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Apolipoprotein J (clusterin) activates rodent microglia *in vivo* and *in vitro*

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Abstract

Apolipoprotein J (apoJ; also known as clusterin and sulfated glycoprotein (SGP)-2) is associated with senile plaques in degenerating regions of Alzheimer's disease brains, where activated microglia are also prominent. We show a functional link between apoJ and activated microglia by demonstrating that exogenous apoJ activates rodent microglia *in vivo* and *in vitro*. Intracerebroventricular infusion of purified human plasma apoJ (~4 μg over 28 days) activated parenchymal microglia to a phenotype characterized by enlarged cell bodies and processes (phosphotyrosine immunostaining). *In vitro*, primary rat microglia were also activated by apoJ, with changes in morphology and induction of major histocompatibility complex class II (MHCII) antigen. ApoJ increased the secretion of reactive nitrogen intermediates in a dose-dependent manner (EC_{50} 112 nM), which was completely blocked by aminoguanidine (AG), a nitric oxide synthase inhibitor. However, AG did not block the increased secretion of tumor necrosis factor-α by apoJ (EC_{50} 55 nM). Microglial activation by apoJ was also blocked by an anti-apoJ monoclonal antibody (G7), and by chemical cleavage of apoJ with 2-nitro-5-thiocyanobenzoate. The mitogen-activated protein kinase kinase and protein kinase C inhibitors PD98059 and H7 inhibited apoJ-mediated induction of reactive nitrogen intermediate secretion from cultured microglia. As a functional measure, apoJ-activated microglia secreted neurotoxic agents in a microglia–neuron co-culture model. We hypothesize that ApoJ contributes to chronic inflammation and neurotoxicity through direct effects on microglia.

Keywords: Alzheimer's, apoJ, clusterin, microglia, neurotoxicity, nitric oxide, tumor necrosis factor-α.
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Apolipoprotein J (apoJ; also known as clusterin and SGP-2) is a ubiquitous glycoprotein found in most biological fluids and tissues where its function remains unresolved (Jones and Jomary 2002; Finch 1999; Holtzman 2004). ApoJ is one of the most abundantly expressed apolipoproteins in the CNS and is secreted by astrocytes, the primary CNS source (Pasinetti *et al*. 1994; Morgan *et al*. 1995). Its diverse biological activities include associations with cell death, cell–cell interactions, macromolecular aggregation, lipid transport, extracellular chaperone activities, and complement-mediated cell lysis. Within the CNS, apoJ mRNA and protein is selectively increased in neocortical brain regions that degenerate in Alzheimer's disease (AD) (May *et al.* 1990; Oda *et al*. 1994; Bertrand *et al*. 1995; Lidstrom *et al*. 1998; Holtzman 2004). ApoJ immunoreactivity is detected in diffuse, neuritic and cerebrovascular β-amyloid (Aβ) deposits (Choi-Miura *et al*. 1992; McGeer *et al*. 1992; Kida *et al*. 1995; Zhan *et al*. 1995; Verbeek *et al*. 1998). Astrocytes near senile plaques express apoJ (May *et al.* 1990) and secrete apoJ *in vitro* (Pasinetti *et al*.

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Abbreviations used: Aβ, β-amyloid; AD, Alzheimer's disease; AG, aminoguanidine; ApoJ, apolipoprotein J; DAB, diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; DMPC, dimyristylphosphatidylcholine; ICV, intracerebroventricular; IFN-γ, interferon-γ; LPS, lipopolysaccharide; LRP-2, low-density lipoprotein receptor-related protein-2; MEK, mitogen-activated protein kinase kinase; MHCII, major histocompatibility complex class II; NTCB, 2-nitro-5-thiocyanobenzoate; PBS, phosphate-buffered saline; PKC, protein kinase C; PT, phosphotyrosine; PTX, pertussis toxin; RNI, reactive nitrogen intermediates; TNF-α, tumour necrosis factor-α.

1994; Morgan *et al*. 1995; LaDu *et al*. 1998; Fagan *et al*. 1999; Han *et al*. 2001; Patel *et al*. 2004).

Bigenic mice (PDAPP × apoJ–/–) reveal an important role for apoJ in generation of fibrillar Aβ (amyloid) plaques and associated neuritic dystrophy (DeMattos *et al*. 2002, 2004). The absence of apoJ (PDAPP × apoJ–/–) decreased fibrillar amyloid plaques and reduced the soluble pool of Aβ without altering total Aβ. Amyloid plaque-associated neuritic dystrophy was also decreased in the absence of apoJ. The activity of apoJ as a chaperone for Aβ may partially explain these observations (Ghiso *et al*. 1993; Matsubara *et al*. 1995; Oda *et al*. 1995; Zlokovic *et al*. 1996; Lambert *et al*. 1998, 2001). In particular, apoJ can promote the aggregation of Aβ into oligomeric assemblies that are highly neurotoxic (Oda *et al*. 1995; Lambert *et al*. 1998; Klein *et al*. 2001).

