during which the oxidation of NADPH to NADP$^+$ was accompanied by a decrease in absorbance at 340 nm. Briefly, 100 μl of assay buffer and 50 μl of co-substrate mixture were added to 20 μl of untreated and treated CASMC homogenate, in triplicate, in a 96-well ELISA plate. Reactions were initiated by the addition of 20 μl cumene

hydroperoxide and the sample absorbances were read every 60 sec for 5 min at 340 nm. The reaction rate per min was determined for the blank reaction and then subtracted from the reaction rates for the analysed samples. GPx activity was then calculated using the extinction coefficient for NADPH at 340 nm ($0.00373 \, \mu M^{-1}$). One unit of GPx was defined as the activity that converts 1 mM of reduced glutathione per litre per min at 25^oC.

2.10. SOD Assay

The SOD assay kit (Merck Biosciences) utilising a tetrazolium salt for detection of O_2^- generated by xanthine oxidase and hypoxanthine was used. Untreated and treated CASMC homogenates were diluted 1:20 with radical detector and assayed in triplicate in a 96-well ELISA plate. Reactions were initiated by the addition of 20 µl diluted xanthine oxidase and the plate was incubated on a shaker for 20 min at room temperature. Sample absorbance values were read at 450 nm and total SOD activity was determined using the equation obtained from the linear regression of the SOD standard curve. One unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the O_2^- (kit range 0.025-0.25 units/ml). In similar experiments, MnSOD activity was detected following inhibition of CuZn-SOD activity via incubations with 3 mM potassium cyanide (BDH Chemicals Ltd) at room temperature for 45 minutes. CuZn-SOD activity was subsequently calculated by the subtraction of MnSOD activity from total SOD activity.

2.11. Evaluation of cell viability

To detect cytotoxicity of ROS generating enzyme inhibitors, CASMC were incubated with the aforementioned compounds for 75 min. A small aliquot was then incubated with 0.1% trypan blue for 4 min and viewed under a light microscope. By counting 100 cells, the percentage of viable cells was calculated.

2.12. Statistical Analysis

To test for directed migration (chemotaxis) the Rayleigh test for unimodal clustering of directions was applied to the data and $p < 0.01$ was chosen as the criterion for rejecting the null hypothesis of random directionality. Where there was significant unimodal clustering the mean direction and its 95% confidence interval were calculated and Tukey's *post hoc* analysis was conducted. Results for proliferation and enzyme activity assays are presented as mean±SEM. Statistical analyses were performed by two-way analysis of variance (ANOVA) followed by Bonferroni-Dunn's *post hoc* analysis and $p < 0.05$ was considered significant.

3. Results

3.1. Effects of Ang II and PDGF-BB on CASMC migration

Ang II (5-50 nM) evoked significant increases in CASMC chemotaxis. To examine the relevance of O_2^- to Ang II-mediated migration, cells were treated with a cell-permeable SOD mimetic (MnTBAP; 50 μM) which significantly attenuated chemotaxis. The prevention of Ang II-induced chemotaxis with losartan (0.01-1 μM) confirmed the involvement of AT_1R in Ang II-induced VSMC migration. Mevastatin (10 nM) also inhibited Ang II-mediated migration (Fig. 1A).

PDGF-BB (5-50 ng/ml) also significantly enhanced CASMC chemotaxis. However, unlike Ang II, the PDGF-evoked responses were not affected by MnTBAP and losartan but markedly inhibited by mevastatin (Fig. 1B).

The cells used in chemotaxis experiments were serum starved for 18 h to exclude the migratory effects of serum in order to detect the specific and directed migration produced by a given atherogen. As the cells in the absence of stimuli such as serum or atherogens generated aberrant undirected migratory responses, they could not be used as positive controls. However, the impact of a given treatment on atherogen-mediated chemotaxis was assessed using the cells exposed to Ang II or PDGF-BB alone as controls.

3.2. Effects of Ang II and PDGF-BB on CASMC proliferation

Ang II (5-50 nM) produced significant increases in cell numbers in a dose-dependent manner as assessed by cell counting (0.88 ± 0.07 x 10^6 vs. 1.21 ± 0.15 x 10^6 and 1.49 ± 0.13 x 10^6 at 5 and 50 nM of Ang II, respectively, n=5, $p<0.05$) and determination of total protein levels (447 ± 16 μg vs. 499 ± 21 μg and 630 ± 31 μg, n=5, $p<0.05$). The relative changes in cell number and protein levels were reflective of the changes in [^{3}H]thymidine incorporation rates (Fig. 2A).

Similar increases in cell numbers (0.88 ± 0.07 x 10^6 vs. 1.24 ± 0.09 x 10^6 and 1.43 ± 0.11 x 10^6 at 5 and 50 ng/ml of PDGF-BB, respectively n=5, $p<0.05$), total protein levels (447 ± 16 μg vs. 510 ± 19 μg and 546 ± 18 μg, n=5, $p<0.05$) and [^{3}H]thymidine incorporation rates were also obtained with PDGF-BB (Fig. 2B).

Treatment of CASMC with MnTBAP (50 μM) reduced cell numbers by approximately 30% in both Ang II- (1.49 ± 0.13 x 10^6 vs. 1.03 ± 0.05 x 10^6, n=5, $p<0.05$) and PDGF-BB-treated (1.43 ± 0.11 x 10^6 vs. 0.90 ± 0.06 x 10^6, n=5, $p<0.05$) cells. Similar decreases were also obtained in [^{3}H]thymidine incorporation rates (Fig. 2A-B). Treatment of cells with a phagocytic inhibitor of NADPH oxidase i.e. apocynin (100 μM) also produced substantial decreases in cell growth (Fig. 2A-B). However, the specific inhibitors of xanthine oxidase (allopurinol, 10-100 μM), cyclooxygenase (indomethacin, 5-50 μM) and mitochondrial complex I inhibitor (rotenone, 5-50 μM) did not alter cellular growth rate (data not shown).

Similar to apocynin, mevastatin (10 nM) and pravastatin (100 nM) also suppressed cellular growth rates in both high dose Ang II- and PDGF-treated cells (Fig. 2A-B).

3.3. Effects of Ang II and PDGF-BB on enzyme activities

Overnight incubation of CASMC with the higher dose of Ang II (50 nM) and both concentrations of PDGF-BB (5 and 50 ng/ml) dramatically decreased NOS activity. Incubation of cells with mevastatin but not losartan or apocynin alone led to marked increases in NOS activity. Mevastatin (10 nM), losartan (1 μM) and apocynin (500 μM) selectively

reversed higher dose Ang II-mediated decreases in NOS activity (Fig. 3A-B). Neither Ang II

(50 nM; 980 ± 120 vs 1100 ± 100 pmol L-citrulline/mg protein/min, n=3, p<0.05) nor PDGF-

BB (50 ng/ml; 980 ± 120 vs 1210 ± 240 pmol L-citrulline/mg protein/min, n=3, p<0.05)

significantly altered iNOS activities while the inhibition of arginase activity by NOHA (100

μM) resulted in approximately 6% increase in both atherogen-induced NOS activities.

