Received Date : 23-Jun-2016
Revised Date : 08-Dec-2016
Accepted Date : 11-Dec-2016
Article type : Original Article

Antihypertensive methyldopa, labetalol, hydralazine, and clonidine reversed TNF-α inhibited eNOS expression in endothelial-trophoblast cellular networks

Bei Xu[a,c], Gabriele Bobek[a], Angela Makris[a,b,c] and Annemarie Hennessy[a, c]

[a]School of Medicine, Western Sydney University; [b]Renal Unit, Liverpool Hospital, Sydney and [c]Vascular Immunology Research Laboratory, The Heart Research Institute, University of Sydney; Sydney, Australia

Address correspondence to: Bei Xu

Vascular Immunology Research Laboratory

The Heart Research Institute

University of Sydney

7 Eliza Street

Newtown, NSW 2042

Australia

Ph: +612 82088900

Fax: +612 95655584

Email: bei.xu@hri.org.au

Antihypertensive methyldopa, labetalol, hydralazine, and clonidine reversed TNF-α inhibited eNOS expression in endothelial-trophoblast cellular networks

Bei Xu[a,c], Gabriele Bobek[a], Angela Makris[a,b,c] and Annemarie Hennessy[a, c]

[a]School of Medicine, Western Sydney University; [b]Renal Unit, Liverpool Hospital, Sydney and [c]Vascular Immunology Research Laboratory, The Heart Research Institute, University of Sydney; Sydney, Australia

Summary

Medications used to control hypertension in pregnancy also improve trophoblast and endothelial cellular interaction *in vitro*. Tumour necrosis factor-α (TNF-α) inhibits trophoblast and endothelial cellular interactions and simultaneously decreases endothelial nitric oxide synthase (eNOS) expression. This study investigated whether antihypertensive medications improved these cellular interactions by modulating eNOS and inducible nitric oxide synthase (iNOS) expression. Human uterine myometrial microvascular endothelial cells (UtMVECs) were pre-incubated with (or without) low dose TNF-α (0.5 ng/ml) or TNF-α plus soluble *fms-like* tyrosine kinase-1 (sFlt-1) (100 ng/ml). The endothelial cells were cultured on Matrigel. After endothelial cellular networks appeared, trophoblast derived HTR-8/SVneo cells were co-cultured in the presence of clinically relevant doses of methyldopa, labetalol, hydralazine or clonidine for 24 hours. Cells were retrieved from the Matrigel to extract mRNA and eNOS and iNOS expression were

examined by quantitative PCR. Methyldopa, labetalol, hydralazine and clonidine reversed the inhibitory effect of TNF-α on eNOS mRNA expression. After pre-incubating endothelial cells with TNF-α and sFlt-1, all the medications except methyldopa lost their effect on eNOS mRNA expression. In the absence of TNF-α, antihypertensive medications did not change eNOS expression. The mRNA expression of iNOS was not affected by TNF-α or any medications. This study shows that selected antihypertensive medications used in the treatment of hypertension in pregnancy increase eNOS expression *in vitro* when induced by the inflammatory TNF-α. The antiangiogenic molecule sFlt-1 may antagonise the potential benefit of these medications by interfering with the NOS pathway.

Key words

Preeclampsia; tumor necrosis factor-α; soluble *fms-like* tyrosine kinase-1; endothelial nitric oxide synthase (eNOS); anti-hypertensive medication; methyldopa; labetalol; hydralazine; clonidine

Introduction

In pregnancy, the uteroplacental vasculature undergoes a profound change where a marked increase in uteroplacental blood flow is achieved by a reduction in vascular resistance. Trophoblasts invade into the uterine spiral arteries transforming them into low resistance vessels [1] [2]. Failure of spiral artery transformation and poor placental invasion of the uterine vasculature by trophoblast cells [3] are implicated in the pathogenesis of preeclampsia, a significant and common complication of pregnancy with characteristic signs of hypertension and proteinuria[4] [5].

Nitric oxide (NO) is a potent vasorelaxant that contributes to the reduction in peripheral vascular resistance during pregnancy [6] [7] [8]. There are three isoforms of nitric oxide synthase (NOS) which produce NO. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed and are dependent on Ca^{2+} and calmodulin to produce small basal levels of NO for vasodilatation and maintenance of vascular tone [9]. In contrast, Ca^{2+} independent inducible NOS (iNOS) is stimulated by proinflammatory molecules to liberate a great amount of NO and cause cell damage [10]. During gestation, eNOS and iNOS are expressed and dynamically regulated in the trophoblast and endothelial cells of the placenta [11] [12] [13]. iNOS has been shown to play a role in promoting trophoblast invasion [14].