ApoJ may have either cytoprotective or cytotoxic properties, depending on the experimental model. In non-neuronal cell lines, overexpression of apoJ inhibited tumour necrosis factor-α (TNF-α)-mediated apoptosis (Sensibar *et al*. 1995; Humphreys *et al*. 1997), whereas the silencing of *apoJ* gene expression increased chemosensitivity and apoptosis (Trougakos *et al*. 2004). ApoJ also protected other non-neuronal cell lines from oxidative stress (Schwochau *et al*. 1998; Viard *et al*. 1999). In contrast, apoJ secreted from astrocytes potentiated neuronal death induced by oxygen/glucose deprivation, and apoJ deficient mice showed less neurodegeneration in a neonatal hypoxia–ischemia model (Han *et al*. 2001).

In the present study, apoJ activated microglia when infused into the brain or when added to cultured microglia. Furthermore, apoJ-activated microglia secreted reactive nitrogen intermediates and TNF-α that might have contributed to the neurotoxicity observed in apoJ-treated neuron–microglia co-cultures. These results demonstrate that apoJ may participate in neurotoxic mechanisms by activating microglia.

Materials and methods

Purification of apoJ

ApoJ was purified from human serum or from culture medium of hamster fibroblasts transfected with a human apoJ cDNA (generously provided by Patrick May, Eli Lilly; Boggs *et al*. 1996) by means of an apoJ antibody affinity column with subsequent HPLC (Oda *et al*. 1994). Briefly, human plasma in 20 mM Tris-HCl (pH 7.4), 0.02% NaN_3, 1 mM EDTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride and 0.4 μM aprotinin was stirred overnight at 4°C. The 5000 *g* supernatant was applied to a DEAE Sephacel column in the same buffer as above and the 0.5-mM NaCl eluate was applied to an apoJ antibody (G7) affinity column (CNBr-activated Sepharose 4B). The apoJ fraction was eluted with 0.1 M glycine (pH 2.5), concentrated with Centricon 30 (Amicon, Beverly, MA, USA) and further purified by gel filtration (Bio-Sil SEC 250) on an HPLC column equilibrated in 10 mM sodium phosphate (pH 6.8), 0.1% sodium dodecyl sulfate and 0.15 M NaCl. After concentration with Centricon 50, the purified apoJ was delipidated during dialysis in 5 M guanidine HCl. Purified apoJ in phosphate-buffered saline (PBS) (5 μg/μL) was stored at −80°C. Unlike ApoE, activities of apoJ are not dependent on lipidation (see Calero *et al*. 1999).

ApoJ infusion

ApoJ reconstituted in myristylphosphatidylcholine (DMPC; Panvera) (Innerarity *et al*. 1986) was administered to rats as an intracerebroventricular (ICV) infusion (Frautschy *et al*. 1996, 1998). ApoJ-DMPC (21 μg/mL, 4.27 μg total) or DMPC liposome (vehicle) was infused via a stainless steel ICV catheter (Plastics One, Roanoke, VA, USA) connected to an Alzet mini-osmotic pump (model 2004; Durect Corp., Palo Alto, CA, USA). The rate of delivery was 6 μL/day for 28 days into the right lateral ventricle (coordinates: medial lateral (M/L) −1.4, anterior posterior (A/P) −0.6, dorsal ventral (D/V) −3.5 from dura) of 9-month-old female Sprague–Dawley rats (six rats per treatment). Surgical and animal care procedures were carried out with strict adherence to the guidelines described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell culture

Primary mixed glial cells were cultured from the cerebral cortex of postnatal day 3 F344 rats as described previously (Giulian and Baker 1986). After 14–21 days, microglia were detached from the cellular monolayer by gentle shaking and replated into (1) Laboratory-Tek four-well chamber slides (NalgeNunc International, Naperville, IL, USA); (2) 96-well plates (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA); and (3) cell culture inserts (pore size 0.4 μm; Costar, Cambridge, MA, USA) for co-culture (Xie *et al*. 2002). Microglial homogeneity was > 98% (Morgan *et al*. 1995). The murine microglial cell line BV-2 (Blasi *et al*. 1990) was plated at 10 000 cells/well in 96-well plates. For microglia–neuron co-cultures (Xie *et al*. 2002), neurons were first cultured from fetal rat cortex (E17) (Rozovsky *et al*. 1994). Before treatment (6–7 days *in vitro*), inserts containing microglia were placed into the neuron-containing wells. Culture medium for microglia, BV-2 and microglia–neuron co-cultures was Dulbecco's modified Eagle's medium (DMEM)–F_{12} ± 10% fetal bovine serum depending on experimental design. Neurons were initially plated in DMEM with B27 supplements.

