Molecular Pharmacology Fast Forward. Published on February 14, 2008 as doi:10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

MOL #42028

SIMVASTATIN PROTECTS AGAINST AMYLOID BETA AND HIV-1 TAT-INDUCED PROMOTER ACTIVITIES OF INFLAMMATORY GENES IN BRAIN ENDOTHELIAL CELLS

Ibolya E. András, GeunBae Rha, Wen Huang, SungYong Eum, Pierre-Olivier Couraud, Ignacio A. Romero, Bernhard Hennig, Michal Toborek

Molecular Neuroscience and Vascular Biology Laboratory, Department of Neurosurgery (I.E.A., G.B.R., W.H., S.Y.E., M.T.) and College of Agriculture (B.H.), University of Kentucky, Lexington, KY; Institut COCHIN, INSERM U567, Paris, France (P-O.C.); Department of Life Sciences, The Open University, Milton Keynes, United Kingdom (I.A.R.)

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Anti-inflammatory properties of simvastatin in HIV-1

Correspondence: Michal Toborek, MD. PhD, Molecular Neuroscience and Vascular Biology Laboratory, Department of Neurosurgery, University of Kentucky Medical Center, 593 Wethington Bldg., 900 S Limestone, Lexington, KY 40536, USA. E-mail: michal.toborek@uky.edu Phone: (859) 323-4094; Fax: (859) 323 2705

Number of text pages: 32

Number of Tables: 0

Number of Figures: 6

Number of references: 40

Number of words in the Abstract: 213

Number of words in the Introduction: 620

Number of words in the Discussion: 1401

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; AIDS, acquired immune deficiency syndrome; APP, amyloid precursor protein; BBB, blood-brain barrier; CCL-2, CC chemokine ligand-2 (also called monocyte chemoattractant protein-1 [MCP-1]), CYP, cytochrome P-450; EGFP, enhanced green fluorescent protein; HBMEC, human brain microvascular endothelial cells; HIV-1, human immunodeficiency virus type 1; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IL, interleukin; NF-κB, nuclear factor-κB; LTR, long terminal repeats; RAGE, receptor for advanced glycation end products; RLU, relative light units; SVGA, a human astrocytic subclone of SVG cells; TNF-α, tumor necrosis factors-α.

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Increased deposition of amyloid beta (Aβ) is characteristic for normal aging and HIV-1-associated alterations of the central nervous system. In addition, both Aβ and HIV-1 are known to induce cellular oxidative stress and disruption of the blood-brain barrier (BBB). Therefore, we hypothesize that Aβ and HIV-1 protein Tat can potentiate their proinflammatory effects at the brain endothelium level. To address this hypothesis, we studied promoter activity of three proinflammatory genes in an *in vitro* BBB model of human brain microvascular endothelial cells (HBMEC) co-cultured with a human astrocyte cell line producing Tat (SVGA-Tat cells) and exposed to Aβ. Treatment of HBMEC with Aβ(1-40) in the presence of SVGA-Tat cells resulted in a significant upregulation of E-selectin, CCL-2, and IL-6 promoter activities and protein levels as compared to the individual effects of Aβ or Tat. In addition, Aβ markedly amplified E-selectin promoter activity in HBMEC co-cultured with HIV-1-infected Jurkat T cells. Simvastatin, the HMG-CoA reductase inhibitor, effectively blocked proinflammatory reactions induced by Aβ in co-cultures with SVGA-Tat cells or with HIV-1-infected Jurkat cells. The present study indicates that a combined exposure to Aβ and Tat or HIV-1 can synergistically potentiate expression of inflammatory genes in brain endothelial cells. In addition, simvastatin may provide a beneficial influence by reducing these effects at the BBB level.

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

A new challenge in the HIV/AIDS epidemic in Western countries is an increase in infection rates among the older population. It is estimated that approximately 15-20% of HIV-1 infected people in the United States are 50 years of age or older (Shah and Mildvan, 2006). This is partly caused by increased survival rates due to more effective antiretroviral therapy. In addition, the rates of new HIV-1 infections are rapidly growing in older people. Due to a variety of sociological, biological, and medical implications, this trend may further increase in the near future. These facts are of significant concerns because elderly patients develop AIDS more rapidly and have higher morbidity and mortality rates. In addition, older patients are more likely to develop HIV-associated dementia. This phenomenon appears not to be related to the length of HIV-1 infection (Valcour and Paul, 2006), suggesting that aging can independently aggravate HIV-1 associated CNS pathology.

Among a variety of mechanisms which can be affected by HIV infection and aging, alterations of the blood-brain barrier (BBB) appear to be especially important. Even normal aging can lead to the BBB disturbances, including disrupted microvascular integrity, higher permeability to albumin, and accumulation and depositions of amyloid beta (Aβ) that precede neuronal degeneration and dementia (Price and Morris, 1999). In addition, disturbances of the BBB are associated with HIV-1 infection and HIV-1 trafficking into the brain (for review see Persidsky and Poluektova, 2006).

It has been hypothesized that the BBB pathology in HIV-1 infection is moderate by soluble mediators released from infected cells. One of such mediators may be HIV-1 protein Tat, which normally serves as a transcriptional transactivator critical for HIV-1 replication (Nath

and Geiger, 1998). In support of its pathogenic role, Tat was detected in the perivascular cells of AIDS patients with HIV-associated encephalitis (Gartner, 2000). Tat can easily cross cell membranes and can affect brain endothelial cells, including stimulation of inflammatory responses and alterations of tight junction protein expression (for review see Toborek et al., 2005). Interestingly, Tat can elevate Aβ levels in the CNS by inhibiting the Aβ-degrading enzyme, neprilysin (Rempel and Pulliam, 2005). This phenomenon may have important implications for older HIV-infected patients. Indeed, HIV-1 infection and prolonged anti-retroviral therapy were demonstrated to contribute to an increase in Aβ deposition in older HIV-infected individuals (Green et al., 2005). In addition, Aβ levels in the CSF were shown to correlate with AIDS dementia complex (Brew et al., 2005).

Aβ is the major constituent of senile plaques and cerebrovascular deposits. It is deposited in the brain parenchyma and cerebral blood vessels in normal aging and Alzheimer's disease (AD). It was demonstrated that the BBB can regulate Aβ transport into the brain via two main receptors, the low density lipoprotein receptor related protein 1 (LRP1) and the receptor for advanced glycation end products (RAGE) (Deane and Zlokovic, 2007). The main forms of Aβ are the 1-40 and 1-42 fragments originating from amyloid precursor protein (APP) by physiological proteolytic processes. Soluble Aβ(1-40) is present mainly in the circulation and in cerebral vascular amyloid. In contrast, Aβ(1-42) is mainly localized to the senile plaques (Seubert et al., 1992). Exposure to Aβ can affect the metabolism of endothelial cells by several mechanisms, including induction of inflammatory responses (Paris et al., 2002; Toborek et al., 2005). Aβ can also stimulate infection of target CD4 positive cells by HIV-1 (Wojtowicz et al., 2002).

Based on these reports, we hypothesize that Aβ and HIV-1 Tat can potentiate their toxic effects. Due to their role in HIV and aging pathology, we focused on the mechanisms of microvascular endothelial injury and the BBB dysfunction. The present study indicates that Aβ(1-40) and Tat can cross-amplify their proinflammatory effects in an *in vitro* model of the BBB.

