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α1-acid Glycoprotein Induced Effects in Rat Brain Microvessel Endothelial Cells

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Introduction

α1-acid glycoprotein (AGP), also known as orosomucoid, is a 41–43 kDa glycoprotein with 45% oligosaccharide component. The peptide moiety is a single chain of 183 (human) or 187 (rat) amino acids with two disulfide bridges (Ceciliani and Pocacqua, 2007). AGP, a positive acute phase protein (APP), is elevated 1–10 times during inflammation in humans and rats while the major plasma protein, albumin (a negative APP) is typically decreased. These changes in plasma proteins have led to drug binding characterization studies of AGP, which shows AGP preferentially binds basic central nervous system (CNS) drugs like imipramine and carbamazepine (Zhang et al., 2007; Zsila and Iwao, 2007). AGP has been reported to have anti-inflammatory, immunomodulatory and vascular protective roles suggesting a role as a biomarker of inflammatory diseases (Curry et al., 1989; Jorgensen et al., 1998; Pukhal'skii et al., 2000; Ceciliani et al., 2007). AGP acts on immune cells that are involved in the inflammatory process, such as decreasing thymocyte proliferation via macrophage derived IL-1 inhibitor (Bories et al., 1990), and modulating lymphocyte proliferation as well as regulate cytokine (IL-1, IL-2, IL-6, and TNF-α) production by leukocytes via mitogen activators (Shiyan and Bovin, 1997). Reducing reactive oxygen species production by neutrophils upon phorbol 12-myristate 13-acetate (PMA) stimulation (Stakauskas et al., 2005) and changing the shape of platelets via Rho/Rho-kinase signaling pathway (Gunnarsson et al., 2009) are also biological characteristics of AGP. In particular, AGP has been shown to protect against inflammation-induced tissue injury through the inhibition of neutrophil activation and reduced prostaglandin E2 generation (Matsumoto et al., 2007), as well as inhibition of TNF-α-induced apoptosis of hepatocytes (Van Molle et al., 1997).

As cytokines play important roles during inflammation, whether AGP can exert its biological functions on endothelial cells via regulation of cytokines is not well known. There is evidence for cross-talk between APPs and pro-inflammatory cytokines like IL-1, IL-6 and TNF-α, which are known to stimulate hepatocytes to increase the expression of AGP and

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other positive APPs during inflammation (Castell et al., 1989; Christian Pous, 1989; Baumann and Gauldie, 1990). Bories et al. showed that AGP can inhibit IL-1 activity by modulating an unknown macrophage derived factor (Bories et al., 1990). While AGP is primarily produced by hepatocytes, it is also produced by endothelial cells where it plays an important role in forming the glycocalyx (Curry et al., 1989; Sorensson et al., 1999). Some studies suggest that AGP is necessary to maintain capillary permeability, likely by increasing the polyanionic charge selectivity of the endothelial barrier as well as by interacting with components of the endothelial glycocalyx (Haraldsson and Rippe, 1987; Curry et al., 1989; Huxley et al., 1993).

Despite the evidence implicating inflammatory cytokines (e.g. TNF-α, IL-1β and IL-6) in disrupting the blood-brain barrier (BBB) (Mark and Miller, 1999; Paul et al., 2003; McColl et al., 2008), little is currently known about the response of brain microvascular endothelial cells to acute phase proteins such as AGP. The BBB is crucial in maintaining brain homeostasis and protecting the CNS from harmful solutes in the peripheral circulation. Tight junction (TJ) proteins and basement membrane proteins are two important components for maintaining the integrity of the BBB. TJ proteins are composed of transmembrane proteins (occludin and claudins) and intracellular zonula occludens (ZO-1, 2, 3) which bind cytoskeletal F-actin. Occludin is the major TJ integral membrane protein, which is highly expressed at brain endothelial cells where the structure and localization of occludin are critical in maintaining BBB functional integrity (i.e., low paracellular permeability) (Furuse et al., 1993; Lochhead et al., 2011). Among the 23 claudin family members, claudin-5 and claudin-2 are located at the BBB where they contribute to barrier “tightness” (Nitta et al., 2003). Tethered to occludin-based strands through ZO-1, junction adhesion molecules (JAM) concentrated at cell–cell adhesion sites facilitate TJ protein reassembly (Itoh et al., 2001). Inflammatory models have shown to disrupt both structural components (occludin and claudin-5) and function of the BBB (Mark and Miller, 1999; Ferrari et al., 2004; Brooks et al., 2005);

To date, the effects of AGP in regulating the expression of TJ proteins (ZO-1 and occludin) and changing the integrity of BBB have not been investigated. Whether AGP can stimulate brain endothelial cells to express anti- or pro-inflammatory cytokines remains largely unknown. As inflammatory cytokines have been shown to alter the BBB, we hypothesized that AGP changes BBB integrity by altering inflammatory cytokines and TJ protein expression. In this study, we used primary rat brain microvessel endothelial cells (RBMECs) to investigate the effects of AGP at the BBB.

Materials and methods

Isolating and Culturing of Primary Rat Brain Microvessel Endothelial Cells (RBMECs)

The RBMECs are a well characterized *in vitro* BBB model and this study has been approved by the Institutional Animal Care and Use Committee (IACUC), Texas Tech University Health Science Center, Amarillo, USA. Primary isolated RBMECs were collected from female Sprague-Dawley rats (200–250g) using a modified enzymatic and centrifugation steps as previously described (Abbott et al., 1992; Miller et al., 1997). RBMECs were seeded onto collagen coated, fibronectin-treated 75 cm^2 tissue culture flasks 6-well culture

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plates or 12-well Transwell® plates (Corning Inc., NY). Culture medium for RBMECs consisted of: 45% minimum essential medium (MEM), 45% Ham's F12 nutrient mix, 10 mM HEPES, 13 mM NaHCO3, 50 μg/ml gentamycin, 10% equine serum, 2.5 μg/ml amphotericin B, and 100 μg/ml heparin. 200 μg/ml endothelial cell growth supplement (Sigma; St. Louis, MO) was added to the medium for first 3 days. Cell cultures were grown in a humidified 37°C incubator w/5% CO_2 and medium was replaced every other day until the monolayers reached confluency (about 11–14 days).