Ang II and PDGF-BB increased NADPH oxidase activity in a dose-dependent fashion

which was significantly diminished by mevastatin, losartan and apocynin (Fig. 4A-B). The

level of changes observed in NADPH oxidase activities measured using the total cellular

homogenates were shown to be representative of those obtained with membrane fractions

(Table 1). To assess the potential toxic effects of ROS-generating enzyme inhibitors that were

used in NADPH oxidase assays to eradicate the contributions of enzymes, other than NADPH

oxidase, to overall O_2^- formation, CASMC viability rates were measured which revealed no

significant difference amongst treatment groups (Table 2).

The levels of mitochondrial (MnSOD) and cytosolic SOD (CuZn-SOD) activities were also

elevated with both atherogens where treatments with mevastatin, losartan and apocynin alone

abolished both SOD activities and significantly decreased them when used in combination

with either atherogen (Table 3).

Atherogenic agents differentially regulated GPx activity that was dose-dependently

decreased by Ang II and unaffected by PDGF-BB. It was shown that, when used alone,

mevastatin but not losartan or apocynin significantly decreased basal GPx activity. Although

these agents significantly decreased GPx activity in the presence of PDGF-BB, only

mevastatin was shown to be suppressive to Ang II (Fig. 5A-B).

3.4. Effects of Ang II and PDGF-BB on O_2^- and nitrite levels

The differences observed in CASMC NADPH oxidase and NOS activity following

overnight exposure to Ang II (5-50 nM) or PDGF-BB (5-50 ng/ml) were mimicked by the

differences observed in the levels of their end products; $O_2^{\cdot-}$ and nitrite, an indirect marker used for estimation of NO production, respectively (Table 4).

3.5. Effects of inhibition of signal transduction pathways on enzyme activities

Suppression of tyrosine kinase (TLCK; 50 μM), MAPK (PD98059; 10 μM) or protein kinase C (Bis-I; 5 μM) using the indicated specific inhibitors led to atherogen- and enzyme-specific changes. Individual inhibition of tyrosine kinase and MAPK diminished both PDGF-BB- and Ang II-mediated GPx activity. However, co-incubation of these inhibitors with Ang II enhanced GPx activities compared to control cells and high dose Ang II-treated cells. Significant increase in GPx activity was also observed in PDGF-BB and TLCK co-incubated versus control cells (Fig. 6A-B). The inhibition of each pathway reduced NADPH oxidase and CuZn-SOD enzyme activities compared to higher dose PDGF and Ang II-treated cells by almost 50% while concomitantly increasing NOS activity in these cells (Tables 5 and 6).

4. Discussion

The major conclusions to be drawn from this study are that Ang II and PDGF-BB distinctly regulate CASMC proliferation, migration and oxidative status through involvement of multiple enzyme systems and signal transduction pathways. These findings provide crucial evidence on the mechanisms whereby these agents may contribute to vasculopathologies in addition to their well-established vasoconstrictor and mitogenic effects.

The migration of SMC to the intimal space constitutes one of the key events in the atherosclerotic lesion formation and is mediated by various growth factors and cytokines. To substantiate these, the present study has shown that both PDGF-BB ($\geq$5 ng/ml) and Ang II ($\geq$5 nM) produce significant CASMC chemotaxis. In SMC, Ang II through AT_1R-mediated induction of NADPH oxidase, increases $O_2^{\cdot-}$ production which in turn scavenges NO and stimulates migration [9-11,20,21]. Indeed, losartan (AT_1R blocker) and MnTBAP have selectively inhibited Ang II- but not PDGF-BB-dependent chemotaxis. These findings imply

that neutralisation of $O_2^{\cdot-}$ is a prerequisite for efficacious inhibition of Ang II-evoked SMC migration and that reduced oxidative stress has no benefit for PDGF-BB-induced migration supporting previous studies with aortic VSMC and those showing a prerequisite for PDGF-β receptor binding for chemotaxis [22,23].

Contrary to losartan, mevastatin abolished CASMC migration regardless of the atherogenic agent used. Our recent study has attributed this effect to the ability of statins to prevent prenylation of small monomeric GTPases as evidenced by recovery of migration following microinjection of isoprenoid intermediate farnesylpyrophosphate (FPP) and further attenuation of migration by Ras/MAPK pathway inhibition via a farnesyltransferase inhibitor [24]. The critical roles of Ras and other small GTP-binding proteins including p21 Rac in the assembly of the NADPH oxidase enzyme system, cell proliferation, NOS activity and thus NO generation may help explain the beneficial effects of statins on preventing and/or regressing atherosclerotic plaque development [25,26].

In addition to cell migration, proliferation which contributes to in-stent restenosis is also considered a key feature of atherosclerosis [27]. Hence, the present study investigated the correlation between atherogenic agents and CASMC proliferation and revealed that both atherogens increased $O_2^{\cdot-}$ production and CASMC proliferation in a dose-dependent manner which were suppressed by MnTBAP, apocynin and mevastatin. Taken with the migration studies these data imply that while ROS modulate both Ang II-mediated CASMC proliferation and migration, they are solely associated with PDGF-induced proliferative processes. It is of note that differential regulation of VSMC proliferation and chemotaxis is not unique to the agents used in this study, as similar effects have been generated with VAS2870, a novel NADPH oxidase inhibitor, in rat thoracic aorta SMC [29]. In this context, a previous study has attributed normal coronary endothelial cell growth to NADPH oxidase-

derived $O_2^{\cdot-}$ bioavailability, a finding supported in this study by inability of other ROS-generating enzymes to alter CASMC proliferation rates [26].

NO inhibits proliferation of several cells and thus prevents atherosclerotic disease progression [28]. Since, similar to previous studies, no significant differences in iNOS activity were detected in CASMC incubated with/out Ang II and PDGF-BB, the enhanced cell growth was investigated in relation to potential differences in eNOS activity and nitrite levels, a surrogate marker of NO [30,31]. These studies revealed that both Ang II and PDGF-BB produced significant dose-dependent reductions in both parameters where treatments of cells with mevastatin but not apocynin or losartan alone enhanced NOS activity. Furthermore, while these agents normalised Ang II-mediated decreases in NOS activity, they failed to reverse those brought about by PDGF-BB which sheds some light on their inability to suppress PDGF-BB-evoked chemotaxis.

Given that NADPH oxidase-derived $O_2^{\cdot-}$ determines the availability of NO, the effects of atherogenic agents on this enzyme activity were investigated which demonstrated dose-dependent elevations with both agents. Interestingly, incubation of CASMC with mevastatin alone also enhanced NADPH oxidase activity. Although the mechanism behind this increase is unknown, it is possible that mevastatin-induced dramatic increases in NO bioavailability may diminish local $O_2^{\cdot-}$ levels thereby stimulating NADPH oxidase activity through a negative feed back mechanism. Indeed, the combinations of either atherogen with mevastatin, apocynin or losartan have led to reduced enzymatic activities compared to sole Ang II or PDGF-BB treatments. In this context, our previous studies have shown using endothelial cells that Ang II increases eNOS activity via concomitant involvement of its type 1 and type 2 receptor subtypes and also enhances cell proliferation and NADPH oxidase which are attenuated by MnTBAP, apocynin or antisense p22-phox (a pivotal subunit of NADPH oxidase) cDNA treatments [32-34].