MATERIALS AND METHODS

Cell cultures

Human brain microvascular endothelial cells (HBMEC): brain endothelial cells used in the present study represent the first stable, fully characterized, and well-differentiated human brain endothelial cell line (Weksler et al., 2005). HBMEC were cultured in EBM-2 medium (Clonetics, East Rutherford, NJ) supplemented with VEGF, IGF-1, EGF, basic FGF, hydrocortisone, ascorbate, gentamycin, and 2.5% fetal bovine serum (FBS) as originally described (Weksler et al., 2005). Collagen type I (BD Biosciences Pharmingen, San Jose, CA) was used for coating the cell culture dishes.

Tat expressing astrocytes: SVGA cells, which represent a human astrocyte cell line, were stably transfected with Tat expression construct (SVGA-Tat cells) as described and characterized previously (Chauhan et al., 2007). Control SVGA cells were transfected with an empty vector. The cells were maintained in Dulbecco's Eagle Modified Medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% heat inactivated FBS (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37ºC and 5% CO_2.

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

Jurkat T cells: Jurkat cells (a human peripheral blood leukemia T cell line) were maintained in RP1640 medium (Invitrogen, Carlsbad, CA) containing 10% FBS (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37ºC and 5% CO_2.

Co-culture models: HBMEC were plated onto polycarbonate membranes of the upper chamber of the Transwell system (pore size 0.4 μm; Corning Costar, Corning, NY). SVGA, SVGA-Tat, or Jurkat cells were cultured in the lower chamber of the system.

Generation of HIV-1 stock and infection of Jurkat cells

HIV-1 stock was generated using 293T cells (American Type Culture Collection, Manassas, VA). The cells were grown in DMEM containing 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). When the cultures reached ~70% confluence, the cells were transfected with the NL4-3 plasmid containing full-length proviral DNA using calcium phosphate. Following transfection, cells were incubated for 24 h in RPMI 1640 medium with 10% FBS and penicillin plus streptomycin (100 U/ml penicillin, and 100 μg/ml streptomycin). Then, culture supernatants were collected, filtered through 0.45 μm filters (Millipore, Bedford, MA), and frozen at -80°C. HIV-1 p24 levels in the supernatants were determined by ELISA (ZeptoMetrix, Buffalo, NY) as the marker of HIV-1 infection.

HIV-1 stock was used to infect Jurkat T cells. Briefly, 4×10^6 Jurkat cells were cultured in T-25 flasks (Corning Costar) in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were infected with viral isolate containing 65 ng HIV-1 p24 and incubated at 37°C and 5% CO_2. The cultures were maintained for up to 18 days with fresh medium added every other day. Non-infected Jurkat cells were used as controls.

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

Treatment factors

Aβ(1-40) (active peptide) and Aβ(40-1) (negative control with the reverse amino acid sequence) were purchased from Anaspec (San Jose, CA) and dissolved in sterile ultra-pure water obtained from ELGA Purelab Classic (Lowell, MA). Freshly solubilized Aβ solutions without pre-aggregation were used in our experiments. Such form of Aβ was demonstrated to induce proinflammatory reactions in isolated rat brain microvessels (Paris et al., 2002).

The cells were treated with Aβ(1-40) at the concentrations of 0.1-1 μM for 20 h in EBM-2 medium. HIV-1 Tat strongly binds to serum proteins; therefore, all experiments in co-cultures with SVGA-Tat cells were performed in EGM-2 medium without serum or growth factors. However, the experiments that involved treatment with Aβ in the presence of HIV-infected Jurkat cells were performed in cell culture media containing 10% FBS. Cells cultured on chamber slides were treated with 1 μM Aβ(1-40) and SVGA or SVGA-Tat conditioned medium. In selected experiments, co-culture systems were pretreated for 15 min with 5 μM or 10 μM simvastatin followed by co-treatment with Aβ(1-40) for 20 h. Both simvastatin and Aβ were added to the upper and lower chambers of the Transwell systems. Simvastatin was left in cell culture media for the duration of Aβ treatment. Our preliminary studies (data not shown) indicated that simvastatin concentrations between 5-20 μM did not affect cell viability.

Promoter constructs

Inflammatory gene promoter constructs (pGL3 E-selectin, pGL3 CCL2, and pGL3 IL-6) were generated based on the pGL3 luciferase basic vector (Promega, Madison, WI). Briefly, 5'-flanking regions of human E-selectin (-1014/+36), CCL2 (-1699/+18), and IL-6 (-1026/+15)

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

were amplified by PCR from human genomic DNA and individually cloned into the pGL3 basic vector by inserting fragments between MluI and NcoI site.

To generate pcDNA3 Tat86 expression construct, HIV Tat(1-86) sequence was amplified using Tat1 BamHI primer, 5'- AGA TCT GGA TCC ATG GAG CCA GTA GAT CCT -3' and Tat86 XhoI primer, 5'- GAA TTC CTC GAG CTA TTC CTT CGG GCC TGT CGG GTC CCC TCG GGA TTG GGA GGT GGG TTG CTT TGA TAG AGA AGC TTG -3' harboring exon II of Tat (underlined) and pNL4-3 template. The amplified fragment was digested with BamHI and XhoI and then cloned into the pcDNA3 vector.

To generate the pGL3 LTR construct, 5'-LTR region of HIV-1 was amplified by PCR from the pNL4-3 plasmid and cloned into the pGL3 basic vector. pEGFP LTR construct was a gift from Dr. Avindra Nath (Johns Hopkins University, Baltimore, MD). Promoter-less pEGFP was generated from the pEGFP LTR construct by eliminating the 5'-LTR region. *Renilla* luciferase pRL-TK control vector was purchased from Promega (Madison, WI).

Transient transfections of HBMEC

HBMEC were cultured on the filter inserts in co-cultures with SVGA, SVGA-Tat, or Jurkat cells and grown to 90% confluence. To prepare a transfection solution, lipofectin (4 µl/ml) was mixed with Opti-MEM medium (both from Invitrogen, Carlsbad, CA) for 30 min. Then, 2 µg/ml of pGL3 E-selectin, pGL3 CCL2, or pGL3 IL-6 promoter constructs was added, followed by incubation for the additional 10 min at room temperature to allow DNA-lipofectin complexes to form.

Filter inserts with HBMEC were removed from the co-culture systems, placed in empty 12 well plates, and washed twice with Opti-MEM to remove serum residues. Then, 250 µl of the

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

transfection solution containing individual promoter gene constructs was added to each filter insert and the cells were incubated for 5 h at 37ºC. Transfection solutions were aspirated and cultures were returned into the Transwell co-culture systems. Cultures were allowed to recover in a normal growth medium for 24 h. Then, the growth medium was replaced with serum free EGM-2 basal medium (for endothelial cells) and DMEM (for astrocytes), and co-cultures were treated with Aβ added into both the upper and lower chambers.

