

**A Study of the Vascular Basis of Alzheimer's Disease: The Role of Beta Amyloid ($A\beta$) Proteins
and Saturated Fatty Acids in Endothelial Dysfunction and Inflammation**

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ABSTRACT

Background: Studies have shown that Alzheimer's Disease (AD) is strongly associated with the presence of atherosclerosis risk factors, including hyperlipidemia (and associated increased free fatty acids), hypertension and diabetes. We tested the hypotheses that β - amyloid proteins ($A\beta$) or palmitic acid (PA), a saturated fatty acid and known atherosclerotic risk factor, cause impaired function and viability of human umbilical vein endothelial cells (HUVEC), and that together, they exert synergistic effects on HUVEC dysfunction.

Methods: HUVEC were exposed for 18-20 hours to vehicle, $A\beta$ 42 ($2\mu\text{M}$) \pm PA ($150\mu\text{M}$) or PA ($150\mu\text{M}$) while some HUVEC were exposed to a 4-hour pre-treatment with PA ($150\mu\text{M}$) followed wash and treatment with vehicle \pm $A\beta$ 42 ($2\mu\text{M}$) for 18-20 hours. Outcomes measured included: (1) nitric oxide (NO) and measures of oxidative stress (superoxide) and nitrosative stress (peroxynitrite), (2) inflammatory and associated markers (interleukins (IL)-6, IL-8, Reaction for Advanced Glycolytic End Products (RAGE) 1 and 2, and Matrix Metalloproteinases (MMP-9) by PCR.

Results: HUVECs exposed to either $A\beta$ or PA showed impaired NO production and increased superoxide and peroxynitrite when compared to vehicle control. Co-treatment with $A\beta$ and PA did not cause a statistically significant change compared to $A\beta$ or PA alone. HUVEC demonstrated variable inflammatory responses following exposure to either $A\beta$ or PA. Treatment with PA resulted in upregulation of RAGE2 gene expression ($p < 0.003$) and trend towards IL-6 overexpression ($p = 0.059$). Co-treatment with both $A\beta$ and PA led to an observed increase in inflammatory responses versus control, but the results did not reach statistical significance.

Conclusion: Independent exposure of HUVECs to $A\beta$ and PA caused decreased nitric oxide production and increased oxidative and nitrosative stress. HUVECs did not demonstrate $A\beta$ -induced endothelial cell inflammation. Co-treatment with $2\mu\text{M}$ $A\beta$ and PA $150\mu\text{M}$ did not result in a synergistic or additive increase in endothelial cell inflammatory responses.

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1. INTRODUCTION/SIGNIFICANCE

Alzheimer's disease (AD) is a leading cause of cognitive impairment that is expected to afflict 80 million people by year 2040, yet there exists no cure to this day¹. Both AD and stroke have been implicated as a cause of cognitive decline in elderly patients and, recent evidence indicates vascular risk factors contribute to the pathogenesis of both AD and stroke²⁻⁸. Half of patients with AD exhibit mixed pathology of AD and infarcts, indicating infarcts commonly coexist with AD in elderly patients³. While the importance of the infarcts and its role in the pathology of AD has not yet been determined, studies have shown that infarcts synergistically lower cognitive functioning in patients with AD³. Pathologically, there is disputed evidence of the role of vascular lesions in cognitive impairment seen in AD and stroke patients. Some clinical evidence implicates cholinergic compromise plays an important pathologic role in AD and vascular cognitive impairment, suggesting cholinesterase inhibitors may provide clinical benefits³. However, there exists overwhelming evidence that the risk of developing AD is directly related to vascular risk factors. Studies have shown that early AD is characterized by vascular dysfunction, cerebral hypoperfusion and interruption of the blood brain barrier⁹⁻¹¹. These changes occur early in AD and may precede cognitive neurodegeneration and beta amyloid deposition.

The knowledge gap surrounding the pathophysiology of Alzheimer's Disease (AD) has important implications especially in finding a viable treatment option for AD. AD represents the predominant cause of dementia and cerebral degeneration in the elderly. Amyloid Beta (A β) protein has been identified as a major contributor to the deposition of senile plaques and plays an important role in the pathology of Alzheimer's¹². Both soluble A β 40 and the less soluble A β 42 have been proven to impair vasomotor function (Fig. 1). The presence of cerebral hypoperfusion, atherosclerosis of the Circle of Willis, and biochemical alterations in cerebral microvessels in patients with AD, implicates vascular dysfunction in the early pathogenesis of AD¹³⁻¹⁵. Clinical and pre-clinical data suggest these deleterious changes in cerebral microvessels may occur prior to the deposition of A β proteins^{9, 11, 16}. However, the precise role of A β , in contributing to vascular dysfunction and the progression of AD, has not yet been fully

elucidated. Epidemiologic studies demonstrate that identified risk factors for AD are the same risk factors associated with atherosclerosis. AD is associated with the presence of atherosclerosis risk factors, such as hyperlipidemia (including increased free fatty acids), hypertension and diabetes, which contribute to alterations in vascular endothelial function¹³⁻²¹. However, mechanisms linking these risk factors to AD are not well understood; and the mechanisms by which they modulate A β -induced vascular dysfunction are not well established. Exposure of vascular endothelial cells to A β is associated with significant damage to the endothelial structure cerebral microangiopathy, and upregulation of A β ¹².