Alpha 1-acid Glycoprotein (AGP) Treatment of RBMECs

Confluent RBMECs monolayers were pre-treated with low serum (0.5%) medium for 6–24 hrs prior to AGP treatment. The acute phase protein, AGP from human plasma (Sigma; St. Louis, MO), was diluted into low serum (0.5%) medium prior to treatment of RBMECs. Confluent monolayers were treated with varying doses of AGP (50 μg/ml or 500 μg/ml) or culture medium alone (control) at various exposure times (1, 3, 6, 12, 24, 48, or 72 hrs) as indicated in the particular experiments; AGP treatment was added to apical side including the Transwell® plates (Corning; Lowell, MA).

Paracellular Permeability of AGP Treated RBMEC Monolayer

After treatment and prior to the permeability study, primary RBMEC monolayers were assessed by transendothelial electrical resistance (TEER) measurement via EVOM resistance meter (World Precision Instruments, Sarasota, FL, USA). Those monolayers with resistance values > 100Ω•cm^2 were used to assess the permeability effects of AGP on RBMEC monolayers. After treatment, RBMEC monolayers were incubated with assay buffer composed of (in mM) 122 NaCl, 3 KCl, 1.4 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 10 HEPES, 10 glucose, and 0.5 K_2HPO_4 for 30 min at 37°C. Permeability across RBMEC monolayers was determined by adding 5 μM sodium fluorescein (NaFl, 376 MW, a paracellular transport marker) in the assay buffer to the apical side (upper compartment of Transwell). Samples (100 μl) were removed from the basolateral side (lower chamber of Transwell) at 0, 60, and 120 min and replaced with fresh assay buffer. The concentration of NaFl was measured by Synergy Mx microplate reader (BioTek, Winooski, VT) at excitation: 485 nm and emission: 528 nm. Concentration of NaFl applied to the apical side was determined by removing samples (50 μl) at time zero. Permeability coefficients (PC) for NaFl were expressed using the equation below as previously described (Mark and Miller, 1999); where V is volume in receiver chamber (1.5 cm^3), SA is surface area of cell monolayer (1.12 cm^2), Cd is the concentration of marker in the donor chamber at time 0, and Cr is the concentration of marker in the receiver at sample time T.

$$\mathrm{PC\,(cm/\,min)} = \frac{\mathrm{V}}{\mathrm{SA}\cdot \mathrm{C_d}} \cdot \frac{\mathrm{C_r}}{\mathrm{T}}$$

Western Blot Analysis of Tight Junction Protein Expression in RBMECs

Following treatment, the RBMECs were solubilized in Cellytic buffer (Sigma) with 1% protease inhibitor cocktail (Research Products International Corp.). Protein concentration of

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cell lysates was quantified using the bicinchoninic acid (BCA) protein assay. RBMECs lysate samples (8 μg total protein) were loaded onto a 4–12% tris-glycine gel (Invitrogen) and protein expression was determined via standard Western blot protocol as previously described (Mark and Davis, 2002). Antibodies used are occludin (1:10,000; Invitrogen), ZO-1 (1:10,000; Invitrogen), or β-actin (1:10,000; Sigma) for primary and anti-mouse or anti-rabbit antibody (1:50,000; Invitrogen) for secondary. The protein bands were developed and visualized using the enzyme chemiluminescence method (Femto ECL; Thermo Science) and developed on X-ray film. Protein expression was semi-quantified by using Image J software (courtesy of N.I.H.) and normalized by β-actin expression and presented as % of control.

Real-time PCR (quantitative PCR)

mRNA was isolated from RBMECs following AGP treatment using an RNeasy Mini Kit (Qiagen). Synthesis of cDNA was performed by reverse transcription of the isolated mRNA using the iScript cDNA synthesis kit (Bio-rad). qPCR was performed on cDNA (corresponding to 35–50 ng RNA per reaction) and amplified through 35 cycles using SYBR Green Master Mix (Applied Biosystems (ABI)) and an ABI 7300 real-time PCR instrument. Specific primer sets were designed using IDT DNA website or published literature (see Table 1) and synthesized by Invitrogen (Carlsbad, CA). GAPDH was amplified as an internal standard and target cDNA was normalized by GAPDH cDNA. The effect of the AGP-treatment was demonstrated as a change (ratio) in mRNA expression for the respective target genes as calculated using the Pfaffl method and the equation below, where controls were RBMECs receiving media alone, treated were AGP-treated RBMECs, and reference is GAPDH gene.

$$\text{Ratio}=\frac{\text{E}_{\text{Target}}^{(\Delta\text{Ct}(\text{Control}-\text{Treated}))}}{\text{E}_{\text{Reference}}^{(\Delta\text{Ct}(\text{Control}-\text{Treated}))}}$$

AP-1 family transcription factor assay

The effect of AGP on the DNA binding activity of two major AP-1 subunits, namely, cFos and cJun, were determined using an ELISA-based assay kit (TransAM® kit, from Active Motif; Carlsbad, CA). In brief, 5 μg of nuclear extract from control or AGP (50 or 500 μg/ml) treated RBMECs for 1–72 hrs were incubated in separate wells of a 96-well plate possessing an immobilized AP-1 consensus sequence [TPA responsive element (TRE) oligonucleotide 5′-TGAGTCA-3′], inactive AP-1 molecules were removed with wash buffer, and the active TRE-bound AP-1 was identified using primary antibodies directed against the c-Fos or c-Jun subunits in conjunction with horseradish peroxidase-conjugated secondary antibodies and a colorimetric substrate quantified by spectrophotometry (Synergy Mx, Bio Tek) at 450 nm with a reference wavelength of 655 nm. Results are presented as fold of the absorbance values of control.

