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Age-related decrease in cerebrovascular-derived neuroprotective proteins: effect of acetaminophen

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Abstract

As the population ages, the need for effective methods to maintain brain function in older adults is increasingly pressing. Vascular disease and neurodegenerative disorders commonly co-occur in older persons. Cerebrovascular products contribute to the neuronal milieu and have important consequences for neuronal viability. In this regard vascular derived neuroprotective proteins, such as vascular endothelial growth factor (VEGF), pigment epithelium-derived factor (PEDF), and pituitary adenylate cyclase activating peptide (PACAP) are important for maintaining neuronal viability, especially in the face of injury and disease. The objective of this study is to measure and compare levels of VEGF, PEDF and PACAP released from isolated brain microvessels of Fischer 344 rats at 6, 12, 18, and 24 months of age. Addition of acetaminophen to isolated brain microvessels is employed to determine whether this drug affects vascular expression of these neuroprotective proteins. Experiments on cultured brain endothelial cells are performed to explore the mechanisms/mediators that regulate the effect of acetaminophen on endothelial cells. The data indicate cerebrovascular expression of VEGF, PEDF and PACAP significantly decreases with age. The age-associated decrease in VEGF and PEDF is ameliorated by addition of acetaminophen to isolated brain microvessels. Also, release of VEGF, PEDF, and PACAP from cultured brain endothelial cells decreases with exposure to the oxidant stressor menadione. Acetaminophen treatment upregulates VEGF, PEDF and PACAP in brain endothelial cells exposed to oxidative stress. The effect of acetaminophen on cultured endothelial cells is in part inhibited by the selective thrombin inhibitor hirudin. The results of this study suggest that acetaminophen may be a useful agent for preserving cerebrovascular function. If a low dose of acetaminophen can counteract the decrease in vascular-derived neurotrophic factors evoked by age and oxidative stress, this drug might be useful for improving brain function in the elderly.

Keywords

Acetaminophen; neuroprotective; endothelial cells; microvessels; VEGF; PEDF; PACAP

Introduction

Advances in medical research and new treatment modalities have significantly increased life expectancy. In 2011, 77 million baby boomers will begin to turn 65, and by 2025, will represent 20.6 percent of the US population (Bierman et al., 2012). As life expectancy has increased, so too has the prevalence of cognitive decline and dementia, largely in the form of

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the neurodegenerative disease, Alzheimer's disease (AD), which now affects almost 50% of adults over the age of 85 in the US (Bishop et al., 2010; Hebert et al., 2003). Despite the fact that aging is a primary risk factor for developing cognitive decline and AD, our knowledge as to age-related mechanisms that contribute to cognitive decline is still incomplete.

Numerous studies link vascular risk factors to cognitive decline and dementia in the elderly (de la Torre, 2010; Helzner et al., 2009; Hofman et al., 1997; Iadecola, 2010; Miller, 1999; Pansari et al., 2002; Rhodin and Thomas, 2001; Schmidt et al., 2000; Sparks et al., 1990). Age-related changes in cerebrovascular structural proteins such as collagen as well as compositional alterations in proteins and lipids have been documented (Alba et al., 2004; Brown and Thore, 2011; Farkas et al., 2006; Kalaria and Pax, 1995; Mooradian, 1994; Uspenskaia et al., 2004). Similarly, age-related functional changes in the cerebrovasculature including altered vascular reactivity, inflammation and abnormal blood-brain barrier transport have also been reported (Banks and Kastin, 1985; Mooradian, 1988; Price et al., 2004; Tripathy et al., 2010). Cardiovascular risk factors negatively affect vascular function suggesting that deleterious age-related changes in brain vessel function directly impact neuronal function. Vascular products contribute to the neuronal milieu and have important consequences for neuronal viability (Grammas, 2011; Grammas et al., 1999). In this regard, brain endothelial cells are an important source of growth factors with neuroprotective properties including vascular endothelial growth factor (VEGF) (Carmeliet, 2003; Kallmann et al., 2002; Kim et al., 2004). Indeed, studies comparing brain-derived endothelial cells to systemically-derived endothelial cells demonstrate that expression of neurotrophic factors is largely a feature of brain-derived not peripheral endothelial cells (Kallmann et al., 2002). In aging and AD the cerebrovasculature releases factors with demonstrated neurotoxic properties (Grammas et al., 1999; Tripathy et al., 2010). The effect of aging on production of vascular-derived neurotrophic proteins is unknown.