Atherosclerosis risk factors, including free fatty acids, have been shown to induce endothelial dysfunction. A β 42 proteins have similarly demonstrated endothelial dysfunction as a result of impaired vasomotor regulation of both peripheral and cerebral vessels¹⁸⁻²⁰. Endothelial cells exposed to A β 42 have been demonstrated to undergo biochemical alterations in cell structure and function²¹. Further, upon exposure to A β 42, endothelial cells increased production of superoxide radicals and reactive oxygen species^{21, 22}. While evidence suggests that there exists an underlying vascular component to the neurodegeneration process in AD, the role of A β 42 in this process has not been fully established. The findings of these studies suggest studying the interaction of atherosclerosis risk factors on A β -induced endothelial dysfunction is significant to the understanding of vascular dysfunction in AD. Previous data have shown that ex-vivo human subcutaneous adipose arterioles from living subjects show impaired dilator response to acetylcholine when exposed to 2 μ M of A β 42, but not scrambled A β 42, suggesting endothelial dysfunction²³. These arterioles demonstrated similar vessel responses when exposed to atherosclerosis risk factors, hyperglycemia and free fatty acids²³. A recent study demonstrated a similar decrease in dilation in response to acetylcholine of rapid-autopsy cadaver leptomeningeal arterioles when exposed to A β 42²⁴.

There is evidence that the neurodegenerative changes in AD may include the involvement of cerebrovascular inflammation. Clinical studies indicate brain microvessels are biochemically altered as a result of a focal neuroinflammatory response^{15,27,28}. In one study, brain microvessels from AD patients showed elevated production of proinflammatory cytokines

including interleukin(IL)-6 and IL-8²⁷. These findings suggest chemokines and inflammatory factors mediate deleterious changes in the microvasculature in AD. It is proposed that the disruption of cerebral microcirculation leads to accumulation of A β protein deposition and cerebral hypoperfusion^{10, 13-16,21,22,29}. Studies have shown that free fatty acids (including palmitic acid) both activate NF- κ B, a proinflammatory transcription factor, resulting in the upregulation of inflammatory cytokine production, and cause endothelial damage in human arterioles^{28, 30}. While evidence has shown that A β 42 or free fatty acids, alone, results in proinflammatory processes in arterioles, *the modulating effects of free fatty acids on A β -induced endothelial inflammation has not been determined.*

Our study addresses a critical knowledge gap in the understanding of the early pathogenesis of AD, specifically the relationship between atherosclerosis risk factors and A β -induced vascular dysfunction. We tested the hypotheses that A β or palmitic acid (PA), a saturated fatty acid and atherosclerotic risk factor, induces endothelial dysfunction and impaired viability of human umbilical vein endothelial cells (HUVEC), through oxidative stress with upregulation of inflammatory cytokines. Secondly, we hypothesized that together, both beta amyloid proteins and PA, will exert synergistic effects on HUVEC function and cell viability.

