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Research Article

Nitric Oxide and Inhibition of PPAR Gamma Regulate the ANG II Induced VSMC Proliferation: Relationship with Gi Protein Levels

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Abstract

Cardiovascular diseases are, nowadays, viewed as the major cause of morbidity and mortality. They are due to complications from hypertension, thrombosis, atherogenesis, and Rest enosis. In these pathologies, increased Vascular Smooth Muscle cells (VSMCs) hypertrophy, migration and proliferation promote their onset and progression. Furthermore, Nitric Oxide (NO), a vasoprotective molecule was shown to decrease the levels of inhibitory G alpha-protein (Giα) in VSMCs, thus indicating their possible involvement in its anti-proliferative effects. Peroxisome Proliferator Activated Receptors gamma (PPAR-γ) is linked to both NO and G-proteins. In this study, the aim was to establish that the anti-proliferative role of NO in VSMCs is mediated by decreased Giα while investigating the role of PPAR-γ as a modulator of these effects. For these purposes, A-10 cells and VSMCs were incubated with the NO donor Sodium Nitro Prusside (SNP) and/or the PPAR-γ antagonist GW9962 (20 μM and 30 μM). Cellular proliferation was assessed by a cell proliferation assay, and Giα and PPAR-γ expression were assessed by western blotting. Results show that SNP and GW9962 decreased the expression of Giα-2 and Giα-3 by 50% with no difference between individual and combination treatments. In addition, SNP had no effect on PPAR-γ activation while GW9962 increased PPAR-γ inactivation by 30-70% with combination treatments having no effect. Furthermore, SNP and/or GW9962 following stimulation with ANG II showed that they decrease the proliferation of VSMCs by21.57%, 23.33%, and 27.54 % while combination treatments do not indicate an interaction between PPAR-γ and NO. In conclusion, these results show that NO and GW9962 may independently inhibit VSMCs proliferation and decrease Giα protein levels.

Keywords: Nitric Oxide, PPAR-γ, Giα, Proliferation, Vascular Smooth Muscle cells

Introduction

NO is a short-lived gaseous free radical generated by the oxidation of L-arginine to L-citrulline in a reaction catalyzed by Nitric Oxide Synthase (NOS). Three distinct genes code for the three is forms of NOS: neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3) [1]. The effect of NO has been extensively studied in remodeling of the vasculature. In fact, demonstrated that the induction of angiogenesis by VEGF-E in human umbilical vein endothelial cells requires the activation of endothelial iNOS, and that the angiogenesis-promoting role of NO is independent of cGMP [2]. It has also been shown, by Pyriochou et al. to promote angiogenesis independently of cGMP in rat aortic endothelial cells [3].

Heterotrimeric GTP-binding proteins are composed of α, β, and γ subunits convert extracellular receptor mediated signals into

intracellular signals [4].Activation of G-protein coupled receptors leads to the replacement of GDP by GTP in the alpha subunit causing, therefore, the dissociation of Gα from the other two subunits [5]. The activated Gα and Gβγ subunits are involved in the regulation of the activity of various effectors like adenylatecyclases, phosphodiesterases, phospholipases, ion channels, and Mitogen Activated Protein Kinases (MAPKs) [6]. The implication of Gi-proteins in proliferative events has been thoroughly studied. Li et al. showed that the enhanced levels of Gi-proteins in SHR are implicated in ANG II induced hyper proliferation in A-10 VSMCs [7]. Sandoval et.al also reported similar results when they noted that the ANGII- induced increase in oxidative stress transactivates Epidermal Growth Factor Receptor, which, through downstream Mitogen Activated Protein Kinase signaling contributes to the enhanced expression of Gi-proteins and results in proliferation of A-10 VSMCs [8,9].

It has been previously shown that NO modulates Gi protein expression and Adenylyl Cyclase signaling in VSMCs. Bassil et al. showed that treatment of A-10 SMC with an NO donor resulted in a decrease of Giα-2 and Giα-3 levels while having no effect on the expression levels of Gsα proteins. The decreased level of Giα proteins was reflected in a reduction in both receptor dependent and receptor independent Gi function [10]. In addition Bassil et al. also showed that the effects of nitric oxide on Giα were mediated by the highly reactive oxygen species ONOO- and not via cGMP dependent pathway [11].