Neuroprotective proteins are important for maintaining neuronal viability, especially in the face of injury and disease. A number of proteins have been shown to possess neuroprotective/neurotrophic properties including VEGF, pigment epithelium-derived factor (PEDF), and pituitary adenylate cyclase-activating polypeptide (PACAP) (Barnstable and Tombran-Tink, 2004; Shintani et al., 2005; Sköld and Kanje, 2008). VEGF has direct effects on neuronal growth, axonal outgrowth and neuroprotection (Jin et al., 2000; Sanchez et al., 2010; Sondell et al., 2000). Application of VEGF protects neurons against ischemic, hypoxic, and excitotoxic injury (Jin et al., 2000; 2001; Matsuzaki et al., 2001). Mice that express reduced VEGF levels develop adult-onset motor neuron degeneration, similar to the human neurodegenerative disease amyotrophic lateral sclerosis (ALS), and VEGF overexpression delays neurodegeneration and prolongs survival in ALS mice (Oosthuysen et al., 2001; Wang et al., 2007). PEDF is a multifunctional protein with neurotrophic, anti-oxidative and anti-inflammatory properties (Barnstable and Tombran-Tink, 2004; Gettins et al., 2002; Houenou et al., 1999; Sanchez et al., 2012). PACAP38 is a multifunctional anti-inflammatory and anti-apoptotic neuropeptide widely distributed in the nervous system (Arimura et al., 1991; Somogyvari-Vigh and Reglodi, 2004). *In vitro* studies involving exposure of neuronal cultures to various neurotoxins including amyloid beta, hydrogen peroxide and glutamate reveal the strong neuroprotective effects of PACAP38 (Onoue et al., 2002; Sanchez et al., 2009; Shintani et al., 2005).

Levels of neuroprotective/neurotrophic proteins in the brain are not static but are dynamically regulated by aging, inflammation and exercise (Cortese et al., 2011; Gomez-Pinilla et al., 2008). Many of the beneficial effects of diet and exercise on the brain appear to be related to increases in expression of neuroprotective proteins (Gomez-Pinilla et al., 2008; Lau et al., 2011). Therapeutic approaches for improving brain function and staving off neurodegenerative disease are focused on reducing oxidative stress and inflammation

through diet/life style changes and drug treatment (Ahlskog et al., 2011; Gorelick, 2010). Acetaminophen is a widely used over the counter antipyretic and analgesic drug with antioxidant and anti-inflammatory properties (Maharaj et al., 2006; Mancini et al., 2003; Tripathy and Grammas, 2009). Work from our laboratory also shows that low dose acetaminophen reduces inflammatory protein release from cultured brain endothelial cells exposed to oxidant stress (Tripathy and Grammas, 2009).

The objective of this study is to measure and compare levels of VEGF, PEDF and PACAP released from the cerebrovasculature of 6, 12, 18, and 24 months of age Fischer 344 rats. Furthermore, addition of acetaminophen to isolated brain microvessels is employed to determine whether this drug affects vascular expression of neuroprotective proteins. Finally, *in vitro* experiments are performed to explore the mechanisms/mediators that regulate the effect of acetaminophen on endothelial expression of VEGF, PEDF and PACAP.