We have previously established an *in vitro* model of trophoblast invasion, where we have shown that the proinflammatory molecule TNF-α, found in higher levels in the sera of preeclamptic women [15] inhibits the integration of trophoblast cells into endothelial cell networks, and simultaneously decreases eNOS expression [16] [17]. The NO donor, sodium nitroprusside dehydrate, reversed the inhibitory effects of TNF-α on integration and eNOS expression [17]. More recently we have shown that antihypertensive medications administered selectively to control hypertension in pregnancy, such as methyldopa, labetalol, hydralazine and clonidine, can improve trophoblast interaction with endothelial cellular networks. These medications are the most commonly used for everyday treatment of hypertension in pregnancy [18]. However, whether these antihypertensive medications modulate the interaction between trophoblast and endothelial cells via the NOS pathway is yet to be investigated. It has been reported that clonidine, a central acting α_2-adrenergic

agonist, induces vascular relaxation by a nitric oxide-dependent mechanism [19] and enhances iNOS mRNA expression [20]. The vasodilator hydralazine has been reported to reduce NO production and iNOS gene expression in macrophages [21] but to have no effect on any NOS protein expression in the brain [[22]. There are no reported studies on the effect of the indirectly acting α_2-adrenergic agonist methyldopa or of labetalol, the combined α-$_{1/\beta}$ adrenergic antagonist, on NOS activity or expression.

The aim of this study is to investigate whether the antihypertensive medications typically used during pregnancy; methyldopa, labetalol, hydralazine and clonidine, alter the interaction between human trophoblast and endothelial cells via modulation of eNOS and iNOS expression. Further, we aim to investigate whether the presence of the antiangiogenic molecule soluble fms-like tyrosine kinase-1 (sFlt-1), which is found in elevated levels in preeclamptic women and thought to be involved in its pathogenesis [23] attenuates the eNOS and iNOS response.

Results

Concurrent administration of anti-hypertensives reverses the TNF-α induced reduction of eNOS expression in co-cultured cells

The addition of the proinflammatory cytokine TNF-α to the co-cultured endothelial and trophoblast cells resulted in a decrease of the eNOS mRNA expression to 35±6% of control co-cultured cells (Figure 1, **p<0.01). Concurrent addition medications; methyldopa, labetalol, hydralazine and clonidine, not only prevented

the inhibitory effect of TNF-α on eNOS expression (Figure 1, ***p<0.001), but also increased eNOS expression to that of 109±15% (methyldopa) 144±26% (labetalol), 286±73% (hydralazine) and 210±48% (clonidine) of control co-cultures. In the absence of TNF-α, none of the medications had an effect on eNOS expression (Figure 1). In the absence of endothelial cells, trophoblast HTR-8 cells alone cultured on matrigel did not express eNOS (see supplemental data).

Anti-hypertensives prevent reduced eNOS expression in co-cultured cells after endothelial cell pre-exposure to TNF-α

In order to simulate a situation when the inflammatory pathway has already been activated prior to addition of the anti-hypertensives, endothelial cells were pre-exposed to TNF-α prior to co-culture. Preincubating UtMVECs with TNF-α before endothelial cellular networks formed also resulted in an inhibition of eNOS mRNA expression. This was noted with co-cultured cells (Figure 2, 51±19% of control cells, *p<0.05), and with the addition of the selected medications methyldopa, labetalol, hydralazine and clonidine. Under these conditions a significant augmentation of eNOS mRNA expression compared to control cells was only observed with labetalol (170±26%), hydralazine (192±43%) and clonidine (146±15%) but not methyldopa (87±15%) (Figure 2, *p<0.05, **p<0.01, ***p<0.001 respectively). A comparison of the effects of medications on eNOS expression when endothelial cells were pre-incubated with TNF-α or when TNF-α was added concurrently with medications was shown in Table 1.

The anti-angiogenic molecule sFLT-1 modulates the eNOS response to TNF-α and anti-hypertensives

As the anti-angiogenic molecule sFLt-1 was also found to be elevated in the plasma of women with preeclampsia, we assessed the eNOS response of the co-culture system when the endothelial cells were pre-incubated with both TNF-α and sFlt-1. The inhibition of eNOS expression observed with TNF-α alone was negated. Labetalol (87±13%), hydralazine (101±16%) and clonidine(121±20%), except methyldopa (129±18%, *$p<0.05$) diminished their effects on eNOS expression (Figure 3).

TNF-α or anti-hypertensives have no effect on iNOS expression

The mRNA expression of iNOS was not significantly affected by TNF-α or any of the medications. The addition of methyldopa, labetalol, hydralazine or clonidine did not alter the iNOS mRNA expression either in the presence or absence of TNF-α. This was the case regardless of whether endothelial cells were pre-incubated with TNF-α or it was added concurrently. The addition of sFlt-1 to the TNF-α pre-incubated cells did not alter mRNA expression of iNOS (data not shown).

Discussion

In this study, we found that antihypertensive medications commonly used for blood pressure control in pregnancies; methyldopa, hydralazine, clonidine and labetalol, counteracted the inhibitory effect of TNF-α on eNOS mRNA expression *in vitro*. In the absence of the inflammatory molecule TNF-α, these medications had no effect on eNOS expression. Additionally, the co-presence of sFlt-1 along with TNF-α

prevented both the inhibitory effect of TNF-α and the attenuation of these medications on eNOS expression.