PPAR-γ has also been implicated in the protection of the vasculature by preventing hypertensive remodeling. Cipolla et.al has shown the effectiveness and clinical relevance of PPAR-γ in improving vascular function. Female Sprague Dawley rats were treated with N6-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor or L-NAME and PPAR-γ activator rosiglitazone. The hypertrophic remodeling and the enhanced myogenic activity caused by L-NAME were reversed by rosiglitazone without having any effect on blood pressure [12].

Recent research suggested that NO acts on PPAR-γ via protein nitration, which is a marker of ONOO- formation in the presence of NO. In fact, the NO- donating moiety of the non-steroidal anti-inflammatory drug NCX 2216, as shown by Bernardo et al., is responsible for PPAR-γ nitration and activation in microglia cells [13]. Nitration of tyrosine residues has also been demonstrated as an important regulator of PPAR-γ activity. Shibuya et al. proved that ONOO- induced nitration of tyrosine residues on PPAR-γ during inflammation in RAW 265 a macrophage-like cell line [14].

PPAR-γ signaling has also been extensively associated with G-proteins. A study by Knowles et al. showed that niacin which possesses an anti-lipolytic effect involving inhibitory G-protein signaling induces PPAR-γ expression and transcriptional activation in macrophages via HM74 and HM74a induction of prostaglandin synthesis pathways [15].

On another level, a study by Jeninga et al. indicates that PPAR-γ is involved in the regulation of the anti-lipolytic human G-protein-coupled receptor 81 which regularly couples to Gi members of the G-protein family [16]. These studies suggest a possible relation between NO, PPAR-γ, and Gi in VSMCs, and the possibility that the anti-proliferative effect of NO might be due to the implication of PPAR-γ activity and associated with G-protein signaling.

Materials and Methods

Cell Culture: Primary VSMCs from rat aorta and A-10-SMCs were cultured as described previously [17]. Cells were plated in 7.5 cm2 flasks and incubated at 37°C in 95% air and 5% CO2 humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) (with glucose, L-glutamine, and sodium bicarbonate) containing 1% antibiotics and 10% heat-inactivated fetal Bovine serum (FBS) from Gibco, Invitrogen. The cells were passaged upon reaching confluence with 0.5% trypsin containing 0.2% EDTA and used between passages 5 and 15 as described previously [17]. Confluent cell cultures were starved by incubation for 3 h in DMEM without FBS at 37°C to reduce the interference by growth factors present in the serum. These cells were then incubated with ANG II (10-7 M) for 2 hrs and /or (SNP) (0.5 mM) for 24 h at 37°C. After incubation, cells were washed twice with ice-cold homogenization buffer. The cells were homogenized in a homogenization potter. The homogenate was for immunoblotting experiments.

The involvement of PPAR-γ in the signaling pathway of NO in the VSMCs of the aorta was examined by the use of the specific inhibitors GW9962 (20 µmol/L) and (30µmol/L) for PPAR-γ. Cell Count: Cell viability and cell counting was assessed with the trypan blue exclusion technique [17].

Western Blots: Western blotting for Gi, PPAR-γ, and pPPAR-γ was performed as previously described [4]. After the SDS-PAGE, the separated proteins were electrophoretically transferred to a nitrocellulose membrane with a semidry transblot apparatus at 15 V for 45 min. The proteins on the membrane were stained with Rouge Ponceau S. The membranes were then blocked with 5% milk, washed twice in phosphate-buffered saline (PBS), and incubated in PBS containing 5% milk. The blots were then incubated with antibodies: Giα-2, Giα-3,PPAR-γ and pPPAR-γ anti-rabbit antibodies, and GAPDH anti-mouse anti-body against GAPDH in PBS containing 5% dehydrated milk and 0.2% Tween 20 at 4°C for overnight. The antibody–antigen complexes were detected by incubating the membranes with goat anti-rabbit IgG and goat anti-mouse IgG conjugated with horseradish peroxidase for 1 h at room temperature. All antibodies were purchased from Santa-Cruz,

Santa Cruz, USA. The blots were then washed three times with PBS before reaction with enhanced-chemiluminescence Western-blotting detection reagents purchased from GEAmersham, Europe. The autoradiograms were quantified by densitometricscanning using a gel image reader.