Methods

Microvessel isolation and brain endothelial cell cultures

Cortical microvessel preparations were obtained from 6, 12, 18 and 24 month old Fisher 344 rat brains, as we have previously described (Diglio et al., 1982). Briefly, brains were isolated from groups of 18 rats and cerebral cortices were placed in cold Hanks' Balanced Salt Solution (HBSS), scissor minced, and homogenized. The homogenate was centrifuged at 3000 g for 15 min at 4°C, the supernatant discarded and the pellet resuspended in cold HBSS containing 15% dextran and 5% fetal bovine serum (FBS). The suspension was then centrifuged at 4500 g for 20 min at 4°C. The supernatant discarded and the pellet filtered through a 150 µm and onto a 53 µm nylon mesh sieve. Microvessels were collected off the 53 µm sieve in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and dimethyl sulfoxide and stored frozen in liquid nitrogen until use. The purity of the microvessel preparations was assessed by phase contrast microscopy.

Brain endothelial cells were obtained from rat brain microvessels, as previously described (Diglio et al., 1982, 1993). Endothelial cell cultures were obtained from cerebrovascular explant cultures. Isolated microvessels were suspended in 10 ml HBSS containing 0.05% collagenase type II for 60 min at 37°C with periodic shaking. The suspension was centrifuged at 200 g for 10 min and the resultant pellet was then resuspended in DMEM plus 10% FBS and the dissociated microvessel preparation consisting of individual cells and vessel fragments was aliquoted into 100 mm tissue culture dishes and incubated overnight at 37°C in a humidified 5% CO₂-95% air atmosphere. After incubation, the cultures were washed with HBSS to remove non-adherent cells and vessel fragments. Fresh complete medium was then added, and the cultures were monitored daily using phase-contrast microscopy. Endothelial cell cultures were identified and serially cloned using penicylinders with 0.25% trypsin. The purity of the endothelial cultures was confirmed using antibodies to endothelial cell surface antigen Factor VIII. Cells (passages 7–10) were placed in serum-free media prior to treatments.

Preparation of conditioned media and lysates

Microvessels from 6, 12, 18 and 24 month old rats were thawed and centrifuged at 2000 g for 15 min. Microvessels (50 µg/sample) were washed three times with cold HBSS and resuspended in 500 µl serum-free DMEM containing 1% lactalbumin hydrolysate (LAH) with or without 100 µM of acetaminophen for 8 h at 37°C in a 5% CO₂ incubator and then centrifuged (2000 g). The supernatant and pellet were collected and used for ELISA and western blotting, respectively.

Measurement of VEGF, PEDF and PACAP38 by ELISA

Indirect ELISA was used to detect VEGF, PEDF and PACAP38. Briefly 100 µl of supernatant collected from conditioned media were placed onto custom-coated ELISA 96-well plates with sodium bicarbonate buffer (0.1 M) and incubated overnight at 4°C, as described in our published protocol (Sanchez et al., 2008; Tripathy et al., 2010). Plates were blocked with 1% bovine serum albumin (BSA) for 45 min at 37°C. Following washing to remove unbound BSA, plates were incubated with 200 µl of primary antibodies for VEGF, PEDF (ab1316 and ab14993, AbCam, Cambridge, MA) or PACAP38 (sc25439, Santa Cruz Biologicals, Santa Cruz, CA) diluted (1:1000) in carbamate buffer and incubated at 37°C for 1 h. After extensive washing to remove unbound antibody, plates were incubated with 200 µl goat anti-rabbit IgG coupled with horseradish peroxidase (Bio-Rad, Hercules, CA 1:1000 dilution) and incubated for 45 min at 37°C, in the dark. The reaction was developed by adding 200 µl/well of *o*-phenylene diamine H₂O₂ (Pierce, Chemicon, CA, USA) for 20 min. Optical density was measured 450 nm using a microplate ELISA reader (BIO-RAD). Samples were assayed in triplicate.