Transient transfections of astrocytes

SVGA cells were transfected using lipofectamin 2000 or lipofectin (both from Invitrogen, Carlsbad, CA). Briefly, SVGA cells were seeded on the 6 well or 24 well plates and grown to ~90% confluence. Prior to transfections, Opti-MEM medium was mixed with lipofectamin 2000 (10 µl/well for the 6 well plates) or lipofectin (1µl/well for the 24 well plates) for 30 min. Then, pEGFP LTR, pEGFP (both 500 ng/well for the 6 well plates), pcDNA3, pcDNA3 Tat86 (2 µg/well for the 6 well plates or 400 ng/well for the 24 well plates), and pRL-TK (10 ng/well for the 24 well plates) were added to prepare individual transfection solutions. The cells were washed with Opti-MEM, the specific transfection mixtures were added to each well, and transfections were performed for 4 h at 37ºC. Then, cultures were washed and allowed to recover in growth medium for 20 h. Astrocytes transfected with the EGFP constructs were imaged using an inverted fluorescence microscope. In cultures transfected with other constructs, luciferase activity was determined as described below.

Luciferase activity assay

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

Firefly and renilla luciferase activities were measured using the Dual Luciferase Assay System (Promega, Madison, WI). Briefly, cells were washed with phosphate buffered saline and incubated on a shaker for 30 min at 24ºC with passive lysis buffer. Cell lysates were transferred to the Eppendorf tubes and centrifuged to remove cell debris. Then, 10 µl of the cell extracts were mixed with 100 µl luciferase assay reagent containing luciferin plus ATP and luminescence was measured in a luminometer with dual automatic injector (Turner Designs, Sunnyvale, CA). The samples were then mixed with the Stop and Glo reagent and *renilla* luciferase activity was determined as an internal control.

All transfection results from astrocytes were expressed as the ratio of the firefly to *renilla* luciferase activity. On the other hand, Aβ used for treatment of HBMEC interfered with the *renilla* luciferase assay. Therefore, firefly luciferase activity in HBMEC was normalized to total protein levels and the transfection results were expressed as relative light units (RLU)/µg protein.

Immunofluorescence microscopy

HBMEC cultured on Permanox chambered plastic slides were fixed with ethanol for 30 min at 4^{o}C. After washing with PBS and blocking with 3% bovine serum albumin in PBS for 30 min, samples were incubated overnight at 4˚C with rabbit polyclonal anti-E-selectin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The excess of primary antibody was removed, slides were washed with PBS, and incubated with Texas Red-conjugated secondary antibody (Santa Cruz Biotechnology) for 2 h at room temperature. After washing with PBS, slides were mounted using ProLong Gold Antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA) to visualize the nuclei. Specimens were covered with coverslips and

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

evaluated under an epifluorescence Nicon Eclipse E600 microscope. The images were captured using a Spot CCD camera system.

Enzyme-Linked Immunosorbent Assay (ELISA)

Concentrations of CCL-2 and IL-6 were quantified using commercially available human CCL-2 and IL-6 Immunoassay kits (R&D Systems, Minneapolis, MN). Assays were performed using aliquots of cell culture media collected from both the luminal and abluminal chambers of the Transwell co-culture systems.

Statistical Analysis

Data were analyzed using SigmaStat 2.0 (SPSS Inc., Chicago, IL, USA). One- or two-way ANOVA was used to compare responses among treatments. Treatment means were compared using Bonferroni's least significant procedure and $p<0.05$ was considered significant.

RESULTS

Morphological characterization of HBMEC and SVGA-Tat cells

HIV-1 can infect astrocytes, which then produce viral proteins such as Tat. Astrocytes constitute the most abundant cell type in the brain and viral proteins produced by infected astrocytes are likely to have significant neuropathological effects. Therefore, the present study was based on co-culture systems of HBMEC with astrocytes expressing HIV Tat protein (SVGA-Tat cells) or normal SVGA cells used as controls.

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

The phase contrast micrographs in **Figure 1** depict confluent HBMEC and SVGA cells. HBMEC cultured on cell culture dishes exhibited typical monolayer morphology of elongated, fusiform shape of endothelial cells. Experimental design of the present study required HBMEC cultures on filter inserts. Therefore, we also determined that HBMEC cultured on microporous filters had normal morphology. In addition, confluent SVGA-Tat and SVGA control cells exhibited typical morphological characteristic of astrocyte cultures.

Tat expression by SVGA-Tat astrocytes

In order to confirm expression and activity of Tat protein in our culture systems, SVGA-Tat and control SVGA astrocytes were transfected with pEGFP LTR (**Figure 2A**) or pGL3 LTR (**Figure 2B**) promoter constructs. SVGA-Tat cells transfected with pEGFP LTR (**Figure 2A**, middle panel) exhibited markedly stronger EGFP fluorescence as compared to control SVGA cells (**Figure 2A**, left panel). In contrast, transfections with the promoter-less pEGFP construct revealed only background fluorescence both in SVGA-Tat and control SVGA cells. To further confirm the specificity of these responses, control SVGA cells were transiently transfected with pcDNA3 Tat86 and pEGFP LTR constructs. As illustrated (**Figure 2A**, right panel), this procedure resulted in efficient and specific LTR transactivation, similar to that in SVGA-Tat astrocytes.

In order to further characterize SVGA-Tat cells, Tat transactivation was quantified by transfection with the pGL3 LTR construct, followed by measuring of luciferase activity. Transfections with the pGL3 basic vector were performed as negative controls. A strong induction of luciferase activity was observed in SVGA-Tat cells but not in control SVGA cells

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

(**Figure 2B**). Strong transactivation of LTR promoter was also observed in control SVGA cells transiently transfected with the pcDNA3 Tat86 and pGL3 LTR constructs (positive control).

Tat and Aβ selectively amplify promoter activities and protein levels of proinflammatory mediators in HBMEC

Co-culture systems of HBMEC with SVGA-Tat astrocytes were employed to investigate the combined effects of Aβ and Tat on promoter activity of selected proinflammatory genes. HBMEC co-cultured with SVGA-Tat or control SVGA cells were transfected with the firefly luciferase reporter constructs containing human E-selectin, CCL2, or IL-6 promoter sequence. Then, both HBMEC and astrocytes were exposed to 0.1, 0.5, or 1 μM Aβ(1-40) for 20 h. In co-cultures of HBMEC with control SVGA cells, Aβ induced a marked increase in the promoter activity of E-selectin (**Figure 3A**) and CCL2 (**Figure 3B**); however, it did not affect promoter activity of IL-6 (**Figure 3C**). On the other hand, exposure to Aβ in co-cultures with SVGA-Tat cells markedly amplified promoter activities of all studied proinflammatory genes (**Figures 3A-3C**). These effects were achieved by 1 μM Aβ for E-selectin and IL-6 promoters, and by 0.5 μM Aβ for CCL2 promoter.

To verify the specificity of the Aβ(1-40) effects, co-cultures were also exposed to the reversed sequence Aβ peptide (Aβ40-1) at the concentration of 1 μM. As illustrated in **Figures 3A-3C**, Aβ(40-1) did not affect transactivation of the inflammatory gene promoters.

In addition to promoter activities, we determined the effects of Aβ and/or SVGA-Tat on protein levels of E-selectin, CCL2, and IL-6 (**Figures 4A-4C**). Based on the results of the dose-response experiments presented in Figures 3A-3C, Aβ was used at the concentration of 1 μM to stimulate E-selectin and IL-6 protein expression and at 0.5 μM to induce CCL2 protein

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

production. As illustrated in **Figure 4A** (second panel from left), treatment with Aβ alone slightly elevated E-selectin immunoreactivity. Similar effects were also apparent in HBMEC exposed to conditioned medium from SVGA-Tat cultured (third panel from left). However, the most marked increase in E-selectin immunoreactivity was observed in HBMEC treated with Aβ in the presence of conditioned medium from SVGA-Tat cells (right panel). Potentiation of pro-inflammatory effects of Aβ and SVGA-Tat were also observed in experiments in which levels of secretary CCL2 and IL-6 proteins were determined in cell culture media (**Figures 4B** and **4C**, respectively).