Immunocytochemistry and image analysis

Paraffin coronal sections (8 μm) 2.0 mm posterior to the infusion site (correcting for cannulation injury responses) were steamed for 30 min with Antigen Revealing Buffer (Vector Laboratories, Burlingame, CA, USA) and then incubated with or without (no primary control) phosphotyrosine (PT) antibody (1 : 1000; Sigma, St Louis, MO, USA). Primary antibody was followed by horse anti-mouse secondary antibody, ABC-Elite kit (Vector Laboratories) and metal diaminobenzidine (DAB) visualization (Pierce, Woburn, MA, USA). Immunostaining of fixed sections with PT antibody specifically labels microglia (Karp *et al*. 1994; Korematsu *et al*. 1994). After coverslipping, image analysis was performed and percentage area stained and cell density (cell body per mm^2) per brain region calculated (Frautschy *et al*. 1998). Regions analyzed by 2 × 2 ANOVA were the cortical layers frontal (I/II, II, IV, V/VI), parietal

(I/II, II,IV, V/VI), temporal (I/II, II,IV, V/VI) and entorhinal (III/IV). A *t*-test was used to evaluate the change in PT immunoreactivity in the corpus callosum. Data were collected from triplicate adjacent sections equidistant from the cannula site for each animal. Statistical analysis was performed using a 2 × 2 ANOVA (treatment × region) and Fisher's protected least squares difference to determine differences between preplanned comparisons (StatView 4.5, Abacus, Berkeley, USA).

After treatment, cultured microglia grown on four-well chamber slides were rinsed in PBS, fixed in fresh, buffered (pH 7.4) 4% paraformaldehyde (Fisher, Pittsburgh, PA, USA) for 1.5 h at room temperature (20°C), dehydrated in increasing concentrations of ethanol, and stored at − 70°C. After blocking with 1% normal rabbit serum (Sigma) and 1% bovine serum albumin (Sigma) in PBS, slides were incubated overnight at 4°C with primary antibody OX6 [mouse anti-rat major histocompatibility complex class II (MHCII) at 1 : 50 dilution; Serotec, Raleigh, NC, USA]. After washing in PBS, secondary biotinylated antibody (rabbit anti-mouse; Serotec) was added to the slides and incubated for 1 h at room temperature. Vectastain ABC Kit (Vector Laboratories) was used for the binding of avidin-biotinylated horseradish peroxidase to the secondary biotinylated antibodies, and DAB was used as the substrate. Morphology of immunostained microglia was assigned as resting (flattened cell body bearing processes), or activated (rounded, amoeboid cell body without processes).

Nitrite measurement

Nitric oxide in conditioned media was assayed indirectly as its stable metabolite nitrite using the Griess reagent and measurement at 550 nm (Ding *et al*. 1988; Xie *et al*. 2002).

TNF-α assay

TNF-α in conditioned media of cultured microglia was assayed by ELISA (Genzyme; Cambridge, MA, USA).

Immunotitration of apoJ

ApoJ (2000 nM) was incubated with monoclonal mouse anti-human apoJ antibody G7 (Quidel, San Diego, CA, USA) or with control antibody (mouse anti-cow glial fibrillary acidic protein; Boehringer Mannheim, Indianapolis, IN, USA) at antibody : ApoJ molar ratios of 2 : 1, 1 : 1, 0.5 : 1 and 0.1 : 1 for 30 min at 37°C, then diluted 10-fold in culture media for microglial treatment.

Chemical cleavage of apoJ

2-Nitro-5-thiocyanobenzoate (NTCB; Sigma) was used to cleave apoJ at cysteinyl residues (Smith 1996). Briefly, NTCB at a 10-fold excess over the sulfhydryl groups in apoJ (2 mg/mL, pH 8.0) was incubated at 37°C for 20 min. Cleavage occurred after adjusting the pH to 9.0 and incubating at 37°C for 16 h. Cleaved peptides were recovered by ultrafiltration (Centricon-3; Millipore, Bedford, MA, USA) and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as fragments of 7–15 kDa.

Neuron viability

Following treatment, culture inserts containing microglia were removed and neurons were stained with 10 μg/mL fluorescein diacetate (Sigma). Fluorescein diacetate is membrane permeable and freely enters intact cells, where it is hydrolyzed by cytosolic esterase and converted to membrane-impermeable fluorescein, with a green fluorescence exhibited only by live cells. Because neuron death occurs directly below the microglial inserts (Xie *et al*. 2002), images at the center of each well ($n = 8$) were taken with a Nikon (Tokyo, Japan) TE300 fluorescence microscope and analyzed by IP Laboratory imaging software (Scanalytics, Fairfax, VA, USA). Viable neurons were quantified by the area of green fluorescence. The total area analyzed was 30% of the area directly under the culture inserts.