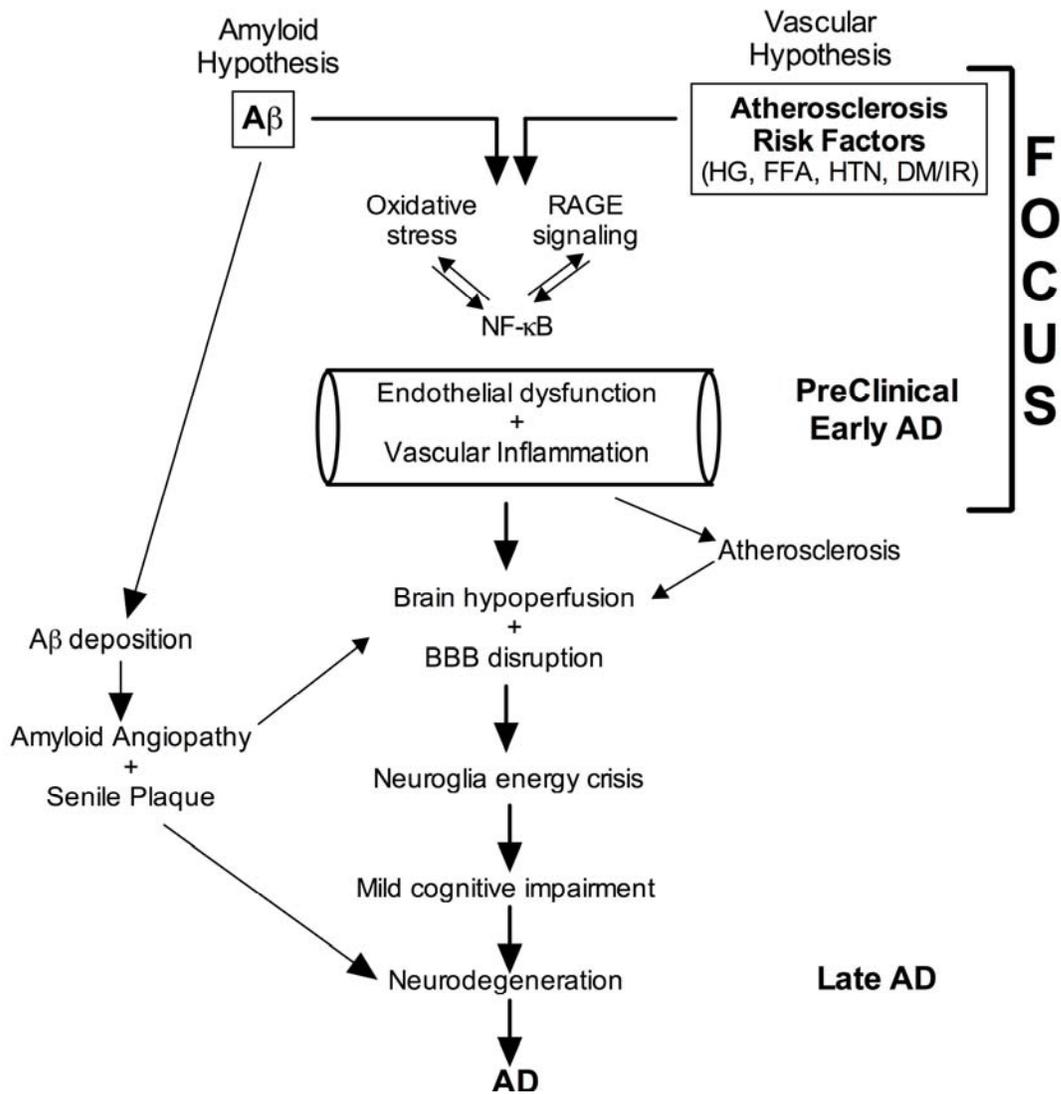


Figure 1. A schematic representation of the basis of the study. The vascular hypothesis of the pathophysiology of Alzheimer’s Disease identifies the role of atherosclerotic risk factors and the deposition of beta amyloid proteins in the development of endothelial dysfunction and vascular inflammation.

2. RESEARCH METHODS AND MATERIALS

2.1 Materials and Reagents

Human umbilical vein endothelial cells (HUVEC, primary cells), cell culture media EGM-2 and supplements were purchased from Lonza (Allendale, NJ). Beta-Amyloid (1-42) peptides were obtained from Anaspec (Fremont, CA). Palmitic acid as well as all other agents not otherwise specified were obtained from Sigma-Aldrich (St. Louis, MO).

2.2 HUVEC endothelial cell culture.

HUVECs were maintained in endothelial cell basal medium (Lonza) containing fetal bovine serum (2%) and VEGF growth factors in T-225ml culture flasks. Cells were routinely cultured in EGM-2 complete medium under standard cell culture conditions (37° C, humidified, 95% air, 5% CO₂). HUVECs were reseeded every 2-3 days to maintain a density of 0.25x10⁶ – 1x10⁶ cells/ml. Cell confluence of growth flasks was determined by direct microscopic observation. Cells were grown to approximately 80% confluence of the culture flask surface prior to reseeding in 6-well plates and/or tissue culture 10cm² tiny flasks for experimentation purposes.

2.3 HUVEC endothelial function.

HUVECs were passaged 24-48 hours into tissue culture 10cm² tiny flasks prior to exposure to vehicle, Aβ₄₂ (2μM) ± PA (150μM), PA (150μM) for 18-20 hours or HUVECs were exposed to a 4 hour pre-treatment with PA (150mM) followed by treatment ± Aβ₄₂ (2μM) for 18-20 hours. The dose of Aβ₄₂ was determined because this is less than but similar to the reported concentration (~30,000 ng/g tissue) found in cortical tissue of patients with AD³¹. Responses of HUVECs when exposed to Aβ₄₂, palmitic and co-treatment with both were measured. To determine endothelial cell nitric oxide production, NO head gas was measured using Sievers NO Analyzer (General Electric, Boulder, CO) and normalized to cell count as per previously published protocols²³.

To measure superoxide production, HUVECs were passaged 24-48 hours into tissue culture 10cm² tiny flasks prior to exposure to vehicle, Aβ₄₂ (2μM) ± PA (150μM), PA (150μM) for 18-20 hours or exposed to a 4 hour pre-treatment with PA (150μM) followed by treatment ± Aβ₄₂

(2 μ M) for 18-20 hours. The treatment was then replaced with pre-warmed media containing 25 μ M dihydroethidium (Life Technologies, Eugene, OR), a fluorescent marker of superoxide production³². After 1 h, cells were washed with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and lifted with trypsin (Lonza) and suspended in 500 μ M 10% Dulbecco's modified eagle medium (Invitrogen, Carlsbad, CA) and transferred to flow cytometry tubes. Cells were washed twice with HEPES buffer and fluorescent signal read on the Beckman-Coulter (Brea, CA) FC 500 flow cytometer excited with 488nm laser and read on the FL-3 channel.

To measure peroxynitrite production, treated HUVECs were separately washed with cold Phosphate-buffered Saline (PBS), stained with 20 μ M of coumarin boronic acid pinaolate ester (Cayman Chemical, Ann Arbor, MI), a fluorescent probe that reacts with directly with peroxynitrite³³, for 15 minutes and fixed with 4% paraformaldehyde in PBS and cold 100% methanol and then washed a final time. Images were obtained on EVOS FL Auto (Life Technologies) using DAPI light cube (excitation 357/44 nM, emission 447/60 nM). Images were analyzed using ImageJ 1.49 analysis software (National Institute of Health, Bethesda, MD).

