Combining metformin and sulfasalazine additively reduces the secretion of antiangiogenic factors from the placenta: Implications for the treatment of preeclampsia

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ABSTRACT

Introduction: The antiangiogenic factors soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sENG) are elevated in preeclampsia and have been implicated in its pathogenesis. We have previously demonstrated metformin and sulfasalazine independently reduce antiangiogenic factor secretion. Here we examined whether combining metformin and sulfasalazine may be more effective than either alone in reducing placental expression and secretion of antiangiogenic and angiogenic factors and the expression of markers of endothelial dysfunction.
Methods: We performed functional experiments using primary human placenta to explore the effect of metformin and sulfasalazine, at lower doses than previously explored, individually and in combination, on sFlt-1 and sENG secretion and placental growth factor (PlGF) and vascular endothelial growth factor (VEGFα) expression. Using primary endothelial cells we induced dysfunction using cytokine tumor necrosis factor-α (TNF-α) and assessed the effect of low dose combination treatment on the expression of vascular cell adhesion molecule-1 (VCAM-1) and Endothelin-1 (a potent vasoconstrictor).
Results: We demonstrated combination metformin and sulfasalazine was additive in reducing sFlt-1 secretion from cytotrophoblasts and placental explants. Combination treatment was also additive in reducing sENG secretion from placental explants. Furthermore, combination treatment increased cytotrophoblast VEGFα mRNA expression. Whilst combination treatment increased PlGF mRNA expression this was similar to treatment with sulfasalazine alone. Combination therapy reduced TNFα induced endothelin-1 mRNA expression however did not change VCAM expression.
Discussion: Low dose combination metformin and sulfasalazine reduced cytotrophoblast sFlt-1 and sENG secretion, increased VEGFα expression and reduced TNFα induced endothelin-1 expression in primary endothelial cells. Combination therapy has potential to treat preeclampsia.

1. Introduction

Preeclampsia is a common complication of pregnancy characterized by hypertension and multisystem organ involvement [1,2]. An increase in antiangiogenic factors FMS-like tyrosine kinase-1 (sFlt-1) [3–5] and soluble endoglin (sENG) [6] and reduced angiogenic markers placental growth factor (PlGF) and VEGF resulting in endothelial dysfunction are strongly implicated in its pathogenesis [7–9]. Repurposing medications that target the production or secretion of these proteins has been used to identify potential treatments for preeclampsia.

We have identified esomeprazole [10], metformin [16] and sulfasalazine [11] as medications that individually reduce sFlt-1 secretion with varying effects on other cellular processes altered in preeclampsia. We are pursuing clinical trials for each of these medications with the clinical trial for metformin (trial number PACTR201608001752102) [12] and sulfasalazine (trial number ACTRN12617000226303)

currently in progress. We demonstrated in a randomized controlled trial that esomeprazole was not an effective treatment for preeclampsia [13]. Perhaps this may have resulted from insufficient drug concentrations reaching the placenta and vascular tissues.

An effective strategy used clinically to improve drug efficacy is to combine agents to obtain an additive or synergist effect. We have investigated the effect of combining esomeprazole and metformin at doses that individually had no effect on sFlt-1 secretion and demonstrated a significant reduction in antiangiogenic factor secretion and endothelial dysfunction [14]. In this study we examined whether combining metformin and sulfasalazine at doses that individually did not reduce sFlt-1 secretion might be additive or indeed synergistic in reducing sFlt-1 and sEng secretion, enhancing VEGF or PlGF, and reducing endothelial dysfunction, compared to either alone.

2. Materials and methods

We performed functional experiments to investigate whether sulfasalazine and metformin were additive in their ability to reduce antiangiogenic or increase angiogenic factor expression and/or secretion from primary human placental tissues. Specifically, we examined the effect of metformin and sulfasalazine independently and in combination on the placental secretion or expression of sFlt-1, sFlt-1 variants e15a and i13, sENG, PlGF and VEGFα. We also explored whether this combination additively reduced key markers of endothelial dysfunction including VCAM and ET1 using primary human endothelial cells.