Simvastatin protects against Tat and Aβ-induced stimulation of promoter activities of proinflammatory genes in HBMEC

Statins have been demonstrated to have pleiotropic effects, including anti-inflammatory and anti-HIV properties. Therefore, we hypothesized that they can protect against Tat and Aβ-induced proinflammatory effects. To address this hypothesis, HBMEC were co-cultured with SVGA-Tat or control SVGA cells and transfected with the pGL3 E-selectin, pGL3 CCL2, or pGL3 IL-6 construct. Then, the co-cultures were pretreated for 15 min with 10 μM (**Figures 5A and 5B**) or 5 μM (**Figure 5C)** simvastatin, followed by exposure to Aβ(1-40) for 20 h. Aβ was used at the concentration of 1 μM to stimulate E-selectin and IL-6 promoters and at 0.5 μM to induce CCL2 promoter. Consistent with data presented in Figures 3 and 4, Aβ in the presence of SVGA-Tat induced a marked increase in E-selectin (**Figure 5A**), CCL2 (**Figure 5B**), and IL-6 (**Figure 5C**) promoter activities. Treatment with simvastatin upregulated the IL-6 promoter activity in control (i.e., not exposed to Aβ) co-cultures of HBMEC with SVGA-Tat cells. Nevertheless, simvastatin effectively protected against upregulation of E-selectin and IL-6

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

promoters in co-cultures of HBMEC with SVGA-Tat and exposed to Aβ. Inhibition of IL-6 promoter activity was achieved by 5 μM simvastatin; however, 10 μM simvastatin was required to block E-selectin promoter activity.

Aβ amplifies HIV-1-stimulated transactivation of E-selectin promoter in HBMEC

In the final series of experiments, we investigated whether exposure to Aβ can stimulate E-selectin promoter activity in the presence of HIV-1. HBMEC were transfected with the pGL3 E-selectin construct and co-cultured with HIV-1-infected or control Jurkat cells for 24 h. Cultures were then pretreated for 15 min with 10 μM simvastatin or vehicle control and exposed to 1 μM Aβ(1-40) for 20 h. The experiments were performed 7, 14, and 18 days after infection of Jurkat cells. The results were similar, therefore individual data from these separate experiments were combined in a single figure (**Figure 6**). Stimulation of HBMEC by HIV-1-infected Jurkat cells resulted in a sufficient increase in E-selectin promoter activity. In addition, treatment with Aβ markedly elevated these effects. Most importantly, pretreatment with simvastatin effectively protected against both HIV-1 and HIV-1 plus Aβ-induced transactivation of the E-selectin promoter construct.

DISCUSSION

The mechanisms of HIV entry into the brain are still unclear; however, they may occur through the so-called Trojan horse mechanism, via HIV-infected monocytes crossing the BBB (Gartner, 2000). Thus, induction of inflammatory reactions which mediate monocyte adhesion

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

to the brain endothelium and transendothelial migration are of particular significance. Therefore, in the present studies we focused on expression of inflammatory mediators, such as E-selectin (an adhesion molecule), CCL2 (a chemokine), and IL-6 (an inflammatory cytokine) in brain endothelial cells. Upregulation of these inflammatory mediators can induce alterations of the BBB integrity by facilitating adhesion and migration of leukocytes into the brain (for review see Buckner et al., 2006). The present study was based on co-culture systems in which HBMEC were exposed to Tat produced by transfected astrocytes. Such co-cultures are clinically relevant, because HIV can infect astrocytes, which then are able to produce viral proteins, such as Tat. Astrocytes along with HBMEC are the structural and functional part of the BBB. In addition, astrocytes are susceptible to Aβ treatment and can influence HBMEC inflammatory responses. Indeed, we observed that Aβ stimulated E-selectin promoter activity in HBMEC co-cultured with SVGA astrocytes (**Figure 3A**) but not with Jurkat cells (**Figure 6**).

The facts that a) deposits of Aβ increase in normal aging (Price and Morris, 1999), b) Aβ can induce vascular alterations (Deane and Zlokovic, 2007), and c) Aβ may be involved in HIV-associated pathology (Wojtowicz et al., 2002), prompted us to study the hypothesis that Aβ and Tat may cross-potentiate their toxic effects and alter the functions of the BBB. Indeed, the results of the present study indicated that co-exposure to Aβ(1-40) and Tat or HIV-1 infected cells can synergistically elevate promoter activity of E-selectin, CCL2, and IL-6. These effects were also confirmed at the protein levels (**Figure 4**). Although the proinflammatory influence of Tat and Aβ in endothelial cells has been recognized (Toborek et al., 2005), the evidence that these factors can exert synergistic proinflammatory effects is the new finding of the present study.

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

As illustrated in **Figure 3**, exposure to Aβ resulted in a dose-dependent increase in promoter activities of E-selectin and IL-6. On the other hand, CCL2 promoter was activated by Aβ at the concentration of 0.1 and 0.5 μM but not at 1 μM. Although Aβ is generally considered a pro-oxidative factor, evidence indicates that Aβ-mediated regulation of cellular oxidation may depend on the ratio of Aβ to other prooxidative factors. Indeed, prooxidative/antioxidative effects of Aβ can be regulated by the molar ratio of Aβ to Cu2+. When Cu2+ is bound to two molar equivalents of Aβ, lipid peroxidation is inhibited and Aβ exerts antioxidant effects (Hayashi et al., 2007). Thus, it is possible that these finely tuned regulations of cellular redox balance might be responsible for the lack of CCL2 stimulation by 1 μM Aβ.

The transfection experiments with the promoter constructs performed in the present study indicate that induction of inflammatory genes by Aβ, Tat, or HIV-1 occurs primarily at the transcriptional levels. In agreement with these results, it has been shown that Tat-induced expression of TNF-α is regulated by an NF-κB-dependent pathway in macrophages and astrocytes (Chen et al., 1997). The NF-κB-dependent pathway is also involved in Tat-induced upregulation of IL-6 and IL-8 expression in human breast cancer cells (Lee et al., 2005). In addition, treatment with Tat can induce DNA binding activities of other transcription factors, such as CREB and AP-1, which are known to regulate inflammatory responses (Kumar et al., 1998; Toborek et al., 2005).

Aβ also appears to induce activity of several transcription factors involved in the regulation of inflammatory gene expression. For example, Aβ treatment resulted in a poly(ADP-ribose) polymerase-1 (PARP-1)-facilitated DNA binding of NF-κB (Chiarugi and Moskowitz, 2003) in microglial cells. Exposure to Aβ stimulated activation of early growth response-1 (Egr-1) transcription factor, which is involved in the expression of cytokines (TNF-α and IL-1β) and

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

chemokines (MIP-1β, CCL-2 and IL-8) in monocytes (Giri et al., 2004). Transcription factor C/EBPdelta was also shown to be involved in Aβ-mediated induction of IL-6 and CCL-2 (Hu et al., 2000). Thus, Tat and Aβ-mediated stimulation of a variety of transcription factors may be responsible for cross-potentiation of inflammatory responses as observed in our study.