2.4 HUVEC cell viability, markers of inflammation and matrix degradation.

HUVECs were passaged 24-48 hours into 6-well plates prior to exposure to vehicle, A β 42 (2 μ M) \pm PA (150 μ M), PA (150 μ M) for 18-20 hours or exposed to a 4 hour pre-treatment with PA (150mM) followed by treatment \pm A β 42 (2 μ M) for 18-20 hours. To measure vascular inflammation, we measured inflammatory cytokines, interleukins (IL)-6 and IL-8, Receptor for Advanced Glycation End Products (RAGE) 1 and 2, and Matrix Metalloproteinases (MMP-9) by Polymerase Chain Reaction (PCR). RAGE 1 and 2 are implicated in endothelial cell inflammation via receptor-mediated mechanisms altering cellular signaling, promoting gene expression and the release of pro-inflammatory molecules. MMP-9 is a protein induced in endothelial cells by cytokine stimulation and is involved in the degradation of extracellular matrix during the development and progression of atherosclerotic lesions^{34, 35}.

Total RNA was isolated from HUVECs using the Aurum mini column method (Bio-Rad Laboratories, Hercules, CA) from cells grown in 6-well plates to approximately 80% confluence.

Total RNA was assessed for purity and quantity by OD260/280 readings. Single-strand cDNA was synthesized using 0.5µg of RNA and iScript cDNA synthesis kit (Bio-Rad). Quantitative Real-time PCR was performed using iQ SYBR Supermix and analyzed by iCycler iQ5 and CFX96 Real-Time Detection Systems (Bio-Rad). Our methodology is consistent with protocols based on previous studies published by members of the Phoenix VA research teams^{30, 36}.

2.4 Data Analyses.

Data are expressed as means±standard error of means, and significant p-value (2-sided) was set at p<0.05. Groupwise analyses were performed using analysis of variance (ANOVA) and post-hoc pairwise analyses were performed using paired Student's t-test or Holm-Sidak method. Unpaired t-test were also used for some comparisons between treatment groups if appropriate.

3. RESULTS

3.1 HUVEC endothelial function.

Independent treatment with A β 42 (2 μ M) and PA (150 μ M) demonstrated a statistically significant decrease in endothelial cell nitric oxide production when compared to vehicle control (relative to control: A β 42 0.66 \pm 0.083, PA 0.76 \pm 0.02, $p=0.01$ and $p<0.001$ respectively) (Figure 2(a)). Co-treatment with A β 42 (2 μ M) and PA (150 μ M) also demonstrated a statistically significant decrease in nitric oxide production when compared to vehicle (relative to control: A β 42+PA 0.73 \pm 0.076, $p=0.01$). However, the change in nitric oxide production of endothelial cells following co-treatment with A β 42 (2 μ M) and PA (150 μ M) was not significantly different when compared to the separate effects of endothelial cells treated with either A β 42 (2 μ M) or PA (150 μ M).

There was no significant difference in superoxide production in HUVECs treated with vehicle, A β 42 (2 μ M), PA (150 μ M), co-treatment with A β 42 (2 μ M) and PA (150 μ M) or a 4-hour pretreatment with PA (150 μ M). None of these effects reached statistical significance because of high variability between experiments, even though care was taken to harvest cells from tissue culture 10cm² tiny flasks at equivalent confluences. Sequential treatment of endothelial cells with PA (150 μ M) for a 4-hour pre-treatment interval and A β 42 (2 μ M) resulted in an 88% increase in superoxide production versus vehicle ($p=0.03$) (Figure 2(b)).

HUVECs treated with A β 42 (2 μ M) showed a statistically significant increase in peroxynitrite production versus vehicle control (Figure 2(c)). Following treatment with A β 42 (2 μ M), peroxynitrite production increased by 34% as compared to vehicle ($p=0.03$). The increased peroxynitrite production in HUVECs exposed to A β 42 (2 μ M) is consistent with the decrease in nitric oxide production. Endothelial cell responses to PA 9150 (μ M), co-treatment with A β 42 (2 μ M) and PA (150 μ M) or a 4-hour pretreatment with PA (150 μ M) \pm A β 42 (2 μ M) did not result in a significant difference from vehicle control.

Results of nitric oxide, superoxide and peroxynitrite production consistently indicated co-treatment of HUVECs with A β 42 (2 μ M) and PA (150 μ M) did not have a statistically synergistic effect on nitric oxide bioavailability.