Western blot

Proteins were extracted from rat microvessels or endothelial cells using lysis buffer containing 150 mM sodium chloride, 50 mM Tris, 1% NP-40, and 2 mM phenylmethyl sulfonylfluoride. Protein samples (20 µg) were mixed with sample buffer (25 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 2% β-mercaptoethanol, and 0.02% bromophenol blue) boiled for 3 minutes, resolved in 10% SDS polyacrylamide electrophoresis mini gel and transferred to PVD membrane. After transfer, the membranes were blocked in Tris-buffered saline containing 0.25% Tween-20 (TBST) and 4% nonfat dry milk at room temperature for 2 h, and subsequently incubated with antibodies to thrombin (1:200, Cat. No: HYB 109-04, Antibody Shop, Denmark) or GAPDH (1:1000, Cat. No: MAB374, Millipore, Billerica, MA) diluted in TBST plus 2% nonfat dry milk overnight at 4°C. Bound antibody was detected using secondary antibodies conjugated to horseradish peroxidase and developed with chemiluminescence.

RT-PCR

Total RNA was extracted from the treated microvessels using the Trizol method (Invitrogen, Carlsbad, CA). RNA (1 µg) was reverse transcribed using oligo dT primers (Roche Applied Science) and amplified by PCR (2 min-95°C, 30 cycles of 45 sec- 94°C, 1 min-60°C, 2min-72°C and final extension of 5min at 72°C) using a Mastercycler system (Eppendorf). Primers used for PCR are shown in Table 1. The PCR products were visualized on a 1.5% agarose gel using UV trans-illumination.

Statistical analysis

Data from each experiment are expressed as mean ± standard deviation (SD). The one-way ANOVA followed by Bonferroni's multiple comparison tests for multiple samples were employed. Statistical significance was determined at $p < 0.05$.

Results

Expression of VEGF, PEDF and PACAP decreases with age

Fisher 344 rats at 6 months of age were used as a baseline (young) age and results were compared to those obtained from rats at 12, 18 and 14 months of age. Brain microvessels isolated from Fischer 344 rats (6, 12, 18 and 24 months of age) were incubated in serum free media for 8 h. VEGF, PEDF and PACAP released into the media were determined by indirect ELISA. Overall, there was a significant ($p < 0.05$ -0.001) decrease in neuroprotective

proteins in microvessels isolated from 24 month old rats compared to 6 month old animals (Fig. 1). However, the pattern and magnitude of the change varied with specific protein and age. Expression of both VEGF and PEDF peaked in animals 12 months of age and then declined (Fig 1a, Fig 1b). In contrast, PACAP levels were highest in young 6 month old rats and progressively declined with age (Fig 1c).

Age-dependent effect of acetaminophen on vascular-derived VEGF and PEDF

Addition of acetaminophen to the isolated microvessels from animals 6 and 12 months of age did not significantly affect levels of VEGF and PEDF released into the media. In contrast, acetaminophen caused a large and significant ($p<0.05$ - 0.01) increase in both VEGF and PEDF in cerebral microvessels isolated from 18 and 24 month old rats (Fig. 2). Addition of acetaminophen to isolated brain microvessels from rats 6, 12, 18, or 24 months of age did not affect levels of PACAP (data not shown).

Acetaminophen improves oxidative stress-induced decreases in VEGF, PEDF and PACAP in cultured brain endothelial cells

Cultured brain endothelial cells were examined for their ability to release VEGF, PEDF, and PACAP and to determine the effect of oxidative stress and acetaminophen on this response. Conditioned media collected after 3 h incubation demonstrated that untreated brain endothelial cells have basal release of VEGF (Fig 3a), PEDF (Fig. 3b) and PACAP (Fig. 3c). Exposure of cultured brain endothelial cells to the oxidant stressor menadione ($25\text{ }\mu\text{M}$) resulted in a significant ($p<0.01$ - 0.001) decrease in all three proteins. Co-incubation of acetaminophen (50 – $300\text{ }\mu\text{M}$) with menadione produced a dose-dependent increase in VEGF and PEDF; the effect was maximum at $300\text{ }\mu\text{M}$ (Fig. 3a, Fig. 3b). The ability of acetaminophen to block the decrease in PACAP evoked by menadione was less pronounced and only significant at $100\text{ }\mu\text{M}$ acetaminophen (Fig. 3c).