Another important finding was that treatment with statins, such as simvastatin, protected against Aβ and Tat or HIV-1-induced inflammatory responses. Statins are drugs which effectively diminish cholesterol biosynthesis by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. In HIV-infected patients, statins have been successfully introduced to improve disturbances in lipid profile (Dube et al., 2003). On the other hand, statins, such as simvastatin can also increase NF-κB activation and superoxide production via heme oxygenase-1-dependent mechanism (Hsieh et al., 2007). Such effects could be responsible for an increase in IL-6 promoter activity observed in simvastatin-treated HBMEC in the presence of SVGA-Tat cells.

Observed in the present study, the protective effects of simvastatin on Tat and Aβ-induced inflammatory responses are supported by literature reports. For example, it was shown that statins downregulated VCAM-1, E-selectin, IL-6, IL-8, CCL-2 expression, and had immuno-modulatory effects in endothelial cells (for review see Greenwood et al., 2006). In monocytes, statins inhibited adhesion and migration (Pozo et al., 2006). Statins also decreased inflammatory responses in microglia and attenuated experimental autoimmune encephalomyelitis (Nath et al., 2004). Anti-inflammatory properties of statins appear to be related to decreased synthesis of several isoprenoid intermediates, which normally are involved in post-translational modification of proteins, including the γ-subunit of heterotrimeric G-proteins and Ras and Rho proteins (Liao, 2005). Isoprenylation of Ras and Rho is required for covalent attachment, subcellular

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

localization, and membrane trafficking of these molecules. The Ras and Rho signaling can stimulate inflammatory reactions; thus, a decrease in isoprenylation prevents activation of these kinases and may induce anti-inflammatory responses (Liao, 2005).

In the present study, simvastatin not only protected against Tat and Aβ-induced inflammatory reactions, but also was highly effective against HIV-mediated stimulation of E-selectin promoter transactivation. The protective effects suggest that statins may be a valuable group of drugs for supplemental therapy in HIV infection. Indeed, a short-term statin treatment was demonstrated to decrease HIV-1 viral load in patients and inhibit HIV-1 infection through downregulation of Rho activity (del Real et al., 2004). However, these effects were not confirmed in another study in which no anti-HIV activity of statins was observed in cell culture or in HIV-positive patients (Moncunill et al., 2005). It also should be noted that most statins are metabolized through the CYP3A4 pathway, which is inhibited by protease inhibitors widely used in anti-retroviral therapy. Thus, interactions between statins and protease inhibitors may result in an increase in tissue statin concentrations, leading to drug toxicity. On the other hand, statins such as pravastatin and fluvastatin are the least influenced by the CYP3A4 pathway and, therefore, might be safer when administered with protease inhibitors (Dube et al., 2003).

Evidence also indicates that statins may provide useful treatment in pathologies related to Aβ pathology, such as AD dementia. Retrospective epidemiological studies revealed that patients with high cholesterol levels and treated with statins less frequently developed AD (Jick et al., 2000). A relative safety of simvastatin was demonstrated in a 5 year clinical study (Pedersen et al., 1996), suggesting that statins might be safely used to prevent the development of AD. The beneficial effects of statins were also shown in patients with mild to moderate AD (Sparks et al., 2006). Finally, experimental studies revealed that statins can protect neurons

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

against Aβ toxicity, increase learning capabilities in mice, and decrease Aβ levels both in cultured hippocampal neurons and *in vivo* in guinea pigs (Fassbender et al., 2001; Li et al., 2006). However, there are also reports which do not support these findings or even suggest that statins can induce cognitive impairment (Padala et al., 2006). Thus, to elucidate these controversies, at least two large multicenter trials have been initiated using simvastatin and atorvastatin (Miida et al., 2005) in an attempt to better understand the effects of statin therapy on dementia.

In summary, Aβ(1-40) and HIV-1 Tat protein cross-amplified promoter activities of three different proinflammatory genes in HBMEC. Similar synergistic effects were observed in HBMEC exposed to Aβ in the presence of HIV-1-infected Jurkat cells. Most interestingly, simvastatin effectively attenuated these effects. The present results indicate that Aβ and HIV Tat may synergistically induce inflammatory reactions in brain endothelial cells. In addition, statins may provide a beneficial influence by reducing these effects at the BBB level.

ACKNOWLEDGEMENT

pNL4-3 was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIH/NIAID. SVGA and SVGA-Tat cells were generous gift from Dr. Avindra Nath, Johns Hopkins University.

REFERENCES

Brew BJ, Pemberton L, Blennow K, Wallin A and Hagberg L (2005) CSF amyloid beta42 and tau levels correlate with AIDS dementia complex. *Neurology* **65**:1490-1492.

Buckner CM, Luers AJ, Calderon TM, Eugenin EA and Berman JW (2006) Neuroimmunity and the blood-brain barrier: molecular regulation of leukocyte transmigration and viral entry into the nervous system with a focus on neuroAIDS. *J Neuroimmune Pharmacol* **1**:160-181.

Chauhan A, Hahn S, Gartner S, Pardo CA, Netesan SK, McArthur J and Nath A (2007) Molecular programming of endothelin-1 in HIV-infected brain: role of Tat in up-regulation of ET-1 and its inhibition by statins. *FASEB J* **21**:777-789.

Chen P, Mayne M, Power C and Nath A (1997) The Tat protein of HIV-1 induces tumor necrosis factor-alpha production. Implications for HIV-1-associated neurological diseases. *J Biol Chem* **272**:22385-22388.

Chiarugi A and Moskowitz MA (2003) Poly(ADP-ribose) polymerase-1 activity promotes NF-kappaB-driven transcription and microglial activation: implication for neurodegenerative disorders. *J Neurochem* **85**:306-317.

Deane R and Zlokovic BV (2007) Role of the blood-brain barrier in the pathogenesis of Alzheimer's disease. *Curr Alzheimer Res* **4**:191-197.

del Real G, Jimenez-Baranda S, Mira E, Lacalle RA, Lucas P, Gomez-Mouton C, Alegret M, Pena JM, Rodriguez-Zapata M, Alvarez-Mon M, Martinez AC and Manes S (2004) Statins inhibit HIV-1 infection by down-regulating Rho activity. *J Exp Med* **200**:541-547.

Dube MP, Stein JH, Aberg JA, Fichtenbaum CJ, Gerber JG, Tashima KT, Henry WK, Currier JS, Sprecher D and Glesby MJ (2003) Guidelines for the evaluation and management of

dyslipidemia in human immunodeficiency virus (HIV)-infected adults receiving antiretroviral therapy: recommendations of the HIV Medical Association of the Infectious Disease Society of America and the Adult AIDS Clinical Trials Group. *Clin Infect Dis* **37**:613-627.

Fassbender K, Simons M, Bergmann C, Stroick M, Lutjohann D, Keller P, Runz H, Kuhl S, Bertsch T, von Bergmann K, Hennerici M, Beyreuther K and Hartmann T (2001) Simvastatin strongly reduces levels of Alzheimer's disease beta -amyloid peptides Abeta 42 and Abeta 40 in vitro and in vivo. *Proc Natl Acad Sci U S A* **98**:5856-5861.

Gartner S (2000) HIV infection and dementia. *Science* **287**:602-604.

Giri RK, Rajagopal V and Kalra VK (2004) Curcumin, the active constituent of turmeric, inhibits amyloid peptide-induced cytochemokine gene expression and CCR5-mediated chemotaxis of THP-1 monocytes by modulating early growth response-1 transcription factor. *J Neurochem* **91**:1199-1210.