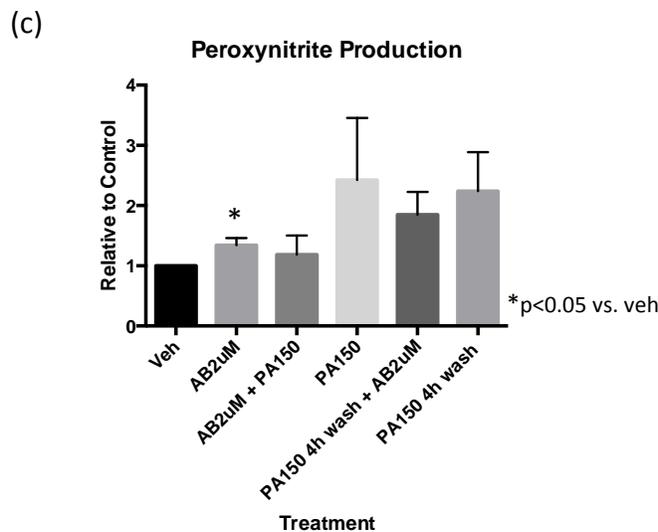
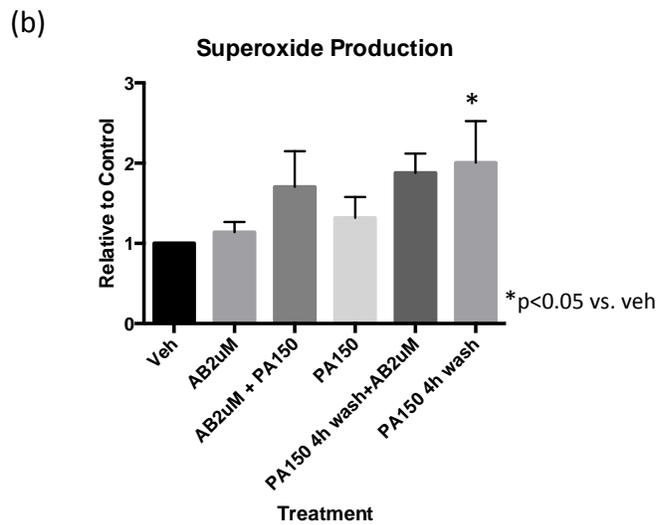
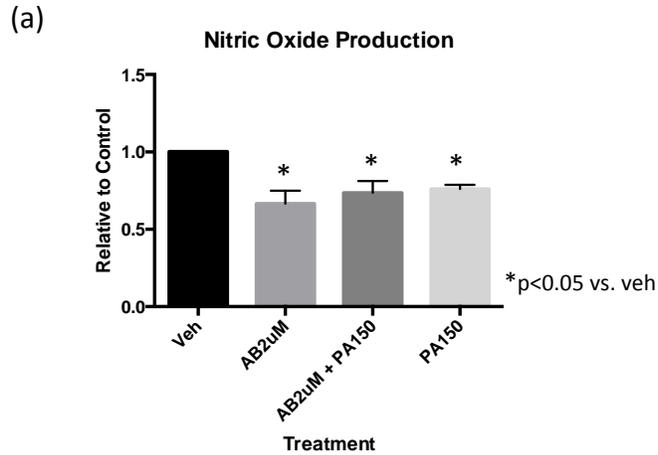


Figure 2. (a) Human umbilical vein endothelial cell nitric oxide production. HUVECs exposed to A β 42, PA, and co-treatment with both A β 42 and PA demonstrated statistically significant decreases in nitric oxide production as compared to vehicle control ($p=0.01$, $p<0$, $p<0.001$, and $p=0.01$ respectively). The decrease in NO production for cells exposed to co-treatment did not result in a significant decrease when compared to the separate effects of A β 42 and PA. (b) HUVEC superoxide production. Sequential treatment of endothelial cells with PA for a 4-hour pre-treatment interval and treated for 18-20h with A β 42 resulted in an 88% increase in superoxide production versus vehicle ($p=0.03$). There was no significant difference in endothelial cell superoxide production among the other treatment groups. (c) Total peroxynitrite production as measured by flow cytometry. HUVECs exposed to treatment with A β 42 (2 μ M), peroxynitrite production increased by 34% as compared to vehicle ($p=0.03$). While the other treatment groups demonstrated an increase in HUVEC peroxynitrite production, the increases did not reach statistical significance.

3.2 HUVEC cell viability and inflammation.

HUVECs treated with PA (150 μ M) upregulated inflammatory responses in HUVECs, leading to a 200-fold increase in IL-8 production, a 20-fold increase in IL-6, 2-fold increases in RAGE1 and RAGE2 and a minor increase in MMP-9 compared to vehicle-treated control cells (Figure 3 (a)(b), 4(a)(b), 5). When compared to vehicle, the amplification of RAGE2 reached statistical significance ($p < 0.003$) (Figure 4(b)) and IL-6 approached significance ($p = 0.059$) (Figure 3(a)). While there was a generalized trend in the amplification of inflammatory cytokines or signaling pathways in HUVECs treated with PA (150 μ M), the increases did not reach statistical significance for IL-8, IL-6, RAGE1 and MMP-9 as a result of high variability between individual experiments.

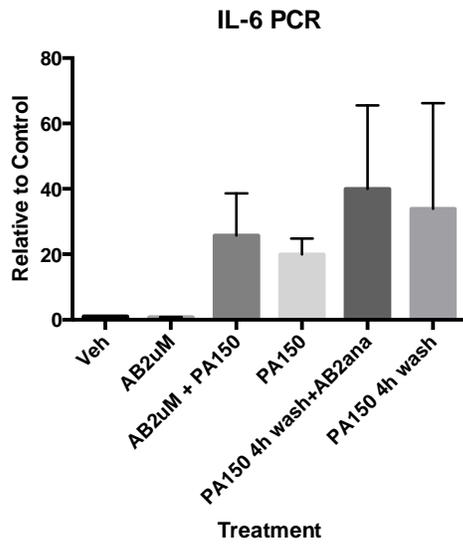
Consistent with these results, HUVECs exposed to PA (150 μ M) for a 4-hour pre-treatment followed by 18-20h treatments without successive A β 42 (2 μ M) treatment, demonstrated amplification of IL-6, IL-8, RAGE1, RAGE2 and MMP-9 when compared to vehicle control (Figure 3(a)(b), 4(a)(b), 5). Similar trends were observed for HUVECs exposed to PA (150 μ M) without the 4-hour pre-treatment. Again, the wide variability between individual experiments resulted in the amplification of the inflammatory mediators not reaching significance.