3. Isolating and treating primary human cytotrophoblast cells

Term placental tissue were collected from women having elective caesarean sections on the background of uncomplicated pregnancies and human cytotrophoblasts were isolated as previously described [15,16]. Cells were plated at 24,000 cells/cm^2 (n = 3–4) and incubated overnight to ensure viable cytotrophoblasts had adhered, washed to remove cell debris and treated. The cells were treated with 200 μM metformin or 200 μM sulfasalazine alone or in combination under 8% O_2 and 5% CO_2 at 37 °C. After 24 h the conditioned media was collected to assess sFlt-1 and sENG secretion and cell lysates were collected for RNA extraction for sFlt-1 variants, e15a and i13, and PlGF and VEGFα expression.

3.1. Culture of placental explants

Placental explants were prepared as previously described [15]. After culturing the explants in media for 24 h, they were treated with 400 μM metformin or 400 μM sulfasalazine alone or in combination for 48 h under 8% O_2 and 5% CO_2 at 37 °C. Conditioned media was collected and sFlt-1, sENG and PlGF levels were assessed. Protein secretion was normalised against placental explant weights. Tissue was collected for RNA extraction and sFlt-1 variants e15a and i13 were determined.

3.2. Isolating and treating primary human umbilical vein endothelial cells (HUVECs)

Umbilical cords were collected from term normal placenta and the cord vein was infused with 10 ml (1 mg/ml) of collagenase (Worthington, Lakewood, New Jersey) and cells isolated as previously described [15]. Cells were used between passage 2 to 4, incubated at 37 °C in 20% O_2 and 5% CO_2 and plated at 24,000 cells/cm^2. They were treated once they achieved 80% confluency n = 3 with 10 ng/ml TNFα (Sigma) to induce endothelial dysfunction for 24 h then treated with 300 μM sulfasalazine (sigma) or 500 μM Metformin alone or in combination n = 3 for 24 h. Cell lysates were collected for RNA for VCAM and ET1 analysis.

3.3. Cell viability assay (MTS assay and calcein stain)

Cell viability assays were performed using CellTiter 96-Aquesous One solution (Promega, Madison WI) according to the manufacturer's instructions or after staining cells with calcein-AM (Merck Millipore, Darmstadt, Germany) fluorescent signal was detected using Fluostar omega fluorescent plate reader (BMG labtech, Victoria, Australia).

3.4. ELISA analysis

Concentrations of sFlt-1, sENG and PlGF were measured in conditioned cell/tissue culture media using the DuoSet VEGF R1/Flt-1 kit (R&D systems by Bioscience, Waterloo, Australia), a DuoSet Human Endoglin CD/105 ELISA kit (R&D systems) or Duoset Human PlGF kit (R&D systems) according to manufacturer's instructions.

3.5. RT-PCR

RNA was extracted from placental explants and HUVECs using a RNeasy mini kit (Qiagen, Valencia, CA) and quantified using the Nanodrop ND 1000 spectrophotometer (NanoDrop technologies Inc, Wilmington, DE). 0.2 μg of RNA was converted to cDNA using Applied Biosystems high capacity cDNA reverse transcriptase kit (Life Technologies) as per manufacturer guidelines.

Gene expressions of *VCAM-1* (Life Technologies), *ET1* (Life Technologies), *PlGF* (Life Technologies) *VEGFα* (Life Technologies) were quantified by real time PCR (RT-PCR) on the CFX 384 (Bio-Rad, Hercules, CA) using FAM-labeled Taqman universal PCR mastermix and its specific primer/probe set (Life Technologies) with the following run conditions: 50 °C for 2 min; 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min (40 cycles). SYBR RT-PCR was carried out to assess gene expressions of *sFlt-1 e15a* and *sFlt-1 i13* and *GAPDH*. Primers were designed as previously described (Geneworks, South Australia, Australia) [17]. Quantitative RT-PCR was performed using the following run conditions: 95 °C for 20 min; 95 °C for 0.01 min, 60 °C for 20 min, 95 °C for 1 min (39 cycles), melt curve 65 °C–95 °C at 0.05 °C increments at 0.05 s.

Data were normalised to GAPDH as a housekeeper and calibrated against the average C_t of the control samples. Results were expressed as fold change from control.

Ethics approval

This study was approved by The Mercy Health Human Research Ethics Committee (Institutional review board number R11/34 and R14/11) and all women gave written informed consent.