Antioxidant enzymes including SOD and GPx also play critical roles in determining the overall intracellular oxidative status. Under physiological conditions, SODs convert $O_2^{\cdot-}$ to H_2O_2 that in turn is metabolised to H_2O by catalase and GPx, a pathway that may be perturbed in pathological conditions [12]. This study has shown that both Ang II and PDGF-BB have increased both CuZn-SOD (the main isotype which accounts for ~80% of total SOD activity) and MnSOD activities, a strong indicator of elevated intracellular oxidative stress, which were significantly attenuated by mevastatin, apocynin or losartan. Considering the suppressive effects of these agents on NADPH oxidase and hence $O_2^{\cdot-}$ production, these results were somewhat expected. However, the data pertaining to GPx activity were rather unexpected and displayed an atherogen-dependent regulation in that while Ang II elicited a dose-dependent decrease in its activity, PDGF-BB did not affect its basal activity. Besides, while mevastatin, losartan and apocynin significantly decreased GPx activity when co-incubated with PDGF-BB, both losartan and apocynin failed to alter Ang II-induced GPx activity.

Variety of signal transduction pathways including tyrosine kinases, protein kinase C and MAPK activated by Ang II and PDGF are implicated in VSMC differentiation, migration and proliferation [35,36]. In this study, selective inhibition of these signal transduction pathways to examine the putative links between their impaired regulation and promotion of pathological cascades have shown an atherogen- and/or enzyme-specific changes in enzyme activities. For example, suppressions of tyrosine kinase and MAPK enhanced Ang II- but not PDGF-mediated GPx activity. In contrast, selective inhibition of each pathway displayed similar effects on NADPH oxidase, CuZn-SOD and NOS activities. The similarities in these responses can in part be explained by the well-documented presence of a cross-talk between G-protein coupled AT_1R and the PDGF receptor tyrosine kinase in vascular SMCs and thus the ability of Ang II to elicit responses unique to growth factor stimulation [15,16,37].

In conclusion, statins, free radical scavengers and ARB may suppress the early events in atherogenesis by markedly inhibiting overall oxidative stress status, SMC migration and/or proliferation. Intracellular antioxidants and ARB suppress Ang II-mediated but not PDGF-BB-induced chemotaxis despite equally blocking cellular proliferation to both agents. Considering the involvement of different signal transduction pathways in the regulation of pro- and anti-oxidant enzyme activities, it is important to reiterate that Ang II and PDGF-BB may induce vascular complications by stimulating CASMC migration and proliferation through agent-specific regulation of oxidative status and utilisation of different signal transduction pathways.

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Legends

Fig. 1. Effects of angiotensin II (A) and PDGF-BB (B) on coronary artery smooth muscle cell migration in the absence or presence of a cell-permeable superoxide dismutase mimetic (MnTBAP), angiotensin II type 1 receptor blocker (losartan) and a statin (mevastatin). $n \geq 30$ cells from 3 independent experiments were used. *$p < 0.01$ (Rayleigh test: chemotaxis detected), †$p < 0.05$ (t-Test: % migrating cells versus the higher dose of Ang II or PDGF-BB).

Fig. 2. Effects of angiotensin II (A) and PDGF-BB (B) on [^{3}H]thymidine incorporation in coronary artery smooth muscle cells in the absence or presence of a cell-permeable superoxide dismutase mimetic (MnTBAP), an NADPH oxidase inhibitor (apocynin) and two statins (mevastatin and pravastatin). Data are expressed as mean ± SEM from 4 different experiments. *$p < 0.05$ compared to control groups. †$p < 0.05$ compared to the higher dose of Ang II or PDGF-BB groups.

Fig. 3. Effects of angiotensin II (A) and PDGF-BB (B) on nitric oxide synthase (NOS) activity in the absence or presence of a statin (mevastatin), an angiotensin II type 1 receptor blocker (losartan) and an NADPH oxidase inhibitor (apocynin). Data are expressed as mean ± SEM from 3 different experiments. *$p < 0.05$ compared to control groups. †$p < 0.05$ compared to the higher dose of Ang II or PDGF-BB groups.

Fig. 4. Effects of angiotensin II (A) and PDGF-BB (B) on NADPH oxidase activity in the absence or presence of a statin (mevastatin), an angiotensin II type 1 receptor blocker (losartan) and an NADPH oxidase inhibitor (apocynin). Data are expressed as mean ± SEM from 3 different experiments. *$p < 0.05$ compared to control groups. †$p < 0.05$ compared to the higher dose of Ang II or PDGF-BB groups.

**Fig. 5.** Effects of angiotensin II (A) and PDGF-BB (B) on glutathione peroxidase (GPx) activity in the absence or presence of a statin (mevastatin), an angiotensin II type 1 receptor blocker (losartan) and an NADPH oxidase inhibitor (apocynin). Data are expressed as mean ±

SEM from 3 different experiments. *p<0.05 compared to control groups. †p<0.05 compared to the higher dose of Ang II or PDGF-BB groups.