Unlike HUVECs exposed to treatment with palmitic acid, A β 42-treated endothelial cells showed reduced amplification of IL-6, IL-8, and MMP-9 when compared to vehicle control, with these differences not reaching significance (Figure 3(a)(b), 5). HUVECs treated with A β 42 (2 μ M) did result in amplification of both RAGE1 and RAGE2, consistent with similar trends in gene expression of RAGE1 and RAGE2 observed for HUVECs treated with PA (150 μ M) and pre-treated with PA (150 μ M).

For HUVECs exposed to co-treatment with A β 42 (2 μ M) and PA (150 μ M) for 18-20h and for the 4-hour pre-treatment, there was a resultant upregulation of inflammatory cytokines IL-6, IL-8, RAGE1, RAGE2, and MMP-9, of which, neither treatment group reached significance when compared to the HUVECs treated as control. While not reaching significance, the addition of PA to HUVECs subject to co-treatment suggests either one of two possibilities. The first being, the addition of atherosclerotic risk factors, such as palmitic acid, enhanced the amplification of

endothelial cell-induced inflammatory responses as measured by IL-6, IL-8, RAGE1, RAGE2 and MMP-9. Secondly, the decrease in gene expression of IL-8, IL-6, and MMP-9 in HUVECs subject to treatment with A β 42 alone may suggest A β 42 may attenuate the inflammatory effects of palmitic acid.

(a)



(b)

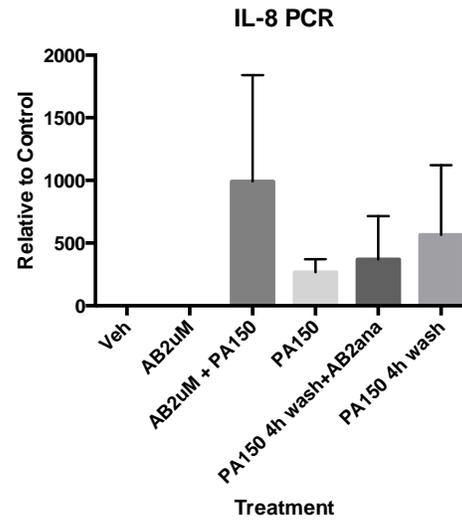
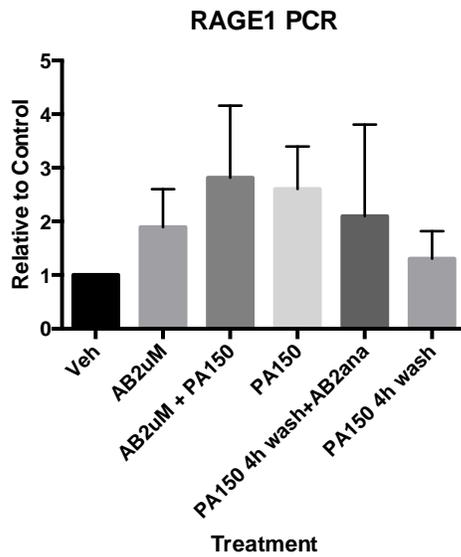


Figure 3. (a) HUVEC IL-6 gene expression as measured by rtPCR. Amplification of IL-6 gene expression was observed for all treatment modalities with the exception of A β 42 (2 μ M), which resulted in a modest decrease in gene expression. The variations among IL-6 amplification when compared to vehicle-treated control cells did not reach statistical significance for any of the treatments groups. IL-6 amplification for HUVECs treated with PA approached statistical significance ($p=0.059$) (b) Endothelial cell IL-8 gene expression. Similar to the results observed for IL-6, there was a non-significant upregulation of IL-8 gene expression for all treatment groups except for the HUVECs exposed to the A β 42 treatment.

(a)



(b)

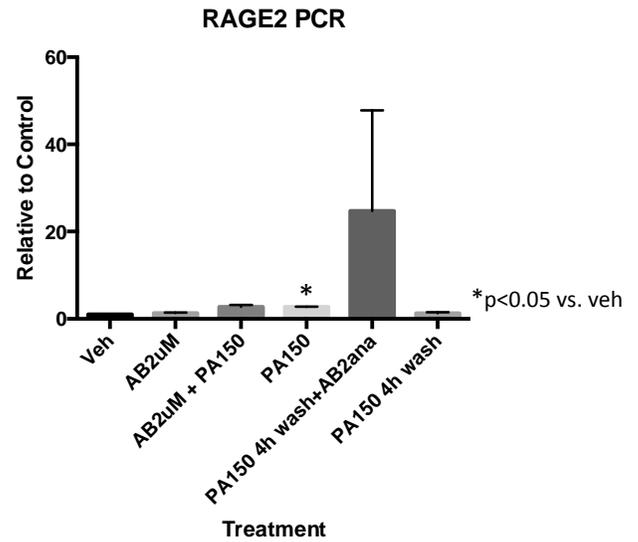


Figure 4. (a) HUVEC RAGE1 gene expression. In general, amplification of RAGE1 was upregulated by all treatment modalities. The increased gene expression did not reach statistical significance due to the wide variability between individual experiments. (b) RAGE2 gene expression. Endothelial cells exposed to treatment with PA resulted in a statistically significant increase in RAGE2 gene expression versus vehicle ($p<0.003$). Co-treatment with A β 42 and PA increased the expression of RAGE2 with the data approaching significance ($p=0.058$).