In this study, prior activation of endothelial cells by TNF-α before trophoblast interaction is designed to mimic a very early stage of pregnancy before preeclampsia is clinically apparent. Whereas concurrent exposure to TNF-α during trophoblast integration into endothelial networks, mimics later stages of placental development. We found that the inhibitory effect of TNF-α on eNOS expression occurs independent of whether there is prior endothelial exposure to TNF-α, or concurrent exposure during trophoblast co-culture. Correspondingly the attenuation of eNOS expression by the hypertensives in the presence of TNF-α occurs regardless of the timing of exposure, suggesting that the hypertensives are modulating a factor downstream on the TNF-α pathway which in turn affects the eNOS response. These results are consistent with our previous study that showed that the selected drugs rescued the inhibition of integration of trophoblast cells into endothelial networks regardless of whether there was prior or concurrent exposure to TNF-α, but that they had no effect on integration in the absence of TNF-α [18].

Antihypertensive medications work by different mechanisms to control blood pressure. Methyldopa and its alternative clonidine are α_2-adrenoceptor agonists that act on the central nervous system to control blood pressure [24]. Clonidine has also been reported to stimulate peripheral α_2 receptors and induce vasodilatation through the activation of α_2 receptors in endothelial cells. This effect can be blocked by L-NAME, an inhibitor of NOS, suggesting that NO pathway may be involved in

this process [25]. The “rescue” effect of clonidine and methyldopa on eNOS mRNA expression in the presence of TNF-α shown in our *in vitro* model also suggests the involvement of endothelial α_2 receptors and the NO pathway in the action of methyldopa and clonidine. Hydralazine relaxes arterial smooth muscle cells by inhibiting intracellular free calcium and consequently reducing peripheral resistance [26]. Our study has found that, in the presence of TNF-α, hydralazine increased eNOS mRNA expression in the co-cultured cells. This is in contrast to other studies where hydralazine was found to have no effect on eNOS production [27] [28] [22]. Non-selective α and β-blocker labetalol, reduces blood pressure by decreasing total peripheral resistance [29]. Whether this effects eNOS activity has not been reported. But other similar anti-hypertensive α and β-blockers such as nebivolol and carvidelol have been shown to have nitric oxide-potentiating vasodilatory effect [30] [31]. Our study suggests that labetalol does not directly affect eNOS mRNA expression alone, but rather that labetalol, hydralazine, methyldopa and clonidine, modulate the eNOS response in the presence of TNF-α.

TNF-α is a proinflammatory mediator that plays a key role in cellular response to inflammation and injury. Elevated levels are associated with the vascular dysfunction of preeclampsia [15, 32] and hypertension [33]. TNF-α is required for trophoblast invasion in pregnancy [34], however in the exaggerated systemic inflammatory response of preeclampsia [35], excessive TNF-α can inhibit trophoblast migration and integration [16] [36] and induce endothelial cell injury [37] [38]. TNF-α plays a pivotal role in both the NO and the reactive oxygen species (ROS) pathways. It has been shown to regulate eNOS by a pathway that involves reduction of eNOS mRNA stability [39]. TNF-α also plays a role in the regulation of

ROS by increasing NADPH oxidase leading to increased levels of the superoxide radical [33].Superoxide can react with NO to form the damaging peroxynitrate (ONOO-) which in turn, by oxidising the essential eNOS cofactor tetrahydrobiopterin (BH4), can lead to uncoupling of eNOS activity [9]. Additionally, superoxide can be converted by superoxide dismutase (SOD) to peroxide ($H_2O_{2)}$, a molecule that has been reported to increase eNOS mRNA transcription and stability [40]. TNF-α may therefore have dual and conflicting effects on eNOS mRNA depending on the level of oxidative stress in the cells and the activity of the other participants in the complex NO and ROS pathways. The differential effect of the various anti-hypertensive medications on eNOS expression, both in the absence and presence of TNF-α and in comparison to each other, may thus be a consequence of their individual mechanisms of action and how this action may intersect with the conflicting pathways of TNF-α regulation of eNOS mRNA. Labetalol, for example, has been shown to inhibit superoxide production [41] and thus may modulate the effect of TNF-α induced NADPH oxidase activity.

In this study, an assessment of the effect of the hypertensive medications on NOS expression was carried out under conditions where both TNF-α and sFlt-1 were present, simulating untreated established preeclampsia. Elevated serum levels of both these molecules are found in preeclampsia [42] [43]. It is well documented that excessive placental sFlt-1 plays a significant role in the pathogenesis of preeclampsia [23, 42]. Experimentally, placental sFlt-1 causes a preeclampsia-like syndrome by antagonizing VEGF signalling, leading to endothelial dysfunction [23]. Recently, it has been reported that reducing sFlt-1 from the plasma of preeclamptic patients by apheresis can decrease and stabilize maternal blood pressure [44].