Fig. 6. Effects of suppression of tyrosine kinase (TLCK), mitogen activated protein kinase (PD98059) or protein kinase C (Bis-I) on angiotensin II (A) and PDGF-BB-mediated (B) changes in glutathione peroxidise activities. Data are expressed as mean ± SEM from 3 different experiments. *p<0.05 compared to control groups. †p<0.05 compared to the higher dose of Ang II or PDGF-BB groups.
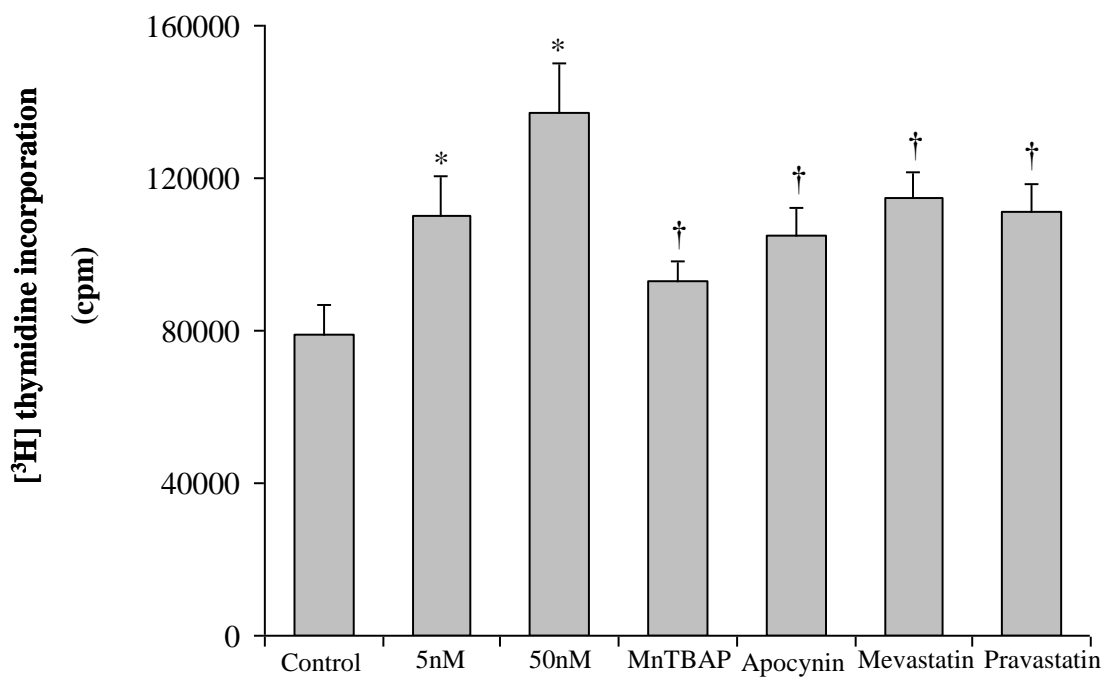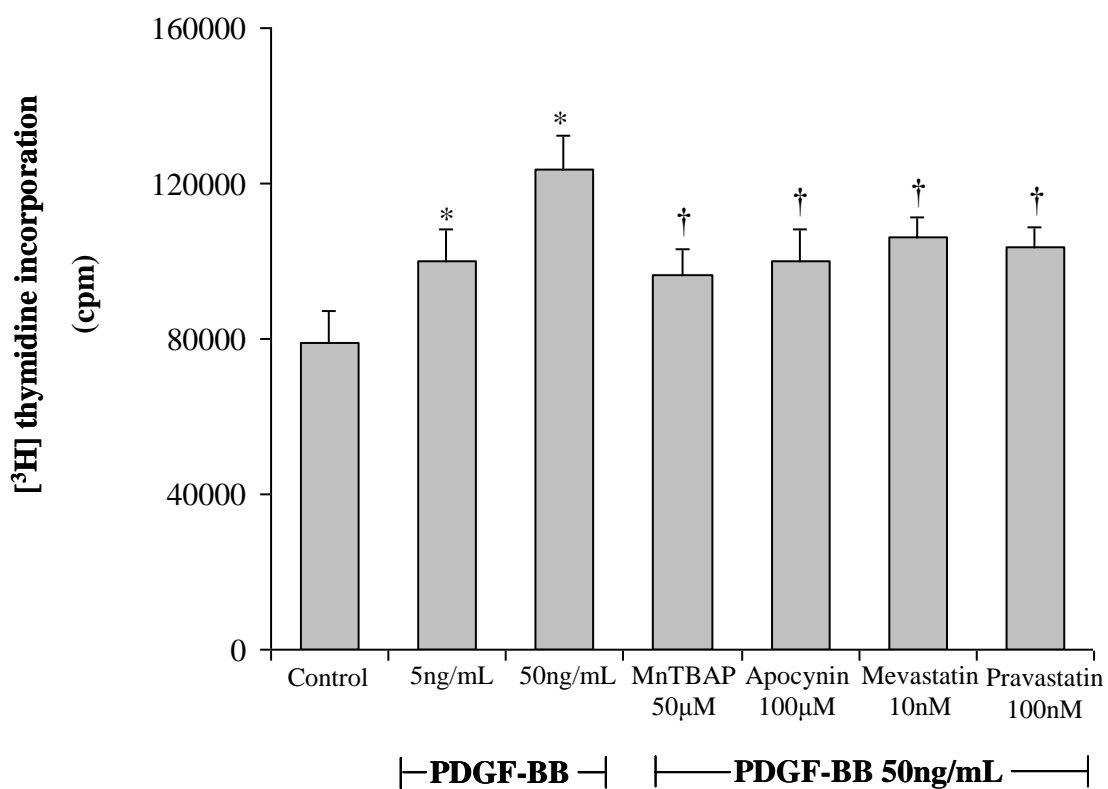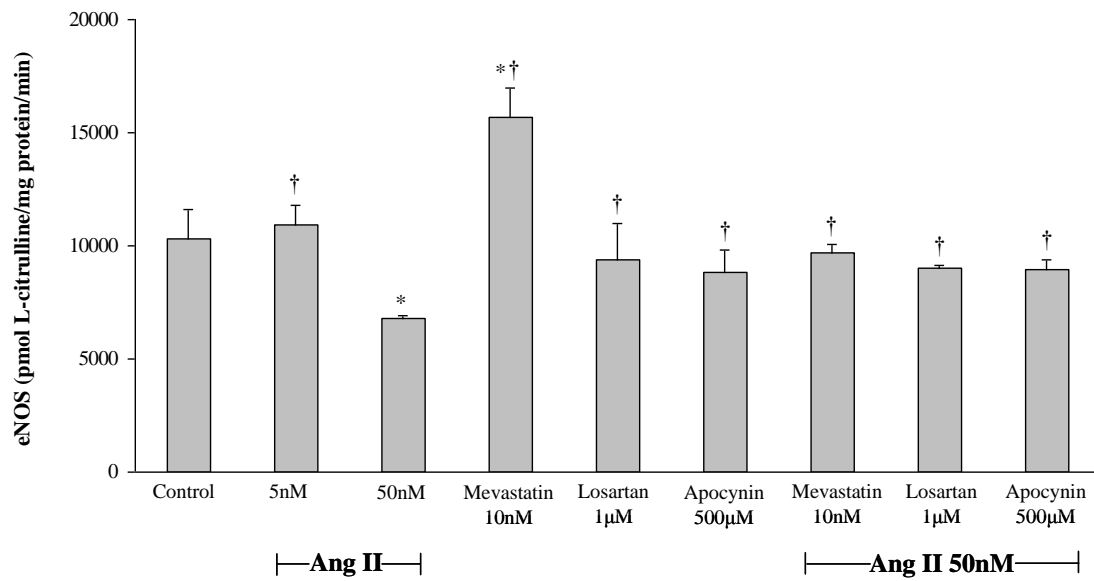
Figure 1