Other reagents

Lipopolysaccharide (LPS; *Escherichia coli* strain O26 : B6) was purchased from Sigma; PD98059, H7 and pertussis toxin (PTX) were from RBI (Natick, MA, USA); and recombinant mouse interferon-γ (IFN-γ) was from R & D Systems (Minneapolis, MN, USA). Hexafluoroisopropanol (HFIP)-treated Aβ1–42 (US Peptide, Fullerton, CA, USA) was dissolved in dimethylsulfoxide (Sigma) to produce a 5-mM stock which was diluted to 80 μmol/L in PBS and incubated at 37°C for 2 h. Before treating cells, this Aβ solution was briefly vortexed. This preparation of Aβ contained diverse monomeric, oligomeric and fibrillar Aβ forms.

Statistical analysis

In vitro experiments were repeated independently three to six times. Data plotted show a representative experiment, unless noted otherwise in figure legends. Some error bars are smaller than the data symbols. Student's *t*-test was used for statistical comparisons ($p < 0.05$). Dose–response data were fitted to a sigmoidal dose–response curve (Prism 3.0; GraphPad Software, San Diego, CA, USA).

Results

Microglia are activated *in vivo* by infusion of apoJ

ICV infusion of apoJ (∼4 μg total) for 28 days caused widespread microglial activation. Microglia in control infusions had small, lightly PT-immunoreactive cell bodies (Figs 1a and c). Microglia in apoJ-infused rats had large, intensely PT-immunoreactive cells bearing thick processes with an activated morphology (Figs 1b and d), particularly near perivascular regions of the cortex (arrows, Figs 1b and d). The total PT-immunoreactive area was increased by ∼30% in cortical layers (Fig. 1e) and ∼85% in the corpus callosum (Fig. 1f) relative to that in DMPC vehicle-infused rats. The density of PT-stained microglia (cell bodies/mm^2) increased similarly (∼35% in cortical layers III–VI; ∼60% in corpus callosum).

ApoJ causes morphologic and phenotypic changes in cultured microglia

In primary rat microglial cultures, human apoJ (400 nM) induced activated morphologic characteristics by 48 h (Fig. 2). ApoJ transformed MHCII-immunopositive microglia from a lightly stained, process-bearing morphology (Fig. 2a) into a darkly stained, process-free amoeboid-like

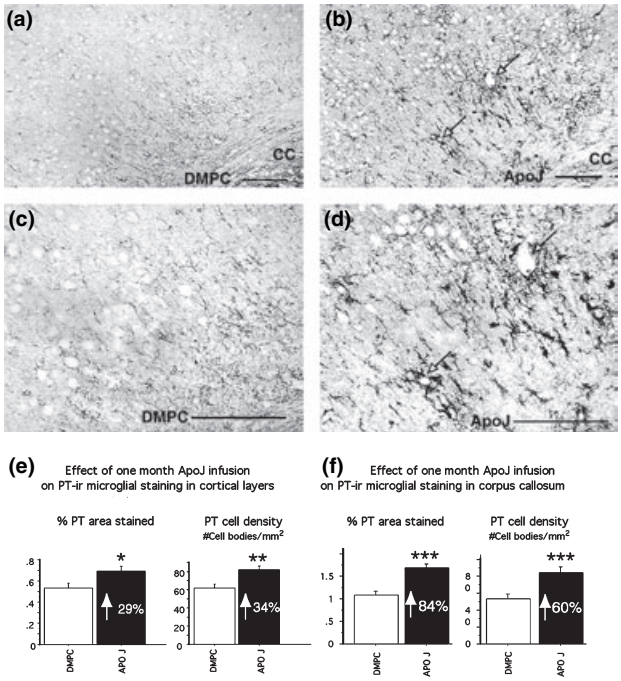

Fig. 1 ICV infusion of apoJ activates microglia, as assessed by PT immunoreactivity. (a, c) Effect of infusion of vehicle (DMPC) on PT immunoreactivity of microglia in corpus callosum (CC) and adjacent cortical layers. Image in (c) is a 2 × magnification of that in (a). Scale bar 100 μm. (b, d) The same region from a rat infused with ApoJ (4.27 μg or 50 pmols, 28 days). Image in (d) is a 2 × magnification of that in (b). Scale bar 100 μm. (e) Quantification of PT-immunoreactive (PT-ir) staining including total area stained (% PT area) and cell density (#cell bodies/mm^2). ApoJ-induced increases in percentage area and microglial cell density were observed only in cortex layers III, IV and V/VI ($p < 0.05$), but not in layer I or II (2×2 ANOVA, treatment × layer). (f) Corpus callosum showed greater increases in microglial PT staining than was observed in cortical layers III–VI. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$.