VEGF has been shown to induce activation of eNOS by cell surface receptor signalling mediated phosphorylation of eNOS [45]. By acting as a decoy receptor sFlt-1 may inhibit this activation. A recent human study has demonstrated that methyldopa decreases placental and serum sFlt-1 in preeclamptic women [46]. Our previous *in vitro* data have shown that some antihypertensive medications can decrease sFlt-1 in the conditioned medium [18]. In the study reported here we show that upon endothelial cells preexposure to both sFlt-1 and TNF-α, the inhibition of eNOS expression observed with TNF-α alone was negated and that of the selected antihypertensive medicines only methyldopa slightly attenuated eNOS mRNA expression. .

The calcium independent inducible NOS (iNOS) can produce a large amount of NO (>1uM) during inflammation, exerting detrimental effects on tissue [47]. Sera derived from preeclamptic patients has been reported to increase mRNA expression of inducible NOS (iNOS) in endothelial cells [48]. In our study, the inflammatory molecule TNF-α did not change iNOS expression in the endothelial cell/trophoblast co-cultured cells, indicating that iNOS is not involved in this *in vitro* model. While clonidine has been reported to induce iNOS expression in macrophage cells [49] none of the selected medications had any effect on iNOS expression in this study.

In summary, our data suggest that, in the presence of the inflammatory molecule TNF-α, some antihypertensive medications used to control blood pressure in pregnancy may have a common pathway to vascular relaxation via influencing nitric oxide production by eNOS. Soluble Flt-1 may antagonise the potential benefit of

these medications in the treatment of hypertension in pregnancy by interfering with the NOS pathway.

Methods

Cell culture

Human uterine microvascular endothelial cells (UtMVEC) (PromoCell, Heidelberg, Germany) were cultured in EGM-2-MV medium (Lonza, Basel, Switzerland) until about 80% confluent. Cells from passage 4-8 were used in this study. Primary extravillous trophoblast HTR-8/SVneo cells (kindly provided by Dr. C.H. Graham from Queen's University, Kingston, ON, Canada) were cultured with RPMI 1640 with 5% bovine calf serum (Invitrogen, Carlsbad, CA, USA) until confluence. All cells were cultured under standard condition at 37^{o}C with 5% CO_2.

Co-culture of endothelial cells and HTR-8/SVneo cells on Matrigel with methyldopa, labetalol, hydralazine, or clonidine, with/without TNF-α.

Tissue culture plates (24-wells) were coated with undiluted Matrigel (300µl/well, In Vitro Technologies, Noble Park, VIC, Australia) and polymerized for 30 minutes at 37^{o}C. UtMVECs (100,000 cells/well) were seeded into each well and endothelial cell tubular networks formed within 4 hours of further incubation at 37°C. HTR-8/SVneo cells (100,000 cells/well) were then added to each well with or without a low dose of TNF-α (0.5 ng/mL) together with methyldopa (5 µg/ml), labetalol (0.5 µg/ml), hydralazine (10 µg/ml) or clonidine (1.0 µg/ml) and co-cultured with the endothelial cellular networks. The chosen doses reflect physiologically equivalent to blood

concentrations during therapeutic use in humans[18]. The control co-cultured cells had neither TNF-α nor medications added. After 24 hours of culture, the cells were retrieved from the cellular networks in Matrigel with Cell Recovery Solution (CRS) (In Vitro Technologies, Noble Park, VIC, Australia) for RNA extraction according to manufacturer's instructions. Each treatment was performed 6 times (n = 6).

Pre-incubation of UtMVECs with TNF-α and sFlt-1

For some experiments the UtMVECs were pre-incubated with low dose of TNF-α (0.5 ng/ml) alone (pre-TNF-α treated group) or TNF-α in combination with sFlt-1 (100 ng/ml) (pre-TNF-α+sFlt-1 treated group) in the culture flask for 24 hours. These cells were then trypsinized, washed 3 times with PBS and were ready for co-culture. No further TNF-α was added during co-culture.

Both UtMVECs and HTR-8/SVneo cells were above 85% viable in all experiments, as monitored by trypan blue staining. None of the medications affected the cellular network formation when applied to endothelial cells alone, nor did they affect trophoblast/endothelial cell integration in the absence of TNF-α [18]

RNA Extraction and Quantitative-PCR (Q-PCR) Analysis

Total RNA was extracted from the co-cultured cell pellets retrieved from Matrigel stated above using Qiagen RNA extraction kit (Qiagen, Doncaster, VIC, Australia) according to manufacturer's instructions. The RNA was resuspended in nuclease-