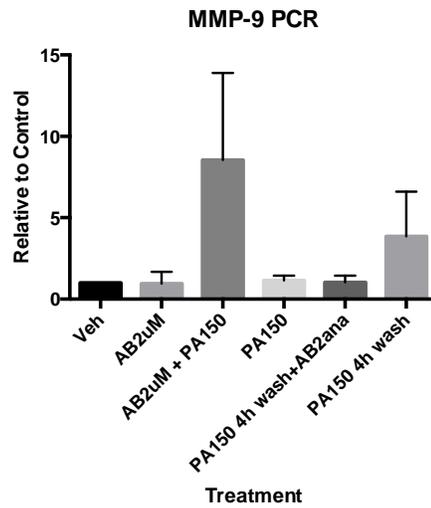


Figure 5. HUVEC MMP-9 gene expression as measured by rtPCR. HUVECs exposed to PA showed greater degree of amplification of MMP-9 from control. Treatment with A β 42 resulted in a slightly lower amplification of MMP-9 when compared with vehicle control. Co-treatment with both A β 42 and PA, and 4-h pre-treatment with PA followed by sequential A β 42 treatment resulted in increased gene expression of MMP-9, with PA mediating endothelial cell inflammatory responses.

4. DISCUSSION

We present the following findings: 1) Exposure to A β 42 and palmitic acid leads to significant endothelial dysfunction that is associated with oxidative/nitrative stress and reduced endothelial cell NO production. Contrary to our hypothesis, with the doses used, there appears to be no additive or synergistic effects when AB42 or PA were co-treated. 2) While there is an observable trend in the upregulation of inflammatory pathways as measured by IL-6, IL-8, RAGE1, RAGE2 and MMP-9, the upregulation in gene expression did not reach statistical significance.

Both Alzheimer's disease and vascular disease are common age-related disease processes. Evidence suggests that there is an association between cerebrovascular atherosclerosis in the Circle of Willis and Alzheimer's disease. The presence of cerebral hypoperfusion, atherosclerosis of the Circle of Willis, and biochemical alterations in cerebral microvessels in patients with AD, implicates vascular dysfunction in the early pathogenesis of AD^{13, 14, 16}. Clinical and pre-clinical data suggest these deleterious changes in cerebral microvessels may occur prior to the deposition of A β proteins^{10, 12, 37}. However, the precise role of A β , in contributing to vascular dysfunction and the progression of AD, has not yet been fully elucidated. AD is associated with the presence of atherosclerosis risk factors, including hyperlipidemia (including increased free fatty acids), hypertension and diabetes, which contribute to alterations in vascular endothelial function^{13-17, 37-41}.

Although various studies have explored the effects of A β proteins and atherosclerosis risk factors on endothelial cells, our study investigated the additive effects of atherosclerosis risk factors and A β proteins on human umbilical vein endothelial cells to determine whether co-treatment amplifies effects on cell function and viability. Our study addressed the knowledge gap surrounding the interaction between A β , identified as the primary component of amyloid plaques in AD, and atherosclerosis risk factors in endothelial cell function, viability and inflammation.

Vascular dysfunction occurs early in the course of AD, preceding clinical diagnosis, yet there exists a lack of understanding surrounding the mechanisms by which the early vascular

dysfunction contributes to the progression of cognitive impairment. Our findings strongly suggest that soluble A β and atherosclerotic risk factors play an important role in AD-associated vascular impairment and dysfunction. Our study demonstrates endothelial cell oxidative and nitrative stress and reduced endothelial cell nitric oxide production may lead to vascular dysfunction induced by exposure to A β and saturated fatty acids. Our results did not support our hypothesis that the presence of both A β and atherosclerotic risk factors, palmitic acid, acted synergistically in contributing to endothelial cell oxidative stress. Our results may necessitate a greater sample size to determine modulating effects of A β and atherosclerotic risk factors and their combined role in vascular dysfunction. Additionally, lower doses that may still be supraclinical levels may show synergistic or additive effects, and should be tested in the future. Importantly our results demonstrate a potential mechanism underlying A β and palmitic acid effects on endothelial cell function. A β and palmitic caused increased superoxide and peroxynitrite production. Peroxynitrite is a reactive nitrogen species, which forms as a result of a reaction between nitric oxide and superoxide. Because of its oxidizing properties, peroxynitrite is an important and potent mediator of protein and DNA damage in endothelial cells. These results strongly suggest that soluble A β and saturated fatty acids independently play an important role in vascular impairment.