3.6. Statistical analysis

A minimum of three technical triplicates were performed for each biological replicate, with a minimum of three biological replicates (each from different patients) performed for each *in vitro* study. When two groups were analysed a *t*-test (parametric) or a Mann-Whitney test (nonparametric) was used and when three or more groups were compared a 1-way ANOVA (parametric) or a Kruskal-Wallis test (non-parametric) was used. Statistical analysis was done using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). All data are expressed as mean ± SEM; P values < 0.05 were considered significant.

4. Results

4.1. Combining metformin and sulfasalazine was additive in reducing sFlt-1 secretion from placental cells/tissues

We have previously demonstrated that metformin [16] and sulfasalazine [11] reduce placental sFlt-1 secretion. We examined whether combining them would additively reduce sFlt-1 secretion. At lower

doses than previously published metformin did not change sFlt-1 secretion whilst sulfasalazine had a significant but modest effect (Fig. 1A) on sFlt-1 secretion from primary cytotrophoblasts. However, combining these low doses of metformin and sulfasalazine additively reduced sFlt-1 secretion beyond either drug alone (Fig. 1A). Importantly, combining these drugs did not affect cell viability determined via MTS assay (data not shown).

Next, we assessed the effects of these medications on sFlt-1 secretion from placental explants. We found lower doses of metformin and sulfasalazine independently did not significantly change sFlt-1 secretion from placental explants (Fig. 1B). In combination however these medications significantly reduced sFlt-1 secretion compared to control (Fig. 1B).

sFlt-1 is transcribed from a number of splice variants. sFlt-1 e15a is human specific and a placentally derived isoform of sFlt-1 [18,19]. We examined the effect of low doses of metformin or sulfasalazine individually and in combination on sFlt-1 e15a mRNA expression. We found metformin or sulfasalazine alone significantly reduced sFlt-1 e15a mRNA expression but this effect was more pronounced when these drugs were administered together to treat primary cytotrophoblast (Fig. 1C). Metformin or sulfasalazine alone or in combination did not change the expression of sFlt-1 e15a in placental explants (Fig. 1D).

sFlt-1 i13 is another sFlt-1 splice variant. It is the most abundant variant in endothelial cells, but is also expressed in primary cytotrophoblast[25,26]. We also assessed the effect of low dose metformin or sulfasalazine on sFlt-1 i13 expression in primary cytotrophoblasts and demonstrated a reduction in sFlt-1 i13 expression, which was more pronounced following combination treatment (Fig. 1E). Low dose metformin or sulfasalazine, either alone or in combination did not affect placental explant sFlt-1 i13 mRNA expression (Fig. 1F).

4.2. Combination low dose metformin and sulfasalazine additively reduced sENG secretion from placental explants but not cytotrophoblasts

We investigated the effects of metformin and sulfasalazine alone, or in combination on the placental secretion of sENG. Metformin and sulfasalazine significantly reduced sENG secretion from primary cytotrophoblasts and combining them did not have an additive effect (Fig. 2A).