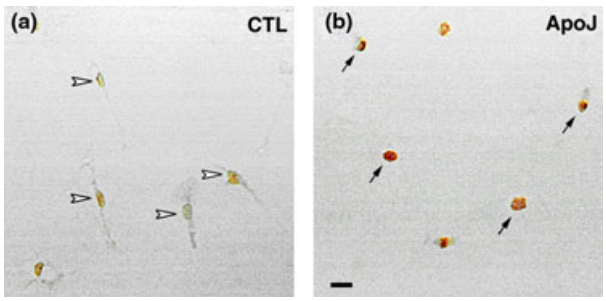

Fig. 2 Activation of cultured rat microglia by apoJ. Rat microglia were treated with 400 nM apoJ for 48 h. Representative photomicrographs of MHCII-immunoreactive microglia in (a) control (CTL) and (b) apoJ-treated microglial cultures are shown. Note the lightly stained, process-bearing microglial morphology (white arrowhead) in control cultures and the more darkly stained, process-free, amoeboid-like microglial morphology (black arrow) in apoJ- treated microglia. Scale bar 10 μm.

morphology (Fig. 2b). ApoJ treatment caused a sixfold increase in the number of microglia with an activated morphology (78 ± 3% vs. 12 ± 3%; $p < 0.0001$). ApoJ at this dose did not affect microglial viability (data not shown).

ApoJ induces nitric oxide release from cultured microglia

Because apoJ potentiates neuronal death *in vitro* (Han *et al.* 2001), we evaluated biochemical features of apoJ-induced microglial activation with apoJ from two sources, plasma and recombinant. Human plasma apoJ caused a dose-dependent microglial production of nitrite, the stable metabolite of nitric oxide (Fig. 3a). Recombinant human apoJ (Boggs *et al.* 1996) had similar activity (data not shown). IFN-γ (40 U/mL) potentiated the effect of apoJ on nitrite production and shifted the EC_{50} from 112 to 31 nM ($p < 0.01$). A comparable EC_{50} (35 nM) was observed for murine BV-2 microglia, when treated with apoJ and IFN-γ (Fig. 3b); however, in BV-2 microglia, apoJ did not induce nitrite release in the absence of IFN-γ. The competitive nitric oxide synthase inhibitor aminoguanidine (AG; 10 mM) completely blocked apoJ-induced nitrite production in both primary microglia and BV-2 cells ($p < 0.0005$ vs. apoJ alone) (Figs 3a and b, open symbols). ApoJ-induced nitrite release from cultured microglia was not sensitive to the presence of fetal bovine serum (data not shown).

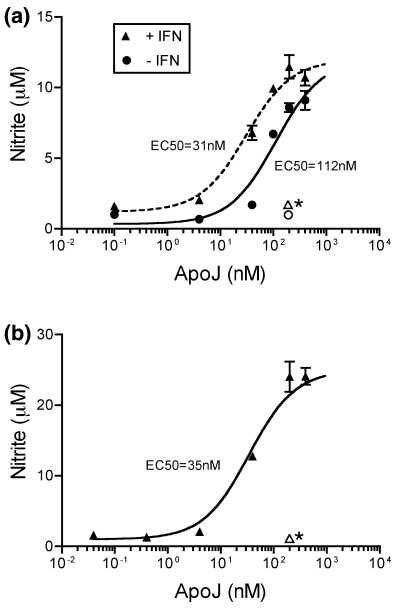

Fig. 3 ApoJ-induced release of nitric oxide from (a) rat microglia and (b) BV-2 microglial cells. Microglia were treated with apoJ (0, 0.4, 4, 40, 200 and 400 nM) with (+ IFN) or without (– IFN) 40 U/mL recombinant mouse IFN-γ. Nitrite concentrations in conditioned media were determined 24 h later. The nitric oxide synthase inhibitor AG (10 mM) was added simultaneously with 200 nM apoJ (open symbols). AG completely blocked apoJ induction of nitric oxide (*$p < 0.0005$ vs. 200 nM apoJ alone; Student's *t*-test). Values are mean ± SEM of three replicates.

ApoJ activation was compared with LPS- and Aβ-induced microglial activation (Fig. 4). Nitrite increases were significant by 8 h and continued for at least 48 h in both apoJ- and LPS-treated BV-2 cells (all treatments included IFN-γ). ApoJ had a more significant effect than aggregated $A\beta_{1-42}$, despite a 20-fold lower molar concentration.

The specificity of the apoJ effect was evaluated by immunotitration. Preincubation of apoJ with the murine anti-human apoJ antibody G7 neutralized the activation of microglia by apoJ in a dose-dependent manner, with an IC_{50} of 223 nM (Fig. 5a). Control antibody or G7 alone had no effect (data not shown).

To further evaluate the specificity of these effects apoJ was chemically cleaved by NTCB at its 10 cysteine residues. The resulting apoJ peptide fragments (7–15 kDa) did not activate primary microglia (Fig. 5b). NTCB-treated LPS and untreated LPS activated microglia similarly, whereas NTCB alone had no effect (Fig. 5b).