Thrombin mediates the effect of acetaminophen on endothelial cell VEGF levels

Based on published studies that have identified thrombin as a multifunctional mediator of endothelial reactivity and a regulator of VEGF expression (Dupuy et al, 2003; Maragoudakis et al., 2002; Naldini et al., 2000), we determined if acetaminophen affected thrombin expression in cultured brain endothelial cells. Endothelial cells were treated with increasing concentrations of acetaminophen (0 – $1000\text{ }\mu\text{M}$) for 24 h. Total RNA isolated was transcribed and PCR performed with thrombin specific primers. There was a significant ($p<0.05$) increase in thrombin expression with $100\text{ }\mu\text{M}$ acetaminophen; this increase was maximal ($p<0.01$) at $500\text{ }\mu\text{M}$ acetaminophen. Addition of the thrombin inhibitor hirudin significantly ($p<0.01$) decreased both basal and acetaminophen-induced thrombin expression (Fig. 4).

To further determine if the increase in VEGF evoked by acetaminophen was mediated, in part, by thrombin, cells were co-incubated with acetaminophen and hirudin for 24 h. RNA isolated from treated cells was reverse transcribed and VEGF expression determined. Acetaminophen (100 – $1000\text{ }\mu\text{M}$) significantly increased VEGF expression that was blocked by co-incubation with hirudin ($p<0.001$) (Fig. 5).

An examination of brain microvessels isolated from Fischer 344 rats (6, 12, 18 and 24 months of age) showed that thrombin levels were significantly ($p<0.01$ – 0.001) decreased in older animals (Table 2).

Discussion

Age-related cerebrovascular dysfunction contributes to stroke, cerebral amyloid angiopathy, and cognitive decline (Vasilevko et al., 2010; Zlokovic, 2008). Data from brain imaging

studies suggest that cerebrovascular dysfunction precedes cognitive decline and the onset of neurodegenerative changes in the age-associated disorder, AD (Bell and Zlokovic, 2009; de la Torre, 2004). The incidence of cerebrovascular disease is highest amongst the elderly (Di Napoli and Shah, 2011). Dysfunction of the cerebrovasculature with aging is further highlighted by studies showing that brain microvessels from aged rodents as well as from aged humans release factors that are toxic to neurons in culture (Tripathy et al., 2010; Grammas et al., 1999). In this regard, the cerebral endothelium is increasingly documented as a source of soluble mediators that affect neuronal viability (Guo and Lo, 2009; Guo et al., 2008; Iadecola, 2004; Navaratna et al., 2011). Several factors known to protect neurons and to support neuronal growth and differentiation are released spontaneously from human cerebral endothelial cells, including VEGF and brain-derived neurotrophic factor (BDNF) (Carmeliet, 2003; Kallmann et al., 2002; Kim et al., 2004). Thus, perturbation of cerebral endothelial function is likely to have important implications in disease and aging. In this regard, a recent study documents a decrease in cerebrovascular-derived BDNF in an animal model of diabetes (Navaratna et al., 2011). In the current study, we show a significant decline in cerebrovascular-derived VEGF, PEDF and PACAP with age. These data are consistent with literature showing an age-related decrease in neuroprotective proteins such as VEGF, PEDF and PACAP in the CNS (Latimer et al., 2011; Van Kirk et al., 2011; Wu et al., 2006).