Figure 2

A

B

├─ **PDGF-BB** ─┤ ├──── **PDGF-BB 50ng/mL** ────┤
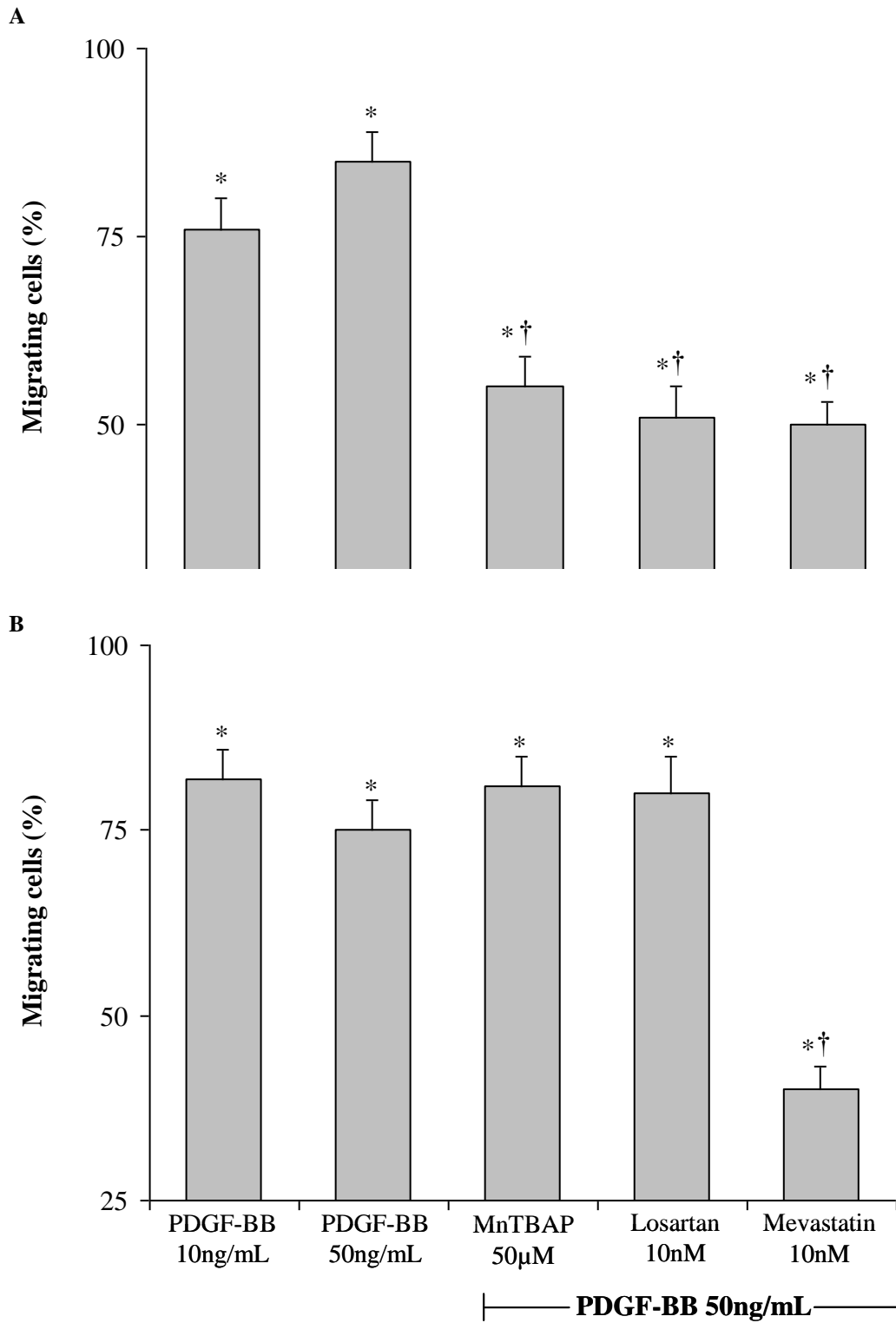
Figure 3

A

B
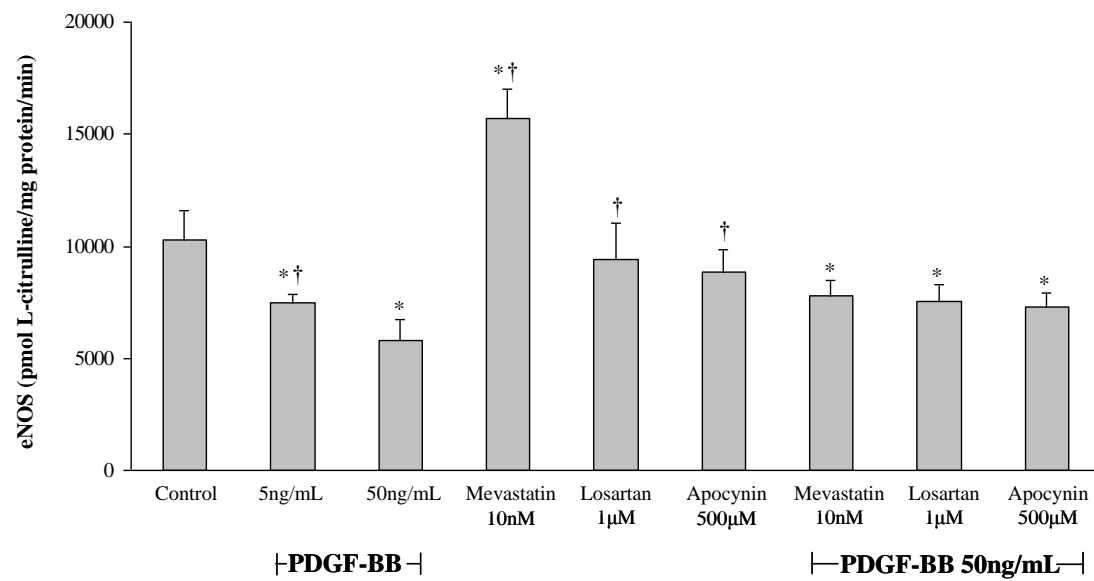
Figure 4
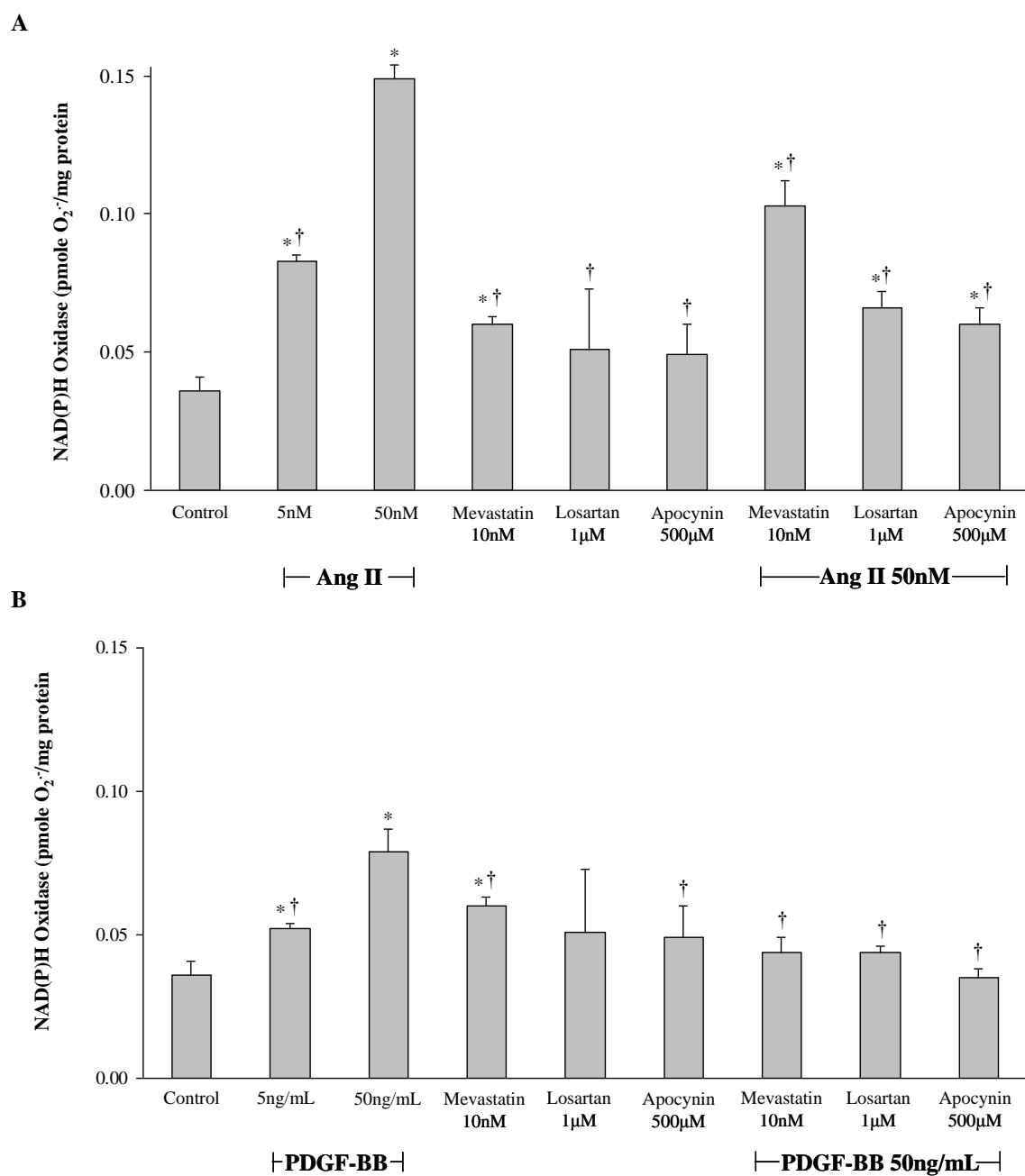
Figure 5