ApoJ stimulation of nitric oxide is attenuated by kinase inhibitors

Signal transduction pathways were evaluated with kinase inhibitors. The MEK inhibitor PD98059 blocked apoJ-induced microglial activation (IC_{50} 2 μM) (Fig. 6a). PKC inhibition by H7 (50 μM) also abolished apoJ-induced microglial nitric oxide production (Fig. 6b). However, PTX (30 ng/mL), an inactivator of G_i/G_o protein, had no effect (Fig. 6c). Microglial viability was not affected by these drugs (data not shown).

ApoJ induces TNF-α secretion from cultured microglia

TNF-α, which is released by activated microglia (Wood 1995; Xie *et al*. 2002), was increased by exogenous apoJ in a dose-dependent fashion (EC_{50} 55 nM) (Fig. 7). The increased TNF-α secretion was not strongly dependent on nitric oxide, because the nitric oxide synthase inhibitor AG only slightly attenuated apoJ-induced TNF-α secretion (Fig. 7, empty circles). The concentration of apoJ in CSF is ~30 nM (Choi-Miura *et al*. 1992), which is close to EC_{50}

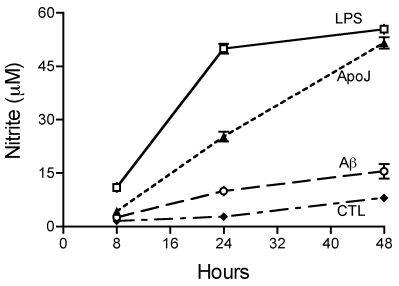

Fig. 4 Time course of nitric oxide production from activated microglia. BV-2 cells were treated with culture medium (CTL), apoJ (400 nM), aggregated $A\beta_{1-42}$ (8 μM) or LPS (100 ng/mL) for 8, 24 or 48 h, followed by nitrite assay. All wells contained 40 U/mL IFN-γ. Values are mean ± SEM of six replicates.

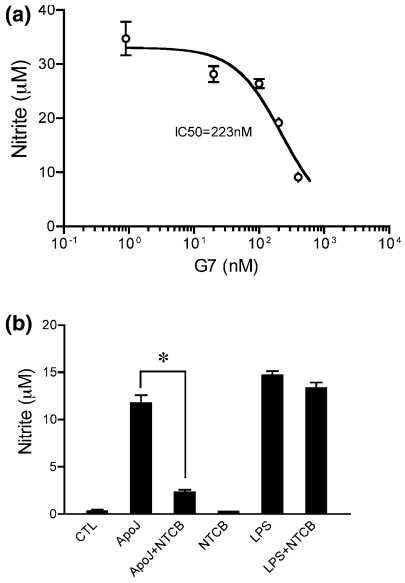

Fig. 5 Inhibition of apoJ activation of microglia by anti-apoJ or chemical cleavage. (a) Rat microglia were treated for 24 h with preincubated apoJ (200 nM) + 0, 20, 100, 200 or 400 nM anti-human apoJ antibody G7, followed by nitrite assay. The IC_{50} for G7 was 223 nM. (b) ApoJ chemically cleaved by NTCB at the cysteine residues (ApoJ + NTCB, containing 100 nM apoJ), untreated apoJ (100 nM), NTCB alone, LPS (100 ng/mL), or LPS + NTCB were added to primary rat microglia cultures and incubated for 24 h, followed by nitrite assay. Values are mean ± SEM of three replicates. *$p < 0.0001$ (Student's *t*-test).

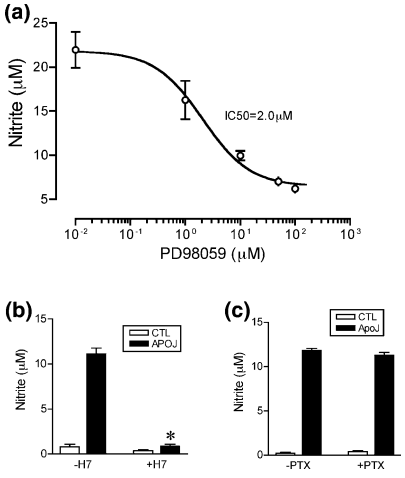

Fig. 6 Protein phosphorylation mediates apoJ-induced microglial activation. Rat microglia were treated with 200 nM apoJ in the absence or presence of various inhibitors. Microglial activation was assayed by quantifying nitrite in conditioned media after 24 h. Values are mean ± SEM of three replicates. (a) PD98059 (0–100 μm), a MEK inhibitor, dose dependently blocked nitric oxide secretion. The IC_{50} of PD98059 was 2.0 μM. (b) H7 (50 μM), a PKC inhibitor, also inhibited apoJ-stimulated nitric oxide secretion (*$p < 0.0001$ vs. apoJ-stimulated in the absence of H7; Student's *t*-test). (c) PTX (30 ng/mL), an inactivator of Gi/Go protein, had no effect.

