4.2.7 Permeability Assay

To determine paracellular permeability, FITC-labelled dextran (10 Invitrogen) was added into the lower chamber of the well, and 10 μl removed from the upper chamber over a 24 h period. Fluorescence was me the aliquots using a fluorimeter (ex λ: 485 nM, em λ: 535 nM; Ther Scientific, Fluoroskan AscentFL) to quantify FITC-dextran movement (perı through the endothelial monolayer.

4.2.8 ELISA

Levels of IL1βb and TNFα in the culture medium post macrophage polarisa quantified using a human IL-1β ELISA MAX™ deluxe set (sensitivity 0. Biolegend) and a TNF-α Duoset® ELISA (sensitivity 15 pg/mL; R&D Conditioned medium was diluted with assay diluent 1:10 and assayed a manufacturer's instructions. The intra and inter assay coefficients of varia <10%.

4.2.9 PP2A Phosphatase activity assay

PP2A phosphatase activity was determined using a PP2A immunoprecipitat kit according to the manufacturer's protocol (PP2A Immunopre Phosphatase Assay Kit; Merck Millipore). In brief, cells were lysed with mod buffer, and 100 μg of protein incubated with 4 μg of anti-PP2A (C subunit, c and protein-A-agarose. The agarose bound samples were centrifuged at 1, incubated with malachite green and the absorbance read at λ 620 nm. Ph activity was expressed as a percentage of the free phosphate of the test s that of the control samples.

4.2.10 Data and Statistical Analysis

RT-PCR data was normalised to GAPDH Ct values and western-blot normalised to β-actin protein abundance. All data were normalised to the ap controls and expressed as a ratio or percentage excluding ELISA data, w expressed as a concentration (pg/mL). Data were analysed using ANOVA (oı way) with *post hoc* analysis (Bonferroni) or an unpaired t test as appropr are represented as a mean ± S.E.M., and a value of $P < 0.05$ was set t statistical significance.

4.3 Results

4.3.1 Macrophage Polarisation

Differentiation of Mθ macrophages to an M1 phenotype was verified through measurement of TNF-α and IL-1β levels. In M1 polarised macrophages TNF-α and IL-1β mRNA expression were increased by 8.3 ± 0.8 fold and a 52.3 ± 2.2 fold ($P < 0.05$;Figure 4.2A and B) respectively compared to the unpolarised macrophages (Mθ macrophage). In conditioned medium from the M1 polarised macrophages TNF-α and IL-1β abundance was higher than in conditioned medium form Mθ macrophages ($P < 0.05$;Figure 4.2C and D). Furthermore, polarisation of the monocytes caused a visual morphological change to a dendritic shape (Figure 4.1).

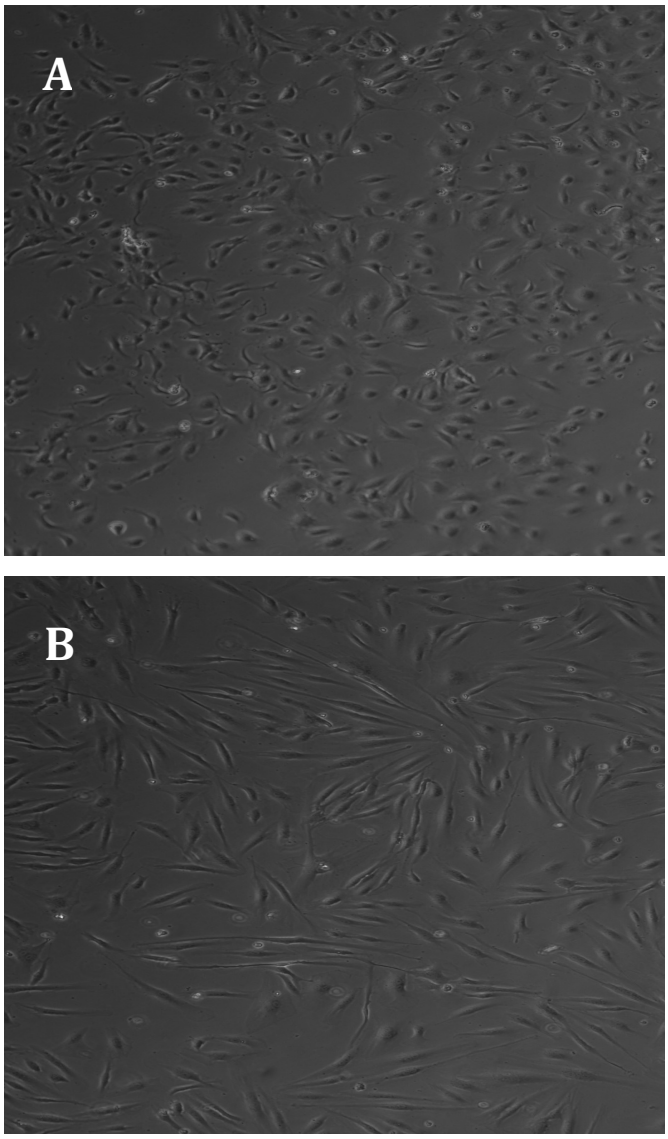

Figure 4.1: M0 and M1 morphology.

Peripheral blood mononuclear cells were isolated from human whole blood. After 7 days incubation the adherent rounded Mθ macrophages (A) are thoroughly washed. Polarisation of Mθ macrophages to an M1 phenotype (B) was achieved by 24 h exposure to LPS (100 ng/mL) and IFN-γ (20 ng/mL).

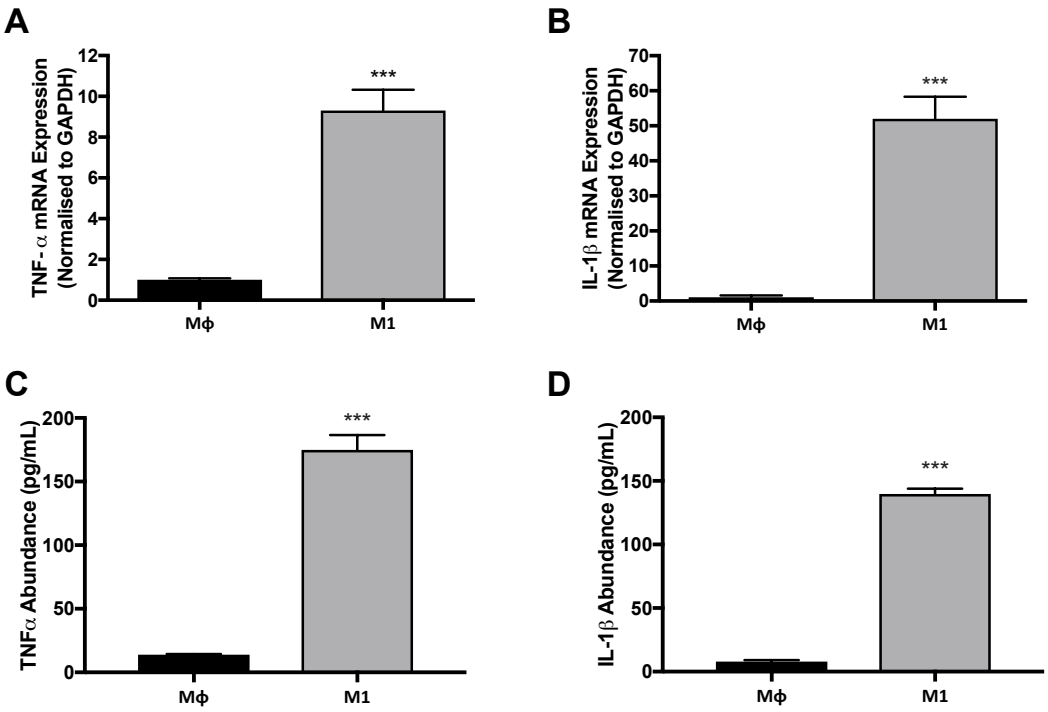

Figure 4.2: Quantification of TNF-α and IL-1β mRNA expression abundance in Mθ and M1 macrophages.

PBMCs were exposed to LPS (100 ng/mL) and IFN-γ (20 ng/mL) for 24 h to initiate polarisation to the M1 (pro-inflammatory) phenotype. TNF-α and IL-1β mRNA expression (A and B) was quantified by RT-PCR and normalised to GAPDH and represented as a mean± S.E.M fold increase. TNF-α and IL-1β abundance in the peripheral blood mononuclear cell (PBMC) culture media (C and D) was quantified using ELISA and presented as mean ± S.E.M pg/mL. * Indicate significance of P < 0.05 (n=5) and were analysed using an unpaired Student t Test.

4.3.2 Effect of Mθ and M1 macrophages on PP2A in hBMEC

PPP2CA mRNA expression in hBMECs was unaltered when co-cultured with Mθ macrophages compared to hBMEC in mono-culture. However, co-culture with M1 macrophages caused a 43 % increase in the mRNA expression of PPP2CA compared to hBMEC in mono-culture, and a 36 % increase compared to those co-cultured with Mθ co-culture ($P < 0.05$; Figure 4.3A). Co-culture of the hBMECs with Mθ and M1 macrophages did not alter the abundance of PP2Ac compared to hBMECs in mono-culture (Figure 4.3B). Mθ macrophages caused an increase in PP2Ac activity by 14% in hBMEC compared to hBMEC mono-culture ($P < 0.05$). However, in hBMEC co-cultured with M1 macrophages PP2Ac activity decreased by 26% and 35% respectively compared to hBMEC in mono-culture and those co-cultured with Mθ macrophages ($P < 0.05$; Figure 4.3C).

4.3.3 Effect of Mθ and M1 macrophage on post-translational modification of PP2A and their modifiers in hBMEC.

As PP2Ac activity in hBMEC was altered independently of a change in PP2Ac abundance, I investigated the effect of Mθ and M1 macrophages on the phosphorylation and methylation status of PP2Ac, which are well documented to modulated its activity [682]. Interestingly, co-culture of hBMEC with Mθ or M1 macrophages increased the abundance of phosphorylated PP2Ac by ~60% in hBMEC compared to hBMECs in mono-culture ($P < 0.05$;

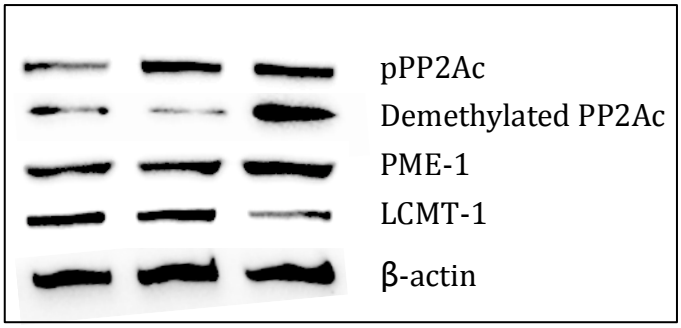

Figure 4.4A). Although Mθ macrophages did not alter the abundance of demethylated PP2Ac in hBMEC, M1 macrophages caused a 2.8 fold increase in

the demethylation of PP2Ac compared to hBMEC alone and those co-cultured with Mθ ($P < 0.05$;

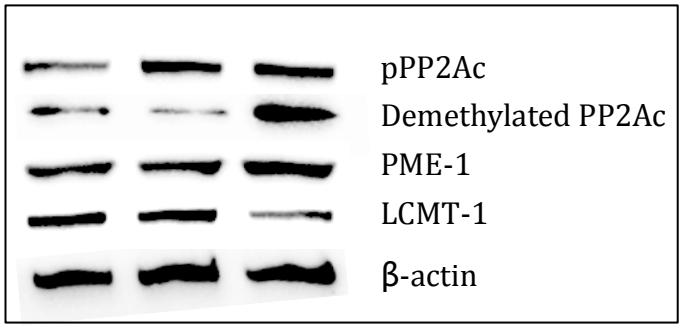

Figure 4.4B). To explain the alteration in methylation state, PME-1 and LCMT-1 abundance were also quantified. The abundance of PME-1 was unaltered when hBMEC were co-cultured with the Mθ or M1 macrophages compared to those in mono-culture (

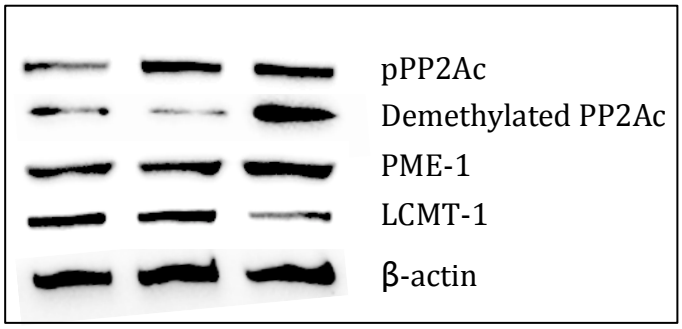

Figure 4.4C). While the abundance of LCMT-1 was not affected in hBMECs co-cultured with Mθ macrophages, it abundance decrease by 70% and 56% compared upon co-culture with M1 macrophages compared to those in mono-culture or co-cultured with Mθ macrophages respectively ($P < 0.05$;

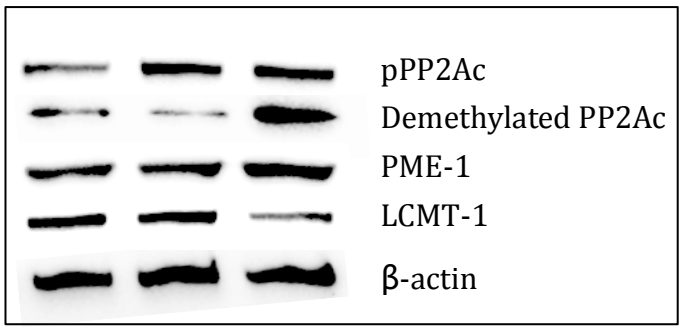

Figure 4.4D).

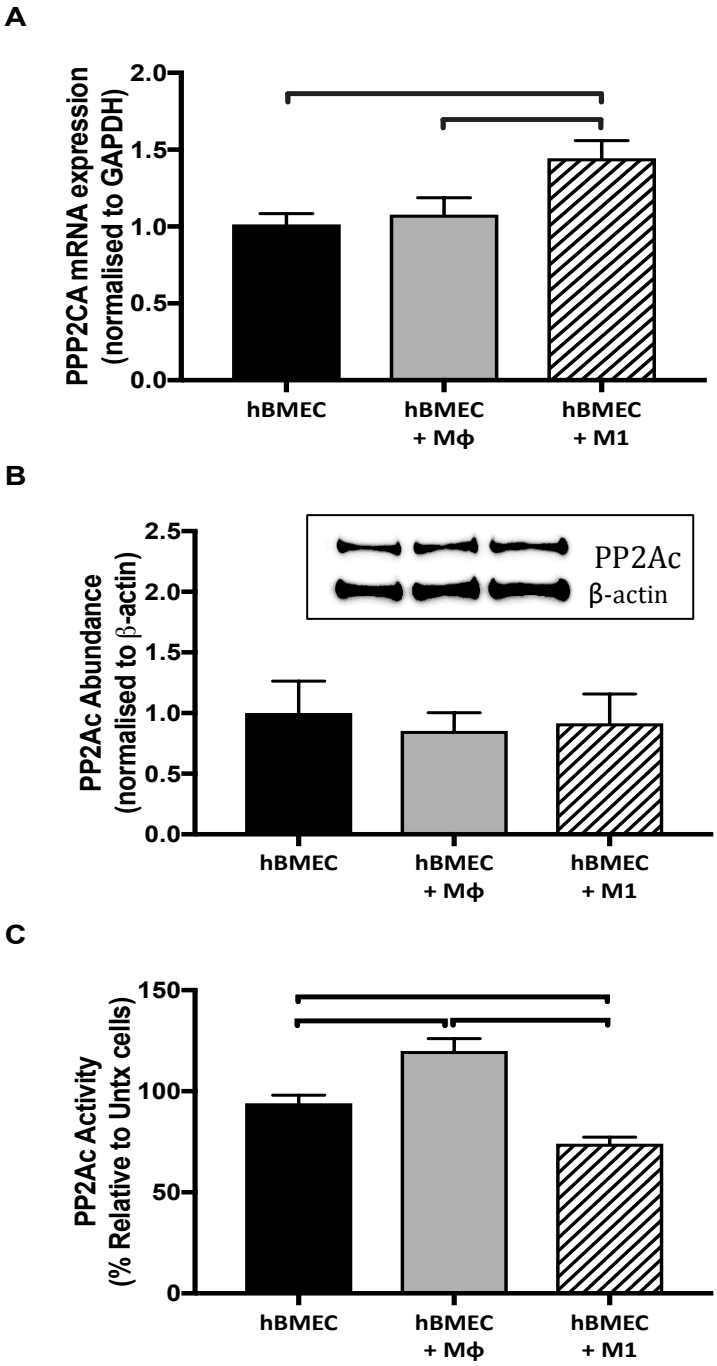

Figure 4.3: Effect of hBMEC, Mθ and M1 macrophage 24 h co-culture on PP2A.

PPP2CA mRNA expression was determined by RT-PCR normalized to GAPDH Ct values (A). PP2Ac abundance was determined by western blot normalised to β-actin expression (B) and PP2Ac activity was determined by an immunoprecipitation activity assay, data represented as a percentage of activity (C). All data sets are presented as a mean ± S.E.M relative to the hBMEC only sample. Data was analysed using a one-was ANOVA with *post hoc* analysis (Bonferroni). Horizontal bars represent statistical significance ($P < 0.05$) (n=5).