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AGP Stability Throughout the Treatment

Culture supernatants were collected at different time points (0, 6, 12, 24, 48, or 72 hrs) from the same well of RBMECs monolayer treated with 50 μg/ml or 500 μg/ml human AGP, to determine stability of AGP in the treatment medium over 72 hrs. Proteins in supernatants were separated by electrophoresis on 10% acrylamide/bis gels and subsequently stained with fluorescent Pro-Q Emerald 300 dye (Molecular Probe) as described (Steinberg et al., 2001). This dye binds to periodate-oxidized carbohydrate groups, forming a green fluorescent signal on glycoproteins. Briefly, after protein fixation in 50% methanol, glycans of gel-separated proteins were oxidized to aldehydes by incubation in 1% periodic acid/3% acetic acid. After washing in 3% acetic acid, gels were incubated in Pro-Q Emerald 300 dye solution. Glycosylated proteins were visualized by 300 nm UV transillumination in Gel Doc 2000 gel documentation system (Bio-Rad). AGP levels were determined by using Image J software (courtesy of N.I.H.) and data are presented as fold of level at time zero (0 hr). This method has high sensitivity for AGP (linear range tested: 12.5–100 ng/lane) and it has been used for quantification of glycoproteins (Ogata et al., 2005).

Measurement of Cytokines Released from RBMECs

Cytokine levels in cell culture supernatant from AGP-treated RBMECs were measured using Quantikine® ELISA kits (R&D systems; Minneapolis, MN) for IL-1β and TNF-α, Platinum ELISA kits (eBioscience; San Diego, CA) for IL-6. Supernatant samples were centrifuged (5000 × *g*, 5 min) and kept frozen until performing the ELISA. Sensitivity for cytokines (IL-1β, TNF-α, and IL-6) are: 31.2 pg/ml, 6.25 pg/ml, and 15.5 pg/ml; respectively.

Briefly, the specific standards and experimental samples were incubated with cytokine-antibody coated ELISA plate. The HRP-conjugated antibody was then added and incubated to form the antibody-antigen-antibody complex. After washing, the substrate was added and incubated prior to stopping the enzymatic reaction. Absorbance was measured by a microplate reader (BioTek Synergy Mx) at 450 nm with 540 nm correction. Cytokine concentrations were calculated based on the respective standard curve.

Statistical Analysis

SigmaStat™ statistical software program was used for statistical analyses. Comparisons of data were made using one- or two-way ANOVA followed by Student Newman Keuls post-hoc analysis. p-values < 0.05 were considered statistically significant.

Results

It should be noted that throughout these studies, two concentrations were used, 50 μg/ml that is high normal serum level of AGP and 500 μg/ml, which represents the 10-fold increase seen during inflammation.

AGP Concentration Maintained Throughout 72 hr

Stability of the AGP in culture conditions was determined by collecting culture supernatants at different time points (0, 6, 12, 24, 48, or 72 hrs) from the same well of RBMECs monolayer treated with 50 μg/ml or 500 μg/ml human AGP. AGP levels were detected by

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Pro-Q Emerald 300 glycoprotein stain. No significant decrease of AGP level was detected in the treatment supernatant at either concentration throughout the 72 hr treatment time (Figure 1).

Effect of AGP on Paracellular Permeability of RBMEC Monolayer

Following AGP treatment on RBMEC monolayers, AGP showed a dose-dependent effect in paracellular permeability of RBMECs. As early as 1 hr, high concentration AGP (500 μg/ml) showed an immediate decrease in permeability which returned to control level by 3 hrs before a second decrease occurred at 6 hrs and lasted until 72 hrs. In contrast, low concentration AGP (50 μg/ml) showed a significant decrease in permeability at 6, 24 and 72 hrs. Interestingly, both concentrations showed the most significant decrease (> 20%) at 24 hrs when compared to control (Figure 2). No permeability change was observed for either concentration at 3 hrs. Furthermore, the high dose of AGP decreased permeability at later times (12, 48 and 72 hrs) when compared to the low dose AGP- treatment. We also measured TEER values (range from 101 to 142 Ωcm^2) of post-treatment monolayers, which mirrored the change of permeability coefficient (PC) values (data not shown). Together, these results suggest that AGP has a direct effect in enhancing the integrity of RBMEC monolayers.

AGP Changes mRNA Expression of Pro-inflammatory Cytokines and Tight Junction Proteins

IL-1β mRNA expression was maximally elevated at 6 hrs (3-fold) following 50 μg/ml AGP treatment, with a strong expression seen at the higher concentration of AGP (500 μg/ml) at 12 hrs and 24 hrs (12-fold and 5-fold, respectively; Table 2).

AGP treatment (500 μg/ml) of RBMECs resulted in a significant increase of IL-6 mRNA expression at 6 hrs (30-fold), 24 hrs (26-fold) and 48 hrs (20-fold) despite no protein detected in the culture supernatant. TNF-α and IL-10 mRNA expression showed mild increases at 6 hrs and 12 hrs following 500 μg/ml AGP treatment, supporting the undetectable levels of protein in the supernatant. Taken together, these results suggest that inflammatory levels of AGP stimulate mRNA expression of IL-1β and IL-6.

In addition to cytokine mRNA expression, we also examined changes in mRNA expression of key TJ proteins (see Table 2). After 24 and 48 hrs of AGP treatment, occludin, ZO-1, claudin-2 and JAM-1 mRNA expression was decreased by 50% or more with both AGP concentrations (50 and 500 μg/ml). A significant increase in ZO-1 mRNA was observed with both AGP concentrations only at the early time point of 3 hrs. In contrast, claudin-5 mRNA expression was increased two fold or more with both AGP concentrations at a later time point of 48 hrs. Together, these results indicate that AGP regulates TJ mRNA expression with diverse time profiles.