We next performed similar experiments on placental explants. Sulfasalazine, but not metformin significantly reduced sENG secretion. Combining these drugs enhanced the reduction of sENG secretion (Fig. 2B).
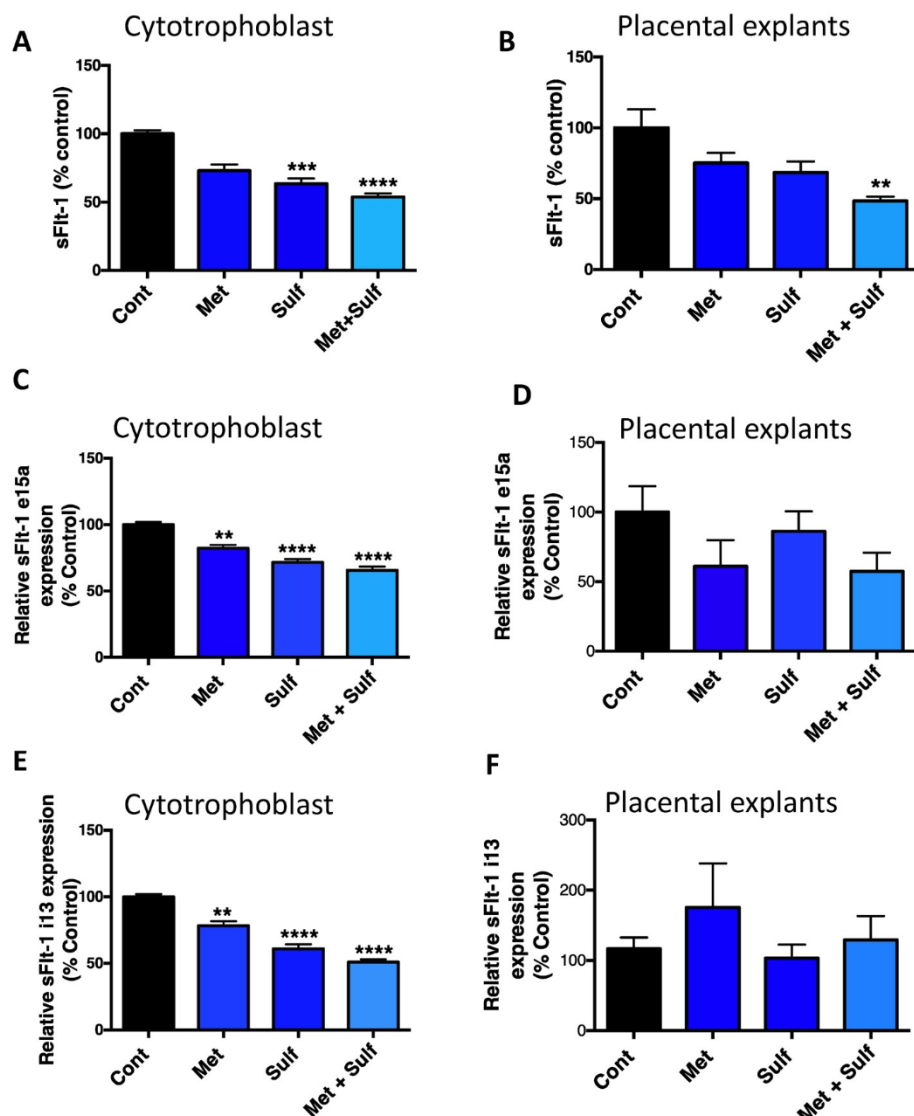

Fig. 1. Combining low dose metformin and sulfasalazine reduces sFlt-1 secretion from placenta. Low dose metformin and sulfasalazine reduced sFlt-1 secretion from cytotrophoblasts (A) and the combination effect was additive when placental explants were treated(B). There was a significant reduction in sFlt-1 e15 mRNA expression with individual treatment of cytotrophoblasts and this was more pronounced in combination (C) whilst the expression of this variant did not change with individual or combination treatment of placental explants (D). Combination treatment had a more pronounced effect on reducing sFlt-1 i13 mRNA expression from cytotrophoblasts (E) compared to individual treatment however these medications had no effect on its expression when placental explants were treated (F). The following treatment doses were used: *Cytotrophoblasts:* Metformin 200 μM, sulfasalazine 200 μM or same doses in combination. *Placental explant tissue:* Metformin 400 μM, sulfasalazine 400 μM or same doses in combination. Treatments were carried out over 24 h for cytotrophoblasts or 48 h for explants. Data are mean fold change from control $\pm$ SEM (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared to control). (n $\geq$ 4).
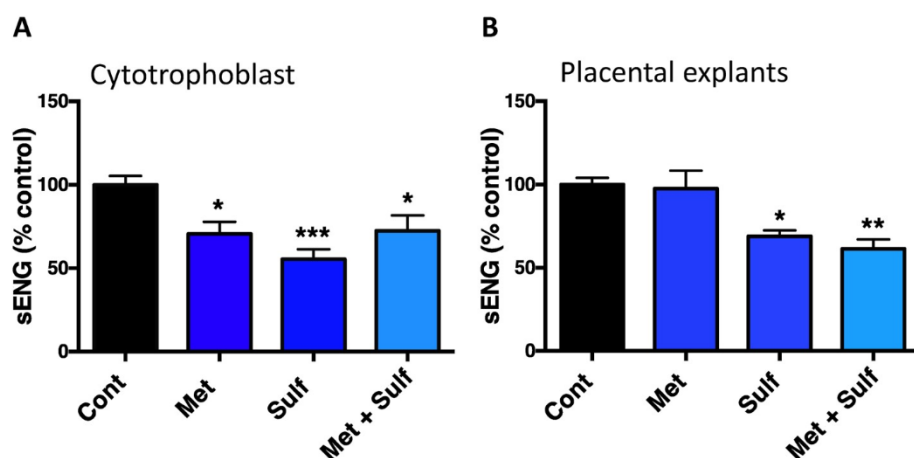
Fig. 2. Combination metformin and sulfasalazine reduce sENG secretion from cytotrophoblasts and the effect was additive in explants. Combination low dose metformin and sulfasalazine reduced sENG secretion from cytotrophoblasts however this was not significant when compared to individual treatment (A) whilst the combination effect was additive in reducing sENG secretion from placental explants (B). The following treatment doses were used: *Cytotrophoblasts:* Metformin 200 μM, sulfasalazine 200 μM or the same doses in combination. *Placental explant tissue:* Metformin 400 μM, sulfasalazine 400 μM or the same doses in combination. Treatments were carried out over 24 h for cytotrophoblasts or 48 h for placental explants. Data are mean fold change from control $\pm$ SEM (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared to control). (n $\geq$ 4).