Cell Proliferation Assay: Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay by Promega was used to examine the proliferation of cells following incubation with proliferative and anti-proliferative agents [18]. Sub confluent primary VSMC were plated in 96-well plates for 24 h and were serum deprived for 24 h to induce cell quiescence. The cells were then incubated with ANG II (10-7 M) to induce proliferation. 10µL per well of Cell Proliferation Assay (MTS)/phenazine methosulfate (PMS) solution were added, and the plates were incubated for 4 hours at 37°C in a humidified, 5% CO2 atmosphere. To measure the amount of soluble formazan produced by cellular reduction of the MTS, the absorbance at 490nm was recorded using an ELISA plate reader. The anti-proliferative effect of NO was assessed by incubation with SNP (0.5 mmol/L) for 2 hrs, and the role of PPAR-γ in the anti-proliferative effects of NO were examined by addition of the specific inhibitors GW9962 (20 µmol//L) and (30 µmol//L).

Statistical Analysis: Values were reported as means ± SE. Graph Pad Prism 5 was used for statistical analysis. Comparisons between groups were made using one way ANOVA in conjunction with the Newman-Keuls multiple comparison tests. Differences between groups were considered statistically significant at $P < 0.05$.

Results

Effect of treatments with SNP and GW9962 on Giα-2

VSMCs (Figure 1) and A-10 cells (Figure 2) were treated with SNP (500 µM) and/or GW9962 (20 µM and 30 µM). Western blotting experiments showed that these treatments uniformly decreased the levels of expression of Giα-2 in VSMCs and A-10 cells Compared to Control Cells (CTL) or those treated with the vehicle DMSO (same volume used to treat cells with GW9962). Treatment with SNP decreased the levels of Giα-2 by 44.3% in VSMCs and 57.6% in A-10 cells. Treatment with GW9962 (20 µM and 30 µM) decreased the levels of expression of Giα-2 by 49% and 53.33% respectively in VSMCs and 62.33% and 58.66% in A-10 cells. The decreased levels of Giα-2 showed no statistical significance between individual and combination treatments with the NO donor and the PPAR-γ antagonist. Treatment with SNP/ GW9962 (20 µM) decreased the levels of Giα-2 to 52% in VSMCs and 54.3% in A-10 cells, while treatment with SNP/ GW9962 (30 µM) decreased the levels of Giα-2 to 49.33% in VSMCs and 52.66% in A-10 cells.

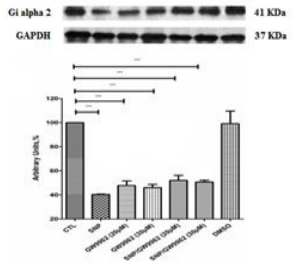

Figure 1: Protein expression levels of Giα-2 in VSMCs. The cells were treated with SNP (0.5 mM) and / or GW9962 (20 µM and 30 µM). The values are the results of 3 independent experiments. ***= significant $p<0.001$

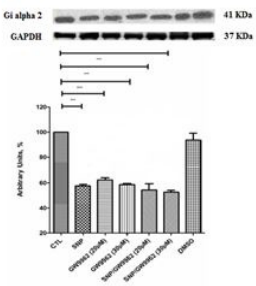

Figure 2: Protein expression levels of Giα-2 in A-10 Cells. The cells were treated with SNP (0.5 mM) and / or GW9962 (20 µM and 30 µM). The values are the results of 3 independent experiments. ***= significant $p<0.001$

Effect of treatments with SNP and GW9962 on Giα-3

VSMCs (Figure 3) and A-10 cells (Figure 4) were treated with SNP (500 µM) and/or GW9962 (20 µM and 30 µM). Western blotting experiments showed that these treatments uniformly decreased the levels of expression ofGiα-3 in VSMCs and A-10 cells compared to control cells or those treated with the vehicle DMSO. Treatment with SNP decreased the levels of Giα-3 by 40.33% in VSMCs and 55.67% in A-10 cells. Treatment with GW9962 (20 µM and 30 µM) decreased the levels of expression of Giα-2 by 47.66% and 46% respectively in VSMCs and 55.33% and 57.0% in A-10 cells. The decreased levels of showed no statistical significance between individual and combination treatments with the NO donor and the PPAR-γ antagonist. Treatment with SNP/ GW9962 (20 µM) decreased the levels of Giα-2 to 52% in VSMCs and 54% in A-10 cells, while treatment with SNP/ GW9962 (30 µM) decreased the levels of Giα-2 to 52.66% in VSMCs and 50.66% in A-10 cells.