AGP Induced Changes of TJ Protein Expression in a Dose- and Time-dependent Manner

In order to follow up the alterations of TJ mRNA expression, TJ protein expression was examined in RBMECs after treatment with AGP (50 μg/ml or 500 μg/ml) for 3, 6, 12, 24, 48 or 72 hrs and compared to control (Figures 3–4). Occludin protein expression showed a

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biphasic dose-dependent response upon AGP treatment as seen in Figure 3. A significant increase in occludin expression was seen with 500 μg/ml AGP treatment after 6 hrs and 12 hrs treatment (2.8- and 1.5-fold), which returned to control level at 24 hrs before increasing again at later time point of 48 hrs and 72 hrs. The time profile for these changes was different with the lower dose of AGP (50 μg/ml) where the occludin expression sharply increased at 6 hrs (3.5 fold), returned to control by 12 hrs before a mild increase again at 48 hrs and 72 hrs.

In contrast to occludin, ZO-1 protein expression demonstrated a different time profile upon AGP treatment. High dose of AGP decreased ZO-1 protein levels at 3 hrs and again at 24 and 72 hrs. RBMECs showed a significant increase in ZO-1 protein expression at early time points (6 and 12 hrs) with both AGP concentrations and a later increase at 48 hrs but only with high dose of AGP (Figure 4). Together, these results show AGP-induced changes in ZO-1 and occludin expression occur in a time- and dose-dependent manner. That inversely correlates with permeability changes seen with the RBMEC.

Transcription Factor AP-1 Involvement of AGP Effects in RBMECs

In order to identify a cell signaling candidate or transcription factor that may be involved in these AGP-mediated effects, we performed a DNA/TF protein microarray (Panomics/Affymetrix, data not shown) where AP-1 was seen to be dramatically decreased at 6 hrs treatment with a high-dose AGP (500 μg/ml). This was followed up by an ELISA based transcription factor assay (Active Motif) using nuclear extract of AGP treated RBMECs (See Figure 5). c-Jun DNA-binding activity was unchanged when compared with control after treatment with normal level of AGP (50 μg/ml; 1–72 hrs). Interestingly, c-Jun DNA-binding activity was significantly increased only at 1 hr after high-concentration AGP treatment (Figure 5, panel B-right). In contrast, both concentrations of AGP activated c-Fos at 1 hr while an inhibition of c-Fos DNA-binding activity was seen at 3 hrs and 12 hrs. Overall, the high-concentration AGP showed a greater impact on c-Fos activity changes than the lower-concentration treatment.

As AP-1 activity negatively regulates ZO-1 gene expression (Chen et al., 2008), both activated c-Fos and c-Jun at 1 hr correlates with the decreased ZO-1 protein at 3 hrs following high-concentration AGP treatment. Furthermore, the inhibition of c-Fos activity at later time points (3 and 12 hrs) may lead to the increase in ZO-1 protein levels seen at 6 and 12 hrs following AGP treatment with both concentrations.

Discussion

This study shows that the positive acute phase protein, alpha-1-acid glycoprotein (AGP) enhances BBB functional integrity in a dose-dependent manner by increasing TJ protein (occludin and ZO-1) expression, to increase cell-to-cell contact, which results in reduced paracellular permeability (“barrier tightness”) of RBMEC monolayers. In contrast to the effects seen with pro-inflammatory cytokines, where BBB structure and functional changes lead to a disrupted or leaky barrier, AGP appears to have direct effects in the brain endothelium to presumably correct these changes and enhance both BBB structure and function. Previous reports have proposed that AGP modulates permeability microvascular

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endothelial cells by increasing the negative charge of the surface glycocalyx layer (Curry et al., 1989; Yuan et al., 2010a; Yuan et al., 2010b). Recently, Yuan et al. showed that high dose (1000 μg/ml) of AGP increased the permeability of positively charged ribonuclease (13.7 kDa) across bEnd3 (mouse cerebral endothelial) cell monolayers while decreasing the permeability of negatively charged α-lactalbumin (14.2 kDa) (Yuan et al., 2010a). They also found that 100 μg/ml AGP in a Ringer-BSA perfusate had similar effects on the *in situ* permeability of charged molecules across pial microvessels with no change in permeability for neutral charged, large dextran (10kDa and 40kDa) molecules (Yuan et al., 2010b). In this study, we show that AGP decreased RBMEC paracellular permeability of a small paracellular marker NaFl (MW 376 Da); most likely by reinforcing the physical barrier of the BBB via TJ regulation. The discrepancies between AGP effects on the endothelium and its integrity to charged and uncharged molecules are likely due to the size of molecular marker and/or the cellular mechanisms affected by AGP. Interestingly, Curry and Yuan both used high concentrations (100–1000 μg/ml) of this acute phase glycoprotein and large size molecules (10 kDa – 40 kDa); while in our experimental paradigm, we used a small size marker (376 Da) and physiologically relevant concentrations of AGP representing high normal (50 μg/ml) as well as an inflammatory relevant concentration (500 μg/ml).

Both *in vitro* and *in vivo* studies have shown permeability changes in brain capillary endothelium have an inverse correlation with TJ protein expression and localization, in particular occludin and ZO-1 proteins (Staddon et al., 1995; Staddon and Rubin, 1996; Mark and Davis, 2002; Jiao et al., 2011). In the current study, alterations in ZO-1 protein expression were seen with both low- and high-concentration AGP treatment in a dose- and time-dependent manner. In contrast, occludin protein showed biphasic increases at early and late phases with both concentrations of AGP treatment. In either case, the higher dose of AGP treatment on primary RBMECs changed TJ protein expression to a larger extent than with the lower dose, indicating that changes in TJ proteins are more dramatic during inflammatory conditions than under normal conditions. Occludin is a critical TJ protein connecting adjacent endothelial cells to form the physical barrier of the BBB (Abbott et al., 2010). In the present study, a biphasic response in occludin protein expression inversely correlated with the decreased paracellular permeability where occludin expression was increased at early time points (6 and 12 hrs) as well as at later time points (48 and 72 hrs), both of which time points paracellular permeability was decreased. The increase of ZO-1, an important TJ accessory protein that links the occludin molecule to the cytoskeleton, supports its role in tightening the barrier at 6, 12 and 48 hrs. It was not surprising that either of these TJ proteins were not increased at 3 hrs as we observed no change in paracellular permeability at this time. However, it is unclear why there was no significant increase in either TJ protein concomitant with the decreased permeability observed at 24 hrs. This might be affected by other cellular mechanisms not examined in the current study, such as changes in kinase or phosphatase activity, which could affect phosphorylation of TJ proteins and impact localization of these proteins. In fact, changes in phosphorylation state of certain amino acid residues have been associated with change in function of proteins (Staddon et al., 1995). In the case of occludin, increased tyrosine phosphorylation has been shown to target the protein for degradation while increased serine/threonine phosphorylation has been correlated with increased membrane-bound occludin resulting in decreased paracellular