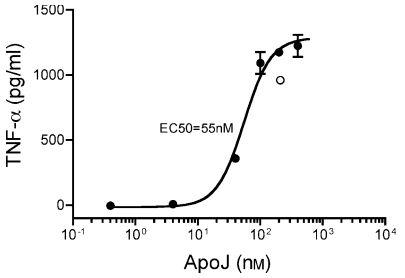

Fig. 7 ApoJ dose dependently induces the release of TNF-α from cultured microglia. Rat microglia were treated with 0, 4, 40, 100, 200 or 400 nM apoJ for 24 h. AG (10 mM) was also added with 200 nM apoJ (empty circle). EC_{50} was 55 nM. Values are mean ± SEM of four replicates.

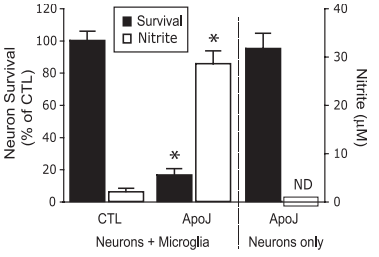

Fig. 8 ApoJ-induced microglial activation leads to neuron death in neuron–microglia co-cultures. Rat neurons were co-cultured with (Neurons + Microglia) or without (Neurons only) microglia. Microglia were grown in the culture insert and neurons on the culture well surface. Co-cultures were treated with 200 nM apoJ for 48 h, after which nitrite was sampled in supernatants and viable neurons were counted. ND, not detectable. Values are mean ± SEM of three independent experiments, each performed in duplicate. $^*p < 0.001$ versus control (CTL) (Student's *t*-test).

values observed *in vitro* (nitric oxide, 112 nM; TNF-α, 55 nM), suggesting that these activities are physiologically relevant.

ApoJ-induced microglial activation causes neurotoxicity in neuron–microglia co-cultures

Neurotoxins secreted by apoJ-activated microglia were assayed in neuron–microglia co-cultures (Xie *et al*. 2002). ApoJ (200 nM, 48 h) activated microglia and increased nitrite (Fig. 8) with extensive neurotoxicity (84 ± 3% reduction in viable neurons; $p < 0.001$) (Fig. 8). In the absence of microglia, apoJ did not affect neuronal viability or nitrite secretion.

Discussion

We have demonstrated a novel activity of apoJ in the activation of microglia and the potential to modulate CNS inflammatory responses. This property was shown both *in vivo* and *in vitro*. *In vitro*, apoJ increased microglial secretion of nitric oxide and TNF-α, and these effects resulted in a microglial-dependent neurotoxic activity for apoJ. These results provide the first evidence that a neurotoxic activity of apoJ can be mediated through direct effects on microglia.

In vivo, chronic ICV infusion (Frautschy *et al*. 1996, 1998) of apoJ was used as a model for the increase in apoJ observed in many neurodegenerative diseases (reviewed in Finch 1999). The ICV infusion of apoJ at ~1.8 pmol/day for 28 days activated microglia above the level seen in vehicle-infused controls. Because reactive astrocytes are the primary source of lesion-induced apoJ increases (Pasinetti *et al*. 1994; Morgan *et al*. 1995), we hypothesize that the secretion of apoJ by reactive astrocytes induces persistent activation of microglia during neurodegenerative events. This is pertinent to AD because regional increases in apoJ levels correlate with regional AD-related inflammation and neurodegenerative pathology, e.g. hippocampus but not cerebellum (May *et al*. 1990; Oda *et al*. 1994; Lidstrom *et al*. 1998). Thus, apoJ-mediated microglial activation may contribute to the chronic microglial activation observed in AD.

In our infusion model, apoJ-activated microglia were especially prominent near the perivascular space within the cortical layers (Fig. 1d). These activated microglia might have been resident or recently recruited. Parenchymal microglia abutting the perivascular space are an important component of the blood–brain barrier (Lassmann *et al*. 1991; Williams *et al*. 2001). At the blood–brain barrier, cerebral vessels display a specific receptor-mediated mechanism for transport of apoJ (and apoJ–Aβ complexes) (Zlokovic *et al*. 1996; Chun *et al*. 1999), which may make these microglia particularly responsive to apoJ. Because apoJ is associated with cerebrovascular Aβ deposits (Kida *et al*. 1995; Verbeek *et al*. 1998), apoJ activation of microglia at this site may intensify inflammation associated with cerebrovascular amyloidosis (Verbeek *et al*. 1998).