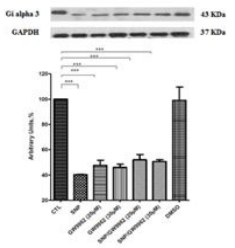

Figure 3: Protein expression levels of Giα-3 in VSMCs. The cells were treated with SNP (0.5 mM) and / or GW9962 (20 μM and 30 μM). The values are the results of 3 independent experiments. ***= significant p<0.001

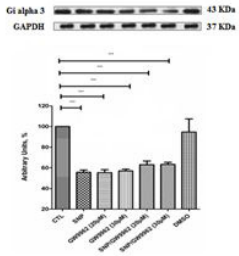

Figure4: Protein expression levels of Giα-3 in A-10 Cells. The cells were treated with SNP (0.5 mM) and / or GW9962 (20 μM and 30 μM). The values are the results of 3 independent experiments. ***= significant p<0.001

Effect of treatments with SNP and GW9962 on the ratio of pPPAR-γ (ser-112)/PPAR-γ

VSMCs (Figure 5) and A-10 cells (Figure 6) were treated with SNP (500μM) and/or GW9962 (20 μM and 30 μM). Western blotting experiments showed that treatment with SNP did not affect the ratio of phosphorylated/unphosphorylated PPAR-γ in comparison to the control group and the group treated with the vehicle DMSO, and hence had no effect on the PPAR-γ activation. In contrast, treatment with PPAR-γ antagonist GW9962 at 20 μM and 30 μM dose dependently increased the ratio of phosphorylated/unphosphorylated PPAR-γ compared to CTL and DMSO treated groups, thus effectively inactivating PPAR-γ. Treatment with GW9962 at 20 μM and 30 μM increased phosphorylation ratios by 37.66% and 70.33% respectively in VSMCs and 36.33% and 78.33% respectively in A-10 cells. It is important to mention that treatments with SNP and GW9962 at both concentrations had no significant effect with respect to treatments with the antagonist alone. Treatment with SNP/ GW9962 (20 μM) increased phosphorylation ratios by 32.33% in VSMCs and 43 % in A-10 cells, while treatment with SNP/ GW9962 (30 μM) increased the ration by 67.66% in VSMCs and by 75.33% in A-10 cells.

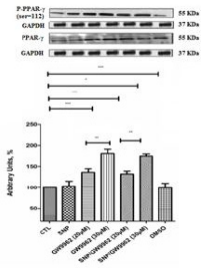

Figure 5: Ratio of p-PPAR-γ (ser 112)/PPAR-γ as an indicator of PPAR-γ inactivation in VSMCs. The cells :ere treated with SNP (0.5 mM) and / or GW9962 (20 μM and 30 μM). The values are the results of 3 independent experiments. **= significant p<0.01, ***= significant p<0.001

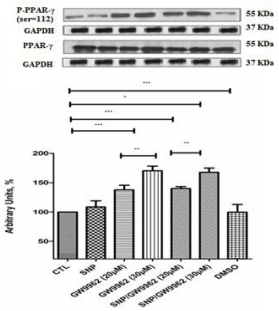

Figure 6: Ratio of p-PPAR-γ (ser 112)/PPAR-γ expression levels as an indicator of PPAR-γ inactivation following treatments in A-10 Cells. The cells were treated with SNP (0.5 mM) and / or GW9962 (20 μM and 30 μM). The values are the results of 3 independent experiments. **= significant p<0.01, ***= significant p<0.001

MTS Cell Proliferation Test

The results of the proliferation test in VSMCs (Figure 7) showed that treatment of quiescent cells with the vehicle DMSO had no effect on cellular proliferation as the absorbance levels at 490 nm remained unchanged. Treatment with ANG II increased the proliferation rate of VSMCs by 26.9%. Treatment with SNP and/or GW9962 following stimulation of quiescent cells with ANG II for 3 hours showed that these treatments alone or in combination decreased the proliferation levels of VSMCs. Treatment with ANGII/SNP decreased the proliferation of VSMCs by 21.57%. No significant difference in proliferation rates was shown between treatments with GW9962 at both concentrations. Treatment with ANGII/GW9962 (20 μM) decreased the proliferation of VSMCs by 23.33%. Treatment with ANGII/GW9962 (30 μM) decreased the proliferation of VSMCs by 27.54%. Treatment with ANGII/SNP/GW9962 (20 μM) decreased the proliferation of VSMCs by 29.03%. Treatment with ANGII/SNP/GW9962 (30 μM) decreased the proliferation of VSMCs by 34.29%.