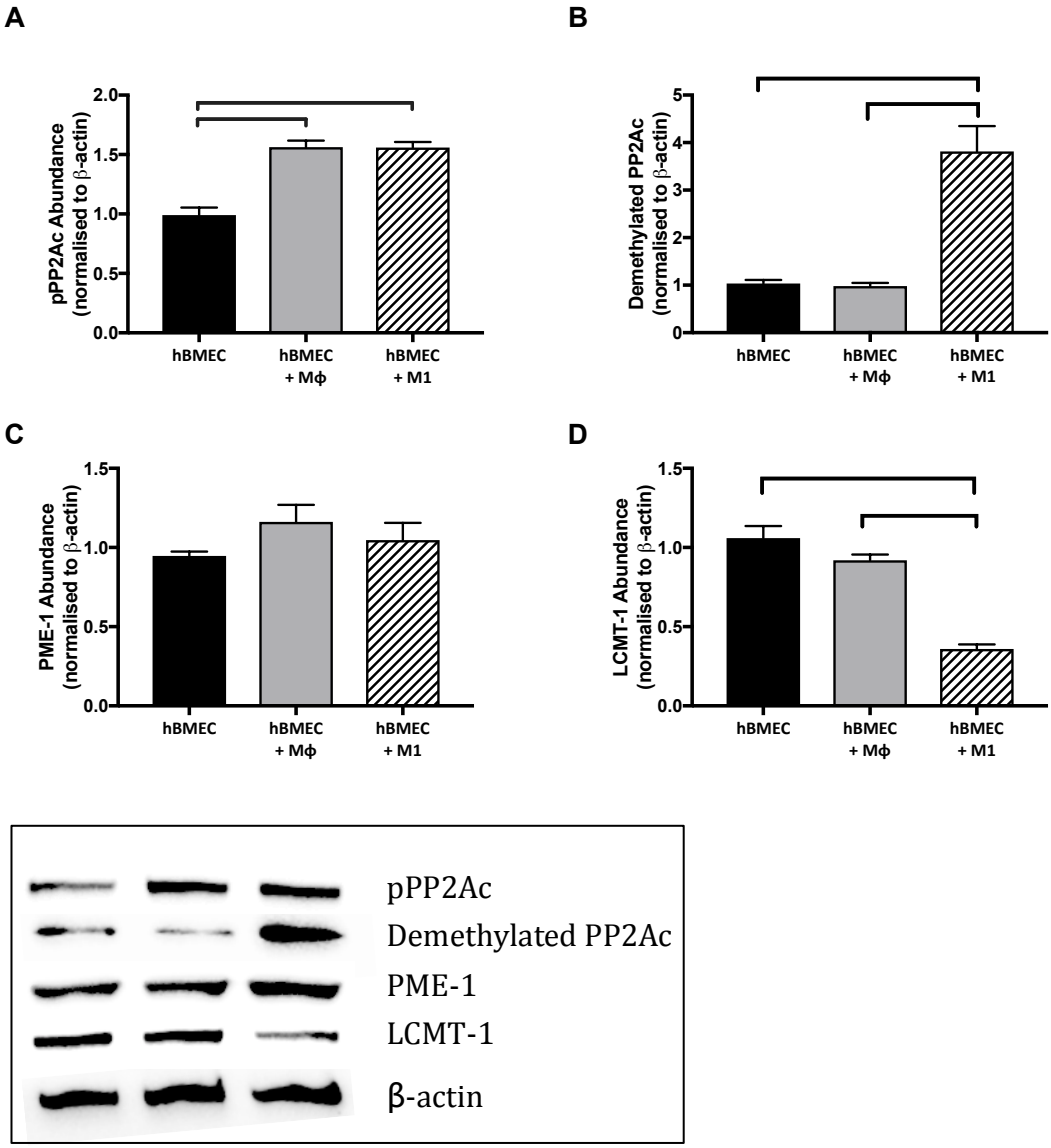

Figure 4.4: Effect of hBMEC, Mθ and M1 macrophage 24 h co-culture on PP2A post-translational modifications;

Phosphorylation (A) and demethylation (B) and modifiers PME-1 (C) and LCMT-1 (D). Abundance was determined by western-blot, normalised to β-actin, represented as a mean ± S.E.M relative to the hBMEC alone samples. Data was analysed using one-way ANOVA *post hoc* (Bonferroni). Horizontal bars represent statistical significance (P < 0.05), (n=5).

4.3.4 Mθ and M1 macrophages alter abundance and expression of VE-cadherin

In hBMEC co-cultured with Mθ macrophages the abundance and mRNA expression of VE-cadherin was increased by 60 % and 2.8 fold respectively compared to hBMEC in mono-culture ($P < 0.05$; Figure 4.5A-C). In contrast, co-culture with M1 macrophages decreased abundance of VE-cadherin decreased by ~ 40% and >60% compared to hBMECs in mono-culture and those co-cultured with Mθ macrophages respectively ($P < 0.05$; Figure 4.5A, C). VE-cadherin mRNA expression was increased by 2.6 fold in hBMEC co-culture with M1 macrophages ($P < 0.05$; Figure 4.5B).

As OA decreased VE-cadherin abundance in chapter 3 (Section 3.3.4) through increased proteasomal degradation, I investigated if this mechanism was also applicable to the effect of M1 macrophages on VE-cadherin abundance in hBMEC. In hBMEC co-cultured with Mθ macrophages MG132 did not alter the Mθ induced increase in VE-cadherin abundance (Figure 4.5C). However, in hBMEC co-cultured with M1 macrophages MG132 (2 μM) prevented the macrophage induced decrease in VE-cadherin abundance (Figure 4.5C).

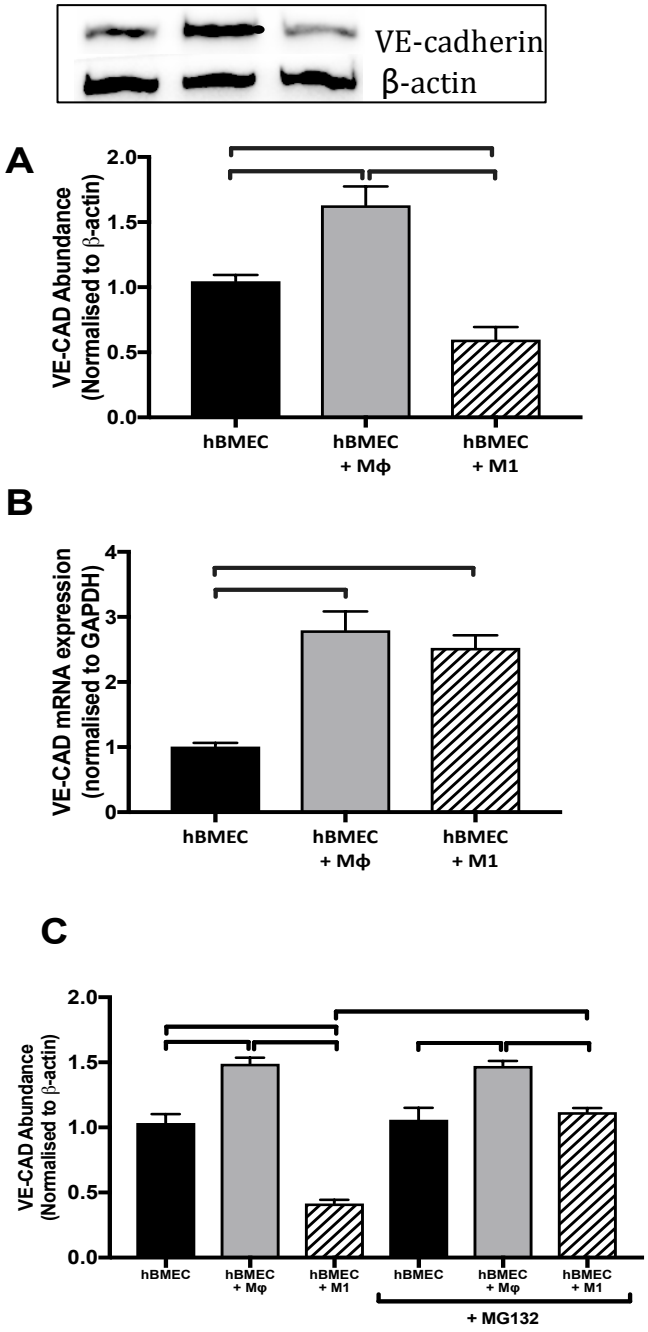

Figure 4.5: Effect of Mθ and M1 macrophage co-culture (24 h) on VE-cadherin in hBMEC.

VE-cadherin abundance was determined by western blot normalised to β-actin (A), VE-cadherin mRNA expression was determined by RT-PCR normalised to GAPDH Ct values (B). The role of proteasomal degradation on M1 induced loss of VE-cadherin abundance (C). Proteasomal degradation was inhibited using MG132 (2 μM). Data represented as mean ± S.E.M relative to the hBMEC mono-culture sample. Data was analysed using one-way ANOVA *post hoc* (Bonferroni). Horizontal bars represent statistical significance ($P < 0.05$), (n=5).

4.3.5 Overexpression of PP2A reverses the loss of VE-Cadherin

Transfection of hCMEC/D3 cells with pPP2Ac increased PP2Ac abundance by ~2 fold ($P < 0.05$; Figure 4.6A) compared to Untx, mock transfected and pCMV6 transfected cells (empty vector). This was accompanied by a >4 fold increase in PP2Ac activity ($P < 0.05$; Figure 4.6B) compared to the un-transfected and transfection control groups.

In hCMEC/D3s transfected with pPP2Ac, co-culture with Mθ macrophages did not alter the abundance of VE-cadherin compared to those in mono-culture in the mock and pCMV6 groups (Figure 4.7). In hCMEC/D3 cells co-cultured with M1 macrophages, mock transfection and transfection with pCMV6, VE-cadherin abundance was decrease by ~70% compared to hCMEC/D3s in mono-culture ($P < 0.05$;Figure 4.7). However, in the hCMEC/D3 pPP2Ac transfected group, co-culture with M1 macrophage did not alter VE-cadherin abundance compared to those in mono-culture (Figure 4.7) and was 58.7 and 60.6% higher than in the corresponding pCMV6 and mock transfected groups ($P < 0.05$; Figure 4.7). Transfection of hCMEC/D3 cells with the empty vector pCMV6 or pPP2Ac did not alter VE-cadherin abundance compared to mock transfected cells in mono-culture.

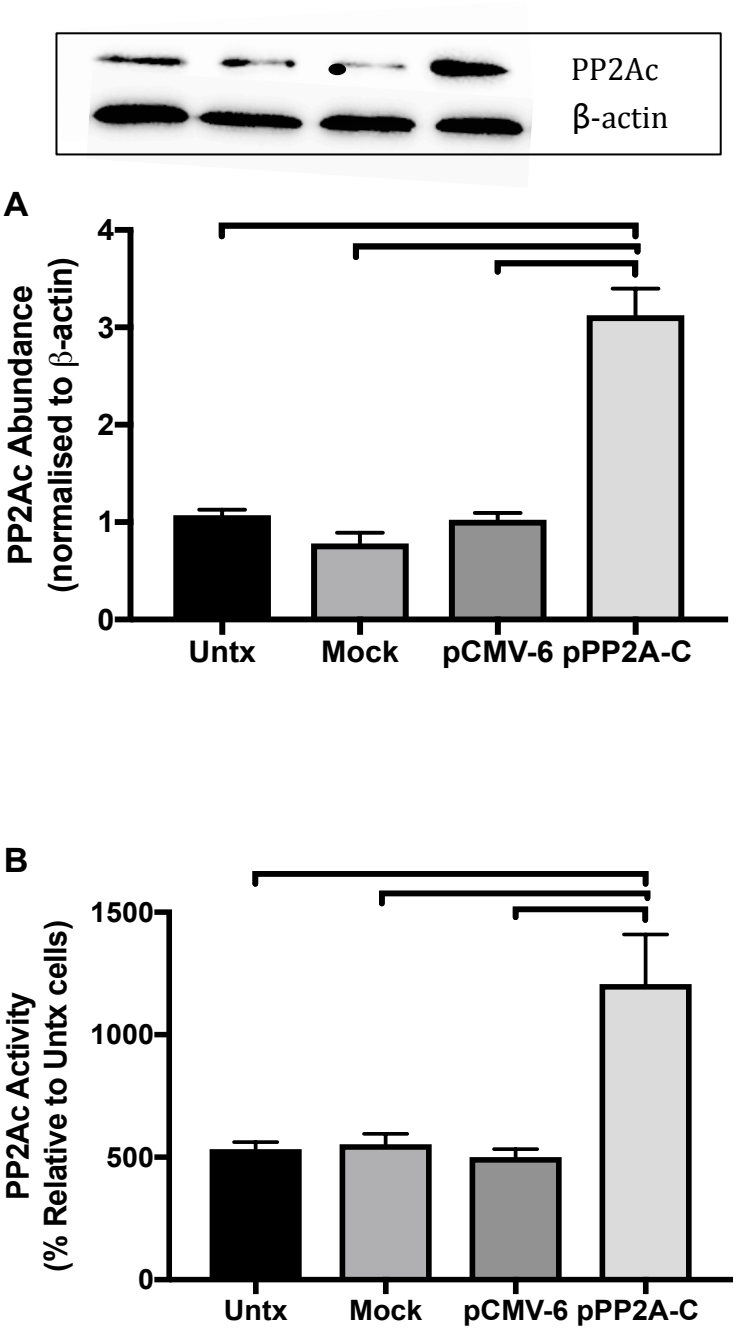

Figure 4.6: Confirmation of PP2A overexpression and associated increase in PP2Ac activity.

hCMEC/D3s were transfected with a pCMV6 PP2Ac plasmid and the relevant controls; mock and empty pCMV6 vector for 72 h. PP2Ac abundance was quantified by Western-blot and normalized to β-actin (A). PP2A activity was determined by PP2Ac immunoprecipitation activity assay (B). Data are represented as mean ± SEM relative to the Untx control; n=5; analysed using one-way ANOVA with post hoc Bonferroni analysis. * Represents statistical significance ($P < 0.05$).

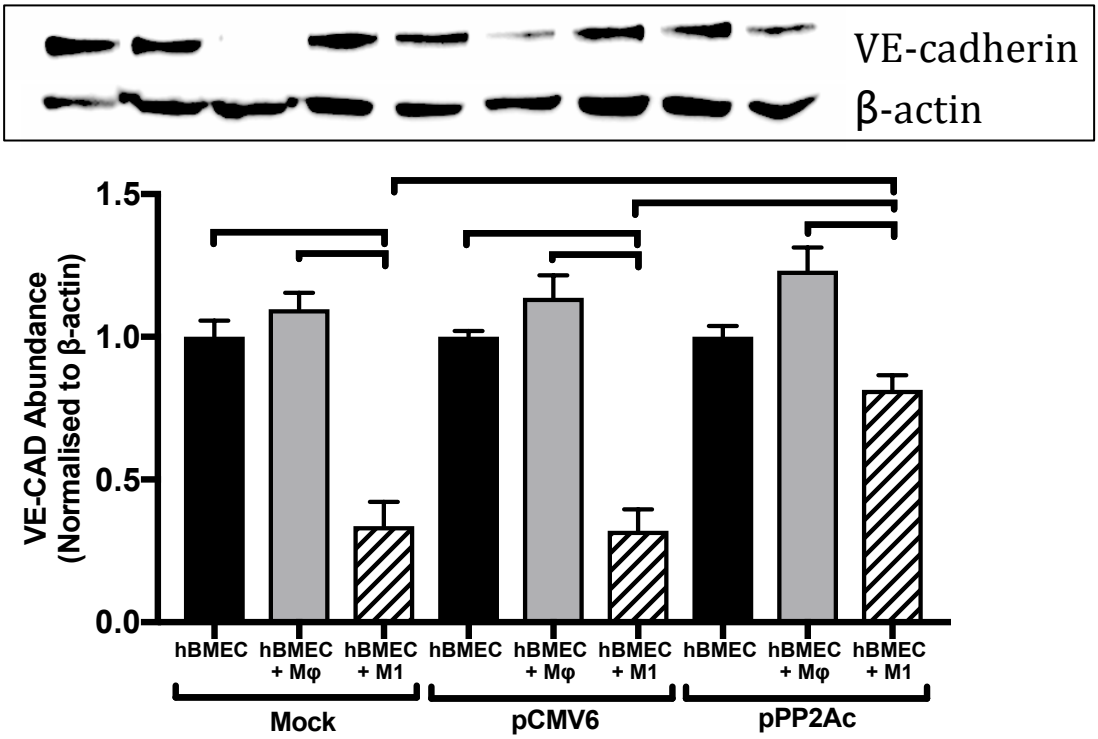

Figure 4.7: Investigating the effect of PP2A overexpression on pro-inflammatory loss of VE-cadherin in hCMEC/D3s.

Cells were transfected with a pCMV6 pPP2Ac plasmid and the relevant controls; mock and empty pCMV6 vector for 72 h. Transfected cells were co-culture samples were incubated with Mθ and M1 polarised macrophages for a further 24 h. VE-cadherin abundance was quantified by Western-blot and normalised to β-actin. Data are represented as mean ± SEM relative to the mock transfected control; n=5; analysed using one-way ANOVA with post hoc Bonferroni analysis. Horizontal bars represent statistical significance ($P < 0.05$).

4.3.6 M1 macrophages increase endothelial cell permeability

Co-culture of hBMEC with M1 macrophages increased paracellular permeability over a 24 h time-course compared to the hBMEC in mono-culture or co-cultured with Mθ macrophages. This increase was first detected at 18 h (4.7 fold) and continued to increase over the 24 h period compared to the those in mono- and Mθ- co-culture ($P < 0.05$; Figure 4.8A). In hBMEC co-cultured with Mθ macrophages paracellular permeability did not alter over the 24 h epoch compared to hBMEC alone (Figure 4.8A). The summary data (area under the permeability time curve) showed that M1 macrophage increased paracellular permeability of hBMEC by 2.6 fold increase compared to hBMEC alone or in co-culture with Mθ macrophages ($P < 0.05$; Figure 4.8B).

4.3.7 Overexpression of PP2A reverses M1 increase in endothelial permeability

Co-culture of hCMEC/D3s with M1 macrophages increased paracellular permeability compared to hCMEC/D3s in mono-culture or co-cultured with Mθ macrophages. This was first detected at 18 h and continued to increase for the duration of the experiment ($P < 0.05$; Figure 4.9A). Over the same period, Mθ macrophages in co-culture with hCMEC/D3 did not alter paracellular permeability compared to hCMEC/D3s in mono-culture (Figure 4.9A).

In hCMEC/D3s cells transfected with pCMV6-AC-PP2A, co-culture with M1 macrophages did not alter paracellular permeability compared to those in mono-culture or co-cultured with Mθ macrophages over the 24 h time period (Figure 4.9.C). However, in mock transfected cells, co-culture with M1 macrophages increased paracellular permeability by ~43% at 18 h and continued to increase for the remaining duration of the experiment relative to those in monoculture or co-cultured with Mθ macrophages ($P < 0.05$; Figure 4.9B). Similarly, in hCMEC/D3 cells transfected with pCMV-6 (empty plasmid), M1 macrophages increased paracellular permeability compared to those in mono-culture or co-cultured with Mθ macrophages ($P < 0.05$; Figure 4.9D).