Reduction in cerebrovascular expression of VEGF, PEDF and PACAP could compromise the ability of the aged brain to respond to injury and/or disease, as the neuroprotective functions of these proteins have been well documented. In this regard, application of VEGF causes axonal outgrowth and protects against ischemic, hypoxic, and excitotoxic injury (Jin et al., 2000; 2001; Khaibullina et al., 2004; Matsuzaki et al., 2001; Sondell et al., 2000). VEGF has also been implicated in neurodegenerative diseases such as Parkinson's, Alzheimer's and multiple sclerosis (Sanchez et al., 2010; Seabrook et al., 2010; Tarkowski et al., 2002; 2006). *In vivo*, the neuroprotective function of PEDF against ischemic damage has been demonstrated in a rat middle cerebral artery occlusion model where infarct volume and degree of edema are significantly reduced in rats transfected to over-express the PEDF gene (Sanagi et al., 2008). PEDF protects cortical neurons from oxidant injury *in vitro* (Sanchez et al., 2012). PACAP has been shown to reduce axonal damage in traumatic brain injury and reduce infarct size in ischemia (Mao, 2011; Reglodi et al., 2002). PACAP is among the neurotrophic genes down regulated in transgenic mouse models of AD. Neuronal viability and the ability of neurons to respond to injury and/or neuroprotective factors reflect complex, multifaceted processes *in vivo*. Although it is difficult to extrapolate data focused on specific proteins (such as VEGF, PEDF, PACAP) to overall neuronal function, the importance of neuroprotective proteins to brain health is supported by studies that show the beneficial effects of exercise on brain function are in part, attributable to increases in neuroprotective proteins, such as VEGF (Gomez-Pinilla et al., 2008; Latimer et al., 2011; Lau et al., 2011). Thus, the ability of acetaminophen to enhance vascular expression of neuroprotective proteins, as we document here, may hold promise for improving vascular and neuronal function in CNS.

Although acetaminophen is a widely used drug, the cellular mechanisms that underlie its therapeutic effects remain incompletely understood. Acetaminophen can reduce cyclooxygenase products, primarily prostaglandin E2 in the CNS and in endothelial cells (Lucas et al., 2005). Acetaminophen has been shown to exert a protective effect on cultured brain endothelial cells exposed to oxidative stress. Incubation of brain endothelial cells with the superoxide-generating compound menadione (Warren et al., 2000) results in decreased cell viability, increased superoxide dismutase activity and increased release of inflammatory proteins including tumor necrosis factor- α and interleukin-1. Pretreatment of endothelial cells with acetaminophen prior to menadione exposure significantly increases survival of

endothelial cells, and reduces menadione-induced superoxide dismutase activity and release of inflammatory proteins (Tripathy and Grammas, 2009). Here we demonstrate that exposure of brain derived endothelial cells to acetaminophen ameliorates menadione-induced decrease in VEGF, PEDF and PACAP release *in vitro*. Because oxidative stress increases with age in the cerebral microcirculation (Tripathy et al., 2010), the ability of acetaminophen to improve age-related decline in microvessel-derived VEGF, PEDF, and PACAP may reflect antioxidant mechanisms.

These data are consistent with literature showing that acetaminophen has tissue-sparing capabilities and can function as an antioxidant. During post-ischemia-reperfusion of the heart, acetaminophen attenuates the damaging effects of peroxynitrite and hydrogen peroxide and limits protein oxidation (Jaques-Robinson et al., 2008). In the brain acetaminophen inhibits superoxide generation, lipid peroxidation and cell damage in the hippocampus in response to quinolinic acid (Maharaj et al., 2006). *In vitro*, acetaminophen protects dopaminergic neurons from oxidative damage evoked by acute exposure to 6-hydroxydopamine as well as hippocampal neurons exposed to amyloid-beta peptide-induced oxidative stress (Bisaglia et al., 2002; Locke et al., 2008). Conversely, a large literature indicates that high dose acetaminophen causes tissue damage by upregulation of oxidative stress and induction of noxious pro-inflammatory proteins (Jaeschke et al., 2003; Williams et al., 2010). Oxidative stress mediated by the metabolite N-acetyl-p-benzoquinoneimine is considered the main cause of acetaminophen-induced toxicity (Liu and Kaplowitz, 2006; Olaleye and Rocha, 2008). Mice deficient in superoxide dismutase are resistant to acetaminophen toxicity due to a reduction in activity of a key acetaminophen metabolizing enzyme (Lei et al., 2006). In the brain, overdose of acetaminophen causes a dramatic decrease of glutathione levels, ascorbic acid levels and superoxide dismutase activity (Nencini et al., 2007). Whether acetaminophen effects are beneficial or noxious appears to be largely dose-dependent.