Green DA, Masliah E, Vinters HV, Beizai P, Moore DJ and Achim CL (2005) Brain deposition of beta-amyloid is a common pathologic feature in HIV positive patients. *Aids* **19**:407-411.

Greenwood J, Steinman L and Zamvil SS (2006) Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. *Nat Rev Immunol* **6**:358-370.

Hayashi T, Shishido N, Nakayama K, Nunomura A, Smith MA, Perry G and Nakamura M (2007) Lipid peroxidation and 4-hydroxy-2-nonenal formation by copper ion bound to amyloid-beta peptide. *Free Radic Biol Med* **43**:1552-1559.

Hsieh CH, Jeng SF, Hsieh MW, Chen YC, Rau CS, Lu TH and Chen SS (2007) Statin-induced heme oxygenase-1 increases NF-kappaB activation and oxygen radical production in cultured neuronal cells exposed to lipopolysaccharide. *Toxicol Sci* in press.

Hu HM, Tian Q, Baer M, Spooner CJ, Williams SC, Johnson PF and Schwartz RC (2000) The C/EBP bZIP domain can mediate lipopolysaccharide induction of the proinflammatory cytokines interleukin-6 and monocyte chemoattractant protein-1. *J Biol Chem* **275**:16373-16381.

Jick H, Zornberg GL, Jick SS, Seshadri S and Drachman DA (2000) Statins and the risk of dementia. *Lancet* **356**:1627-1631.

Kumar A, Manna SK, Dhawan S and Aggarwal BB (1998) HIV-Tat protein activates c-Jun N-terminal kinase and activator protein-1. *J Immunol* **161**:776-781.

Lee YW, Hirani AA, Kyprianou N and Toborek M (2005) Human immunodeficiency virus-1 Tat protein up-regulates interleukin-6 and interleukin-8 expression in human breast cancer cells. *Inflamm Res* **54**:380-389.

Li L, Cao D, Kim H, Lester R and Fukuchi K (2006) Simvastatin enhances learning and memory independent of amyloid load in mice. *Ann Neurol* **60**:729-739.

Liao JK (2005) Effects of statins on 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibition beyond low-density lipoprotein cholesterol. *Am J Cardiol* **96**:24F-33F.

Miida T, Takahashi A, Tanabe N and Ikeuchi T (2005) Can statin therapy really reduce the risk of Alzheimer's disease and slow its progression? *Curr Opin Lipidol* **16**:619-623.

Moncunill G, Negredo E, Bosch L, Vilarrasa J, Witvrouw M, Llano A, Clotet B and Este JA (2005) Evaluation of the anti-HIV activity of statins. *Aids* **19**:1697-1700.

Nath A and Geiger J (1998) Neurobiological aspects of human immunodeficiency virus infection: neurotoxic mechanisms. *Prog Neurobiol* **54**:19-33.

Nath N, Giri S, Prasad R, Singh AK and Singh I (2004) Potential targets of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor for multiple sclerosis therapy. *J Immunol* **172**:1273-1286.

Padala KP, Padala PR and Potter JF (2006) Simvastatin-induced decline in cognition. *Ann Pharmacother* **40**:1880-1883.

Paris D, Townsend KP, Obregon DF, Humphrey J and Mullan M (2002) Pro-inflammatory effect of freshly solubilized beta-amyloid peptides in the brain. *Prostaglandins Other Lipid Mediat* **70**:1-12.

Pedersen TR, Berg K, Cook TJ, Faergeman O, Haghfelt T, Kjekshus J, Miettinen T, Musliner TA, Olsson AG, Pyorala K, Thorgeirsson G, Tobert JA, Wedel H and Wilhelmsen L (1996) Safety and tolerability of cholesterol lowering with simvastatin during 5 years in the Scandinavian Simvastatin Survival Study. *Arch Intern Med* **156**:2085-2092.

Persidsky Y and Poluektova L (2006) Immune privilege and HIV-1 persistence in the CNS. *Immunol Rev* **213**:180-194.

Pozo M, de Nicolas R, Egido J and Gonzalez-Cabrero J (2006) Simvastatin inhibits the migration and adhesion of monocytic cells and disorganizes the cytoskeleton of activated endothelial cells. *Eur J Pharmacol* **548**:53-63.

Price JL and Morris JC (1999) Tangles and plaques in nondemented aging and "preclinical" Alzheimer's disease. *Ann Neurol* **45**:358-368.

Rempel HC and Pulliam L (2005) HIV-1 Tat inhibits neprilysin and elevates amyloid beta. *Aids* **19**:127-135.

Romano M, Diomede L, Sironi M, Massimiliano L, Sottocorno M, Polentarutti N, Guglielmotti A, Albani D, Bruno A, Fruscella P, Salmona M, Vecchi A, Pinza M and Mantovani A (2000) Inhibition of monocyte chemotactic protein-1 synthesis by statins. *Lab Invest* **80**:1095-1100.

Seubert P, Vigo-Pelfrey C, Esch F, Lee M, Dovey H, Davis D, Sinha S, Schlossmacher M, Whaley J, Swindlehurst C and et al. (1992) Isolation and quantification of soluble Alzheimer's beta-peptide from biological fluids. *Nature* **359**:325-327.

Shah S and Mildvan D (2006) HIV and Aging. *Curr Infect Dis Rep* **8**:241-247.

Sparks DL, Sabbagh M, Connor D, Soares H, Lopez J, Stankovic G, Johnson-Traver S, Ziolkowski C and Browne P (2006) Statin therapy in Alzheimer's disease. *Acta Neurol Scand Suppl* **185**:78-86.

Toborek M, Lee YW, Flora G, Pu H, András IE, Wylegala E, Hennig B and Nath A (2005) Mechanisms of the blood-brain barrier disruption in HIV-1 infection. *Cell Mol Neurobiol* **25**:181-199.

Valcour V and Paul R (2006) HIV infection and dementia in older adults. *Clin Infect Dis* **42**:1449-1454.

Weksler BB, Subileau EA, Perriere N, Charneau P, Holloway K, Leveque M, Tricoire-Leignel H, Nicotra A, Bourdoulous S, Turowski P, Male DK, Roux F, Greenwood J, Romero IA and Couraud PO (2005) Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB J* **19**:1872-1874.

Wojtowicz WM, Farzan M, Joyal JL, Carter K, Babcock GJ, Israel DI, Sodroski J and Mirzabekov T (2002) Stimulation of enveloped virus infection by beta-amyloid fibrils. *J Biol Chem* **277**:35019-35024.

FOOTNOTES

Supported by MH63022, MH072567, and NS39254. The authors have no competing financial interests.

Person to receive reprint requests: Michal Toborek, MD. PhD; University of Kentucky Medical Center, 593 Wethington Bldg., Lexington, KY 40536, USA. E-mail: michal.toborek@uky.edu

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS

Figure 1. Morphological characterization of immortalized human brain microvascular endothelial cells (HBMEC), control astrocytic cell line (SVGA), and SVGA overexpressing Tat. Upper panels: Phase contrast micrographs of a confluent HBMEC monolayer with the typical morphology of elongated, fusiform shape of endothelial cells cultured on a cell culture dish (**A**) and on a filter insert (**B**). Endothelial cells cultured on filter inserts have no morphological changes as compared to those grown on cell culture dishes. **Lower panels**: Phase contrast micrograph of confluent control SVGA astrocytes (**C**) and SVGA-Tat astrocytes (**D**). Control SVGA and SVGA-Tat cells exhibit similar morphology characteristic for astrocyte cultures, such as multipolar shape and overlapping morphology.