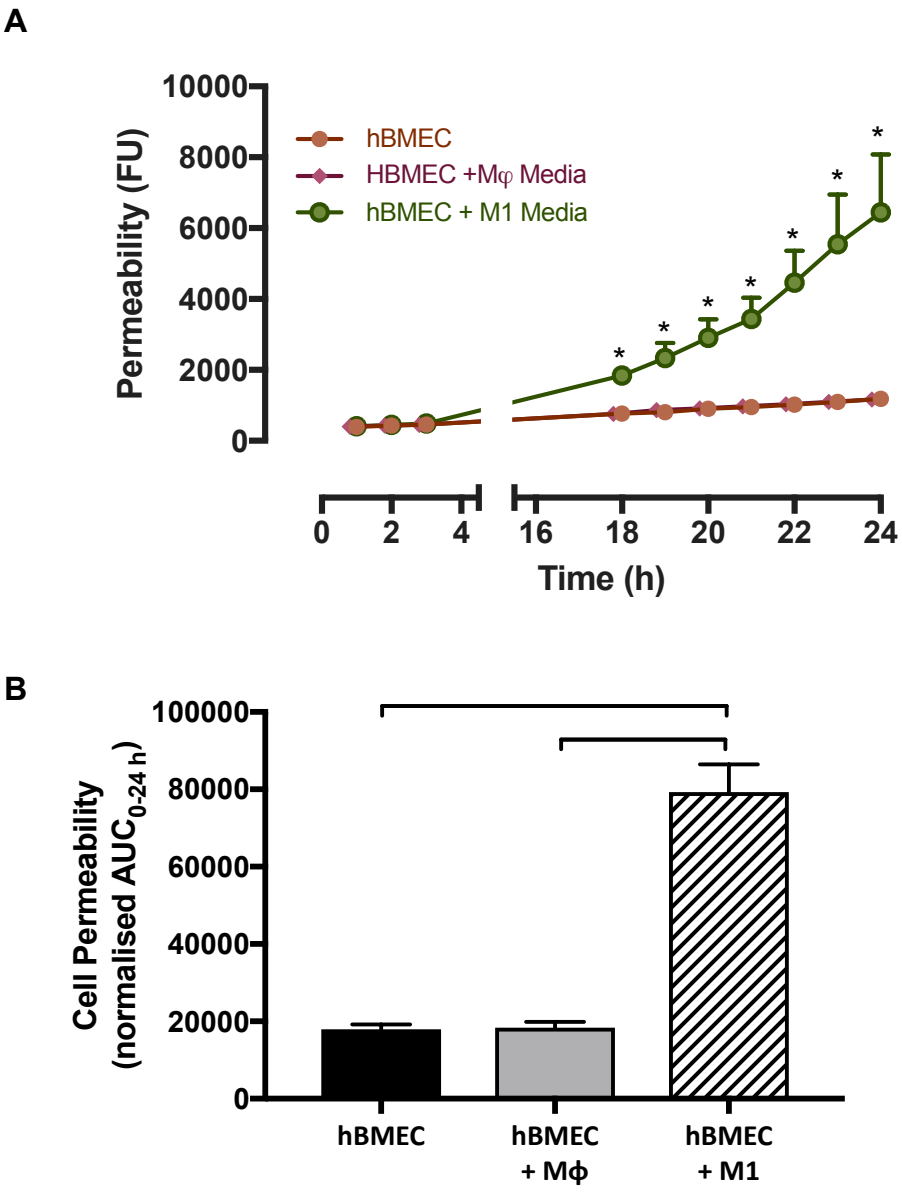

Figure 4.8: Effect of hBMEC, Mθ and M1 macrophage 24 h co-culture on brain microvascular cell permeability.

The movement of a FITC-labelled dextran measured permeability over 24 h through a monolayer of endothelial cells seeded on a transwell plate. hBMECs were cultured alone and as a co-culture with Mθ and M1 macrophage (A), this data was summarized as the AUC normalised to the hBMEC alone sample (B). Data was normalised to the first time point of the hBMEC alone sample (mean ± SEM; n=5). * $P < 0.05$ and horizontal bars represents significant differences from the relevant controls; two-way ANOVA with post hoc Bonferroni analysis.

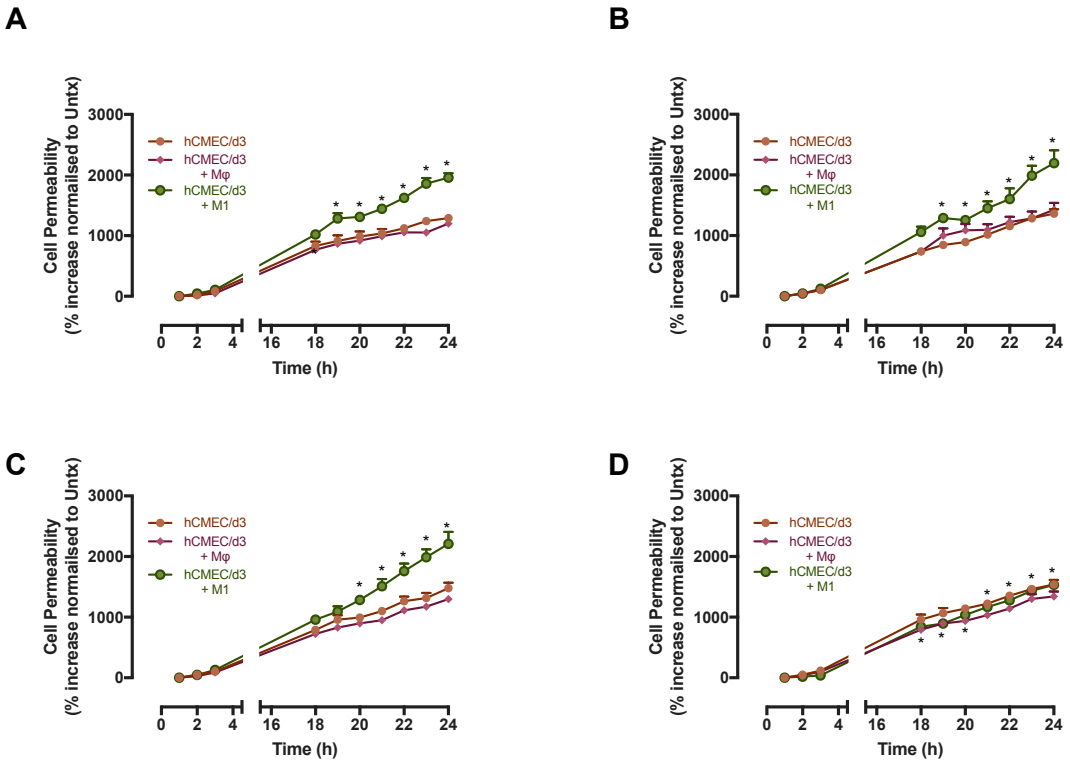

Figure 4.9: PP2A overexpression and associated effect on permeability.

hCMEC/D3s were transfected with a pCMV6-PP2Ac plasmid and the relevant controls; mock and empty pCMV6 vector for 72 h. Permeability was measured by the movement of a FITC-labelled dextran over 24 h through a monolayer of endothelial cells seeded on a transwell plate. hCMEC/D3s (untransfected (A), Mock Transfected (B), pCMV6 transfected (C) and pPP2Ac transfected (D)) were cultured alone and as a co-culture with Mθ and M1 macrophage. Data were normalised to the first time point of the hCMEC/D3s alone sample (mean ± SEM; n=5). Samples were analysed by two-way ANOVA with post hoc Bonferroni.

4.4 Discussion

It is well regarded that the loss of VE-cadherin is associated with an increase in vascular permeability, which can be induced by inflammation. The previous chapter demonstrated that inhibtion of PP2A is associated with loss of VE-cadherin in brain microvascular endothelial cells, however it remains to be established if this is relevant to patho/physiological conditions. To establish this, a hBMEC/macrophage co-culture model was utilised as an *in vitro* model of neuroinflammation. In this study I demonstrate that Mθ and M1 macrophages differentially modulate PP2A activity, Mθ activates while M1 macrophages inhibits.. M1 macrophages increase phosphorylation and decrease methylation of PP2Ac, consistent with a loss of activity through disruption of the holoenzyme in hBMECs. Loss of LCMT-1 is most likely responsible for the demethylation of PP2Ac as PME-1 was not altered. In line with the results of study 1, loss of PP2A induced proteasomal degradation of VE-cadherin. Over-expression of PP2A prevented the proteasomal loss of VE-cadherin induced by co-culture of M1 macrophages with hBMEC. As anticipated, co-culture of hBMECs and hCMEC/D3s with M1 macrophages increased paracellular permeability, an effect prevented by over-expression of PP2Ac.

The presence of Mθ and M1 macrophages provide unique results when investigating the association of PP2A and VE-cadherin. Co-culture models have facilitated the development of *in vitro* models with greater relevance to the *in vivo* state [683-685]. Mθ macrophages increase both PP2A and VE-cadherin abundance compared to the mono-culture suggests a protective role of resident unstimulated macrophages. Highlighting the need to consider the Mθ co-culture when investigating the effect of M1 macrophages. Studies have previously shown similar roles of perivascular and peripheral macrophages in supporting the integrity of the brain endothelial layer (bovine and human cell lines investigated)[686].

The Mθ macrophage-induced increase in PP2A activity is not a result of an increase in PP2A abundance, as abundance remains unaffected in both co-culture models (Mθ and M1). PP2A is a highly stable protein with a long half-life (13-17 h) and frequently abundance remains unaltered [463, 687]. The M1 induced loss of PP2A is likely due to altered assembly or blockage of the active site. Although M1 macrophages increase

the expression of PP2A mRNA this is not translated to protein abundance, suggesting it may be blocked by an miRNA, for example miR-183 [513]. Previous investigations into the role of PP2A in co-culture models demonstrated a decrease in PP2A abundance during inflammation. However all investigations have focused on the effect of PP2A in the inflammatory cells such as THP-1, glial, astrocytes cells in the periphery and brain. [598]. Increasing PP2A activity is associated with an anti-inflammatory and neuroprotection in a Parkinson's disease model [688, 689]. By comparison the present study is the first study to focus on PP2A in the endothelial cell in a co-culture model.

Another aspect that has previously not been investigated is the post-translational modification of PP2A in an inflammatory co-culture model. M1 macrophages induce an increase in phosphorylated PP2A, supporting the loss of PP2A activity. Interestingly hBMECs in the presence of Mθ macrophages also induces an increase in phosphorylated PP2A, which did not attenuate PP2A's increase in activity. One potential explanation is the both increased phosphorylation and demethylation is needed for the M1 induced loss of PP2Ac activity. In comparison Mθ macrophages increase PP2Ac alone without effecting PP2Ac activity. The loss of methylation is due to loss of LCMT-1 in M1 co-culture samples, facilitating PME-1 to remove methyl groups from PP2Ac without the opposing re-methylation. This has not previously been investigated in a similar model however knockdown LCMT-1 studies in rat glioma cells demonstrated a negative effect on PP2A activity [514, 535]. The down-regulation of LCMT-1 in COS7 and HeLa cells also determined that altering the methylation status of PP2A alters the assembly of the PP2A holoenzyme [514]. This loss of LCMT-1 has been suggested as a contributing factor in the loss of PP2A and resulting phosphorylation of tau in Alzheimer's disease (mouse neuroblastoma cell studies)[622, 623]. Adding to this, the activity of PME-1 and LCMT-1 may determine the cellular localisation of PP2A, as LCMT-1 is predominantly located in the cytoplasm and PME-1 in the nucleus [690, 691]. Contradictory to our findings, an investigation into endothelial-mesenchymal transmission in HUVECs demonstrated that TGF-β did not alter PP2Ac methylation and phosphorylation of PP2A. However this was after 72 h incubation, making it difficult to compare the findings of both studies [692]. Hyperglycemia (4 days), often associated with a pro-inflammatory response, also increases in PP2A methylation and activity in BAEC. However, as in our study the

activity and methylation of PP2Ac was reversed by PP2Ac inhibitor, OA [693]. Both studies highlight that PP2A activity and methylation may fluctuate after 24 h and may be of interest in future studies. As there are multiple proteins responsible for the phosphorylation of PP2A it was outside the remit of this study.

The hBMEC and macrophage co-culture model, as previously mentioned, demonstrated a protective role of Mθ macrophages on endothelial integrity through the increase in VE-cadherin abundance. Similar to the effect of OA (10 nM) in the previous chapter, M1 macrophages increase mRNA level of VE-cadherin, which does not correlate to the decrease in protein levels. This may be due to a rapid degradation of VE-cadherin or the presence of a miRNA for example miR-27 preventing the transcription of VE-cadherin [643]. Similar to our findings, use of M1 macrophage associated inflammatory cytokines induces VE-cadherin relocation; increase transendothelial migration and BBB permeability in mouse brain microvascular endothelial cells and hBMECs [233, 694-696]. The co-culture model in this study further supports the role of VE-cadherin during an inflammatory attack on the BBB and association with increased paracellular permeability.

As previously discussed VE-cadherin contains multiple phosphorylation sites on the carboxyl-tail. Considering the association of attenuated PP2A activity and VE-cadherin loss future investigations are required to determine a potential phosphorylation site for PP2A to target in the presence of M1 macrophages. Greater focus has been on the tyrosine sites [256, 678, 679]. Blocking VE-cadherin Y^{658} and/or Y^{685} phosphorylation has been shown to prevent the inflammatory mediator bradykinin, induced permeability and endocytosis of VE-cadherin [327]. While less is known about the effect of VE-cadherin Serine/Threonine phosphorylation, in particular the Ser^{665} residue in an inflammatory model [325, 326]. A previous study in human brain endothelial cells suggests the Ser^{665} is targeted by PP2A. Their results show that the inhibition of PP2A increases Ser^{665} phosphorylation associating with an increase in microvascular permeability [221]. Highlighting Ser^{665} as a potential avenue for future work.

Our results suggest a vital role of PP2A in maintaining the protective endothelial layer in the BBB against inflammation, as demonstrated with the overexpression of PP2Ac attenuating the M1 induced increase in permeability. Conversely, other studies have

suggested that an increase in PP2A is associated with inflammation and induced vascular permeability. Human pulmonary endothelial and mouse skeletal microvascular endothelial studies showed an increase in PP2A after LPS stimulation [652], however when PP2A was inhibited there was no reversal in LPS induced expression of ICAM-1 [697]. Propofol has been used to treat hyperglycaemia and hypoxia/reoxygenation. Its anti-inflammatory effects and benefits to endothelial function have been linked to its inhibitory effects on PP2A in HUVECs [698-700]. A study carried out on rat BMECs also suggests an adverse effect of PP2A, where the increase is PP2A was associated with an increase in permeability [701]. Further supported by the *in vitro* and *vivo* studies using absorbate to decrease PP2A resulting in an associated protection against vascular leak during septic insult [702, 703]. Occludin studies have also demonstrated an increase in PP2A associated with an increase in permeability and the pharmacological inhibition of PP2A reversed the observed effects [651, 704]. However none of the above studies were carried out in hBMECs, and the differing cells may address the conflicting findings.

Our results highlight a potential role of PP2A in regulating both VE-cadherin surface abundance and degradation. Considering the findings from this and the previous chapter, it can be concluded that M1 macrophages and OA (10 nM) both result in the proteasomal degradation of VE-cadherin in hBMECs. Supported by previous studies demonstrating that phosphorylated E-cadherin/VE-cadherin stimulate its removal from the membrane through clathrin-mediated endocytosis [317]. Also a study carried out on ovarian cancer, which utilises an endothelial cell/macrophage co-culture model reported the role of the apoptosis signal regulating-kinase 1 in stimulating proteasomal degradation of VE-cadherin [705]. In conjunction with PP2As association with VE-cadherin abundance suggests an important role of kinase and phosphatase activity in regulating VE-cadherin surface abundance and degradation.

Considering the anti-inflammatory effect of PP2A shown in the literature, it has become a target for pharmacological modulation [605, 706, 707]. We have demonstrated that over-expressing PP2A reverses the M1 induced loss of PP2A activity, VE-cadherin abundance and increased permeability. Tristetraprolin is an anti-inflammatory gene regulator, *in vivo* and *in vitro* demonstrates that is it is also an

agonist of PP2A, contributing to its anti-inflammatory effect [708]. The potential therapeutic benefit was demonstrated in rat subarachnoid haemorrhage *in vivo* study resulting in a neuroprotective and anti-inflammatory effect of Tristetraprolin and increased PP2A [709] A recent publication also demonstrated that LPS induced endothelial cell-monocyte interaction was reduced by resolvin D1 through the blocking of H_2O_2 inactivation of PP2A [710]. Both therapies have the potential to reverse M1 induced loss of VE-cadherin and increased permeability.

In conclusion, the present study has unveiled a strong association between PP2A activity, VE-cadherin and permeability in the presence of macrophages (summarised in Figure 4.10). Highlighting the supportive role of Mθ macrophages through the increase of VE-cadherin and PP2A activity. Also demonstrating the M1 induced increase in hBMEC permeability correlating with the loss of VE-cadherin and PP2A activity. Most likely M1 macrophages attenuate loss of PP2A activity through holoenzyme disassembly. Supported by the increase in phosphorylated PP2A and demethylation induced by M1 macrophages. The ability of PP2A over expression to reverse M1 induced VE-cadherin loss provides strong evidence to the beneficial use of PP2A stimulators against inflammatory effects. Alternatively, this study suggests that targeting LCMT-1 and PP2A methylation may also potentially reverse the M1 induced effects. Our results provide further insight into damage incurred during Neuroinflammation and diseases such as Alzheimer's, which are already associated with a loss of PP2A in the brain.

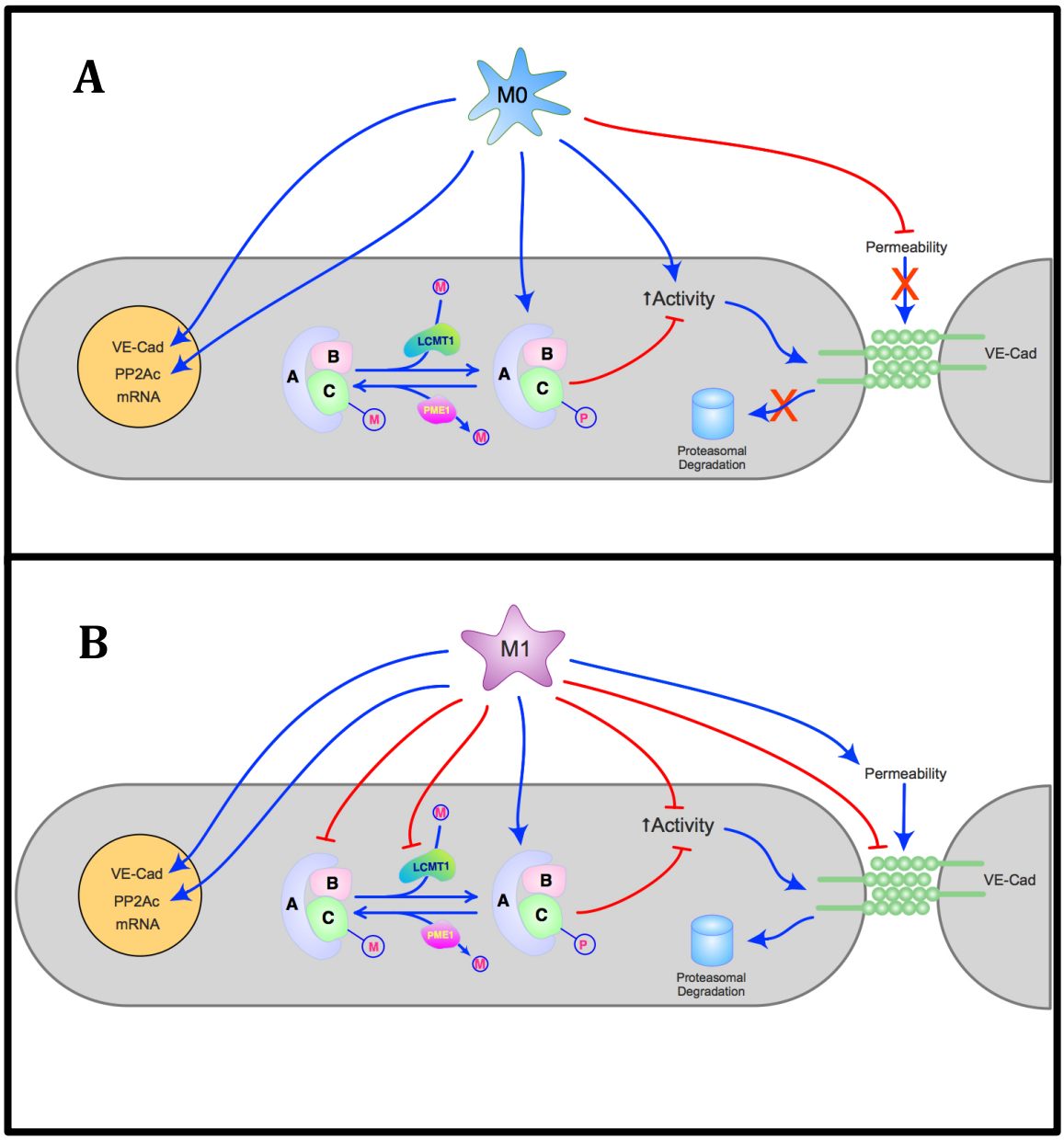

Figure 4.10: Schematic overview of PP2A modulation and VE-cadherin in Human Brain Microvascular Endothelial Cells in a Macrophage Co-culture Model.