4.3. Combination metformin and sulfasalazine additively increase VEGFα, but not PlGF expression

We have previously demonstrated sulfasalazine [11] increases angiogenic factor, PlGF and VEGFα, expression from placental cells and tissues [20]. We found low dose sulfasalazine, but not metformin, significantly increased PlGF expression in cytotrophoblast (Fig. 3A), and combining the treatments did not further add to the effect of sulfasalazine alone (Fig. 3A). Metformin and sulfasalazine, either alone or in combination did not change PlGF mRNA expression in placental explants (Fig. 3B).

Next, we examined the effect of these drugs on the expression of VEGFα. Metformin or sulfasalazine alone increased VEGFα expression in cytotrophoblast but combining them additively increased VEGFα

(Fig. 3C).

4.4. Combining metformin and sulfasalazine is additive in reducing vasoconstrictor ET-1 but does not reduce anti-inflammatory molecule VCAM1

Endothelial dysfunction is an important part of the pathology of preeclampsia and this is also a possible therapeutic target. Increased expression of vascular cell adhesion molecule 1 (VCAM1) on endothelial cells is a key marker of endothelial dysfunction. Upregulated by inflammation, it sequesters leukocytes, erythrocytes and platelets. Endothelin 1 (ET1) is a potent vasoconstrictor where it is released from endothelial cells and can cause hypertension. ET-1 can also be produced in endothelial cells in response to pro-inflammatory cytokines.
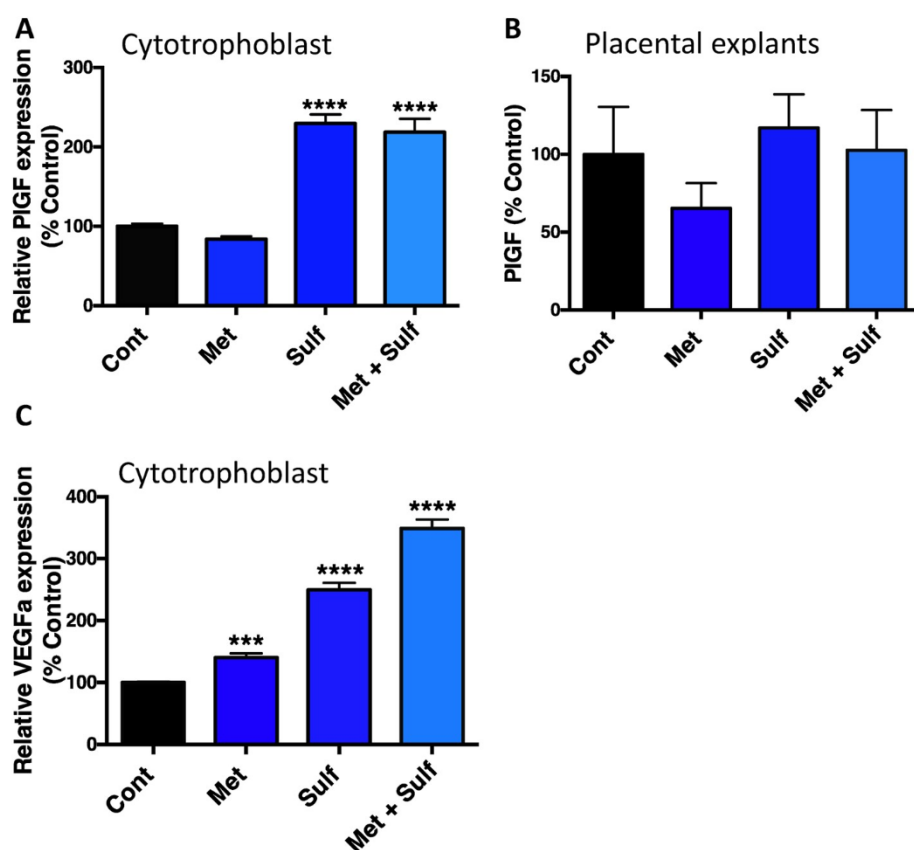