Figure 2. Tat expression by SVGA-Tat astrocytes. A. Control SVGA and SVGA-Tat astrocytes were transfected with the pEGFP LTR (**lower panels**) or promoter-less pEGFP (negative control, **upper panels**) constructs. To confirm specificity of Tat detection, normal SVGA astrocytes were transiently transfected with the pcDNA3 Tat86 expression vector and co-transfected with the pEGFP LTR or the promoter-less pEGFP constructs. EGPF expression was detected by immunofluorescence microscopy.

B. To quantify Tat transactivation, control SVGA and SVGA-Tat astrocytes were transfected with pGL3 LTR or pGL3 basic vector (negative control). As a positive control, SVGA astrocytes were transiently transfected with pcDNA3 Tat86 expression vector (or pcDNA3 empty vector) and pGL3 LTR. To normalize transfection rates, all cells were co-transfected with the *renilla* luciferase reporter plasmid pRL-TK. Luciferase activity was analyzed by dual

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

luciferase assay 20 h post transfections and normalized according to *renilla* luciferase activity. Values are mean ± SEM, n=4-6.

Figure 3. Aβ amplifies Tat-induced promoter activities and of proinflammatory genes in brain endothelial cells. HBMEC co-cultured with control or Tat expressing astrocytes (SVGA and SVGA-Tat cells, respectively) were transfected with the firefly luciferase reporter constructs containing the human E-selectin (**A**), CCL2 (**B**), and IL-6 (**C**) promoter sequences. Cells in the co-culture system were treated with the indicated concentrations of Aβ(1-40) 24 h post transfections. Aβ(40-1) at the concentration of 1 μM was used as a negative control. Luciferase activity was analyzed in HBMEC following a 20 h exposure to Aβ. Values are mean ± SEM, n=4. *Statistically different as compared to the corresponding controls within the HBMEC plus SVGA or HBMEC plus SVGA-Tat co-cultures. †The data in co-cultures of HBMEC with SVGA-Tat are significantly different as compared to co-cultures of HBMEC with control SVGA and exposed to the corresponding concentration of Aβ.

Figure 4. Aβ and Tat synergistically amplify protein expression of proinflammatory mediators in brain endothelial cells. A. HBMEC cultured on chambered slides were exposed to 1 μM Aβ(1-40) and/or conditioned media from SVGA or SVGA-Tat cells for 6 h. E-selectin immunoreactivity was determined by immunofluorescence microscopy. Red color reflects E-selectin-positive immunoreactivity and blue color represents 4',6-diamidino-2-phenylindole (DAPI) staining for DNA that visualizes the nuclei. Arrows indicate intensification of E-selectin immunoreactivity by Aβ and/or SVGA-Tat conditioned media.

Molecular Pharmacology Fast Forward. Published on February 14, 2008 as DOI: 10.1124/mol.107.042028
This article has not been copyedited and formatted. The final version may differ from this version.

B and **C**. HBMEC were co-cultured with SVGA-Tat or control SVGA cells and treated with Aβ(1-40) at 0.5 μM (**B**) or 1 μM (**C**). CCL2 and IL-6 protein levels were determined in the culture media by ELISA. Values are mean ± SEM, n=3-8. *Statistically different as compared to the corresponding controls within the HBMEC plus SVGA or HBMEC plus SVGA-Tat co-cultures. †The data in co-cultures of HBMEC with SVGA-Tat are significantly different as compared to co-cultures of HBMEC with control SVGA and exposed to the corresponding concentration of Aβ.

Figure 5. Simvastatin protects against Aβ and Tat-induced promoter activities of proinflammatory genes in brain endothelial cells. Transfections and luciferase activity assays were performed as described in the legend to Figure 3. In addition, selected cultures were pretreated for 15 min with 10 μM (**A** and **B**) or 5 μM (**C**) simvastatin prior to co-exposure to 1 μM (**A** and **C**) or 0.5 μM (**B**) Aβ(1-40) for 20 h. Values are mean ± SEM, n=4-6. *Statistically different as compared to the corresponding controls within the HBMEC plus SVGA or HBMEC plus SVGA-Tat co-cultures. †Data in co-cultures of HBMEC with SVGA-Tat are significantly different as compared to co-cultures of HBMEC with control SVGA and exposed to the corresponding concentration of Aβ. #Data in co-cultures exposed to simvastatin are significantly different as compared to the corresponding co-cultures without added simvastatin.

Figure 6. Simvastatin protects against Aβ and HIV-1-induced promoter activity of E-selectin in brain endothelial cells. Transfections with the pGL3 E-selectin promoter construct were performed as described in the legend to Figure 3. The next day, the transfected HBMEC were placed in co-cultures with normal or HIV-1-infected Jurkat cells, followed by a treatment

with 1 μM Aβ(1-40) for 20 h. Selected cultures were pretreated for 15 min with simvastatin (10 μM) prior to Aβ(1-40) co-exposure. E-selectin promoter activity was analyzed by luciferase assay and normalized to cellular protein levels. Values are mean ± SEM, n=6-10. *Statistically different as compared to the corresponding controls within the co-cultures of HBMEC with normal or HIV-1-infected Jurkat cells. †Data in co-cultures of HBMEC with HIV-1-infected Jurkat cells are significantly different as compared to co-cultures of HBMEC with normal Jurkat cells and exposed to the corresponding concentration of Aβ. #Data in co-cultures exposed to simvastatin are significantly different as compared to the corresponding controls without added simvastatin.

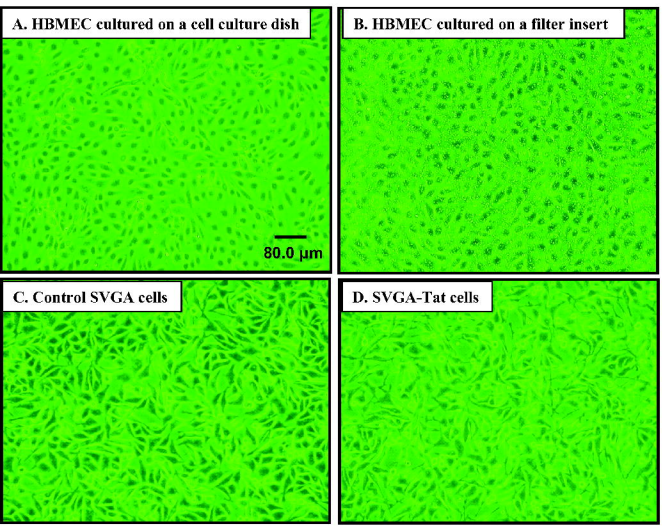
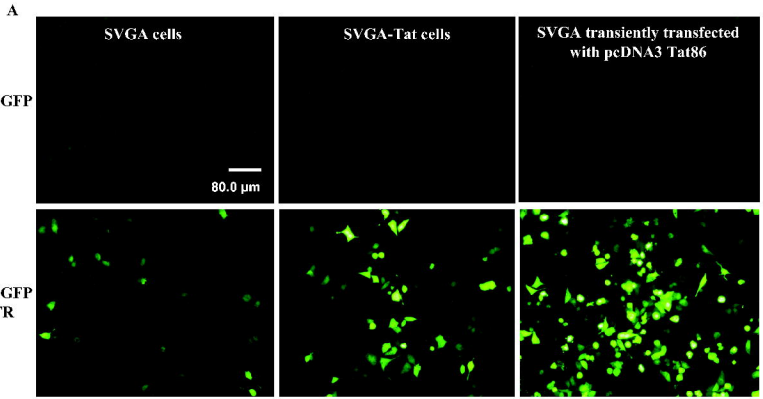
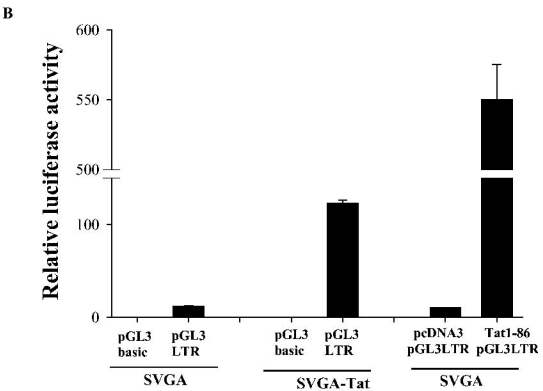