Mθ macrophages cause an increase in the PP2Ac activity and increase in VE-cadherin and without effecting cell permeability. M1 macrophages proteasomal degradation of VE-cadherin and increase in endothelial cell permeability.

Chapter 5

5 Effect of Okadaic Acid and CIP2A on the VE-Cadherin Interactome in Brain Microvascular Endothelial cells

5.1 Introduction

VE-cadherin modulates the integrity of the blood brain barrier through binding of accessory proteins to its cytosolic tail which stabilises the VE-cadherin interactome at the cell surface [711]. These accessory proteins include P120, α, β and γ-catenin [712].

Regarding, P120, it binds to a specific peptide sequence $Y^{645}CEEGGGE^{652}$, in the juxtamembrane region of VE-cadherin [323, 617, 713] to form a connecting bridge between the cell surface and actin filaments through which VE-cadherin regulates cell morphology [714-716]. Additionally, P120 is an important regulator in VE-cadherin expression and internalisation and supports its adhesion to the plasma membrane. P120 mediates internalisation of VE-cadherin by inhibiting RhoA [318] and Src activity, Loss of P120 reduces VE-cadherin membrane abundance and increases endothelial permeability [321, 715, 717].

Binding of α-catenin to VE-cadherin is involved in membrane to actin-filament regulation through direct binding to the β-catenin/VE-cadherin interactome [718-720]. β-Catenin is a multifaceted protein in endothelial cells, whose inclusion in the VE-cadherin interactome is essential for adheren junction strength [721]. In addition to this, unbound β-catenin can translocate from the membrane to the nucleus to mediate the Wnt signalling cascade and alter gene transcription [722].

Importantly, the composition and stability of the VE-cadherin/catenin interactome is regulated by phosphorylation. For example, an increase in phosphorylation of tyrosine residues in VE-cadherin and its accessory proteins result in disassembly and loss of the VE-cadherin interactome from the membrane and actin rearrangement [723]. More specifically, phosphorylation of Tyr^{658} and Tyr^{731} prevents the binding of P120 and β-catenin respectively, disrupting endothelial barrier integrity [678, 724]. Interestingly, amino acid sequence analysis of the intracellular domain of VE-cadherin has uncovered a highly conserved region adjacent to the P120 binding site, which has a unique serine-threonine cluster, distinct from other classical cadherins [325]. This region contains Ser^{665}, whose phosphorylation results in increased VE-cadherin internalisation through the induction the Src-VAV2-Rac-PAK pathway following exposure to VEGF [326].

Not surprisingly, several tyrosine phosphatases including SHP2 [725, 726], DEP-1, PTP1B [323, 727, 728] and VE-PTP [729] have been linked to maintenance of the VE-cadherin interactome by preventing its phosphorylation. However, few studies have investigated the role of Ser/Thr phosphatases in the regulation of VE-cadherin and in particular, the role of PP2A in mediating its internalisation and interaction with accessory proteins. In the previous chapters I have shown that PP2A plays a key role in regulating VE-cadherin abundance in response to pharmacological inhibition of PP2A and during co-culture of brain microvascular endothelial cell with pro-inflammatory macrophages. However, the mechanism by which PP2A modulates this remains to be established. Therefore, the aims of the present chapter were to delineate the mechanism by which PP2A modulates VE-cadherin abundance with regard to its interaction with the key accessory proteins P120, α and β-catenin, and proteasomal degradation.

5.2 Materials and Methods

5.2.1 Cell culture

Human brain microvascular endothelial cells (hBMECs; cell systems, WA, USA) and HCMEC/D3 (gifted from Dr M. Campbell, Trinity College Dublin) were cultured in EndoGRO-MV culture medium containing 5% foetal bovine serum, supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin. hBMECs medium also contained ciprofloxacin (10 μg/mL). Cells were exposed to OA (10 nM) for the relevant experiments. In order to investigate VE-cadherin internalisation endothelial cells were exposed to chlorpromazine hydrochloride (10 μg/mL) for 30 mins prior to OA (10 nM) exposure and a further 24 h. Cells were maintained at 37 ^{o}C in a humified atmosphere containing 5% CO_2. hBMECs were used up to passage 10. All experiments were performed under serum free conditions.

5.2.2 Determination of Protein-Protein Interactions - Pull-down assay

In brief, cultured endothelial cells were lysed freeze-thawing (X3) in 3 mL modified RIPA buffer (section). Following clarification by centrifugation (12,000 RPM), the supernatant was incubated with 1 μg of mouse control IgG and 20 μl of agarose conjugate protein A/G-agarose (25% v/v) and incubated at 4° C for 30 minutes. During this time mouse anti-human VE-cadherin antibody (2 μg) was incubated with 500 μg of total protein for 1 h at 4° C. 20 μl of suspended (25% v/v) agarose conjugate protein A/G was then added to the Ab/protein suspension and mixed overnight at 4° C. Following collection of the beads by centrifugation (12,000 RPM for 5 mins) the protein/agarose pellets were washed in PBS and resuspended in RIPA buffer. The immunoprecipitates (standardised to 1 μg/μL of protein) were the separated by electrophoresis and normalised to total VE-cadherin abundance.

5.2.3 Western blotting

Cells were lysed in modified RIPA buffer (Tris-base 50 mM, NaCl 150 mM, EDTA 2mM and NP-40 0.5 % v/v), supplemented with the protease inhibitor cocktail SIGMAFAST™ and the phosphatase inhibitors sodium orthovanadate (2 mM) and sodium fluoride (5 mM). Samples (20 μg protein) were boiled for 1 min in LDS sample buffer (LDS 5%, Tris HCl 1M, Glycerol 50%, bromophenol blue 2.5 mg, phenol red 2.5 mg, ficoll 400 5% and β-mercaptoethanol 10 %) and placed immediately on ice. Samples were separated using SDS PAGE (8% gel) and transferred to a PVDF membrane using a semi-dry transfer system and transfer buffer (Tris-base 50 mM, glycine 40 mM, methanol 20% v/v, SDS 0.037% w/v, dH_2O). Membrane were blocked using blocking buffer (TBS-T (Tris-base 10 mM, NaCl 100 mM and HCl 1 M and 0.1% Tween-20) containing 5% dried skimmed milk) for 1 h. Membranes were probed overnight at 4 ^oC with 1^o antibodies directed against VE-cadherin, PP2Ac, pPP2Ac, dimethyl-PP2Ac, PME-1 or LCMT-1 (Santa Cruz), and extensively washed in TBS-T prior to probing for 1 h with a polyclonal goat anti-mouse HRP conjugated 2^o Ab (Dako). Membranes were washed, detected using chemiluminescence (3.2 μL of 30% hydrogen peroxide/ 6 mL of 250 mM Luminol, 90 mM 4- iodophenyl boronic acid and 100 mM Tris-HCl)[681] and images captured on a Fusion FX imaging system (Vilber Lourmat). Membranes were stripped using (62.5 mM Tris-HCl, 2% SDS and < 0.02% β-mercaptoethanol) and re-probed with an anti-β-actin HRP conjugated antibody. All antibodies were diluted (1:1000, excluding anti-β-actin (1:3000)) in blocking buffer. Densitometric analysed was performed using the Bio1D software and density normalised to β-actin. The molecular weight of the target proteins were determined from an EZ-RUN™ molecular weight ladder (Fischer Scientific, Dublin, Ireland).

5.2.4 Cell transfection and Overexpression of PP2Ac and CIP2A

hCMEC/3s were seeded in T75 flasks (2 x 10^6 cells per well) 24 h prior to transfection. Transfection was carried out in serum free opti-mem (1 mL; Sigma) using TransIT-X2 (40 μL; Mirus). The transfection reagent, plasmids and opti-MEM mixture was incubated at room temp for 30 mins. Cells were transfected for 72 h with a pcDNA3.1 CIP2A plasmid, pCMV-6 PP2Ac plasmid, pcDNA3.1 or pCMV-6. Overexpression of CIP2A and PP2Ac were confirmed in previous sections (Section 3.3.5 and Section 4.3.7). pcDNA3.1, pCMV-6 and mock-transfection (transfection reagent only) were included in all experiments as controls.

5.2.5 Data and Statistical Analysis

Western-blot data was normalised to β-actin or VE-cadherin for immunoprecipitated samples to determine relative protein abundance. All data were normalised to the appropriate controls and expressed as a ratio or percentage. Data were analysed using ANOVA (one or two-way) with *post hoc* Bonferroni analysis or an unpaired Student t test as appropriate. Data are represented as a mean ± S.E.M., and a value of $P < 0.05$ was set to indicate statistical significance.

5.3 Results

5.3.1 Effect of Clathrin mediated inhibition on OA induced VE-cadherin attenuation

In hBMEC, OA (10 nM) decreased VE-cadherin abundance by >56% compared to the Untx and DMSO controls ($P < 0.05$). Chlorpromazine hydrochloride (CPZ; 10 μg/mL) prevented the loss in VE-cadherin resulting from exposure to OA (0.46 ± 0.11 v 1.09 ± 0.05; $P < 0.05$; Figure 5.1). CPZ alone or in combination with DMSO did not alter the abundance of VE-cadherin. OA's loss of VE-cadherin was also significant compared to the CPZ alone and combination with DMSO ($P < 0.05$;Figure 5.1).

5.3.2 PP2A modulation on VE-cadherin Ser^{665} Phosphorylation

OA (10 nM) elicited approximately a fold increase ($P < 0.05$; Figure 5.2A, B) in the abundance of phosphorylated (Ser^{665}) VE-cadherin in hBMEC and hCMEC/D3 cells compared to their corresponding Untx and DMSO groups. DMSO did not alter abundance of Ser^{665} phosphorylated VE-cadherin compared to the Untx group in either cell line.

In hCMEC/D3 transfected with pcDNA3.1 CIP2A the abundance of Ser^{665} phosphorylated VE-cadherin was doubled compared to the empty vector pcDNA3.1, mock and untransfected groups ($P < 0.05$; Figure 5.2C). In keeping with this, overexpression of PP2A decreased the abundance of Ser^{665} phosphorylated VE-cadherin by ~60% compared to the pCMV6, mock and untransfected groups ($P < 0.05$; Figure 5.2D). Mock transfection and transfection with pcDNA3.1 or pCMV6 plasmids did not alter abundance of Ser^{665} phosphorylated VE-cadherin.

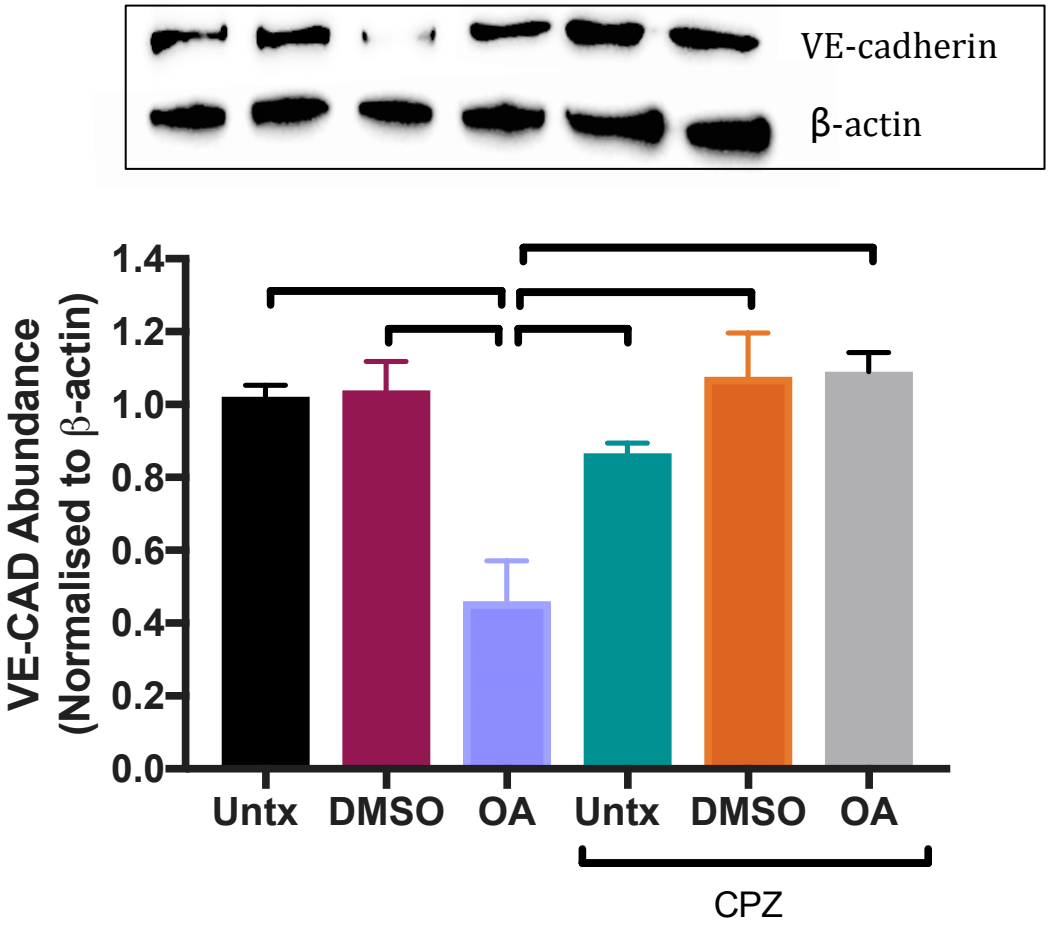

Figure 5.1: Investigation into the role of Clathrin Mediated endocytosis on OA (10 nM) induced loss of VE-cadherin abundance after a 24 h.

Clathrin mediated endocytosis was inhibited using chlorpromazine hydrochloride (CPZ; 10 μg/mL). VE-cadherin abundance was determined by Western-blot and normalised to β-actin. Data are represented as relative expression to the Untx (- CPZ) sample (mean ± SEM; n=5). Samples were analysed using a one-way ANOVA with *post hoc* Bonferroni. P < 0.05 taken to indicate significant differences and are represented by horizontal bars.

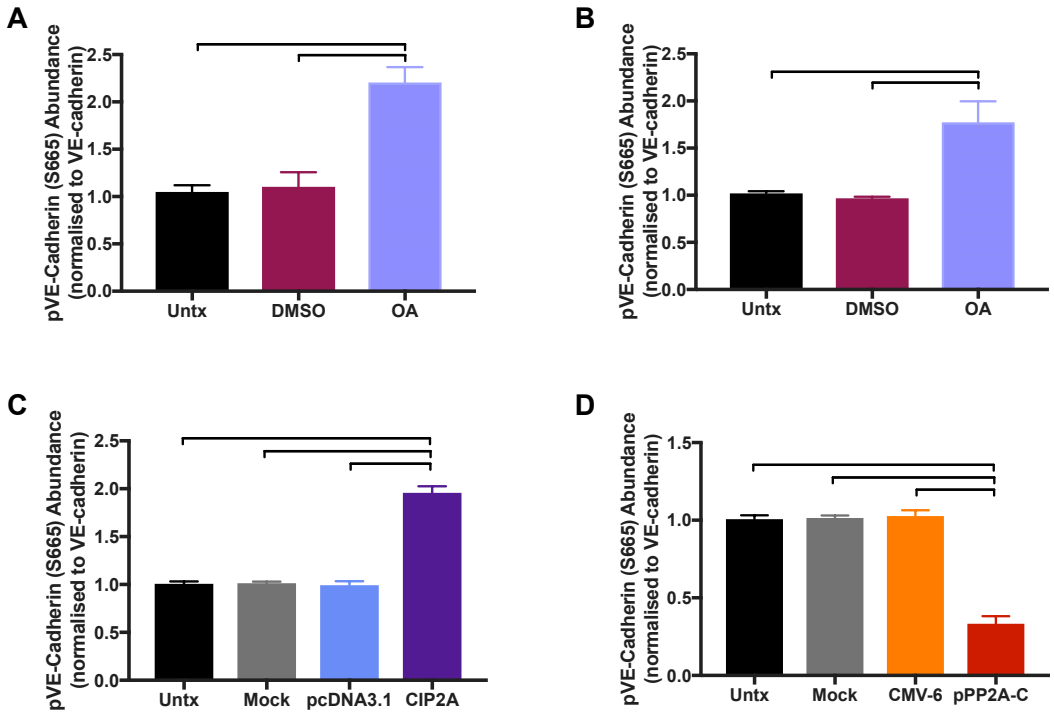

Figure 5.2: Effect of PP2A modulation on Ser^{665} modulation of VE-cadherin.

VE-cadherin was immunoprecipitated from whole cell lysate with VE-cadherin Ab bound to sepharose beads. pVE-Cadherin (S665) abundance was determined by Western-blot, normalised to VE-cadherin. hBMECs (A) and hCMEC/D3s (B) were treated with OA (10 nM) for 24 h to inhibit PP2A. PP2A was also inhibited by the over-expression of CIP2A in hCMEC/D3 for 72 h (C) and PP2A activity was increased by the over-expression of PP2A (D). Data are represented as a mean ± S.E.M relative to the Untx sample. Data were analysed using one-way ANOVA post hoc (Bonferroni). Horizontal bars represent statistical significance ($P < 0.05$; n=5).

5.3.3 Effect of PP2A inhibition on α-Catenin abundance

In hBMECs exposure to OA for 24 h resulted in a 73% and 79% loss in α-Catenin abundance compared to Untx and DMSO groups respectively ($P < 0.05$; Figure 5.3A). OA also resulted in a loss of α-Catenin compared to Untx (-27.52%) and DMSO (32.85%) in hCMEC/D3s ($P < 0.05$; Figure 5.3B). DMSO did not alter the abundance of α-Catenin compared to Untx. In hCMEC/D3 cells, mock or pcDNA3.1 transfection did not alter the abundance of α-catenin compared to the Untx. However, CIP2A overexpression elicited a > 50% loss of α-catenin abundance compared to the Untx, mock and pcDNA3.1 groups ($P < 0.05$; Figure 5.3C).