A
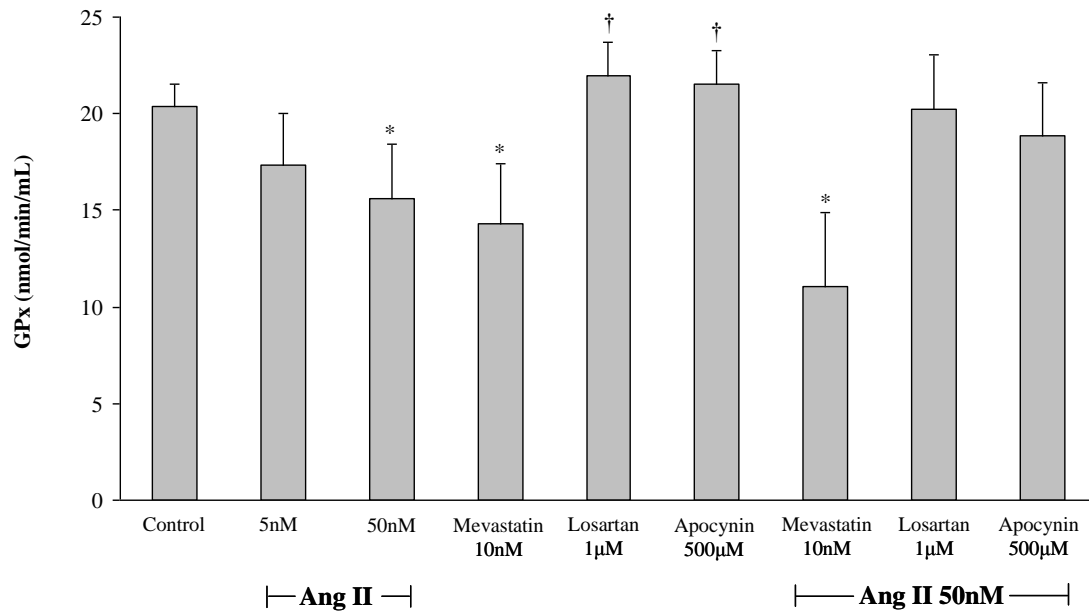
B

Figure 6

A

B

Table 1. The effects of Ang II and PDGF-BB on NADPH oxidase activities of whole cellular and membrane fractions and the effects of mevastatin, pravastatin, losartan and valsartan.
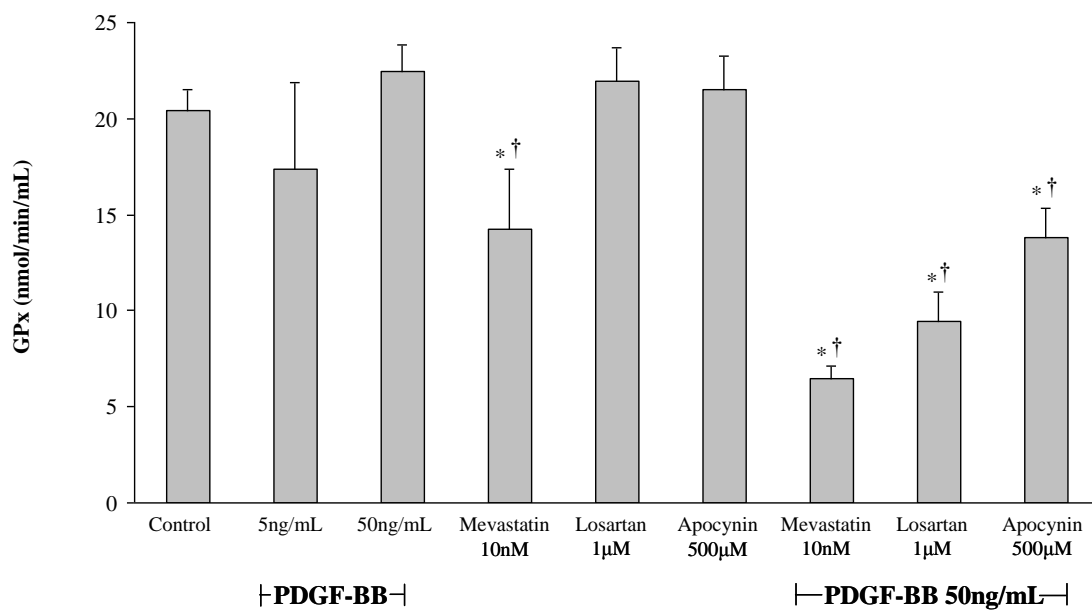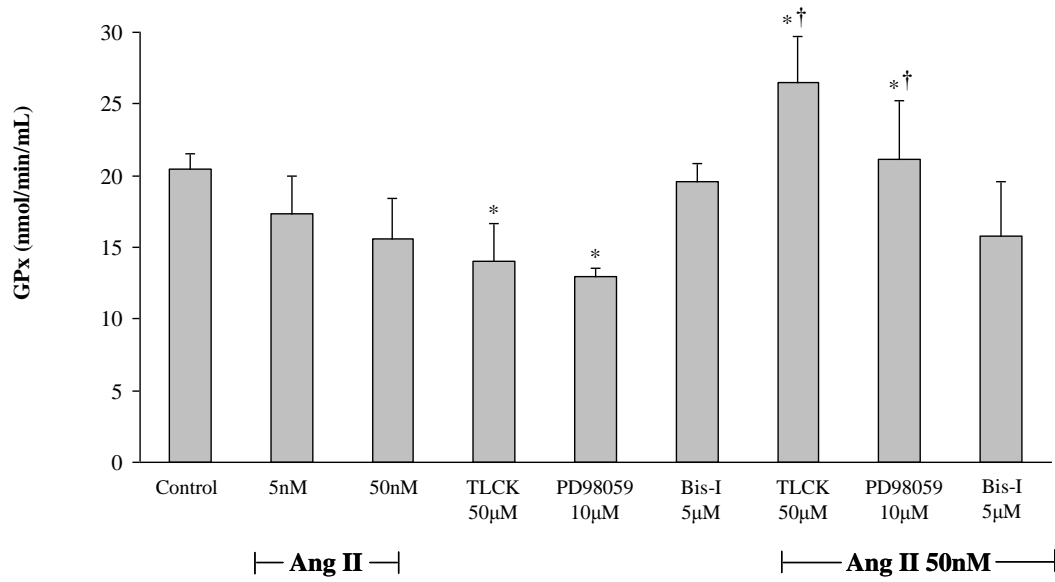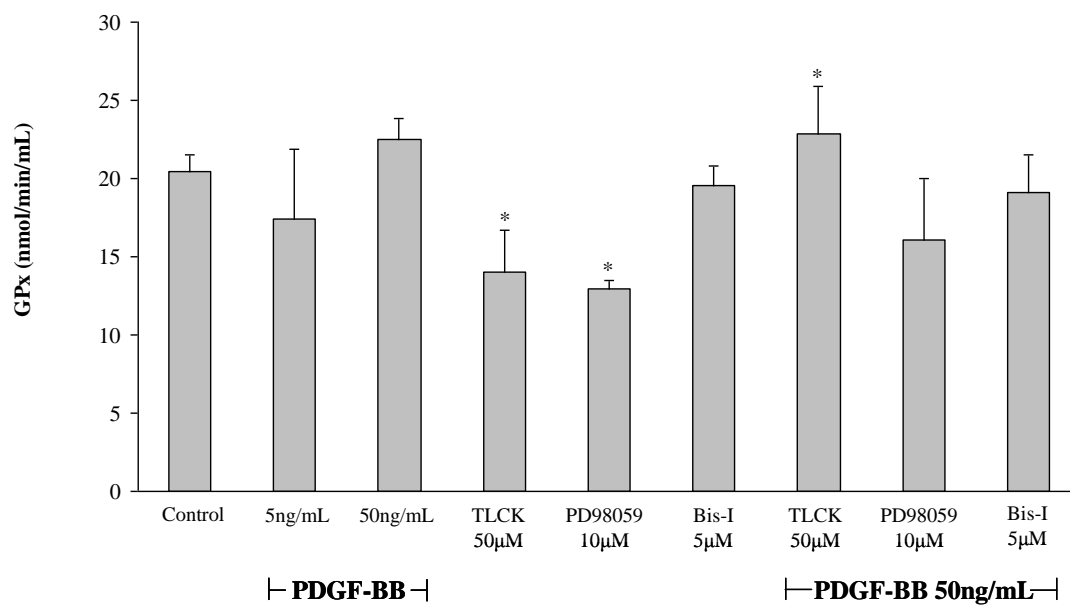
Treatment Group	Cellular (RLU/g.min)	Membrane (RLU/g.min)	Treatment Group	Cellular (RLU/g.min)	Membrane (RLU/g.min)
Ang II 50 nM	210 ± 22	92 ± 13	PDGF-BB 50 ng/mL	145 ± 18	64 ± 8
Mevastatin (10 nM)	139 ± 16*	55 ± 8*	Mevastatin (10 nM)	84 ± 15*	36 ± 4*
Pravastatin (100 nM)	127 ± 14*	52 ± 8*	Pravastatin (100 nM)	86 ± 11*	39 ± 6*
Losartan (1 μM)	89 ± 14*	33 ± 5*	Losartan (1 μM)	76 ± 10*	29 ± 5*
Valsartan (1 μM)	93 ± 11*	36 ± 4*	Valsartan (1 μM)	79 ± 11*	32 ± 4*

The levels of NADPH oxidase activities were measured in coronary artery vascular smooth muscle cells (CASMC) cultured with Ang II or PDGF-BB for 24 hours in the absence or presence of mevastatin, pravastatin losartan and valsartan. Results expressed as mean±SEM from four separate experiments. RLU; relative light unit. *$p<0.05$ difference compared to Ang II or PDGF-BB treatment groups.

Table 2. The effects of Ang II, PDGF-BB and reactive oxygen species generating enzyme inhibitors on coronary artery smooth muscle cell viability.

Treatment Group	Viable Cells (%)
Control	92 ± 4
Ang II (50 nM)	94 ± 5
PDGF-BB (50 ng/ml)	93 ± 5
Rotenone (50 μM)	91 ± 3
L-NAME (100 μM)	90 ± 3
Allopurinol (100 μM)	93 ± 5
Indomethacin (50 μM)	94 ± 4
NOHA (100 μM)	92 ± 3

Percentage viable cells were determined by trypan blue exclusion experiments through counting 100 cells. Data are expressed as mean $\pm$ SEM from 4 different experiments.

Table 3. The effects of Ang II and PDGF-BB on superoxide dismutase enzyme capacities and the effects of a statin (mevastatin), an ARB (losartan) and an NAD(P)H oxidase inhibitor (apocynin).