free water (20-40 µL). The RNA concentration was quantified by spectrophotometry (Nanodrop Technologies, Rockland, DE, USA). Specific cDNA was reverse transcribed from 100 ng of total RNA using iSCRIPT (Bio-Rad, Regents Park, NSW, Australia), according to the manufacturer's protocol. An aliquot of each cDNA sample (1µl) was amplified by real-time PCR in reaction mixtures containing primers and iQ SYBR Green Supermix. Amplification was performed in a Bio-Rad iQ5 thermocycler (Bio-Rad) using the following protocol: 95 °C for 30 secs, annealing temperature (Ta) of specific primer sets for 30 secs and 72 °C for 30 secs. Real time data was measured by quantifying fluorescence during the elongation phase at 72 °C in each cycle. After the completion of amplification, the threshold cycle (CT) was calculated using iCycler iQ Real Time PCR detection system software version 3.0A (BioRad, Gladesville, NSW, Australia). Primer pairs sequences of the assessed genes are eNOS (forward, 5'- GGACTTCATCAACCAGTAC-3', reverse, 5'-GATGTAGGTGAACATTTCC-3'), iNOS (forward, 5'-CAAGCAGCAGAATGAGTC-3', reverse, 5'- GCCTTATGGTGAAGTGTG -3') [17]. Relative changes in mRNA gene expression were determined by the ΔΔCT method using β-actin (forward, 5'-ATGTGGCCGAGGACTTTGATT-3', reverse, 5'-AGTGGGGTGGCTTTTAGGATG-3') as the housekeeping gene [50, 51]. Expression levels were reported as a percentage of the non-treated control group.

Statistical Analysis

Data are expressed as mean ± sem % of control. Statistical analysis was performed using Graphpad Prism version 5.0d for Mac OSX (GraphPad Software, San Diego California USA). Differences between groups were assessed by non-parametric Mann–Whitney *U* tests. Although the overall significance was set at 0.05, the

Sharpened Bonferroni method was used to adjust the individual α level when multiple comparisons were performed and $p<0.05$ being considered significant.

Acknowledgements

The present work was supported by NHMRC grant 1025258 from National Health and Medical Research Council Australia and PEARLS (Preeclampsia Research Laboratories.

Author contributions

Bei Xu designed and performed all the experiments, acquired and interpreted data, wrote manuscript and acted as corresponding author. Gabriele Bobek contributed to manuscript writing, data interpretation and critical revision. Angela Makris helped to write, evaluate and edit the manuscript. Annemarie Hennessy supervised development of work, helped in data interpretation and critical revision.

Competing Interests' Statement

There are no conflicts of interests to be declared.

References

1. Thaler, I., et al., *Changes in uterine blood flow during human pregnancy.* Am J Obstet Gynecol, 1990. **162**(1): p. 121-5.
2. Osol, G. and M. Mandala, *Maternal uterine vascular remodeling during pregnancy.* Physiology (Bethesda), 2009. **24**: p. 58-71.

3. Moffett-King, A., *Natural killer cells and pregnancy.* Nat Rev Immunol, 2002. **2**(9): p. 656-63.
4. Ali, S.M. and R.A. Khalil, *Genetic, immune and vasoactive factors in the vascular dysfunction associated with hypertension in pregnancy.* Expert Opin Ther Targets, 2015. **19**(11): p. 1495-515.
5. Granger, J.P., et al., *Pathophysiology of hypertension during preeclampsia linking placental ischemia with endothelial dysfunction.* Hypertension, 2001. **38**(3 Pt 2): p. 718-22.
6. Sladek, S.M., R.R. Magness, and K.P. Conrad, *Nitric oxide and pregnancy.* Am J Physiol, 1997. **272**(2 Pt 2): p. R441-63.
7. Nathan, C. and Q.W. Xie, *Nitric oxide synthases: roles, tolls, and controls.* Cell, 1994. **78**(6): p. 915-8.
8. Matsubara, K., et al., *Nitric oxide and reactive oxygen species in the pathogenesis of preeclampsia.* Int J Mol Sci, 2015. **16**(3): p. 4600-14.
9. Forstermann, U. and W.C. Sessa, *Nitric oxide synthases: regulation and function.* Eur Heart J, 2012. **33**(7): p. 829-37, 837a-837d.
10. Albrecht, E.W., et al., *Protective role of endothelial nitric oxide synthase.* J Pathol, 2003. **199**(1): p. 8-17.
11. Krause, B.J., M.A. Hanson, and P. Casanello, *Role of nitric oxide in placental vascular development and function.* Placenta, 2011. **32**(11): p. 797-805.
12. Schiessl, B., et al., *Expression of endothelial NO synthase, inducible NO synthase, and estrogen receptors alpha and beta in placental tissue of normal, preeclamptic, and intrauterine growth-restricted pregnancies.* J Histochem Cytochem, 2005. **53**(12): p. 1441-9.
13. Dotsch, J., et al., *Increase of endothelial nitric oxide synthase and endothelin-1 mRNA expression in human placenta during gestation.* Eur J Obstet Gynecol Reprod Biol, 2001. **97**(2): p. 163-7.
14. Harris, L.K., et al., *S-nitrosylation of proteins at the leading edge of migrating trophoblasts by inducible nitric oxide synthase promotes trophoblast invasion.* Exp Cell Res, 2008. **314**(8): p. 1765-76.
15. Conrad, K.P., T.M. Miles, and D.F. Benyo, *Circulating levels of immunoreactive cytokines in women with preeclampsia.* Am J Reprod Immunol, 1998. **40**(2): p. 102-11.
16. Xu, B., et al., *TNF-alpha inhibits trophoblast integration into endothelial cellular networks.* Placenta, 2011. **32**(3): p. 241-6.
17. Xu, B., et al., *Nitric oxide (NO) reversed TNF-alpha inhibition of trophoblast interaction with endothelial cellular networks.* Placenta, 2014. **35**(6): p. 417-21.
18. Xu, B., et al., *Antihypertensive drugs methyldopa, labetalol, hydralazine, and clonidine improve trophoblast interaction with endothelial cellular networks in vitro.* J Hypertens, 2014. **32**(5): p. 1075-83; discussion 1083.
19. Molin, J.C. and L.M. Bendhack, *Clonidine induces rat aorta relaxation by nitric oxide-dependent and -independent mechanisms.* Vascul Pharmacol, 2004. **42**(1): p. 1-6.
20. Venturini, G., et al., *Selective inhibition of nitric oxide synthase type I by clonidine, an anti-hypertensive drug.* Biochem Pharmacol, 2000. **60**(4): p. 539-44.
21. Leiro, J.M., et al., *Antioxidant activity and inhibitory effects of hydralazine on inducible NOS/COX-2 gene and protein expression in rat peritoneal macrophages.* Int Immunopharmacol, 2004. **4**(2): p. 163-77.
22. Kimura, Y., et al., *Long-acting calcium channel blocker, azelnidipine, increases endothelial nitric oxide synthase in the brain and inhibits sympathetic nerve activity.* Clin Exp Hypertens, 2007. **29**(1): p. 13-21.
23. Maynard, S.E., et al., *Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia.* J Clin Invest, 2003. **111**(5): p. 649-58.