Fig. 3. Combination metformin and sulfasalazine increase placental growth factor expression and their effect is additive on vascular endothelial growth factor α (VEGF α) expression in cytotrophoblasts.
Whilst metformin did not change PlGF expression, sulfasalazine potently upregulated its expression and this increase was maintained with combination treatment (A) in cytotrophoblasts. Metformin and sulfasalazine individually and in combination had no effect on PlGF expression in placental explants (B). Metformin and sulfasalazine independently increased VEGFα expression and their effect was additive in cytotrophoblasts (C). The following treatment doses were used: *Cytotrophoblasts:* Metformin 200 μM, sulfasalazine 200 μM or same doses in combination. *Placental explant tissue:* Metformin 400 μM, sulfasalazine 400 μM or same doses in combination. Treatments were carried out over 24 h for cytotrophoblasts or 48 h for explants. Data are mean fold change from control $\pm$ SEM (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared to control). (n $\geq$ 4).

Importantly, the cytokine TNFα is increased in preeclampsia, as are both VCAM1 and ET1.

We have previously demonstrated sulfasalazine [11] and metformin [16] potently reduce VCAM1 in the presence of TNFα. On administering TNFα to primary endothelial cells we demonstrated a potent induction of VCAM1 expression. Metformin or sulfasalazine, either alone or in combination did not affect VCAM1 expression (Fig. 4A). The drugs alone also did not change ET1 expression but significantly reduced ET1 expression when combined (Fig. 4B).

5. Discussion

Preeclampsia is a serious complication of pregnancy with no medical treatment. Previously, we have demonstrated metformin [16] and sulfasalazine [11] independently reduce the secretion of key anti-angiogenic factors, sFlt-1 and sENG and markers of endothelial dysfunction specific to preeclampsia [11,21]. In this study we examined the effect of combining metformin and sulfasalazine at doses previously shown too low to exert an effect on sFlt-1 secretion. Whilst combination therapy additively reduced sFlt-1 secretion and upregulated VEGFa expression from placenta, the low doses were individually effective at reducing sENG secretion and upregulating PlGF expression from placenta and there was no additive effect from the combination.

We had previously shown that metformin at doses of 1000 μM[16] and sulfasalazine at doses of 500 μM[11] only marginally reduced sFlt-1 secretion from placental cells. Using much lower doses of metformin and sulfasalazine (200 μM in cytotrophoblast and 400 μM in placental explants) we demonstrated combination treatment was additive in reducing sFlt-1 secretion from cytotrophoblasts and placental explants. This could be due to the addition of two drugs that both target sFlt-1 production. Furthermore, these drugs likely reduce sFlt-1 through different pathways. Previously we demonstrated metformin likely targets sFlt-1 secretion through blocking complex 1 of the mitochondrial electron transport chain [16] whilst sulfasalazine likely exerts its effect by reducing signaling of the epidermal growth factor receptor (EGFR) [22]. This suggests that combining medications with seemingly disparate modes of action is advantageous at reducing sFlt-1 secretion.

Interestingly we found sulfasalazine alone reduced sENG secretion and increased PlGF secretion from cytotrophoblasts and placental explants whilst metformin had no additional effect. Perhaps this too reflects differences in the mode of action of these drugs. It seems sulfasalazine targets the EGFR pathway [22] and we have demonstrated blocking aspects of this pathway reduces sENG secretion. Metformin is perhaps targeting the mitochondria and we have shown inhibiting complexes of the electron transport chain do not change sENG secretion. Perhaps the EGFR pathway may also regulate PlGF secretion however exploring this was outside the scope of this study.