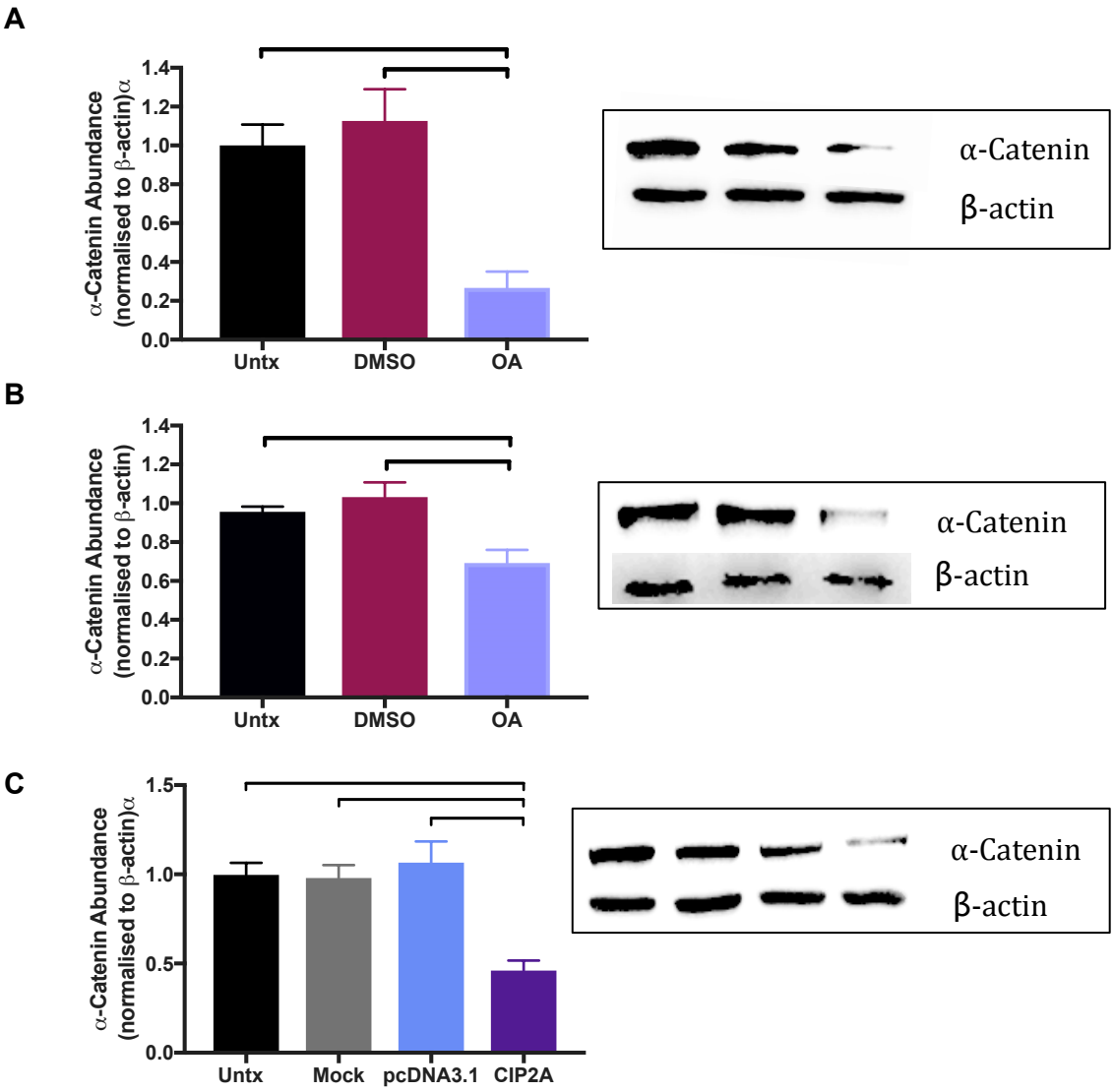

Figure 5.3: Effect of inhibition of PP2A on abundance of α-catenin.

hBMECs (A) and hCMECs (B) were exposed to OA (10 nM) for 24 h to inhibit PP2A activity. The effect of PP2A inhibition was confirmed by the over-expression of CIP2A for 72 h (C). α-catenin abundance was determined by Western-blot, normalised to β-actin, represented as a mean ± S.E.M relative to the hBMEC alone samples. Data were analysed using one-way ANOVA *post hoc* (Bonferroni). Horizontal bars represent statistical significance ($P < 0.05$; n=5).

5.3.4 Effects of PP2A modulation on β-catenin abundance

Exposing hBMECs and hCMEC/D3s to OA (10 nM) or DMSO for 24 h did not alter the abundance of β-catenin compared to the Untx group (Figure 5.4A and B). Similarly, overexpression of CIP2A in hCMEC/D3s did not alter abundance of β-catenin compared to Untx. Mock transfection and transfection with pcDNA3.1 had no effect on β-catenin abundance (Figure 5.4C).

5.3.5 Effects of PP2A modulation on P120 abundance

OA (10 nM) decreased P120 abundance by 57.98% and 77.93% in hBMEC compared to the Untx and DMSO groups respectively ($P < 0.05$). DMSO did not alter the abundance of P120 compared to Untx (Figure 5.5A). In hCMEC/D3s, OA reduced P120 abundance compared to the Untx (-38.6) and DMSO (-45.8%) groups ($P < 0.05$; Figure 5.5B). Similarly, in hCMEC/D3s overexpression of CIP2A reduced P120 abundance by 50% ($P < 0.05$; Figure 5.5C) compared to untreated, mock transfected and pcDNA3.1 transfected groups. Mock and pcDNA3.1 transfection for 72 h did not alter the abundance of P120 compared to the Untx.

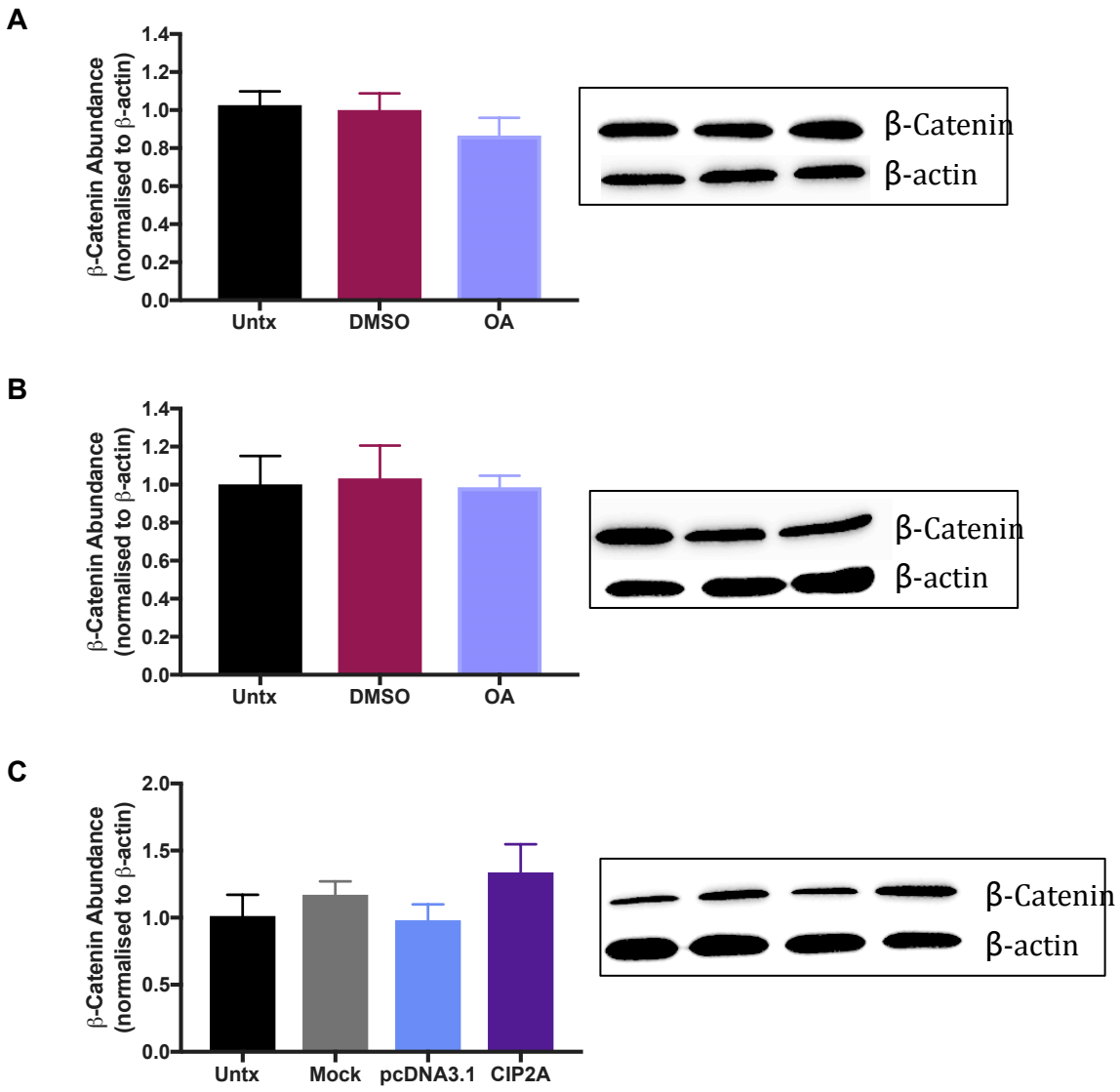

Figure 5.4: Effect of PP2A inhibition on abundance of β-catenin.

hBMECs (A) and hCMECs (B) were exposed to OA (10 nM) for 24 h to inhibit PP2A activity. The effect of PP2A inhibition was confirmed by the overexpression of CIP2A for 72 h (C). β-catenin abundance was determined by Western-blot, normalised to β-actin, and presented as a mean ± S.E.M relative to the hBMEC alone samples. Data were analysed using one-way ANOVA *post hoc* (Bonferroni). Horizontal bars represent statistical significance ($P < 0.05$; n=5).

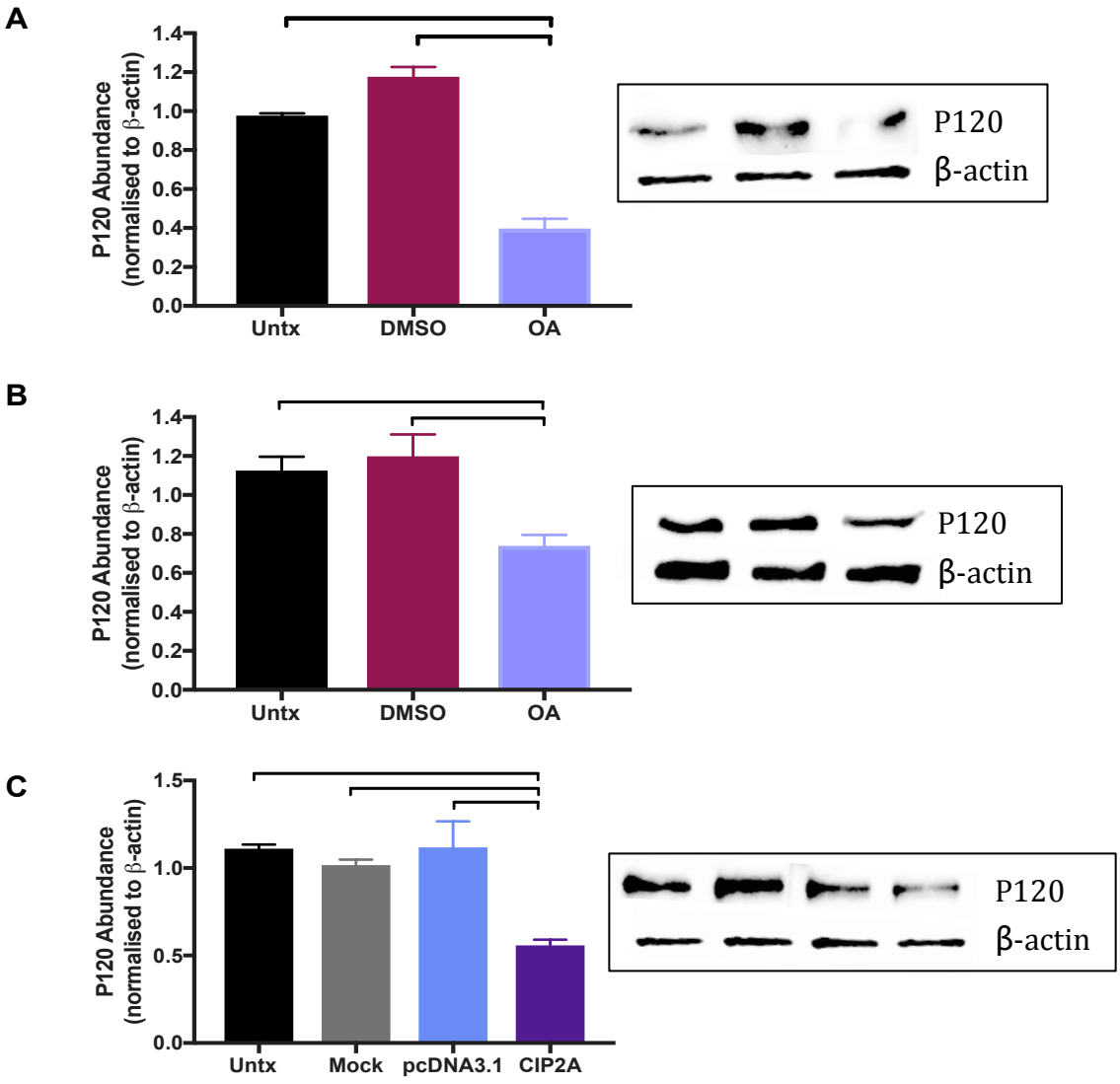

Figure 5.5: Effect of PP2A inhibition on the total abundance of P120.

hBMECs (A) and hCMECs (B) were exposed to OA (10 nM) for 24 h to inhibit PP2A activity. The effect of PP2A inhibition was confirmed by the over-expression of CIP2A for 72 h (C). P120 abundance was determined by Western-blot, normalised to β-actin, represented as a mean ± S.E.M relative to the hBMEC alone samples. Data were analysed using one-way ANOVA *post hoc* (Bonferroni). Horizontal bars represent statistical significance ($P < 0.05$; n=5).

5.3.6 α-Catenin binding to VE-cadherin post PP2A modulation.

In hBMECs, OA (10 nM for 24 h) reduced α-catenin bound to VE-cadherin by 69 % and 74 % compared to the Untx and DMSO groups respectively ($P < 0.05$; Figure 5.6A) Combination of OA and MG132 prevented the loss of VE-cadherin bound α-catenin ($P < 0.05$; Figure 5.6A), returning it to baseline. In hCMEC/D3, OA (10 nM) tended to decrease (~30%) α-catenin bound VE-cadherin abundance compared to the Untx and DMSO groups, however it failed to reach significance (Figure 5.6B). Combining MG132 (2 μM) and OA (10 nM) increased the abundance of α-catenin bound to VE-cadherin compared to the Untx (124.9%) and DMSO (99.82%). When compared to the OA alone the addition of MG132 increased the abundance of α-catenin attached to VE-cadherin by 227.93% ($P < 0.05$; Figure 5.6B). hCMEC/D3s were mock transfected for 72 h which did not alter the abundance of α-catenin bound to VE-cadherin compared to the Untx. CIP2A was overexpressed resulting in a 56.15% loss in α-catenin compared to the empty vector pcDNA3.1 and a ~50% loss compared to the Untx and mock transfected samples ($P < 0.05$; Figure 5.6C).

5.3.7 β-Catenin binding to VE-cadherin after PP2A modulation

OA inhibits the binding of β-catenin to VE-cadherin by 43.99% and 51.92% compared to the Untx and DMSO samples respectively in hBMECs ($P < 0.05$). Combination of OA and MG132 results in a 149.59% increase in β-catenin expression relative to OA alone ($P < 0.05$). There was no alteration in VE-cadherin bound β-catenin expression when comparing the combination of OA and MG132 to Untx and DMSO (Figure 5.7A). OA (10 nM) exposure to hCMEC/D3s resulted in the loss of β-catenin bound to VE-cadherin by ~60% compared to the Untx and DMSO samples ($P < 0.05$). The addition of MG132 reversed this disassociation resulting in a ~70% increase in β-catenin abundance compared to the Untx and DMSO samples and >120% increase compared to OA (10 nM) alone ($P < 0.05$; Figure 5.7B). CIP2A overexpression in hCMEC/D3s resulted in a 69.28% loss in β-catenin associated with VE-cadherin compared to the pcDNA3.1 empty vector. The loss of β-catenin was 73.13% and 75.97% loss when compared to the Untx and mock transfected ($P < 0.05$; Figure 5.7C).

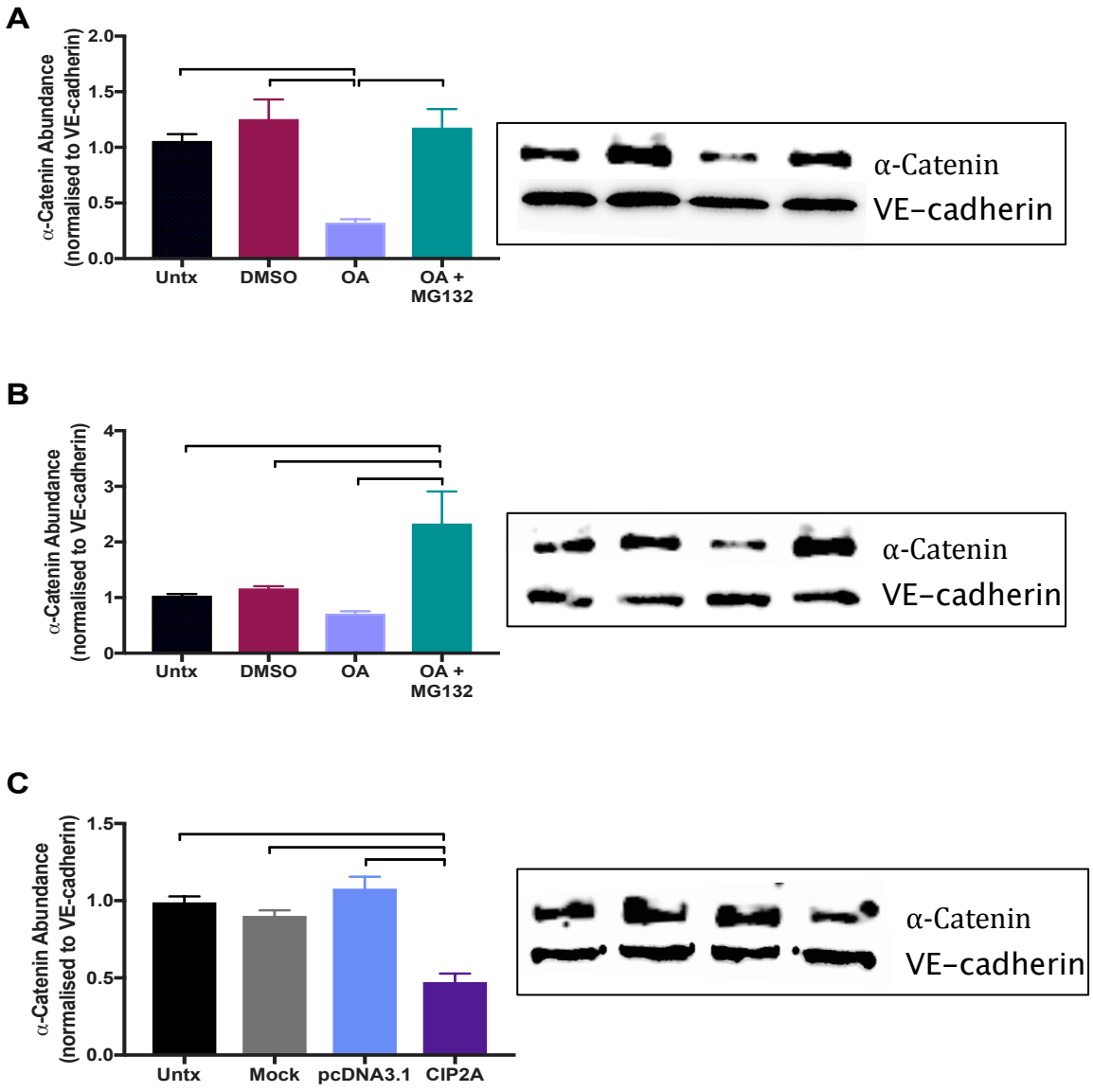

Figure 5.6: Effect of PP2A inhibition on α-catenin abundance in the VE-cadherin interactome.

hBMECs (A) and hCMECs (B) were exposed to OA (10 nM) for 24 h to inhibit PP2A activity, cells were also treated with a combination of OA (10 nM) and MG132 (2 μM) to prevent proteasomal degradation. The effect of PP2A inhibition was confirmed by the overexpression of CIP2A for 72 h (C). VE-cadherin was immunoprecipitated from whole cell lysate with VE-cadherin Ab bound to sepharose beads. α-catenin abundance was determined by western-blot, normalized to VE-cadherin, represented as a mean ± S.E.M relative to the hBMEC alone samples. Data was analysed using one-way ANOVA *post hoc* (Bonferroni). Horizontal bars represent statistical significance ($P < 0.05$), (n=5).