24. Gavras, I., A.J. Manolis, and H. Gavras, *The alpha2 -adrenergic receptors in hypertension and heart failure: experimental and clinical studies.* J Hypertens, 2001. **19**(12): p. 2115-24.
25. Pimentel, A.M., et al., *The role of NO-cGMP pathway and potassium channels on the relaxation induced by clonidine in the rat mesenteric arterial bed.* Vascul Pharmacol, 2007. **46**(5): p. 353-9.
26. Knowles, H.J., et al., *Novel mechanism of action for hydralazine: induction of hypoxia-inducible factor-1alpha, vascular endothelial growth factor, and angiogenesis by inhibition of prolyl hydroxylases.* Circ Res, 2004. **95**(2): p. 162-9.
27. Kazakov, A., et al., *Inhibition of endothelial nitric oxide synthase induces and enhances myocardial fibrosis.* Cardiovasc Res, 2013. **100**(2): p. 211-21.
28. Toba, H., et al., *Calcium channel blockades exhibit anti-inflammatory and antioxidative effects by augmentation of endothelial nitric oxide synthase and the inhibition of angiotensin converting enzyme in the N(G)-nitro-L-arginine methyl ester-induced hypertensive rat aorta: vasoprotective effects beyond the blood pressure-lowering effects of amlodipine and manidipine.* Hypertens Res, 2005. **28**(8): p. 689-700.
29. Lund-Johansen, P. and O.M. Bakke, *Haemodynamic effects and plasma concentrations of labetalol during long-term treatment of essential hypertension.* Br J Clin Pharmacol, 1979. **7**(2): p. 169-74.
30. Korkmaz, O., et al., *Labetalol, nebivolol, and propranolol relax human radial artery used as coronary bypass graft.* J Thorac Cardiovasc Surg, 2015. **149**(4): p. 1036-40.
31. Szajerski, P., et al., *Radical scavenging and NO-releasing properties of selected beta-adrenoreceptor antagonists.* Free Radic Res, 2006. **40**(7): p. 741-52.
32. Wang, Y. and S.W. Walsh, *TNF alpha concentrations and mRNA expression are increased in preeclamptic placentas.* J Reprod Immunol, 1996. **32**(2): p. 157-69.
33. Zhang, H., et al., *Role of TNF-alpha in vascular dysfunction.* Clin Sci (Lond), 2009. **116**(3): p. 219-30.
34. Pijnenborg, R., et al., *Immunolocalization of tumour necrosis factor-alpha (TNF-alpha) in the placental bed of normotensive and hypertensive human pregnancies.* Placenta, 1998. **19**(4): p. 231-9.
35. Redman, C.W. and I.L. Sargent, *Pre-eclampsia, the placenta and the maternal systemic inflammatory response--a review.* Placenta, 2003. **24 Suppl A**: p. S21-7.
36. Bauer, S., et al., *Tumor necrosis factor-alpha inhibits trophoblast migration through elevation of plasminogen activator inhibitor-1 in first-trimester villous explant cultures.* J Clin Endocrinol Metab, 2004. **89**(2): p. 812-22.
37. Schinzari, F., et al., *Tumor necrosis factor-alpha antagonism improves endothelial dysfunction in patients with Crohn's disease.* Clin Pharmacol Ther, 2008. **83**(1): p. 70-6.
38. Yamaoka, J., et al., *Cytotoxicity of IFN-gamma and TNF-alpha for vascular endothelial cell is mediated by nitric oxide.* Biochem Biophys Res Commun, 2002. **291**(4): p. 780-6.
39. Yan, G., et al., *Tumor necrosis factor-alpha downregulates endothelial nitric oxide synthase mRNA stability via translation elongation factor 1-alpha 1.* Circ Res, 2008. **103**(6): p. 591-7.
40. Cai, H., et al., *Induction of endothelial NO synthase by hydrogen peroxide via a Ca(2+)/calmodulin-dependent protein kinase II/janus kinase 2-dependent pathway.* Arterioscler Thromb Vasc Biol, 2001. **21**(10): p. 1571-6.
41. Kouoh, F., et al., *In vitro and ex vivo antioxidant activities of labetalol on rabbit neutrophil respiratory burst.* Adv Ther, 2004. **21**(3): p. 178-85.
42. Levine, R.J., et al., *Circulating angiogenic factors and the risk of preeclampsia.* N Engl J Med, 2004. **350**(7): p. 672-83.
43. Lau, S.Y., et al., *Tumor necrosis factor-alpha, interleukin-6, and interleukin-10 levels are altered in preeclampsia: a systematic review and meta-analysis.* Am J Reprod Immunol, 2013. **70**(5): p. 412-27.
44. Thadhani, R., et al., *Pilot study of extracorporeal removal of soluble fms-like tyrosine kinase 1 in preeclampsia.* Circulation, 2011. **124**(8): p. 940-50.