Receptors for apoJ on parenchymal brain cells have not been clearly identified. Specific interactions are implied by the similar EC_{50} values of apoJ for microglial activation (nitric oxide, 112 nM; TNF-α, 55 nM) and the effects of blocking ApoJ using specific antiserum and peptide cleavage. The amphipathic structure and heparin-binding domains of ApoJ are also consistent with its binding to cellular membranes. However, the only identified membrane receptor with a high affinity for apoJ is low-density lipoprotein receptor-related protein-2 (LRP-2), also known as megalin and gp330 (Kounnas *et al*. 1995). We detected LRP-2 mRNA in cerebral microvessels and choroid plexus, but not in neurons or glia (Chun *et al*. 1999). Because LRP-2 immunoreactivity is detected in some neurons of AD brains (LaFerla *et al*. 1997), it is possible that a soluble form of LRP-2 is released by microvessels and acquired by other cells enabling responses to apoJ.

In CSF and conditioned medium from cultured astrocytes, a fraction of the total apoJ is associated with lipids in distinct

high-density lipoprotein-like particles that are low in apoE (LaDu *et al*. 1998; Fagan *et al*. 1999). The present *in vivo* infusions utilized apoJ reconstituted into liposomes (DMPC) to enhance the distribution and potency of the infusate (Frautschy *et al*. 1996, 1998). Some ApoJ activities, such as inhibition of complement-mediated cell lysis and binding to Aβ, do not depend on the presence of lipids (Calero *et al*. 1999). In the present *in vitro* experiments, delipidated ApoJ protein alone sufficed to activate microglia. It is cogent that apoJ activates cultured microglia similarly with or without serum, which suggests a minimal role of lipids or other soluble factors acting as carriers for apoJ.

In vitro, both nitric oxide and TNF-α accumulated in the medium from apoJ-treated microglia (Figs 3 and 4). The increase in nitric oxide was blocked by AG, a competitive inhibitor of nitric oxide synthase, implying that apoJ-induced nitric oxide production requires *de novo* synthesis. However, induction of TNF-α secretion by apoJ was not blocked with AG, suggesting that TNF-α induction occurs independently of nitric oxide synthase activation. Because both TNF-α and nitric oxide are implicated in microglial-dependent neurotoxicity (Meda *et al*. 1995; Chao *et al*. 1995; Wood 1995; Combs *et al*. 2001; Xie *et al*. 2002), apoJ-induced microglial-dependent neurotoxicity (Fig. 8) may be mediated through these agents. This mechanism may be pertinent to AD as TNF-α and nitric oxide synthase are increased in AD brain (Heneka *et al*. 2001; Tarkowski *et al*. 1999). Therefore, in AD, apoJ may promote neurotoxicity by increasing microglial secretion of TNF-α or nitric oxide.

We also showed that ApoJ initiates a signal cascade involving PKC and MEK, as the kinase inhibitors H7 and PD98059 blocked apoJ-induced microglial activation (Fig. 6). A similar pathway has been reported for activation of microglia by fibrillar Aβ involving a tyrosine kinase-based mechanism (McDonald *et al*. 1998; Combs *et al*. 1999; Bamberger *et al*. 2003). *In vivo* microglial activation was associated with increased microglial PT levels after exposure to apoJ (Fig. 1). Together, the *in vivo* and *in vitro* responses suggest similar mechanisms of microglial activation by apoJ.

Other reports have shown that ApoJ may contribute to neurotoxicity by acting directly on neurons. For example in a neonatal hypoxia–ischemia mouse model (Han *et al*. 2001) apoJ accumulated in dying neurons. The nuclear localization of apoJ in necrotic neurons is consistent with an alternative nuclear form of apoJ contributing to cell death (Yang *et al*. 2000; Leskov *et al*. 2003). The decreased neurotoxicity in apoJ-deficient mice in the hypoxia–ischemia model is consistent with apoJ-dependent neurotoxic pathways; however, the microglial contribution was not examined. The present data suggest that apoJ-deficient mice should have reduced microglial responses in parallel to decreased neurotoxicity observed in this model.

In contrast, there is evidence for protective functions of apoJ. For example, apoJ was neuroprotective in an Aβ1–40-induced toxicity model using mixed glial–neuronal cultures (Boggs *et al*. 1996). However, apoJ did not protect against either H_2O_2 or kainic acid excitotoxicity. The authors concluded that inhibition of Aβ aggregation by apoJ was the principal mechanism underlying the protective effects. Nonetheless, endogenous apoJ also needs consideration because cultured astrocytes secrete abundant apoJ (Pasinetti *et al*. 1994; Morgan *et al*. 1995; Han *et al*. 2001), as well as other neuroprotective agents (Ridet *et al*. 1997). Because of these other protective factors secreted by astrocytes, we excluded astrocytes from our experimental paradigm.

These findings have important mechanistic implications for brain aging and inflammatory neurodegenerative diseases such as AD. The increase in apoJ with age (Senut *et al*. 1992; Patel *et al*. 2004) may exacerbate age-related diseases such as AD. Understanding the mechanism by which apoJ activates microglia might be useful for understanding the inflammatory and neurotoxic activities in AD that are both dependent and independent of Aβ. Ongoing experiments may resolve unique and shared activation pathways for both apoJ and Aβ.

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