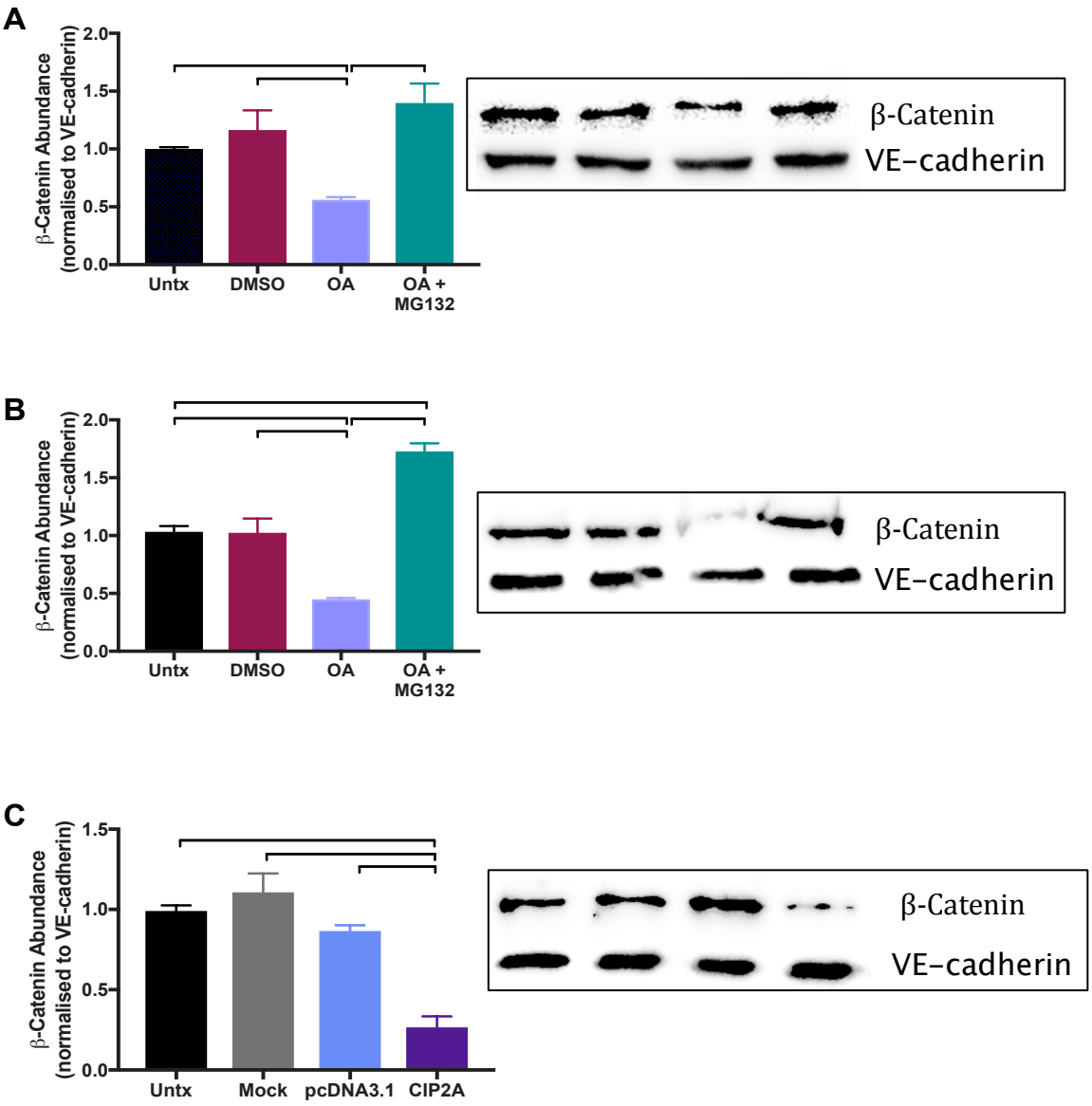

Figure 5.7: Effect of PP2A inhibition on β-catenin abundance in the VE-cadherin interactome.

hBMECs (A) and hCMECs (B) were exposed to OA (10 nM) for 24 h to inhibit PP2A activity, cells were also treated with a combination of OA (10 nM) and MG132 (2 μM) to prevent proteasomal degradation. The effect of PP2A inhibition was confirmed by the overexpression of CIP2A for 72 h (C). VE-cadherin was immunoprecipitated from whole cell lysate with VE-cadherin Ab bound to sepharose beads. β-catenin abundance was determined by western-blot, normalized to VE-cadherin, represented as a mean ± S.E.M relative to the hBMEC alone samples. Data was analysed using one-way ANOVA *post hoc* (Bonferroni). Horizontal bars represent statistical significance ($P < 0.05$), (n=5).

5.3.8 P120 binding to VE-cadherin post PP2A modulation.

OA did not alter P120s binding to VE-cadherin when exposed to OA or the combination of OA and MG132 in either endothelial cell lines compared to Untx or DMSO (Figure 5.8A and B).

Mock transfection of hCMEC/D3s for 72 h prior to VE-cadherin pull down also did not alter the abundance of P120 associated with VE-cadherin compared to the Untx control. Finally this abundance was not altered when hCMEC/D3s were overexpressed with CIP2A and PP2A or their corresponding empty vectors pcDNA3.1 and CMV-6 (Figure 5.8C).

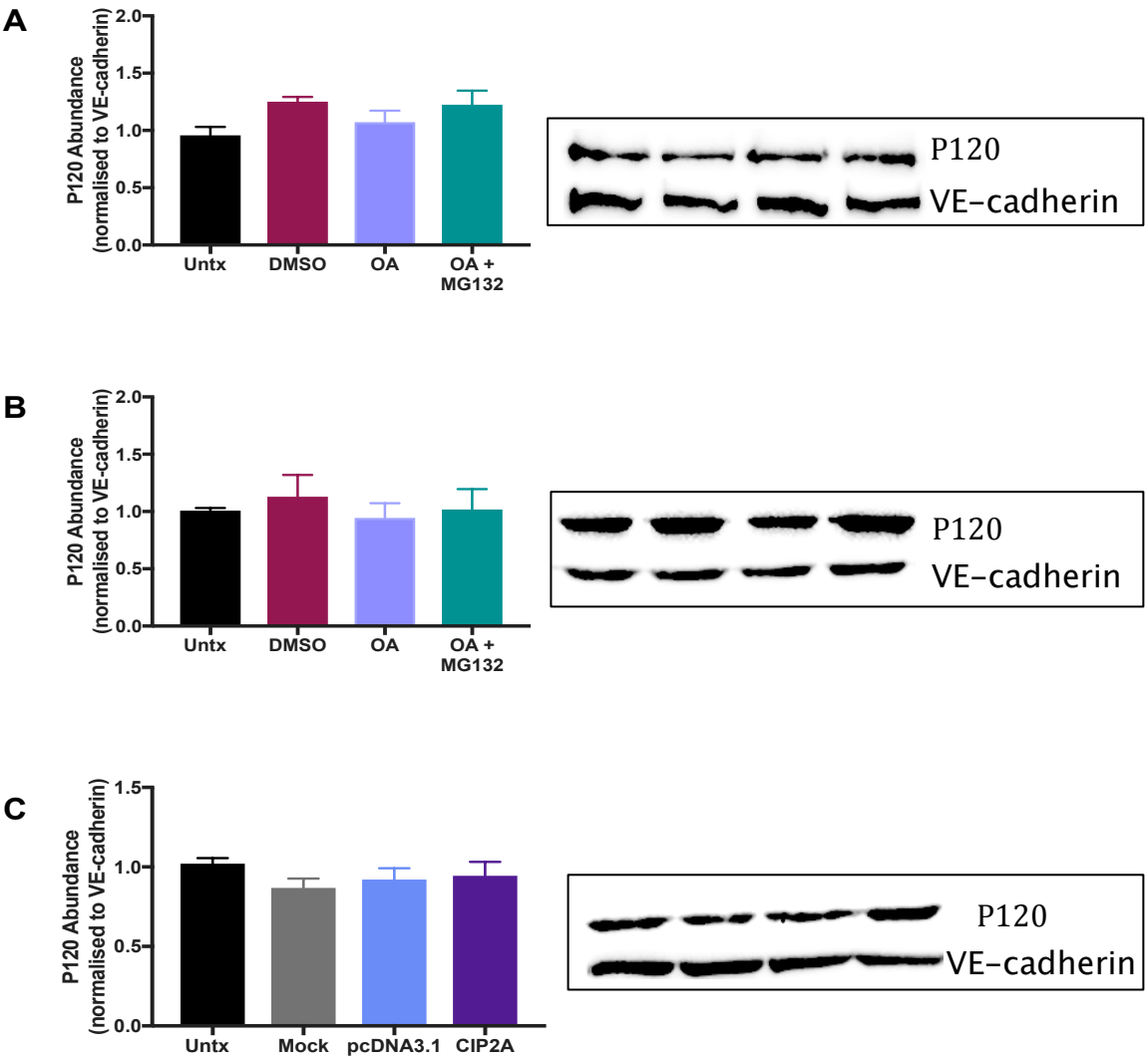

Figure 5.8: Effect of PP2A inhibition the binding of P120 to VE-cadherin.

hBMECs (A) and hCMECs (B) were exposed to OA (10 nM) for 24 h to inhibit PP2A activity, cell were also treated with OA (10nM) and MG132 (2 µM) to prevent proteasomal degradation. The effect of PP2A inhibition was confirmed by the overexpression of CIP2A for 24 h (C). VE-cadherin was immunoprecipitated from whole cell lysate with VE-cadherin Ab bound to sepharose beads. P120 abundance was determined by western-blot, normalized to β-actin, represented as a mean ± S.E.M relative to the hBMEC alone samples. Data was analysed using one-way ANOVA *post hoc* (Bonferroni). Horizontal bars represent statistical significance ($P < 0.05$), (n=5).

5.3.9 PP2Ac's association with VE-cadherin after PP2A modulation.

OA (10nM) decreases the binding of VE-cadherin to PP2Ac by 43.36% and 49.53% compared to the Untx and DMSO respectively in hBMECs ($P < 0.05$). Combining OA with MG132 reversed the disassociation of PP2Ac from VE-cadherin by increasing PP2Ac abundance by 166.74% compared to OA alone ($P < 0.05$, Figure 5.10A). PP2Ac also binds to VE-cadherin in hCMEC/D3s. 24 h exposure of hCMEC/D3s to OA (10 nM) resulted in a 65.52% and 63.46% loss in PP2A abundance bound to VE-cadherin compared to Untx and DMSO respectively ($P < 0.05$). MG132 prevented the loss of PP2Ac's association with VE-cadherin resulting from OA (Figure 5.10B). Mock transfection of hCMEC/D3's and transfection with the empty vectors pcDNA3.1 and CMV-6 did not alter the association of PP2Ac with VE-cadherin compared Untx. While over expression of CIP2A reduced PP2Ac binding by >50% compared to the control samples; Untx, Mock and pcDNA3.1 ($P < 0.05$; Figure 5.10C).

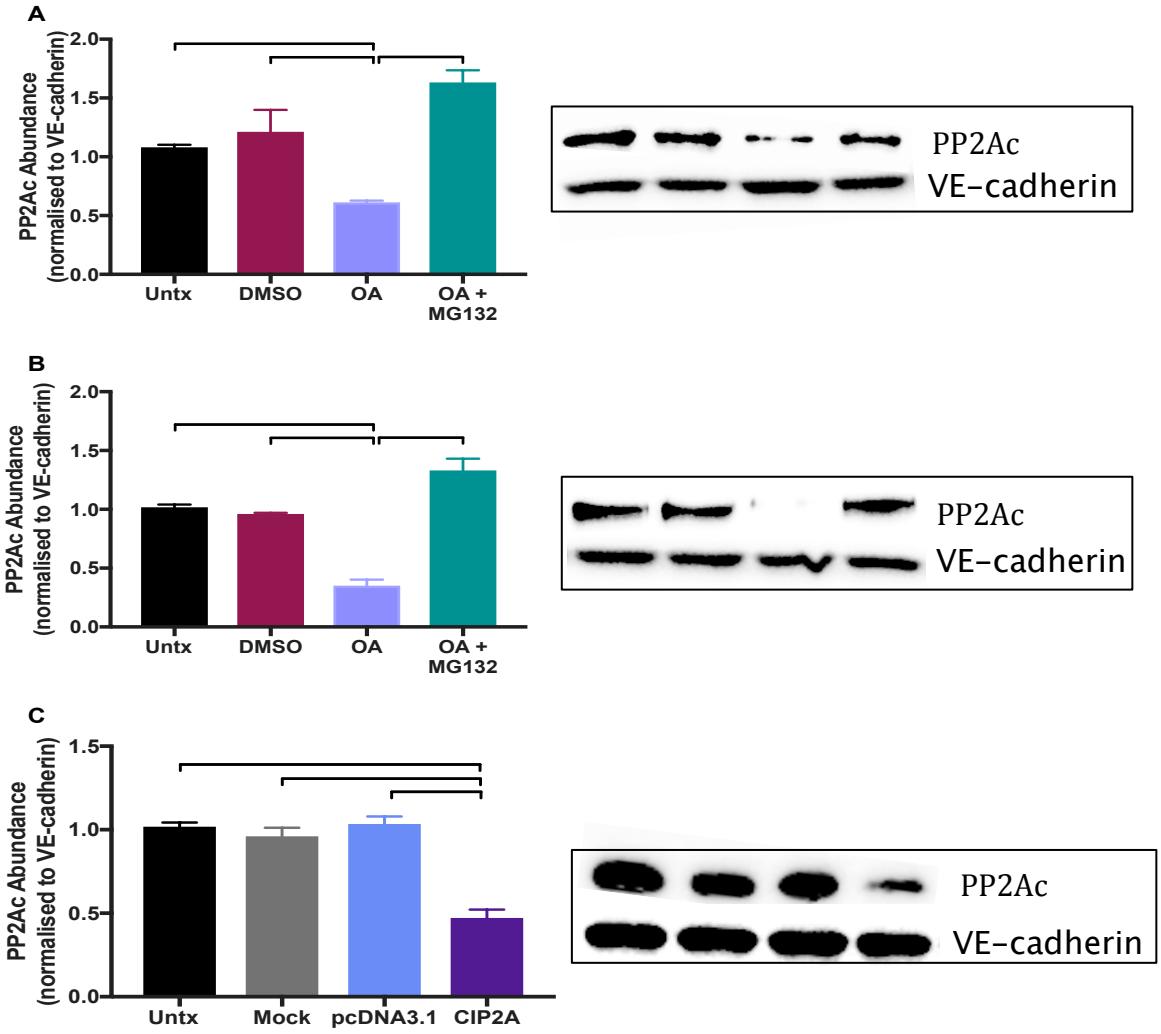

Figure 5.9: VE-cadherin binding to PP2Ac and how modulation of PP2Ac activity effects their interaction.

VE-cadherin was immunoprecipitated from whole cell lysate with VE-cadherin Ab bound to sepharose beads. PP2Ac abundance was determined by western-blot, normalised to VE-cadherin. hBMECs (A) and hCMEC/D3s (B) were treated with OA (10 nM) for 24 h to inhibit PP2A, cells were also treated with a combination of OA (10 nM) and MG132 (2 μM) to prevent proteasomal degradation. PP2A was also inhibited by the over-expression of CIP2A in hCMEC/D3 for 72 h (C). Data are represented as a mean ± S.E.M relative to the Untx sample. Data was analysed using one-way ANOVA *post hoc* (Bonferroni). Horizontal bars represent statistical significance ($P < 0.05$), (n=5).

5.3.10 The effect of PP2A modulation on VE-cadherin Interactome Ubiquitination

OA (10 nM) exposure to hBMECs prior to VE-cadherin pull-down resulted in a >90% increase in ubiquitin expression compared to the Untx and DMSO pull-down samples ($P < 0.05$). DMSO had no effect (Figure 5.10A). In hCMEC/D3s OA (10 nM) increased the abundance of ubiquitin associated with the VE-cadherin pull-down by ~110% compared to the Untx and DMSO samples ($P < 0.05$). DMSO had no effect (Figure 5.10B).

Overexpression of hCMEC/D3s with CIP2A resulted in a ~95% increase in the ubiquitination of the VE-cadherin interactome compared to the Untx, mock and pcDNA3.1 transfected samples ($P < 0.05$) while mock and pcDNA3.1 did not alter the abundance of ubiquitin (Figure 5.10C). PP2A was also overexpressed in hCMEC/D3s however this did not alter the ubiquitin attached to the VE-cadherin pull-down. The mock and CMV-6 samples also had no effect compared to the Untx (Figure 5.10D).