45. Gelinas, D.S., et al., *Immediate and delayed VEGF-mediated NO synthesis in endothelial cells: role of PI3K, PKC and PLC pathways.* Br J Pharmacol, 2002. **137**(7): p. 1021-30.
46. Khalil, A., et al., *Effect of antihypertensive therapy with alpha methyldopa on levels of angiogenic factors in pregnancies with hypertensive disorders.* PLoS ONE, 2008. **3**(7): p. e2766.
47. Mariotto, S., M. Menegazzi, and H. Suzuki, *Biochemical aspects of nitric oxide.* Curr Pharm Des, 2004. **10**(14): p. 1627-45.
48. Matsubara, K., et al., *Role of nitric oxide and reactive oxygen species in the pathogenesis of preeclampsia.* J Obstet Gynaecol Res, 2010. **36**(2): p. 239-47.
49. Su, N.Y., P.S. Tsai, and C.J. Huang, *Clonidine-induced enhancement of iNOS expression involves NF-kappaB.* J Surg Res, 2008. **149**(1): p. 131-7.
50. Bustin, S.A., *Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays.* J Mol Endocrinol, 2000. **25**(2): p. 169-93.
51. Schefe, J.H., et al., *Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's CT difference" formula.* J Mol Med, 2006. **84**(11): p. 901-10.

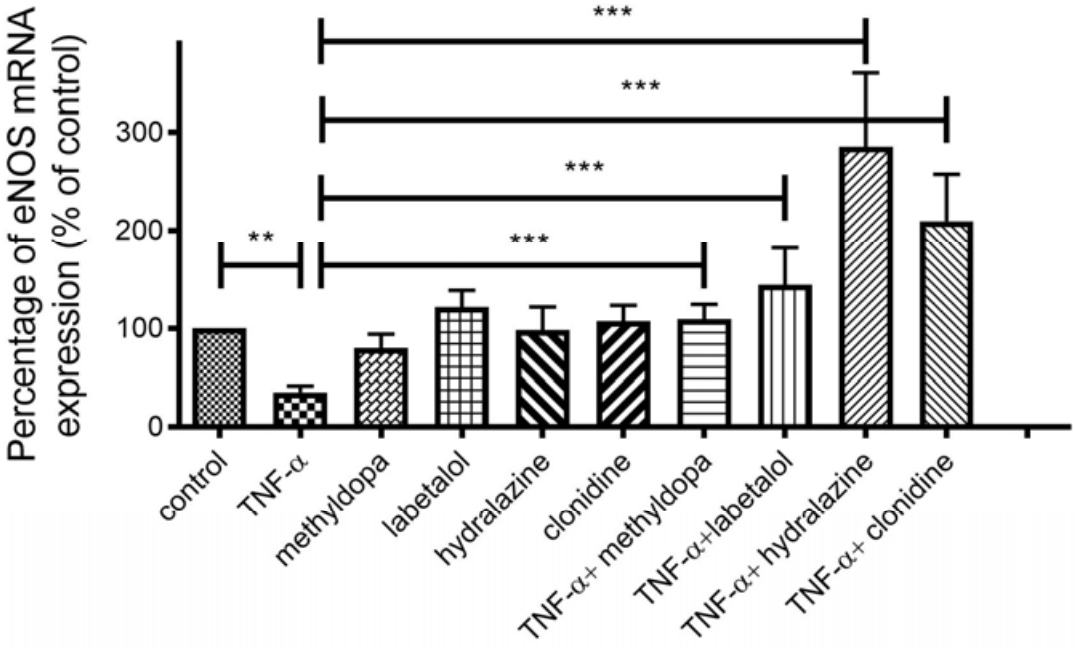
Table1. Comparison of antihypertensive medications on the relative expression of eNOS mRNA in endothelial/trophoblast co-culture system

medication	**TNF-α pre-incubated with endothelial cells**	**TNF-α added concurrently with medications**
methyldopa	87 ± 15 %	109 ± 15 %
labetalol	170 ± 26 % *	144 ± 38 %
hydralazine	192 ± 43 % **	286 ± 74 % **
clonidine	146 ± 15 %	210 ± 48 %