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permeability (reviewed by Cummins, 2012). In addition to phosphorylation of TJ proteins, other potential mechanisms that could underlie the decreased permeability we have seen at 24 hrs may be through increased cAMP and/or intracellular Ca^{2+} levels, both of which have been shown to tighten the brain endothelial barrier (reviewed by Deli et al., 2005). These cellular mechanisms will require further investigation in our experimental paradigm. While we have been unsuccessful in quantifying protein expression of other TJ proteins (claudin-2 and claudin-5) and adhesion molecule (JAM-1) proteins, we have seen changes mRNA expression of these TJ proteins following AGP treatment, these changes may also contribute to functional changes observed with AGP in RBMECs monolayers.

As an intracellular signaling molecule, ZO-1 is required for the formation of adherens junctions as well as acts to regulate cingulin mediated myosin cytoskeleton changes (Cordenonsi et al., 1999; Ikenouchi et al., 2007). The increased level of ZO-1 protein observed at 6 and 12 hrs may be acting to regulate either adherens junctions or secondary TJ accessory proteins to enhance the functional tightness of the barrier. In this study, we showed that the high concentration AGP treatment increased TJ protein expression and decreased permeability to a larger extent than the treatment with the low concentration. This suggests that during inflammation the increased circulating AGP works to enhance BBB integrity. Overall, our findings herein indicate that AGP acts directly on brain endothelial cells by changing expression of TJ proteins in a concerted beneficial manner that is both time- and dose-dependent. Therefore, our results suggest a protective role by AGP during normal conditions and more so during an inflammatory state, which may be necessary to offset the effects of pro-inflammatory cytokines that disrupt the BBB integrity and decreased expression of TJ proteins.

While the physiological level of AGP in Sprague-Dawley (SD) rat has been reported to be 200–300 μg/ml (Schmid, 1975), we have detected levels of 25–30 μg/ml in naïve SD animals. We found that the average AGP serum levels rise 10- to 30-fold (300 – 900 μg/ml) in the female SD rats with an inflammatory-related chronic liver fibrosis. These values correspond to the two AGP concentrations (50 and 500 μg/ml) used for the treatment on the RBMEC monolayers. In our *in vitro* study, there was no significant degradation in AGP levels during the 72 hr treatment of RBMECs, as indicated by quantification of AGP in supernatant using Pro-Q® Emerald 300 glycoprotein stain (Figure 1). Not surprising, decreased levels of circulating AGP with a terminal half-life of 19.3 ± 1.5 hr was reported in an *in vivo* study of administered human AGP in male Holtzman rats (Keyler et al., 1987). While the source of increased AGP in the present study with the high-dose AGP-treated RBMEC is unclear (Figure1), it may be a stimulatory reaction by RBMECs to secret AGP.

Proinflammatory cytokines like IL-6 and IL-1β have been studied for their regulatory effects of AGP production in the liver. In this study, we examined the potential for cross-talk, between cytokines and acute phase proteins, by measuring cytokine expression in AGP-treated RBMECs. While the high concentration of AGP stimulated a dramatic increase of IL-6 mRNA expression in RBMECs, the protein level of IL-6 in cell culture supernatant at all-time points tested was below detection, using Quantikine® ELISA kits (R&D systems; Minneapolis, MN). Therefore, AGP may inhibit the translation and/or accelerate degradation process of IL-6 mRNA, which leads to the undetectable protein in culture supernatant of

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RBMEC monolayer at the time points tested (3–72 hr). Although mRNA changes were detected, protein levels of IL-1β and TNF-α were also not detected using the same methodology. Sensitivity of the ELISA kits for cytokines (IL-1β, TNF-α, and IL-6) are: 31.2 pg/ml, 6.25 pg/ml, and 15.5 pg/ml; respectively. Interestingly, Boutten et al. reported that AGP potentiates LPS-induced secretion of IL-1β, IL-6 and TNF-α by human monocytes and macrophages while AGP alone showed little or no effect on the production of the three cytokines (Boutten et al., 1992). The different AGP effects to cytokine production may be due to different responses from the various cell types or the necessity for a co-stimulatory factor like LPS. To our knowledge, this is the first report that AGP directly stimulates proinflammatory cytokine expression in brain endothelial cells at the RNA levels.

In order to gain an understanding of how AGP exerts its effects on the brain microvasculature, we also looked at transcription factor (TF) and DNA binding activity. Our results showed that AGP had a direct effect in DNA binding activity of transcription factor activator protein (AP)-1. Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra1, Fra2) proteins are primary components of the AP-1 transcription factor family (Karin et al., 1997). Specifically, c-Jun proteins can form AP-1 homodimers or heterodimers with c-Fos. These dimers can directly bind to their target promoters at specific DNA elements such as TGAGTCA (classical AP-1 site) to regulate target gene expression (Glover and Harrison, 1995). The heterodimer (c-Jun: c-Fos) has a higher binding affinity to the DNA consensus sequence compared to the homodimer (c-Jun: c-Jun) and the AP-1 DNA binding activity is regulated by phosphorylation of c-Fos and c-Jun. Overall, the high dose of AGP, which correlates to an inflammatory state, showed a larger change in c-Fos activity than the lower dose of AGP, i.e. the normal physiological level. AP-1 regulates many proinflammatory cytokine genes (TNF-α, IL-1 and IL-6 etc.) and plays a pivotal role during chronic inflammation (Ip and Davis, 1998). This is supported by the findings in another *in vitro* study, which showed that AGP possesses pro- and anti-inflammatory activities by stimulating or inhibiting TNF-α and IL-10 production via LPS stimulated peripheral blood mononuclear leukocytes (Pukhal'skii et al., 2000). In the present study, RBMECs had an acute response on c-Fos activation after 1hr at both AGP doses, while only the high concentration showed activation of both c-Fos and c-Jun. This c-Fos/c-Jun activation most likely leads to formation of high-affinity heterodimers, with increased DNA-binding activity, results in the decreased expression of ZO-1 protein seen at 3 hrs. The significant decrease in c-Fos activity seen at 3 hrs inversely correlated with the increase in ZO-1 mRNA observed at 3 hrs and subsequent protein expression at 6 and 12 hrs. These results are supported by Chen et al., who reported AP-1 activation was shown to decrease ZO-1 expression (mRNA and protein), in Caco-2 intestinal epithelial cells, via transcriptional repression of the ZO-1-promoter (Chen et al., 2008). Together, these results show that pro-inflammatory AGP has a direct effect on AP-1 binding activity and downstream transcription regulation of TJ proteins in the brain microvessel endothelium. Whether these changes in AP-1 occur due to its phosphorylation, requires further investigation.