The notion that endothelial cells are an early and direct target of acetaminophen is supported by work showing acetaminophen-induced sinusoidal endothelial cell injury precedes hepatocellular injury (Ito et al., 2003). The relative effects of acetaminophen on brain vasculature vs. brain parenchyma have not been determined. However, expression of COX-3, a new acetaminophen-sensitive isoform, is very high in brain tissue, with the cerebral endothelial cells showing the highest level of COX-3 expression (Kis et al., 2003) suggesting that the cerebrovasculature is an important target for acetaminophen action. It is likely that the cellular mechanisms used by acetaminophen to produce toxicity may, in a lower dose range, mediate its therapeutic effects. In this regard, toxicity resulting from high dose acetaminophen is in part mediated by thrombin (Ganey et al., 2007; Sparkenbaugh et al., 2011). Thus, we explored whether thrombin is a mediator of acetaminophen's beneficial effects on brain endothelial cells. Our data show that the increase in VEGF evoked by acetaminophen is inhibited by co-incubation with the selective thrombin inhibitor hirudin. Thrombin is a multifunctional angiogenic/inflammatory mediator (Dupuy et al., 2003; Maragoudakis et al., 2002; Naldini et al., 2000). This pleiotropic protein is a central mediator of angiogenesis in endothelial cells and has been shown to increase VEGF and NO (Dupuy et al., 2003; Olszewska-Pazdrak et al., 2010). Thus, induction of thrombin by acetaminophen is not unexpected. However, similar to acetaminophen, thrombin can have both beneficial and noxious effects, depending on concentration. High levels of thrombin are directly neurotoxic both *in vitro* and *in vivo* (Festoff et al., 2000; Mhatre et al., 2004; Reimann-Philipp et al., 2001). However, low doses of thrombin attenuate neuronal cell death *in vitro* and protect neurons from ischemic injury (Smirnova et al., 2001; Striggow et al., 2000; Vaughan et al., 1995). Acetaminophen, via thrombin, likely initiates a cascade of mediators/processes in endothelial cells because thrombin can affect numerous structural (integrins) and functional (matrix metalloproteinases) proteins in the vasculature (Dupuy et

al, 2003; Maragoudakis et al., 2002; Naldini et al., 2000). The precise nature of these mediators remains to be determined.

Conclusions

Endothelial dysfunction, and its attendant release of bioactive species, is increasingly implicated in disease pathogenesis. The activated endothelial cell in various clinical states has become a major target of human therapeutics (Schnitzer, 1998). Thus, targeting brain endothelial cell function in aging with the goal of increasing neuroprotective proteins is worthwhile. Vascular disease and neurodegenerative disorders commonly co-occur in older persons (Schneider and Bennett, 2010). As the population ages, the need for effective methods to maintain or even improve brain function in older adults is increasingly pressing. The data presented herein suggest that age-related changes in brain microvessels may contribute to an environment that is suboptimal for neuronal function. Although the data presented here do not directly demonstrate that acetaminophen's ability to alleviate age-related decreases in neurotrophic factors will improve neuronal health, the close proximity of neurons and non-neuronal cell types in the neurovascular unit allows intercellular interactions that likely directly affect CNS function (Zlokovic, 2008). The results of the current study, as well as previous work, suggest that acetaminophen may be a useful agent for preserving cerebrovascular function. In the current *in vitro* study, we utilized micromolar doses of acetaminophen. However, it should be borne in mind that *in vitro* experiments typically require higher concentrations of drug to achieve the desired effect. The next step is to determine an optimal dosing pattern *in vivo*; one which evokes the beneficial but not toxic effects of acetaminophen. The dosing challenge is particularly vexing in the elderly where drug metabolizing enzymes often function differently than in younger populations. If a low dose of acetaminophen can stabilize the brain vasculature and enhance its neuroprotective function, acetaminophen could be a useful tool for improving brain function in the elderly.