Recent evidence suggests that β -amyloid (A β)-induced inflammatory reactions may partially drive the pathogenesis of Alzheimer's disease. Additional data also implicate similar inflammatory processes in cerebral amyloid angiopathy. To evaluate the roles of A β in the inflammatory processes in vascular tissues, we have tested the ability of A β to induce inflammatory responses in cultured human endothelial cells. Our results suggest that palmitic acid, as an atherosclerotic risk factor, can function as an inflammatory stimulator to activate endothelial cells and induce an amplified inflammatory cascade through interactions among cytokines, IL-6, IL-8, receptors RAGE1 and RAGE2 and MMP-9, an important enzyme in mediating proteolysis of the extracellular matrix.

Our results related to inflammatory responses of HUVECs treated with A β varied widely and did not demonstrate a significant trend in the upregulation of inflammatory responses. In fact,

there was an observable, nonsignificant trend that demonstrated a decrease in cytokine IL-6 and IL-8 production of endothelial cells treated with A β alone as compared to vehicle control. This result is inconsistent with results⁴² from previous studies and may be related to the wide variability of independent experiments in conjunction with our small sample size. Interestingly, HUVECs exposed to co-treatment with A β and PA demonstrated a greater fold of increase over vehicle control cells when compared to the fold of increase for A β or PA alone. It is possible that the PA-induced inflammatory pathway may act to synergistically modulate the inflammatory effects of A β . While our data did not reach significance, additional experiments to increase sample size may contribute to more conclusive results. While we believe human umbilical vein endothelial cells have been shown to be a good surrogate for modeling the blood brain barrier, HUVECs may behave differently compared to human brain microvascular endothelial cells. Future experimentations may focus on testing primary cell lines that more closely model the blood brain barrier.

Collectively, these findings support the hypothesis that A β -induced oxidative stress and decreased nitric oxide bioavailability in endothelial cells could play a significant role in the pathogenesis of AD. However, our results fail to prove that A β and saturated fatty acids exert a synergistic effect on endothelial cell dysfunction. The results do not support the hypothesis that A β or palmitic acid induces endothelial dysfunction of human umbilical vein endothelial cells through inflammatory-mediated pathways. As mentioned previously, additional studies may establish whether A β and PA promote pro-inflammatory gene expression in endothelial cells and if there exists an synergistic effect.

Limitations of our study include a small sample size and the acute exposure treatment times given the short-term viability of human umbilical vein endothelial cells. Although human umbilical vein endothelial cells are not sourced from brain tissue, this human cell line is utilized in this project given the inability to harvest cerebral blood vessels. Human umbilical vein endothelial cells have been shown to serve as an appropriate surrogate for in vitro modeling of the blood brain barrier^{43,44}.

5. FUTURE DIRECTIONS

Evidence suggests there exists an underlying vascular component to the neurodegeneration process in AD. Atherosclerosis risk factors, including free fatty acids, have been shown to induce endothelial dysfunction, inflammation, and impaired cell viability. A β 42 proteins have similarly demonstrated endothelial dysfunction, inflammation, and impaired cell viability as a result of impaired vasomotor regulation of both peripheral and cerebral vessels.

While it is now understood that vascular dysfunction and impaired blood brain barrier function occur early in Alzheimer's disease, a critical delay in understanding the pathophysiology behind early AD remains. The results of our study served to address the knowledge gap surrounding the interaction between A β , identified as the primary component of amyloid plaques in AD, and atherosclerosis risk factors in endothelial cell function. Future implications of this study may further emphasize the need to develop a greater understanding of the common pathophysiological mechanisms underlying vascular impairment and Alzheimer's disease. From a therapeutic standpoint, evidence in this study may suggest that strategies proven to delay the progression of atherosclerosis may be useful for preventing the development of Alzheimer's disease.

6. CONCLUSIONS

Atherosclerosis and Alzheimer's disease, independently, comprise two of the largest public health burdens in aging and elderly populations. Current evidence including clinical, epidemiological and experimental data indicate that together, they appear to act in an additive or potentially synergistic fashion in the progression of AD. Most hypotheses linking atherosclerosis and the progression of AD focus on the vascular biology of amyloid beta proteins. While risk factors for atherosclerosis have been demonstrated to increase inflammatory signaling, the simultaneous presence of atherosclerosis risk factors and amyloid beta proteins, have been shown to increase oxidative stress, pro-inflammatory cytokines and lead to endothelial cell dysfunction. Thus, the presence of atherosclerosis risk factors plays a role in AD pathophysiology by enhancing endothelial dysfunction and vascular inflammation. Our study attempted to address the lack of understanding surrounding the interplay of atherosclerosis risk factors and amyloid beta protein deposition on the development and progression of AD.

The data presented are consistent with, but does not prove, the original hypothesis that A β and palmitic acid, an atherosclerosis risk factor, both independently induce endothelial dysfunction through reduced nitric oxide production and pro-inflammatory cytokine production. The trends observed from our results may indicate a possible synergistic effect on these relationships, but further studies may be required to elicit the combined effects of A β and PA.

The present investigation further adds to our knowledge of the mechanism by which amyloid beta protein deposition and atherosclerosis risk factors, including palmitic acid, induce endothelial dysfunction by causing decreased nitric oxide bioavailability, as well as nitrosative and oxidative stress. Further studies relating to the mechanism of inflammatory gene regulation by A β and atherosclerosis risk factors could ultimately lead to the establishment of an inflammatory mechanism of the vascular dysfunction underlying Alzheimer's disease.

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