In conclusion, this study has shown that AGP has direct effects on permeability, TJ protein expression, and transcriptional factor regulation in our *in vitro* BBB model, i.e. primary RBMECs. In particular, we show AGP enhances brain endothelial function by reinforcement of the monolayer integrity via increased TJ protein expression. Furthermore, our results

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suggest that the changes in TJ protein expression may, in part, occur via regulation of AP-1 DNA binding activity on the primary brain endothelial cells. Further studies will help in understanding the intracellular signaling pathways and how AGP exerts its protective role on the BBB.

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Research highlights

1. AGP decreased 20% paracellular permeability across RBMEC monolayers.
2. RBMECs showed a biphasic response in occludin expression following AGP treatment.
3. AGP changed ZO-1 expression of RBMECs in a dose- and time-dependent manner.
4. AGP significantly changed transcription factor AP-1 DNA binding activity.

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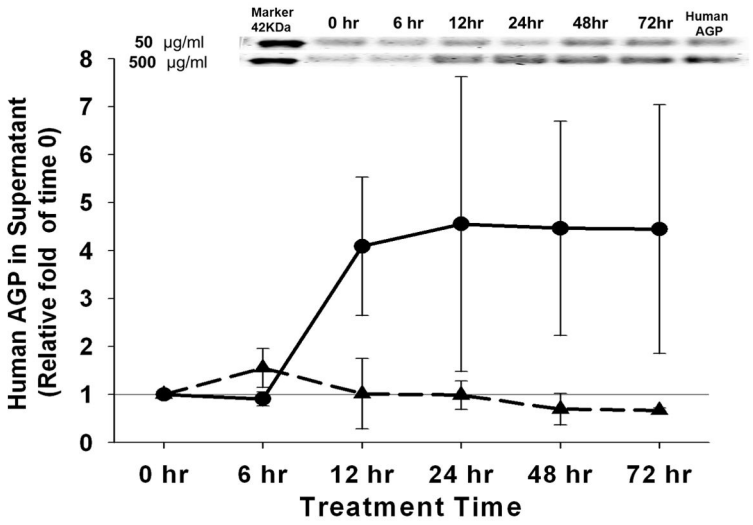

Figure 1. Human AGP Concentration in the Supernatant of RBMECs Treatment

Emerald 300 glycoprotein staining was performed on supernatant collected from RBMECs treated with α-1-acid glycoprotein (AGP) for 6, 12, 24, 48 and 72 hrs. AGP levels in the supernatant of RBMECs treated with 50 µg/ml (long dash line) and 500 µg/ml (solid line) are presented as fold of the human AGP band density at time zero (0 hr). Representative blots are shown above the plot; n=4.

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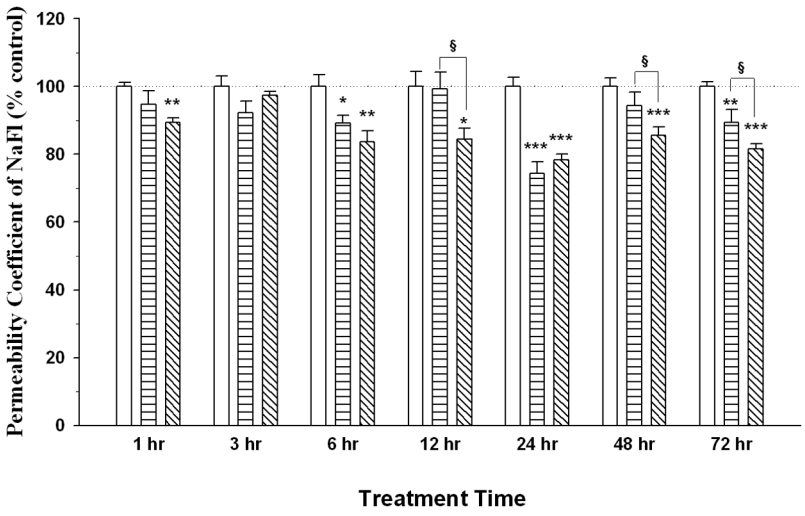

Figure 2. Permeability Study in AGP Treated RBMEC Monolayers

Sodium fluorescein (NaFl) permeability study was performed on RBMECs monolayers treated with α-1-acid glycoprotein (AGP) for 1, 3, 6, 12, 24, 48 and 72 hrs. Permeability coefficient (PC) values for AGP treatment at 50 μg/ml (horizontal bars) and 500 μg/ml (diagonal line) are presented as % of control monolayers (open bars). Absolute PC values for controls are $9.2 \pm 3.5 \times 10^{-4}$ cm/min. Data represents mean +/− SEM with statistical analysis using one-way ANOVA followed by Student Newman–Keuls post-hoc analysis. * indicates p-value <0.05; ** indicates p-value <0.01; *** indicates p-value <0.001 when compared to corresponding control; § indicates p-value <0.05 when compared to 50 μg/ml AGP at respective time points; n = 4–8.