Treatment Group	CuZn-SOD (mU/mg protein)	Mn-SOD (mU/mg protein)	Treatment Group	CuZn-SOD (mU/mg protein)	Mn-SOD (mU/mg protein)
Basal	38 ± 2†	5 ± 1†	Basal	38 ± 1†	5 ± 1†
Ang II 5 nM	110 ± 10*†	44 ± 3*†	PDGF-BB 5 ng/mL	81 ± 4*†	36 ± 1*†
Ang II 50 nM	129 ± 8*	60 ± 2*	PDGF-BB 50 ng/mL	140 ± 16*	66 ± 3*
Mevastatin 10^{-7} mM	8 ± 1*†	2 ± 0.5*†	Mevastatin 10^{-7} mM	8 ± 1*†	2 ± 0.5*†
Losartan 10^{-6} mM	12 ± 1.1*†	3 ± 0.5*†	Losartan 10^{-6} mM	12 ± 1.1*†	3 ± 0.5*†
Apocynin 500 µM	6 ± 1*†	1 ± 0.5*†	Apocynin 500 µM	6 ± 1*†	1 ± 0.5*†
Ang II 50 nM + Mevastatin	83 ± 6*†	7 ± 3†	PDGF-BB 50 ng/mL + Mevastatin	99 ± 10*†	15 ± 3*†
Ang II 50 nM + Losartan	61 ± 11*†	21 ± 4*†	PDGF-BB 50 ng/mL + Losartan	81 ± 7*†	10 ± 4*†
Ang II 50 nM + Apocynin	31 ± 3*†	22 ± 5*†	PDGF-BB 50 ng/mL + Apocynin	62 ± 3*†	5 ± 2†

The levels of superoxide dismutase enzyme activities were measured in coronary artery vascular smooth muscle cells (CASMC) cultured with Ang II or PDGF-BB for 24 hours in the absence or presence of mevastatin, losartan and apocynin. Results expressed as mean±SEM from three separate experiments. ARB: angiotensin II type I receptor blocker; SOD: superoxide dismutase. *$p < 0.05$ difference compared to basal untreated group. †$p < 0.05$ difference compared to higher dose Ang II or PDGF-BB treatment groups.

Table 4. The effects of Ang II and PDGF-BB on nitrite and O_2^- production

Treatment Group	Nitrite (nmol/mg protein)	Superoxide anion (pmole O_2^-/mg protein)
Basal	29 ± 2	0.015 ± 0.002
Ang II 5 nmol/L	$33 \pm 3^{\dagger}$	$0.036 \pm 0.003*^{\dagger}$
Ang II 50 nmol/L	$21 \pm 2^{*}$	$0.057 \pm 0.004*$
PDGF-BB 5 ng/ml	$20 \pm 3^{*}$	$0.024 \pm 0.002*^{\dagger}$
PDGF-BB 50 ng/ml	$18 \pm 2^{*}$	$0.033 \pm 0.002*$

The levels of nitrite and O_2^- were measured in coronary artery smooth muscle cells (CASMC) cultured with indicated concentrations of Ang II and PDGF-BB for 24 hours. Results are expressed as mean±SEM from five separate experiments. *$p<0.05$ difference compared to basal untreated group. $^{\dagger}p<0.05$ difference in each treatment group.

Table 5. The effects of Ang II on NAD(P)H oxidase, CuZn-SOD and NOS activities in the absence or presence of a serine protease inhibitor tosyl-L-lysine chloromethyl ketone (TLCK), a MAPK inhibitor (PD98059) and a protein kinase C inhibitor (Bis-I).

Treatment Group	NAD(P)H Oxidase (pmol O_2^-/mg protein)	CuZn-SOD (mU/mg protein)	NOS (pmol L-citrulline/mg protein/min)
Basal	0.036 ± 0.005	38 ± 2	10290 ± 1296.1
Ang II 50nM	$0.149 \pm 0.005^{*}$	$129 \pm 8^{*}$	$6802.8 \pm 138^{*}$
TLCK 50μM	$0.038 \pm 0.007^{\dagger}$	$51 \pm 8^{*\dagger}$	$12952.8 \pm 924.4^{*\dagger}$
PD98059 10μM	$0.034 \pm 0.005^{\dagger}$	$37 \pm 3^{\dagger}$	$13178.9 \pm 1248.3^{*\dagger}$
Bis-I 5μM	$0.042 \pm 0.002^{\dagger}$	$47 \pm 4^{\dagger}$	$15720.6 \pm 277.9^{*\dagger}$
Ang II 50nM + TLCK 50μM	$0.064 \pm 0.008^{*\dagger}$	$102 \pm 7^{*}$	$12146.7 \pm 704.4^{*\dagger}$
Ang II 50nM + PD98059 10μM	$0.050 \pm 0.004^{*\dagger}$	$85 \pm 11^{*\dagger}$	$10548.9 \pm 2010.6^{\dagger}$
Ang II 50nM + Bis-I 5μM	$0.036 \pm 0.002^{\dagger}$	$119 \pm 13^{*}$	$12234.6 \pm 487.6^{*\dagger}$

The enzyme activities were measured in coronary artery smooth muscle cells (CASMC) cultured with Ang II for 24 hours in the absence or presence of indicated signal transduction pathway inhibitors. Results expressed as mean ± SEM from three separate experiments. NOS: nitric oxide synthase; SOD: superoxide dismutase. *$p<0.05$ difference compared to basal untreated group. †$p<0.05$ difference compared to Ang II 50nM treatment group.

Table 6. The effects of PDGF-BB on NAD(P)H oxidase, CuZn-SOD and NOS activities in the absence or presence of a serine protease inhibitor tosyl-L-lysine chloromethyl ketone (TLCK), a MAPK inhibitor (PD98059) and a protein kinase C inhibitor (Bis-I).

Treatment Group	NAD(P)H Oxidase (pmol O_2^-/mg protein)	CuZn-SOD (mU/mg protein)	NOS (pmol L-citrulline/mg protein/min)
Basal	0.036 ± 0.005	38 ± 2	10290 ± 1296.1
PDGF-BB 50ng/mL	$0.079 \pm 0.008^*$	$140 \pm 16^*$	$5797.3 \pm 948.8^*$
TLCK 50μM	$0.038 \pm 0.007^†$	$51 \pm 8^{*†}$	$12952.8 \pm 924.4^{*†}$
PD98059 10μM	$0.034 \pm 0.005^†$	$37 \pm 3^†$	$13178.9 \pm 1248.3^{*†}$
Bis-I 5μM	$0.042 \pm 0.002^†$	$47 \pm 4^†$	$15720.6 \pm 277.9^{*†}$
PDGF-BB 50ng/mL + TLCK 50μM	$0.038 \pm 0.001^†$	$63 \pm 1^{*†}$	$10664.9 \pm 745.3^†$
PDGF-BB 50ng/mL + PD98059 10μM	$0.034 \pm 0.005^†$	$81 \pm 8^{*†}$	$9789.5 \pm 361.2^†$
PDGF-BB 50ng/mL + Bis-I 5μM	$0.042 \pm 0.005^†$	$53 \pm 9^{*†}$	$11345.6 \pm 1047.1^†$

The enzyme activities were measured in coronary artery smooth muscle cells (CASMC) cultured with PDGF-BB for 24 hours in the absence or presence of indicated signal transduction pathway inhibitors. Results expressed as mean ± SEM from three separate experiments. NOS: nitric oxide synthase; SOD: superoxide dismutase. *$p<0.05$ difference compared to basal untreated group. †$p<0.05$ difference compared to PDGF-BB 50ng/ml treatment group.