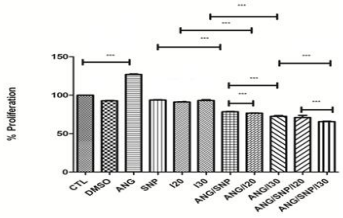

Figure 7: Proliferation rates of VSMCs. Cells were pre-treated with ANG II (10-7 M) for 3 hrs followed by SNP (0.5 mM) and/or GW9962 (20 μM and 30 μM) for 24hrs. The values are the results of 3 independent experiments. I20= GW9962 20 μM, I30= GW9962 30 μM, **= significant $p<0.05$, ***= significant $p<0.001$

Discussion

In this study, the aim was to establish that the anti-proliferative role of NO in VSMCs is mediated by decreased Giα while investigating the possible role of PPAR-γ as a modulator of these effects. Our results show that treatments of both VSMCs and A-10 cells with SNP and GW9962 decreased the levels of Giα-2 and Giα-3. The relationship between NO donor treatments and Gi-protein levels and function was investigated by Bassil et al. with results comparable to those reported in our experiments [10]. The observed decrease in Giα-2 and Giα-3 levels along with results from the proliferation test showing that treatments with SNP and GW9962 had no effect on cell proliferation compared to the control group can lead to the conclusion that the anti-proliferative effect of both NO and PPAR-γ inhibitor in VSMCs is probably due to the observed decrease in Gi expression. In this regard, it has been already proven that the decrease in Gi protein expression and its related decrease in activity have anti-proliferative effects in the vasculature [7-9]. This suggestion concerning the relationship between anti-proliferative effects of both NO and PPAR-γ inhibitor in VSMCs and the decrease in Gi-protein levels has to be further elucidated by treatment by RNA interference or with the inhibitor pertussis toxin [10].

Another key aspect that our results help establish when comparing the proliferation rates of the control group to those treated with SNP and GW9962 and the vehicle DMSO without prior stimulation with ANG II is the fact that these treatments are not cytotoxic since the proliferative rates remained unchanged across these groups. This observation is further validated by cell counting using the trypan blue exclusion technique, which indicated that cellular viability was not affected by these treatments (data not shown). On the other hand, proliferation test data showed that treatment of quiescent cells with ANG II markedly increased the proliferation of both VSMCs. These observations are compatible to previous reports which confirmed the proliferative role of ANG II in the vasculature [7-9]. Interestingly MTS test results showed that both treatments with SNP and GW9962 after stimulation with ANG II decreased cell proliferation rates compared to control groups, and that the combination of SNP/GW9962 had no significant change in proliferation rates compared to individual treatments. These observations have led us to conclude that PPAR-γ may not mediate the anti-proliferative effect of NO in VSMCs.

Experimental results regarding the anti-proliferative role of NO are in agreement with many studies in the literature. A recent study showed that treatment of human aortic cell cultures with synthetic secondary amines containing an aromatic nitro group, and thus acting as sustainable NO donors, decreased the proliferation of these cells, an effect enhanced by incubation with the arginase inhibitor S-2-amino-6-boronic acid (ABH) [19]. Zuckerbraun et al. showed that increase expression of iNOS decreased the proliferation of VSMCs via p42/p44 mitogen-activated-protein kinase and p21waf1/cip1 [20]. Furthermore, Hashimet. All have shown that the L-NAME induced increase in Giα protein level was reversed by Losartan, an Angiotensin Type I receptor antagonist [21]. It is of importance to mention that the anti-proliferative effects of PPAR-γ activation are observed following prolonged treatments with potent proliferative and agents such as ANGII, PDGF, or bFGF and establishment of an advanced proliferative state or after induction of vascular injury [22]. In fact, Alexis et. Al provides an explanation for the role of Bcrkinase in the cross-talk between ANG II and PPAR-γ. In their study, these authors investigated the role of the Bcr kinase; a well-known serine-threonine kinase expressed in many cell types, and activated by PDGF, which is highly expressed in the neointima after vascular injury. The conducted experiments demonstrated that Bcr was stimulated by treatment with ANG II, and that its overexpression abrogated PPAR-γ activity. More importantly, its down regulation resulted in the recovery of PPAR-γ activity [23]. In contrast, the proliferative effect of PPAR-γ ligands in the vasculature was reported by Xiao et.al that showed that treatment of VSMCs with the PPAR-γ activator was able to significantly increase proliferation in VSMCs. A similar effect was observed in VSMCs that over expressed PPAR-γ. In contrast, GW9662 treatment and silencing PPAR-γ were able to markedly inhibit VSMCs proliferation [24]. Furthermore, the involvement of PPAR-γ in the promotion of angiogenesis has been widely reported. Biscetti et al. demonstrated that selective activation of PPAR γ leads to tube formation in endothelial/VSMCs co-culture system. This effect was shown to be mediated via a VEGF dependent mechanism, and reversed following treatment with a PPAR-γ inhibitor [25].