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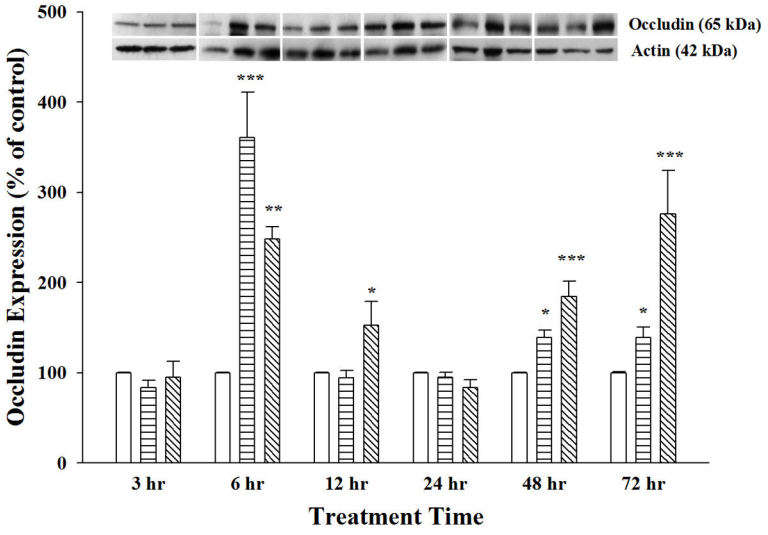

Figure 3. Biphasic Expression of Occludin Protein

Occludin protein expression was determined on cell lysates collected from RBMECs treated with α-1-acid glycoprotein (AGP) for 3, 6, 12, 24, 48 and 72 hrs, using Western blot analysis. AGP treatment at 50 μg/ml (horizontal bars) and 500 μg/ml (diagonal line) were compared to control monolayers (open bars). Occludin protein expression was normalized by β-actin expression with representative blots shown above the bar graph. Statistical analysis was done using one-way ANOVA with Student Neuman–Keuls post-hoc analysis. * indicates p-value <0.05; ** indicates p-value <0.01; *** indicates p-value <0.001 when compared with respective control; n = 4–9. Two-way ANOVA results indicate that occludin protein levels are significantly different following 50 μg/ml AGP treatment versus 500μg/ml treatment at 6, 12 and 72 hrs.

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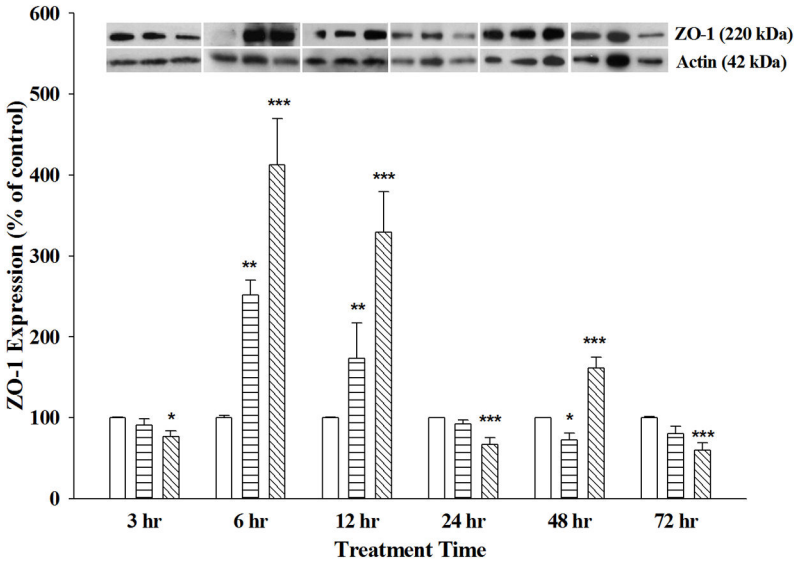

Figure 4. ZO-1 Protein Expression Changes in AGP-treated RBMECs

ZO-1 protein expression was determined on cell lysates collected from RBMECs treated with α-1-acid glycoprotein (AGP) for 6, 12, 24, 48 and 72 hrs, using Western blot analysis. AGP treatment at 50 μg/ml (horizontal bars) and 500 μg/ml (diagonal line) were compared to controls (open bars). ZO-1 protein expression was normalized by β-actin expression with representative blots shown above the bar graph. Statistical analysis used One-way ANOVA with Student Neuman Keuls post-hoc. * indicates p-value <0.05; ** indicates p-value <0.01; *** indicates p-value <0.001 compared to respective control; n = 3–7. Two-way ANOVA results indicate that ZO-1 protein levels are significantly different following 50 μg/ml AGP treatment versus 500μg/ml treatment at 6 and 12 hrs.

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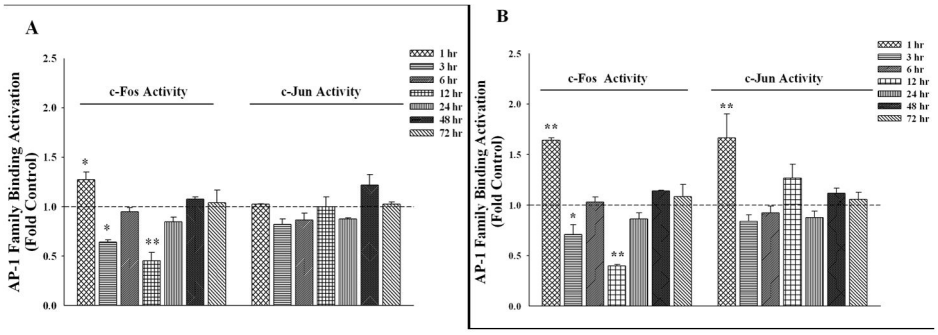

Figure 5. AGP Effects on AP-1 Family DNA Binding Activity in RBMECs

c-Fos and c-Jun DNA binding activity was examined using nuclear extract collected from RBMECs treated with α-1-acid glycoprotein (AGP); 50 μg/ml (A) or 500μg/ml (B) for 1, 3, 6, 12, 24, 48, and 72 hrs (See *materials and methods*). AGP stimulates/inhibits nuclear c-Fos (left side) and c-Jun (right side) activity toward their consensus sequence (TRE) in a time- and concentration-dependent manner. Both 50 μg/ml (A) and 500μg/ml (B) AGP increased c-Fos activity at 1 hr while inhibition of c-Fos was seen at 3 and 12 hrs. An increase of c-Jun activity was observed following high-concentration AGP treatment at 1 hr while the low-concentration AGP did not change c-Jun activity during the treatment period tested. * indicates p-value <0.05; ** indicates p-value <0.01; *** indicates p-value <0.001, compared with respective control; n = 2–4.