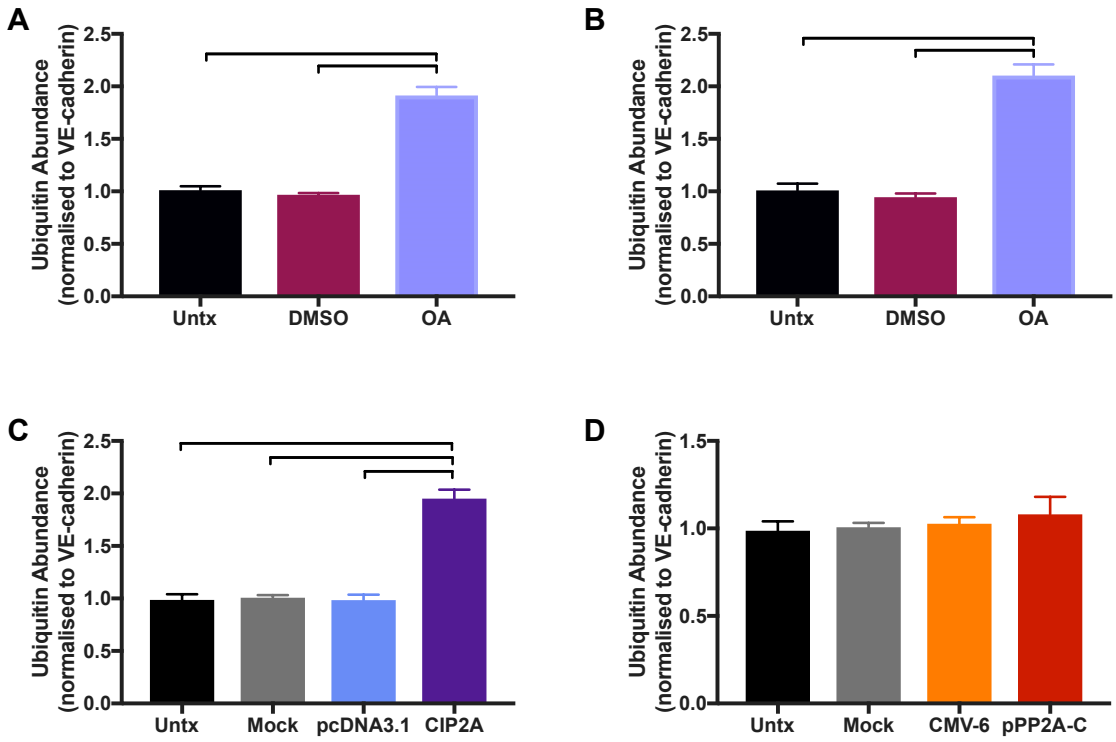

Figure 5.10: Effect of PP2A activity modulation on ubiquitination abundance on VE-cadherin and its binding proteins.

VE-cadherin was immunoprecipitated from whole cell lysate with VE-cadherin Ab bound to sepharose beads. Ubiquitin abundance was determined by western-blot, normalised to VE-cadherin. hBMECs (A) and hCMEC/D3s (B) were treated with OA (10 nM) for 24 h to inhibit PP2A. PP2A was also inhibited by the over-expression of CIP2A in hCMEC/D3 for 72 h (C) and PP2A activity was increased by the overexpression of PP2A in hCMEC/D3s (D). Data are represented as a mean ± S.E.M relative to the Untx sample. Data was analysed using one-way ANOVA *post hoc* (Bonferroni). Horizontal bars represent statistical significance ($P < 0.05$), (n=5).

5.3.11 The effect of PP2A overexpression on VE-cadherin accessory proteins

Overexpression of PP2Ac to counteract the effects of OA. PP2A overexpression did not alter the abundance of α and β-catenin (Figure 5.11A and B) but did however result in a >130% increase in P120 abundance compared to the Untx, mock and CMV-6 transfections ($P < 0.05$; Figure 5.11C).

5.3.12 The effect of PP2A overexpression on the VE-cadherin interactome

Overexpression of PP2A resulted in a 2.1 fold increased association of α-catenin with VE-cadherin compared to the relevant empty vector CMV-6 ($P < 0.05$). This increase is similar to the 2.0 and 2.4 fold increase when compared to the Untx and mock transfection controls respectively ($P < 0.05$; Figure 5.12A).

PP2A overexpression resulted in a 66.85% increase in β-catenin abundance compared to its empty vector control ($P < 0.05$), this increase is 74.94% and 56.45% compared to the Untx and mock transfection controls ($P < 0.05$). The mock and empty vector transfections did not alter the abundance of β-catenin associated with VE-cadherin compared to the Untx sample (Figure 5.12B).

PP2A overexpression increased the binding of P120 to VE-cadherin by > 130% compared to the Untx, mock and CMV-6 transfected samples ($P < 0.05$; Figure 5.12C). Finally the over expression of PP2A increased the abundance of PP2A binding to VE-cadherin by ~2.5 fold compared to the Untx, mock and CMV-6 transfections ($P < 0.05$; Figure 5.12D). Mock and empty vector control CMV-6 did not alter the abundance of P120 and PP2A binding to VE-cadherin compared to the Untx (Figure 5.12C and D).

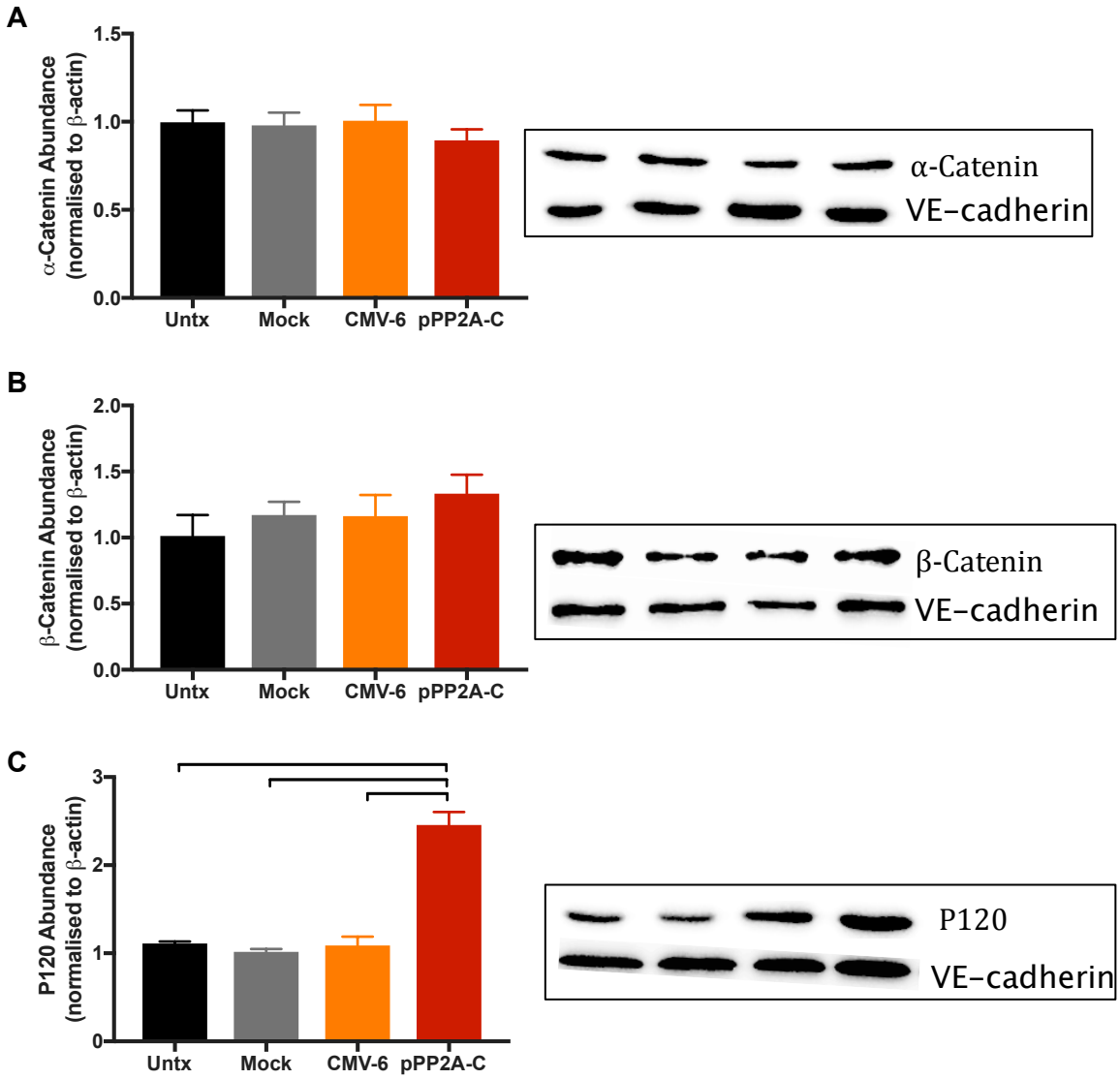

Figure 5.11: Effect of PP2Ac overexpression on VE-cadherin accessory proteins.

hCMEC/D3s transfected with a pPP2A-c plasmid for 72 h to overexpress. α (A), β-catenin (B) and P120 abundance (C) was determined by western-blot, normalised to β-actin. Data are represented as a mean ± S.E.M relative to the Untx sample. Data was analysed using one-way ANOVA *post hoc* (Bonferroni). Horizontal bars represent statistical significance ($P < 0.05$), (n=5).

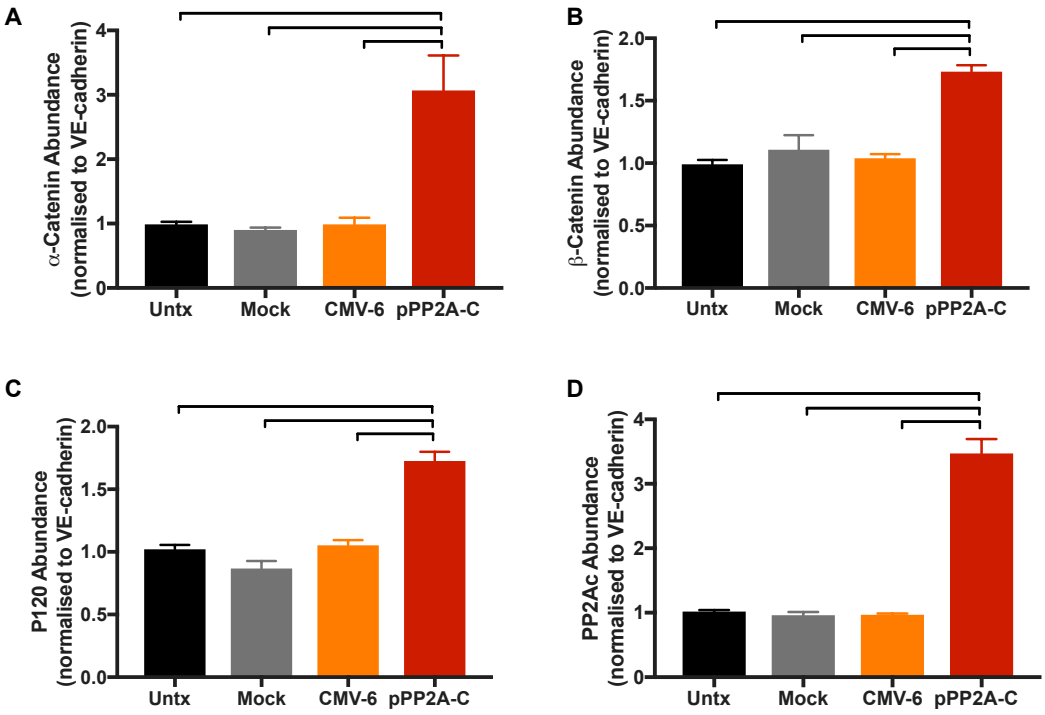

Figure 5.12: Effect of PP2Ac overexpression on VE-cadherin interactome integrity.

hCMEC/D3s transfected with a pPP2A-c plasmid for 72 h to overexpress PP2Ac and increase PP2A activity. VE-cadherin was immunoprecipitated from whole cell lysate with VE-cadherin Ab bound to sepharose beads. α (A), β-catenin (B) and P120 abundance (C) was determined by western-blot, normalised to VE-cadherin. Data are represented as a mean ± S.E.M relative to the Untx sample. Data was analysed using one-way ANOVA *post hoc* (Bonferroni). Horizontal bars represent statistical significance ($P < 0.05$), (n=5).

5.4 Discussion

This study has given us a unique look at how PP2A may regulate the accessory proteins associated with VE-cadherin, while also investigating the VE-cadherin/Catenin interactome (summarised in Figure 5.13). Brain endothelial cells exposure to OA (10 nM) (previously shown to inhibit of PP2A activity; section 3.3.2) has resulted in an associated loss in P120 and α-catenin without affecting the abundance of β-catenin. My investigations into the composition of the VE-cadherin interactome provides evidence that PP2A binds to VE-cadherin and the inhibition of PP2Ac activity induces disassociation of PP2A. OA (10 nM) and CIP2A overexpression is also associated with the dissociation of α and β-catenin from VE-cadherin without effecting P120 binding. However, the disassociation of α-catenin may be due to the loss of whole cell lysate abundance. As we confirmed PP2A binds to VE-cadherin, its inhibition and disassociation may be responsible for the increase in Ser^{665} phosphorylation, associated increase in ubiquitination and internalisation through clathrin-mediated endocytosis. Preventing proteasomal degradation stabilises the VE-cadherin interactome suggesting PP2A may also the labelling of α and β-catenin, targeting them for degradation. With the aim of reversing the above effects, increasing PP2A has the ability to stabilise the VE-cadherin interactome by increasing the association of α and β-catenin to VE-cadherin.

There are two known methods of VE-cadherin endocytosis; clathrin and caveolae mediated. Clathrin mediated endocytosis is the more dominant method first to be utilised by the cell [332, 730]. The results of this study suggest that clathrin mediated is the sole method of OA (10nM) induced endocytosis of VE-cadherin. This increase in endocytosis is also associated with an increase in the ubiquitination of the VE-cadherin interactome. Contrary to the findings of this study, P120 has been designated as a key regulator of VE-cadherin degradation. Its disassociation results in the endocytosis of VE-cadherin, induced by the Kaposi sarcoma-associated ubiquitin ligase K5, which target two membrane proximal lysine residues for ubiquitination [324]. The transmembrane ubiquitin ligase of Kaposi Sarcoma, K5/Mir-2 has also been linked to the ubiquitination and proteasomal degradation of not only VE-cadherin but also α, β and γ-catenin [731]. Although total abundance of P120 is decreased after OA (10 nM) exposure, there is no alteration in the abundance bound

to VE-cadherin indicating that P120 is not a key regulator in OA (10 nM) mediated degradation.

This study has focused on the specific regulation of the Ser^{665} site on VE-cadherin. Gavard and Gutkind first described the importance of this phosphorylation site and its role in VEGF induced recruitment of β-arrestin and subsequent internalisation and degradation of VE-cadherin [325]. This group further highlighted a potential role in PP2A in targeting the Ser^{665} site [221]. In conjunction with our results we hypothesis that the phosphorylation of Ser^{665} is a determining factor for VE-cadherin endocytosis regulated by PP2A similar to the regulation by β-catenin recruitment [732].

Loss of accessory proteins has been associated with the degradation of VE-cadherin. As discussed above the loss of P120 results in the internalisation of VE-cadherin and an increase in permeability. [321, 715, 717]. With regards to the findings of this study, OA (10 nM) and CIP2A overexpression is associated with a decrease in total P120 abundance. An array of studies has been carried out into the role of P120 in regulating the protein expression of other proteins including VE-cadherin. However the regulation of P120 abundance is not well documented and to our knowledge this it the first study to show the effect of OA (10 nM) and CIP2A overexpression in P120 degradation.

Previous studies have highlighted the phosphorylation of P120 at Ser^{879}. Such as the transmigration of cancer cells mediated by thrombin exposure has been linked to the phosphorylation of Ser^{879} [733]. PKCα has also been shown to activate P120 by phosphorylating the Ser^{879} motif in response to LPS or thrombin. Resulting in the disassembly of the VE-cadherin interactome. Preventing this phosphorylation resulted in the protection of mouse lung vessels from thrombin damage [734, 735]. Together suggesting a potential PP2A target for the down-regulation of P120 however does not explain how P120 and VE-cadherin interaction remained unchanged.

α-catenin is also a key regulator of the VE-cadherin interactome. The results of this study strongly suggest that not only does the inhibition of PP2A (through OA (10 nM) and overexpression of CIP2A) result in loss if total α-catenin abundance but also results in the disassociation from VE-cadherin. Previous research has indicated that α-catenin within the VE-cadherin interactome undergoes phosphorylation [736-740]. An increase in Tyr phosphorylation of α-catenin by inhibition of SHP-2 is associated with VE-cadherin disassociation [710, 726, 741]. However with regards to potential targets for PP2A, phosphoproteomic screening in both human and mouse samples unveiled multiple phosphosites, most commonly S^{641} (mouse-[742, 743] human-[744-746]). Following mass-spectrometry investigations four clustered Ser and Thr residues between the linker and c-terminal region in α-catenin, which undergo phosphorylation were confirmed. This sequence area is highly conserved between species [747-749]. Investigations into the role of kinases in the regulation of these residues determined that the Ser^{641} is phosphorylated by CK2, which promotes the phosphorylation of Ser^{652}, Ser^{655} and Thr^{658} by CK1 [750]. However the role of phosphatases remain undetermined and this is the first study suggesting PP2A as a potential regulator.

The results of this study suggest that an increase in α-catenin phosphorylation associated with inhibition of PP2A through OA (10 nM) and CIP2A over-expression results in its degradation and disassociation from the VE-cadherin interactome. However OA (10 nM) did not alter α-catenin binding to the VE-cadherin interactome in hCMEC/D3s over 24 h and this disparity requires further investigation.

Extensive research has been carried out into the association of PP2A and β-catenin in regards to WNT/β-catenin signalling pathway [751-753] but nothing is known about their association into the assembly of the VE-cadherin interactome. β-catenin has an array of roles in cell signalling and regulation, theses role change from cell to cell type, dependent on the primary role of the cell and the cell fate [754]. This study provides compelling evidence that PP2A does not effect total abundance of β-catenin however PP2A does regulate binding of β-catenin to VE-cadherin and the regulation

of endothelial monolayer. Previous studies have suggested that β-catenins are strongly regulated by its phosphorylation status [755]. The specific point at which β-catenin is phosphorylated determines the function and fate of β-catenin. Focusing on particular targets for PP2A, the phosphorylation of Ser^{834}, Ser^{836} or Ser^{842} has been shown to increase the binding of β-catenin to E-cadherin, contradicting the results seen in this study [756, 757]. However phosphorylation of Ser^{846} by CK1 results in the loss of binding and internalisation of E-cadherin [738]. The Ser^{45}/Thr^{41} sites, found at the plasma membrane attenuates the VE-cadherin–dependent cell-cell junction to increase endothelial cell permeability [758]. Cleavage of VE-cadherin as a result of the presence of ovarian cancer microparticles also increases β-catenin disassociation and phosphorylation of Ser^{552} and Ser^{657}, translocation of β-catenin to the nucleus for gene activation or the phosphorylation of Ser^{33-37} and Thr^{41}-Ser^{45} resulting in the proteasomal degradation of β-catenin [759]. Together these studies provide potential targets for PP2A activity on β-catenin to support the results of this study. However, further studies are required to determine which site is involved in PP2As regulation of β-catenins dissociation from VE-cadherin.