A. HBMEC cultured on a cell culture dish
80.0 µm
B. HBMEC cultured on a filter insert
C. Control SVGA cells
D. SVGA-Tat cells

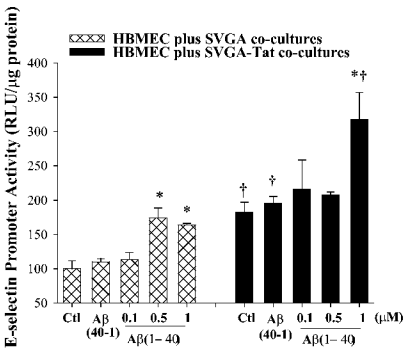
Figure 1



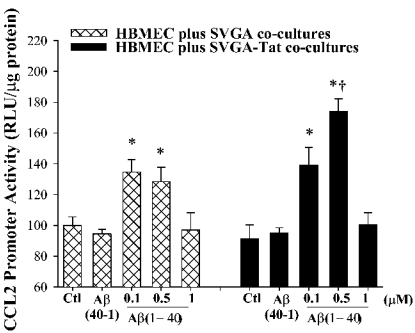


Figures 2A and 2B

A



B



Figures 3A and 3B

C

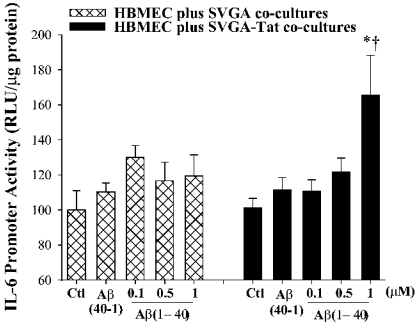

HBMEC plus SVGA co-cultures
HBMEC plus SVGA-Tat co-cultures
IL-6 Promoter Activity (RLU/μg protein)
200
180
160
140
120
100
80
60
*†
Ctl
Aβ (40-1)
0.1
0.5
1
Aβ(1–40)
(μM)

Figure 3C

A

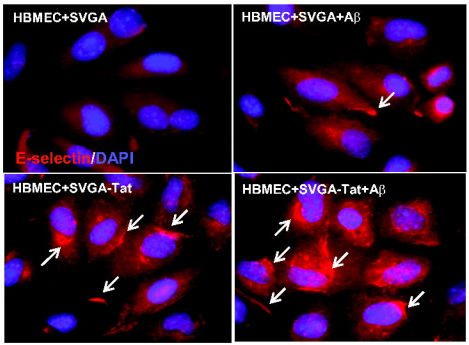
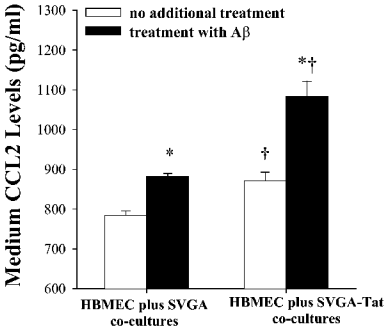
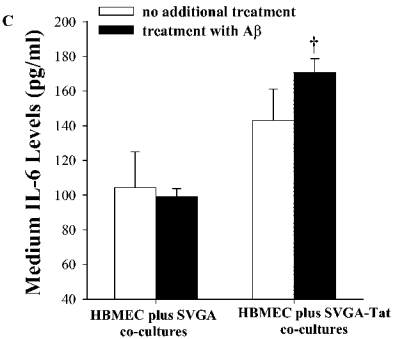

HBMEC+SVGA
HBMEC+SVGA+Aβ
E-selectin/DAPI
HBMEC+SVGA-Tat
HBMEC+SVGA-Tat+Aβ

Figure 4A

B

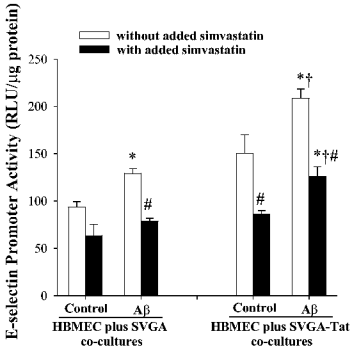


C

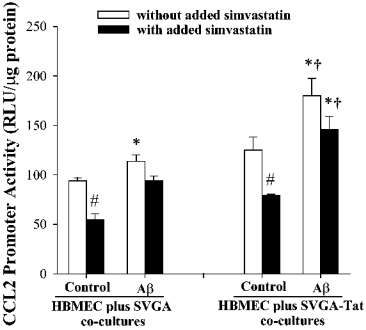


Figures 4B and 4C

A



B



Figures 5A and 5B

C

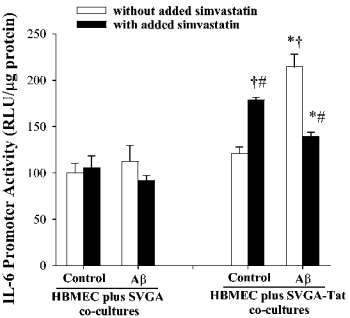

without added simvastatin
with added simvastatin
IL-6 Promoter Activity (RLU/μg protein)
250
200
150
100
50
0
*†
†#
*#
Control
Aβ
Control
Aβ
HBMEC plus SVGA co-cultures
HBMEC plus SVGA-Tat co-cultures

Figure 5C

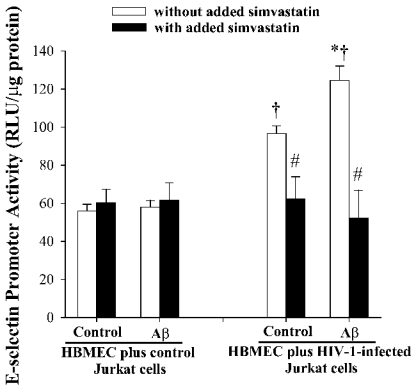

without added simvastatin
with added simvastatin
E-selectin Promoter Activity (RLU/μg protein)
140
120
100
80
60
40
20
0
*†
†
#
#
Control
Aβ
Control
Aβ
HBMEC plus control Jurkat cells
HBMEC plus HIV-1-infected Jurkat cells

Figure 6