The interaction between PPARs and G-proteins has not been extensively studied in the literature. A study by Knowles et al. showed that niacin which possesses an anti-lipolytic effect involving inhibitory G-protein signaling induces PPAR-γ expression and

transcriptional activation in macrophages via HM74 and HM74a induction of prostaglandin synthesis pathways [15]. Similarly, another study indicates that PPAR-γ is involved in the regulation of the anti-lipolytic human G-protein-coupled receptor 81 which regularly couples to Gi members of the G-protein family [16].

Another important finding in our study was that the anti-proliferative effects of SNP and GW9962 individual treatments showed no important significant effect compared to co-incubation with both treatments. These observations may indicate that the anti-proliferative effect of NO is not mediated by PPAR-γ. However, these findings were not able to provide a conclusive answer as to whether the anti-proliferative effect observed following inhibition of PPAR-γ is independent of NO or if PPAR-γ is located upstream of NO signaling pathway. These results are in agreement with those of the western blot experiments which showed that treatment with SNP had no effect on the ratio of pPPAR-γ/PPAR-γ compared to untreated controls. Also, the treatment with SNP in combination with GW9962 showed no difference in the proliferation assay when compared to treatments with GW9962 alone. In this regard, several studies have reported an interaction between NO and PPAR-γ. In this regard, Cipola et al highlighted the effectiveness and clinical relevance of PPAR-γ in improving vascular function. Female Sprague Dawley rats were treated with L-NAME or L-NAME and PPAR-γ activator rosiglitazone. The hypertrophic remodeling and the enhanced myogenic activity caused by L-NAME were reversed by rosiglitazone without having any effect on blood pressure [12]. These studies may indicate that the signaling pathway of PPAR-γ may be independent or in upstream of Nitric Oxide. From another side, others have found that GW9962 elicited PPAR-γ specificity based on its direct and indirect inhibitory effects on the expression of metabolic genes known to be under the control of PPARs [26]. Finally, an inhibition of Gi-protein levels by siRNA or shRNA or activity by pertussis toxin will be needed to better understand the role of Gi-protein in this signaling mechanism.

Conclusion

In conclusion, this research project gave new insights into the role of NO and PPAR-γ in the proliferation of VSMCs and the potential relationship with Gi-protein levels. Although our results were not able establish a conclusive evidence as to the interaction between NO and PPAR-γ, but they showed that both NO donors and inhibition of PPAR-γ in non-pathological conditions regulate the decrease of VSMCs proliferation, and hence may serve as potential therapeutic targets in the prevention of the onset of vascular diseases.

Declarations section

List of abbreviations

- NO - Nitric Oxide
- VSMCs - Vascular Smooth Muscle cells
- Giα - G alpha-protein
- PPAR-γ - Peroxisome Proliferator Activated Receptors gamma
- SNP - Sodium Nitroprusside
- GW9962 - PPAR-γ antagonist
- NOS - Nitric Oxide Synthase
- nNOS or NOS-1 - Neuronal NOS
- iNOS or NOS-2 - Inducible NOS
- eNOS or NOS-3 - Endothelial NOS
- VEGF- Vascular endothelial growth factor
- SHR –Spontaneous hypertensive rats
- SMC – Smooth muscle cells
- cGMP – Current Good Manufacturing Practices
- MAPKs - Mitogen Activated Protein Kinases
- ANGII – Angiotensin II
- ONOO - Peroxynitritre
- PPAR-γ - Peroxisome Proliferator Activated gamma
- L-NAME - N6-nitro-L-arginine methyl ester
- DMEM - Dulbecco's modified Eagle's medium
- FBS - fetal Bovine serum
- GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
- PBS - phosphate-buffered saline
- MTS – Cell Proliferation Assay
- PMS – phenazine methosulfate
- DMSO - Dimethyl sulfoxide
- CTL - Control
- ABH - S-2-amino-6-boronic acid
- PDGF, or bFGF – platelet derived growth factor
- bFGF- Basic fibroblast growth factor

Ethics and Consent statement: Not applicable.

Consent to Publish: Not applicable.

Availability of Data and Materials: PubMed and Medline.

Authors' contributions: Ms. Lama Hamadeh is the first author, she did the techniques and wrote the thesis for her master degree. Dr. Marcel Bassil is her thesis director and mentor.

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Competing Interest: The authors declare that they have no competing interests.

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