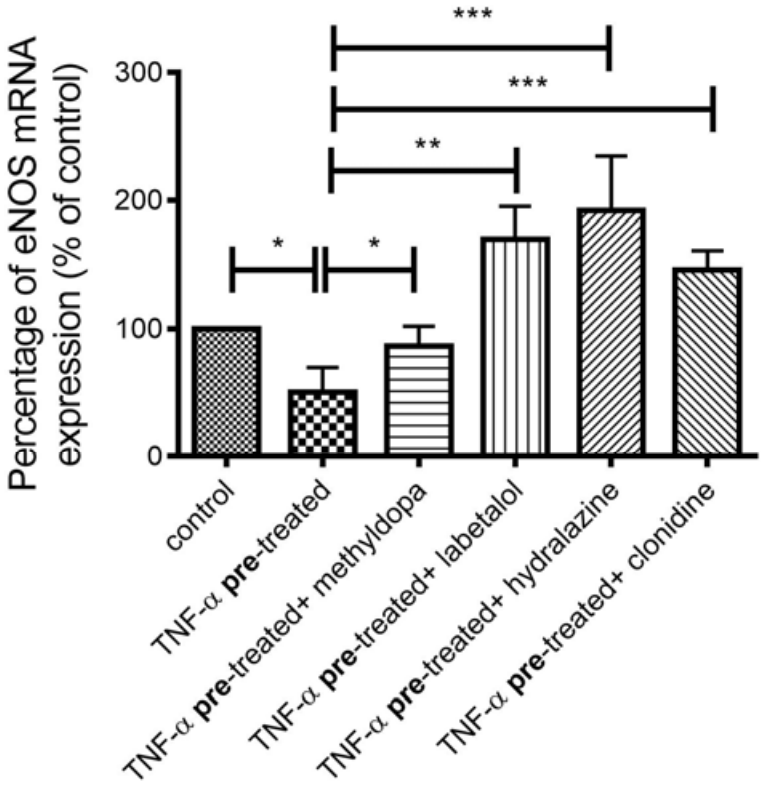
Data is expressed as mean ± sem and levels are relative to control cells co-cultured with no additions and normalised to ß-actin expression levels.

* Indicated a significant difference compared with methyldopa.

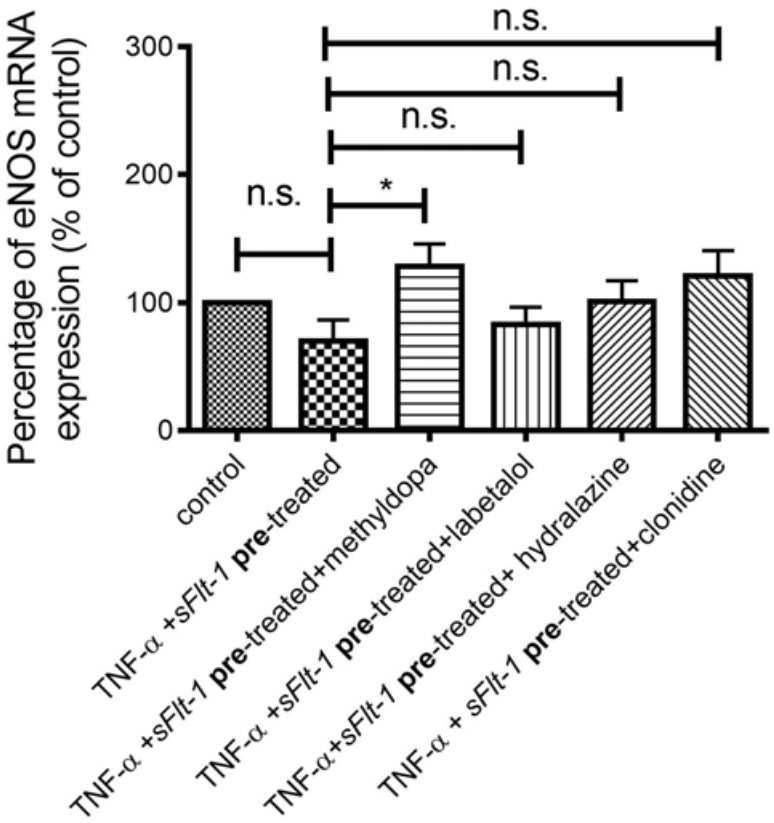
*p <0.05, **p<0.01.



**p< 0.01, ***p< 0.001. (n=6)



*p<0.05, **p<0.01, ***p<0.001. (n=6)



*p<0.05, n.s.= not significant. (n = 6)