An alternative view by Konstantoulaki *et al* who have demonstrated that thrombin injury decreased the phosphorylation of VE-cadherin and β-catenin, which caused interactome disassembly. The study does not however investigate which phosphorylation sites may be involved suggesting it may be alternative site to those involved in the role of PP2A in brain microvascular integrity [760].

The overexpression of PP2A resulted in the increase binding of VE-cadherin with P120, α and β-catenin along with increased PP2A binding. This suggests PP2A as a potential therapeutic target to prevent VE-cadherin loss and interactome disassembly. Forskolin used for its anti-leukemic effect also causes an increase in PP2A activity [761], Noda *et al* demonstrated that forskolin also increases VE-cadherin abundance, cell-cell adhesion and stabilises the VE-cadherin to actin filament connection [762].

In conclusion, this study highlights a vital role for PP2A in the regulation of VE-cadherin and the VE-cadherin interactome (summarised in Figure 5.13). This study has provided multiple potential targets to prevent the disassembly of the interactome. Beginning with the Ser^{665} phosphosite. The regulation of the accessory proteins need further investigations as to which protein alteration is at the beginning of a cascade of events or are multiple events required before permeability is induced. A localised target may be the induction of PP2Ac activity to increase α- and β-catenin interaction with VE-cadherin or the prevention of degradation or internalisation as this maintained endothelial cell integrity.

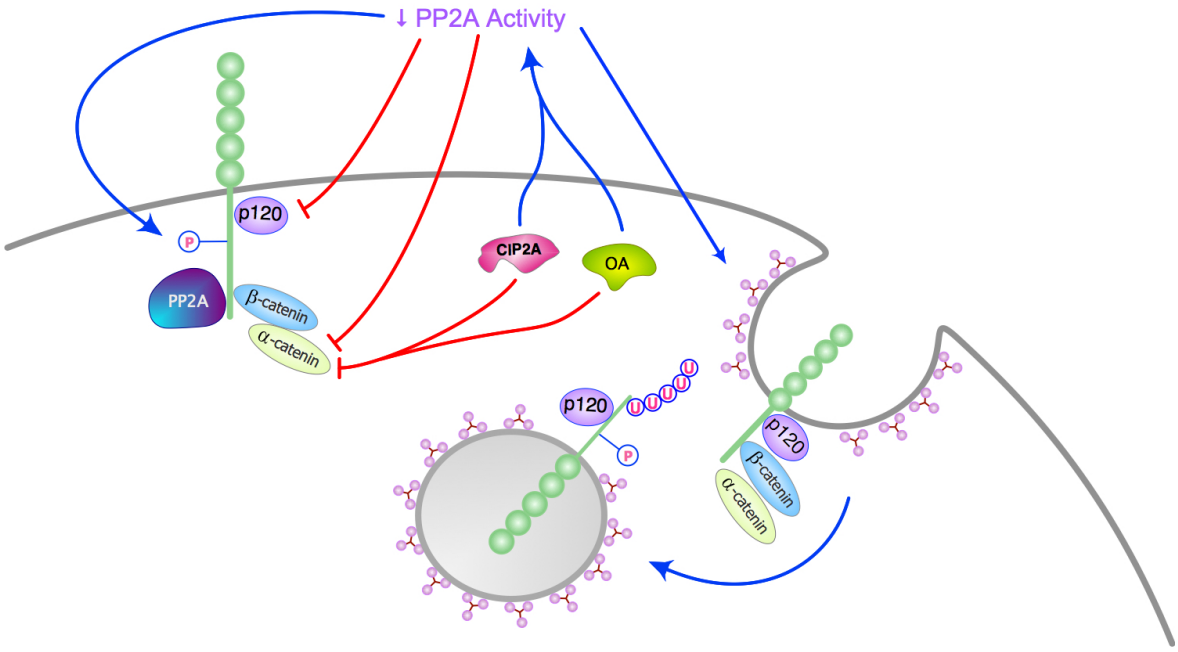

Figure 5.13: Schematic summary of the effect of OA (10 nM) and CIP2A on the VE-cadherin interactome.

OA (10 nM) resulted in an associated loss in P120 and α-catenin without affecting the abundance of β-catenin in brain endothelial. PP2A binds to VE-cadherin and the inhibition of PP2Ac activity induces disassociation of PP2A. OA (10 nM) and CIP2A inhibit PP2A activity and dissociate α and β-catenin from VE-cadherin without effecting P120 binding. PP2A inhibition increases Ser^{665} phosphorylation, increase in ubiquitination and internalisation through clathrin-mediated endocytosis.

Chapter 6

6 Conclusion and Future Direction

6.1 Conclussion

PP2A is the most abundant phosphatase in the brain. It plays an integral role in cellular function and hence why I have investigated its potential role in regulating VE-cadherin abundance and downstream paracellular permeability. VE-cadherin is a critical component of the functioning endothelium, connecting neighbouring endothelial cells and attenuating paracellular permeability. Throughout this thesis I have both increased and decreased PP2A activity resulting in a related alteration in VE-cadherin abundance and permeability of brain microvascular endothelial cells (hBMEC and hCMEC/D3). The loss of PP2A activity, through the methylation of its catalytic subunit by PME-1 and loss of LCMT-1, results in phosphorylation of VE-cadherin (Ser^{665}). Although further work is required to determine what the initial step of the sequence of events is, I have determined that loss of PP2A activity results in VE-cadherin interactome disassembly and proteasomal degradation. The co-culture model suggests that a similar mechanism is involved in the pro-inflammatory induced increase in microvascular permeability.

Loss of PP2A has previously been linked to multiple neurological diseases such as Alzheimer's disease. I have recreated this with the use of pharmacological modulators (OA), overexpression of endogenous inhibitors (CIP2A and SET) and the pro-inflammatory (M1) macrophages. CIP2A and SET overexpression was used to support the effects of OA on human brain microvascular endothelial cells, while also adding physiological relevance. PP2A activity was not attenuated due to a loss of abundance, but instead was a result of post-translational modification of the catalytic subunit. OA and M1 macrophages induced a loss of LCMT-1, resulting in reduced methylation of Leu^{309} and preventing efficient binding of the catalytic and scaffolding unit to the PP2Ac active site. Supported by previous studies in which LCMT-1 is down-regulated in COS7 and HeLa cells resulting in the increase in PP2A methylation and loss of activity [514, 533, 691]. PME-1 opposes the effect of LCMT-1. As PME-1 remained unaffected by OA and M1 macrophages, PME-1 continued to remove the methyl group from the PP2Ac motif (TPDYFL) unopposed [532].

OA and M1 also induced phosphorylation of the PP2Ac Tyr^{307} contributing to the attenuation of activity. Phosphorylation of PP2Ac has previously been shown to

prevent the assembly of the PP2A holoenzyme [466, 467]. Although not determined in the present study, PKC and GSK-3β can phosphorylate PP2Ac [464, 514, 515]. It would be of interest to investigate the mechanism by which OA and M1 macrophages induced phosphorylation of PP2Ac in future studies.

The established loss of PP2A activity led us to conclude an association between PP2A activity and VE-cadherin abundance. In the first study, OA (10nM) reduced the abundance of VE-cadherin to an undetectable level. Previous studies have demonstrated that OA results in the redistribution of VE-cadherin in epithelial and endothelial cell lines. However, these studies exposed cells to a lower concentration of OA (5 nM), this along with the different cell lines may provide an explanation as to why VE-cadherin is not lost [620, 627]. The use of CIP2A and SET further support the association between inhibition of PP2A activity and the loss of VE-cadherin. Previous studies into CIP2A's effect on E-cadherin in renal and laryngeal carcinoma cells support our findings [638, 639]. However, this is not the case in a previous report on SET, where an increase in SET expression was associated with an increase in E-cadherin abundance [576]. This disparity may be attributed to the use of different cell lines, all of which were cancer cell lines (clear cell renal cell carcinoma, laryngeal cancer and human colon carcinoma) and requires further investigation. As a result of this, CIP2A was chosen as the primary endogenous inhibitor in subsequent investigations.

I further reveal how PP2A alters both post-translational modification of VE-cadherin along and the VE-cadherin interactome. Although I have not determined what the sequence of events are, I provide further understanding into the mechanism of VE-cadherin degradation. Inhibition of PP2A activity (through CIP2A and OA) results in the phosphorylation of VE-cadherin Ser^{665}. In conjunction with the M1 macrophage induced loss of VE-cadherin, Ser^{665} phosphorylation acts as a potential target for the loss of M1 induced loss of VE-cadherin. My data also show that the over-expression of PP2A both reduces Ser^{665} phosphorylation and reverse M1 induced VE-cadherin loss supporting these findings.

Gavard and Gutkind demonstrated that the Ser^{665} is involved in β-arrestin induced internalisation of VE-cadherin [325]. Loss of PP2A and phosphorylated Ser^{665} also results in the breakdown of the VE-cadherin interactome. Inhibition of PP2A by CIP2A and OA results in the loss of α-catenin and P120 abundance in the brain microvascular endothelial cell. Adding to this I show using a VE-cadherin pull-down assay, that inhibition of PP2A caused disassociation of α and β- catenin from VE-cadherin, while P120 remained bund. Eventually resulting in the degradation of both VE-cadherin and P120. This was supported by a previous immune-precipitation study of the Bα subunit of PP2A. Supporting our finding that PP2A binds to VE-cadherin and β-catenin, more resulting in increased phosphorylation of β-catenin at Ser^{552} [620]. Considering β-catenin is not degraded following inhibition of PP2A it may be translocated to the nucleus, where it has a role in regulating transcription. Previous reports demonstrated the binding of PP2A to β-catenin, and phosphorylation of both VE-cadherin and β-catenin prevents their assembly [113, 763]. Interestingly, a potential target for PP2A on β-catenin is Ser^{552}. Ser^{552} phosphorylation causes β-catenin disassociation from the cell membrane and increases transcriptional activity, which conflicts with our findings [620, 764, 765]. Also a structural investigation into E-cadherin and β-catenin binding suggests that phosphorylation of Ser^{684} promotes the binding of VE-cadherin to β-catenin [757]. The disparities in β-catenin regulation through phosphorylation require further investigation, specifically the involvement of PP2A. My results still provide further insight into PP2A's association with the VE-cadherin interactome and degradation, unveiling a potential mechanism for the M1 induced loss of VE-cadherin and induced permeability, which also requires further investigation.

As evident from the use of MG132 (proteasomal degradation inhibitor), loss of PP2A activity, through PP2A inhibitors and presence of M1 macrophages, is associated with the proteasomal degradation of VE-cadherin. Supported by previous reports, VE-cadherin is internalised by clathrin-mediated endocytosis [317, 325, 766]. Followed by the ubiquitination of VE-cadherin and its associated proteins. Although determining the ligase responsible for VE-cadherin degradation, a previous study has suggested the involvement of the K5 ligase [324].

The overall physiological relevance to our findings is PP2As association with VE-cadherin, which has a resulting effect on hBMEC paracellular permeability. Early studies attributed OA induced permeability to its alteration in the cells cytoskeletal structure. OA caused morphological changes to endothelial and epithelial cells. Initially attributed to the depolymerisation and destabilisation of microtubules along with the phosphorylation of PP2A sensitive microtubule-associated proteins [767, 768]. The study carried out by Kasa *et al* demonstrated that OA (5nM) not only altered cytoskeletal structure in pulmonary endothelial cells but also resulted in the redistribution of VE-cadherin and β-catenin from the membrane to the cytosol. The role of PP2A was confirmed by depletion of the Bα subunit, which altered both cytoskeleton and induced permeability in pulmonary endothelial cells [620]. Studies conflicting with our results demonstrated that OA (5nM) altered BPAEC morphology, without effecting permeability [654]. While in HPECs, OA does not affect cytoskeleton arrangement or permeability [769]. This may be explained by the use of lower OA concentrations (5nM compared to 10nM used throughout this thesis).

As expected M1 macrophages increased paracellular permeability of human brain microvascular endothelial cell. As the M1 macrophage induced effect coincides with the effects of PP2A inhibitors we suggest PP2A as a mediator of M1 induced permeability. Supported by the depletion of Bα, which both mimicked and exacerbated the effect of thrombin in pulmonary endothelial cells [620].

As PP2A is associated with the attenuation of VE-cadherin, and importantly the physiologically relevant M1 macrophage induced loss of VE-cadherin. Combating this may have a therapeutic advantage. PP2A stimulators are already used to treat neurological dysfunction. Our results demonstrate FTY-720 increases both PP2A activity and VE-cadherin abundance. Considering LCMT-1 is responsible for the loss of PP2A activity and FTY-720 increases LCMT-1 abundance, FTY-720 may potentially reverse the adverse effects associated with loss of PP2A activity. Overexpression of PP2Ac in the co-culture model attenuated the M1 induced loss of VE-cadherin and increase in permeability. Targeting PP2A to benefit the integrity of the endothelial cell is supported by the previous investigations demonstrated that the overexpression of PP2A (both the catalytic and structural subunit) has the ability to

reverse thrombin and nocodazole induced endothelial dysfunction and cytoskeleton rearrangement [770].

Overall, the results of this thesis demonstrated a strong association of PP2A activity and abundance of VE-cadherin (as summarised in Figure 6.1). Gaining physiologically relevant understanding on how pro-inflammatory macrophages regulate PP2A, VE-cadherin and induce permeability. The inhibitors of PP2A (OA, CIP2A and SET) and M1 macrophages stimulate the PP2Ac demethylation and phosphorylation, decreasing PP2A activity. Reduced PP2A activity results in the increased phosphorylation of VE-cadherin (Ser^{665}) and disassociation of PP2A, α- and β- catenin from the VE-cadherin interactome. VE-cadherin undergoes clathrin mediated endocytosis, ubiquitination and proteasomal degradation associated with loss of PP2A activity. P120 and α-catenin are also degraded as a result of PP2A inhibition. This series of events results in an increase in endothelial cell paracellular permeability. Alternatively, Mθ and overexpression of PP2A results in increased methylation and dephosphorylation of PP2Ac consistent with increased PP2A activity. As PP2A activity is maintained, so is the composition of the VE-cadherin interactome, thus maintaining endothelial barrier integrity. This novel insight into the role of PP2A in the regulation of the VE-cadherin interactome not only suggest a potential mechanism for M1 macrophage induced regulation but also provide potential therapeutic targets for the prevention of endothelial barrier dysfunction.

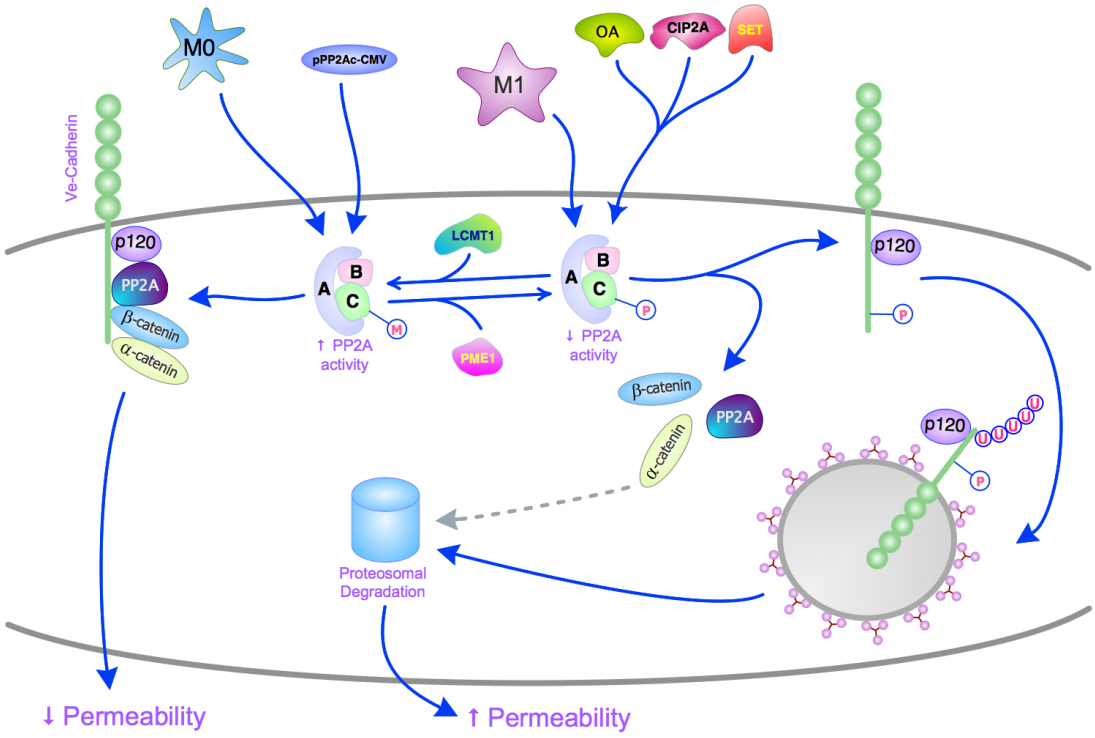

Figure 6.1: Summary of PP2A modulation and presence of macrophages on PP2A's regulation of VE-cadherin in human brain microvascular endothelial cells.

OA, CIP2A, SET and M1 macrophages stimulated the demethylation and phosphorylation of PP2A, in turn decreasing PP2A activity. Reduced PP2A activity results in the phosphorylation of VE-cadherin (Ser^{665}) and disassociation of PP2A, α- and β- catenin from VE-cadherin. VE-cadherin undergoes clathrin mediated endocytosis, ubiquitination and proteasomal degradation. P120 and α-catenin are degraded as a result of PP2A inhibition. This results in an increase in endothelial cell paracellular permeability. Alternatively, Mθ and overexpression of PP2A results in an increase in PP2A activity through the methylation and dephosphorylation of the PP2A catalytic subunit. As PP2A activity is maintained, so is the integrity of the VE-cadherin interactome. Thus maintaining endothelial barrier integrity.

6.2 Future direction

Throughout the work presented in this thesis which, investigates the role of PP2A in regulating VE-cadherin in human brain microvascular endothelial cells, several questions arose which warrant further investigation.

As phosphorylation of the Ser^{665} increased during the inhibition of PP2A and loss of VE-cadherin, it is of particular interest. Point mutation analysis would determine if the phosphorylation of this site is responsible for the degradation of VE-cadherin and disassociation of α- and β- catenin. Also with regards to the macrophage co-culture, the potential involvement of Ser^{665} phosphorylation still remains unknown.

Looking at the overall role of PP2A in the human brain microvascular endothelial cell. Carrying out a phosphoenrichment and proteomic study into the phosphorylated proteins after PP2A inhibition. In particular to determine if the phosphorylation of P120, α- or β- catenin is altered after PP2A inhibition or any other junctional proteins, which may be contributing to the increase in paracellular permeability and seeking the ubiquitin ligase.

Chapter 7

7 References

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