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Highlights

- Expression of VEGF, PEDF and PACAP decreases with age.
- PEDF and VEGF increase at 18 and 24 months of age in rat brain microvessels.
- Acetaminophen improves oxidative stress-induced decreases in VEGF, PEDF and PACAP.
- Thrombin mediates the effect of acetaminophen on endothelial cell VEGF levels.

Figure 1.

Brain microvessels isolated from Fischer 344 rats (6, 12, 18 and 24 months of age) were incubated in serum-free media with 1% LAH for 8 h. Conditioned media were analyzed by ELISA to measure (a) VEGF, (b) PEDF and (c) PACAP. Results are expressed as means $\pm$ SD from three separate experiments performed in triplicate. *p<0.05, **p<0.01, ***p<0.001 vs. 6 months of age.

Figure 2.

Brain microvessels isolated from Fischer 344 rats (6, 12, 18 and 24 months of age) were incubated in serum-free media with 1% LAH or in serum-free media plus 1% LAH and 100 μ M of acetaminophen (APAP) for 8 h. Conditioned media were analyzed by ELISA to measure (a) VEGF and (b) PEDF. Results are expressed as means $\pm$ SD from three separate experiments performed in triplicate. # p <0.05, ## p <0.01 vs. without APAP.

Figure 3.

Cultured brain endothelial cells were incubated with the oxidant stressor menadione (25 μM) in the presence or absence of increasing concentrations of acetaminophen (APAP; 0–300 μM) for 3 h and (a) VEGF, (b) PEDF and (c) PACAP released into the media determined by ELISA. Results are expressed as the mean ± SD from three separate experiments performed in triplicate. ##p<0.01, ###p<0.001 vs. cells without APAP or menadione; **p<0.01, ***p<0.001 vs. cells incubated plus menadione with and without APAP.

Figure 4.

Brain endothelial cells were incubated with increasing concentrations of acetaminophen (APAP, 100—1000 μM) with and without thrombin inhibitor hirudin (12 Units/ml) for 24 h. Total RNA extracted was reverse transcribed and amplified with thrombin primers. Loading equivalency was confirmed using GAPDH. Relative intensity of the bands is shown in bar graph. Results are expressed as means $\pm$ SD from three separate experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$ vs. control (no APAP or hirudin).

Figure 5.

Brain endothelial cells were incubated with increasing concentrations of acetaminophen (APAP, 100–1000 μ M) with and without thrombin inhibitor hirudin (12 Units/ml) for 24 h. Total RNA extracted was reverse transcribed and amplified with VEGF primers. Loading equivalency was confirmed using GAPDH. Relative intensity of the bands is shown in bar graph. Results are expressed as means $\pm$ SD from three separate experiments performed in triplicate. ** p < 0.01, *** p < 0.001 vs. control (no APAP or hirudin).

Table 1

Primers used for reverse-transcription PCR

Gene	Sequence	Amplicon size
Thrombin	Fwd 5' TGG GAG AGG AGA ACC ATG AC Rev 5' AGG GTG GGT ACA GAA TGC AG	339
VEGF	Fwd 5' GCC CAT GAA GTG GTG AAG TT Rev 5' TTT CTT GCC CTT TCG TTT TT	360
GAPDH	Fwd 5' ATG GGA AGCTGG TCA TCA AC Rev 5' GGA TGC AGGGAT GAT GTT CT	440

Table 2

Expression of thrombin in brain microvessels with age

Protein	6 month	12 month	18 month	24 month
Thrombin	716.3 ± 35.6	460.2 ± 61.1 **	531.1 ± 50.3 **	210.2 ± 54.3 ***

Protein lysates from isolated brain microvessels derived from Fischer 344 rats (6, 12, 18 and 24 months of age) were examined by western blot analysis for thrombin. Loading equivalency was confirmed using GAPDH. Results are expressed as means ± SD from three separate experiments performed in triplicate.

**
p<0.01,

p<0.001 vs. 6 month.