Disappointingly combination treatment only had a modest effect on reducing key markers of vascular dysfunction. Combination treatment resulted in a reduction in ET1 expression but had no effect on VCAM expression. This suggests the doses used (300 μM sulfasalazine or 500 μM Metformin) were too low to exert a significant effect individually or in combination or the time course of the treatment on was not optimal. Perhaps in future studies, higher doses of combination medications might produce an effect.

In conclusion, we have demonstrated that combination metformin and sulfasalazine were additive in reducing sFlt-1, upregulating angiogenic factor VEGFα and reducing ET-1 expression. Given this data, we suggest that combining metformin and sulfasalazine may be beneficial for treating preeclampsia.
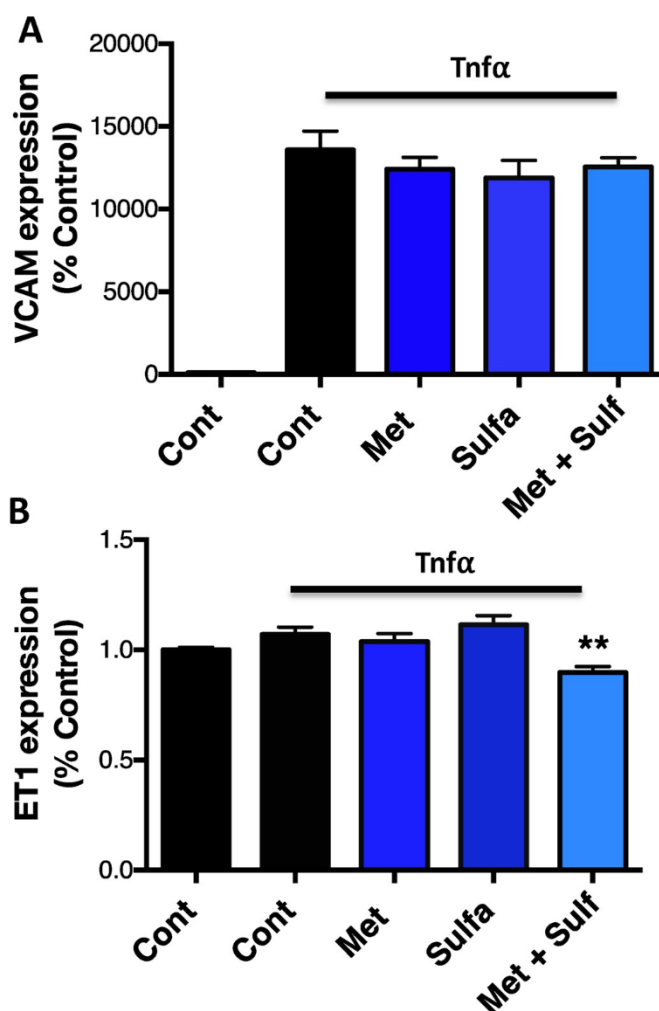
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Fig. 4. Combination metformin and sulfasalazine do not change vascular cell adhesion molecule 1 (VCAM1) expression however additively reduce endothelin 1 (ET1) expression in primary endothelial cells treated with cytokine TNFα. Endothelial dysfunction was induced in primary endothelial cells using cytokine TNFα. Metformin and sulfasalazine independently and in combination did not change VCAM expression when it was induced in primary endothelial cells using TNFα (A). Whilst individual treatment did not change endothelin 1 expression combination treatment had an additive effect on reducing ET1 expression from primary endothelial cells treated with metformin and sulfasalazine (B). The following treatment doses were used: *Cytotrophoblasts:* Metformin 500 μM, sulfasalazine 300 μM or same doses in combination. Treatments were carried out over 24 h. Data are mean fold change from control ± SEM (**p < 0.01 compared to control). (n ≥ 34).

Author contributions

FCB, NJH, TKL, and ST designed the experiments and wrote the main manuscript. SB, PC, MD, and TN were involved in data generation. All authors reviewed the manuscript.

Declaration of competing interest

The authors report no conflict of interest.

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