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Table 1

Primer sets for Real-time PCR

Target	Forward (5′ to 3′)	Reverse (5′ to 3′)	Size (bp)	Reference
Cytokines				
IL-10	GCCAAGCCTTGTCAGAAATGA	TTTCTGGGCCATGGTTCTCT	75	*(Gayle et al., 2004); NM_012854*
TNF-α	TGGCCAATGGCCATGGATCTCAA	ATGAAGTGGCAAATCGGCTGAC	147	*IDT; NM_012675.2*
IL-1β	TGTCCTGTGTGATGAAAGACGG	TTGGGTATTGTTTGGGATCCA	69	*IDT; NM_031512.2*
IL-6	AGCCACTGCCTTCCCTACTT	GCCATTGCACAACTCTTTTCTC	154	*(Lin et al., 2009); NM_012589.1*
TJ Proteins				
Occludin	GTTTACTGGCAGAACTCGAC	CCAGCATCTGTCTAGGTTTTC	247	*IDT; NM031329.2*
ZO-1	GGCTGTCTCAACTCCTGTAA	CGCCAGCTACAAATATTCCG	299	*IDT; NM_001106266.1*
Claudin-2	TATGTTGGTGCCAGCATTGT	ACTCCACCCACTACAGCCAC	266	*(Abuazza et al., 2006); NM_001106846.2*
Claudin-5	CGCTTGTGGCACTCTTTGT	ACTCCCGGACTACGATGTTG	168	*(Wongdee et al., 2010); NM_031701.2*
JAM-1	ACCCCGTGACAGCCTTTGATA	TCAGCTCCACATCATCCATGT	105	*IDT; NM_053796.1*
GAPDH	TGACTCTACCCACGGCAAGTTCAA	ACGACATACTCAGCACCAGCATCA	141	*IDT; NM_017008.3*

IDT: Integrated DNA Technologies.

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Table 2

Real-time PCR (qPCR) Data

Treatment	50 μg/ml AGP					500 μg/ml AGP				
Target Gene	**3 hrs**	**6 hrs**	**12 hrs**	**24 hrs**	**48 hrs**	**3 hrs**	**6 hrs**	**12 hrs**	**24 hrs**	**48 hrs**
Cytokines										
IL-10	1.17 ± 0.1	1.34 ± 0.3	0.72 ± 0.1	1.03 ± 0.2	0.97 ± 0.0	**0.47** ± 0.0	1.68 § ± 0.0	1.89 † ± 0.2	1.02 ± 0.1	**0.49** * ± 0.1
TNF-α	**0.25** ± 0.1	**1.99** † ± 0.1	1.22 ± 0.2	0.74 ± 0.1	1.18 ± 0.3	**0.40** ± 0.0	1.71 § ± 0.1	1.69 § ± 0.1	0.97 ± 0.3	0.97 ± 0.0
IL-1β	0.84 ± 0.1	**3.42** § ± 1.1	0.93 ± 0.1	**0.29** ± 0.1	**2.02** ± 0.6	**2.27** ±0.9	1.81 ± 0.7	**12.36** † ± 0.4	**4.55** † ± 0.3	1.46 ± 0.2
IL-6	0.96 ± 0.0	**2.46** ± 0.1	**3.06** ± 0.1	**2.45** ± 0.5	**2.63** ± 0.4	1.49 ±0.6	**30.45** † ± 4.4	**8.56** § ± 1.0	**26.32** † ± 2.9	**20.09** § ± 3.7
TJ Proteins										
Occludin	1.43 ± 0.4	0.90 ± 0.0	1.60 § ± 0.2	1.01 ± 0.2	0.64 ± 0.2	0.66 ± 0.1	0.82 ± 0.1	0.58 * ± 0.1	**0.47** † ± 0.2	**0.36** † ± 0.1
ZO-1	**2.19** * ±0.7	0.66 * ± 0.1	1.22 ± 0.1	0.75 ± 0.2	0.73 ± 0.1	**3.4** § ± 0.9	0.97 ± 0.1	0.90 ± 0.2	**0.38** † ± 0.1	**0.39** † ± 0.1
Claudin-2	1.45 ± 0.1	**0.48** † ± 0.2	1.08 ± 0.1	**0.24** † ± 0.1	**0.29** † ± 0.0	0.73 ± 0.0	0.76 ± 0.1	1.09 ± 0.0	**0.48** † ± 0.0	**0.22** † ± 0.0
Claudin-5	1.20 ± 0.1	1.01 ± 0.1	1.34 ± 0.4	1.89 ± 0.4	**5.87** † ± 0.7	0.54 ± 0.1	1.36 ± 0.1	0.54 * ± 0.1	0.85 ± 0.2	**2.42** § ± 0.3
JAM-1	1.08 ± 0.2	0.64 ± 0.1	1.60 ± 0.2	**0.28** † ± 0.1	**0.38** § ± 0.2	0.65 ± 0.3	**0.45** § ± 0.1	1.44 * ± 0.2	**0.47** § ± 0.1	**0.32** † ± 0.1

Results have been calculated using the Pfaffl method to produce a ratio (treatment: control) that represents mean ± SEM for the effect of the treatment on mRNA expression for the respective target gene with GAPDH as the reference gene.

* indicates p-value <0.05;

§ indicates p-value <0.01;

† indicates p-value <0.001, compared with respective control; ratio means larger than two or less than 0.5 are bolded; n= 3–6.