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Globular adiponectin induces a pro-inflammatory response in human astrocytic cells

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ABSTRACT

Neuroinflammation, mediated in part by activated brain astrocytes, plays a critical role in the development of neurodegenerative disorders, including Alzheimer's disease (AD). Adiponectin is the most abundant adipokine secreted from adipose tissue and has been reported to exert both anti- and pro-inflammatory effects in peripheral tissues; however, the effects of adiponectin on astrocytes remain unknown. Shifts in peripheral concentrations of adipokines, including adiponectin, could contribute to the observed link between midlife adiposity and increased AD risk. The aim of the present study was to characterize the effects of globular adiponectin (gAd) on pro-inflammatory cytokine mRNA expression and secretion in human U373 MG astrocytic cells and to explore the potential involvement of nuclear factor (NF)-κB, p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK) and phosphatidylinositide 3-kinases (PI3 K) signaling pathways in these processes. We demonstrated expression of adiponectin receptor 1 (adipoR1) and adipoR2 in U373 MG cells and primary human astrocytes. gAd induced secretion of interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1, and gene expression of IL-6, MCP-1, IL-1β and IL-8 in U373 MG cells. Using specific inhibitors, we found that NF-κB, p38MAPK and ERK1/2 pathways are involved in gAd-induced induction of cytokines with ERK1/2 contributing the most. These findings provide evidence that gAd may induce a pro-inflammatory phenotype in human astrocytes.

1. Introduction

Inflammatory processes are triggered in the brains of Alzheimer's disease (AD) patients as well as rodent models of the disease [1–3]. Although inflammation is likely not the initiating event causing AD pathology, neuroinflammation appears to play a critical role in disease progression [2]. Neuroinflammation is primarily mediated by activated glial cells including microglia and astrocytes [4,5]. Astrocytes are the most abundant glial cell type in the brain; their activation is characterized by excessive production of pro-inflammatory mediators (e.g., cytokines, free radicals), which can lead to neuronal damage and death [6,7].

Adiponectin is an adipokine secreted predominantly by adipocytes from peripheral fat tissues [8]. Adiponectin circulates in the bloodstream at high concentrations (μg/ml range) and has profound physiological effects on distant tissues, including improving insulin sensitivity [9,10] and vascular function [11]. Adiponectin circulates in trimer, hexamer and high-molecular weight forms [12] as well as the globular form, which is produced after proteolytic cleavage of full-length adiponectin monomers by neutrophil elastase [13]. Different isoforms of adiponectin have been shown to play distinct biological roles in peripheral tissues [14,15]. It is generally accepted that adiponectin is an anti-inflammatory adipokine, as reported in multiple cell types including pig primary adipocytes and 3T3-L1 adipocytes [16], human aortic endothelial cells [17,18] and macrophages [19–21]. Adiponectin is inversely associated with adiposity, resulting in lower circulating levels of adiponectin in obesity. Reduced adiponectin is therefore thought to contribute towards a chronic low-grade inflammatory state in

Abbreviations: AD, Alzheimer's disease; adipoR, adiponectin receptor; CNS, central nervous system; DMEM-F12, Dulbecco's modified Eagle medium nutrient mixture F-12 Ham; ERK, extracellular signal-regulated kinase; gAd, globular adiponectin; JNK, c-Jun N-terminal kinase; LSD, least significant differences; MAPK, mitogen-activated protein kinase; MCP, monocyte chemoattractant protein; mRNA, messenger RNA; NF, nuclear factor; PI3 K, phosphatidylinositide 3-kinases; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; SEM, standard error of the mean; TNF, tumor necrosis factor.

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obesity [22,23]. However, there is also evidence challenging this traditionally accepted viewpoint. The structure of globular adiponectin (gAd) shows remarkable similarity to tumor necrosis factor (TNF)-α, indicating that gAd could possess pro-inflammatory properties [24]. Indeed, gAd induces TNF-α and interleukin (IL)-6 secretion in both human and murine macrophages [25] and upregulates pro-inflammatory genes including monocyte chemoattractant protein (MCP)-1, vascular cell adhesion molecule (VCAM)-1, E-selectin, IL-6, and IL-8 in vascular endothelial cells [26].

Adiponectin receptors are widely distributed in the central nervous system (CNS) [27]. A recent study has reported expression of adiponectin receptors in rat astrocytes [28] but, to our knowledge, adiponectin receptor expression and the functional effects of adiponectin have not been studied in human astrocytes. Population-based studies have shown that high circulating adiponectin is associated with increased future risk of AD [29] and elevated plasma and cerebrospinal fluid adiponectin has been reported in older adults with mild cognitive impairment [30]. These data suggest that elevated adiponectin may be linked with AD and related dementias.

Studies on the effects of adiponectin on cellular mechanisms involved in AD are limited. It has been recently reported that high concentrations of adiponectin (10 μg/ml) were protective against amyloid beta induced neurotoxicity in Sw-APP transfected SH-SY5Y cells under oxidative stress conditions [31]. To the best of our knowledge, the effects of adiponectin on neuroinflammation have not been studied so far. The aim of this study was: (1) to confirm expression of adiponectin receptor 1 (adipoR1) and adipoR2 in human astrocytes; (2) to explore the effects of gAd on cytokine (IL-6, MCP-1, IL-1β and IL-8) mRNA expression and secretion (IL-6 and MCP-1) in U373 MG astrocytoma cells; and (3) to explore the potential involvement of nuclear factor (NF)-κB, p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK) and phosphatidylinositide 3-kinases (PI3 K) signaling pathways in these processes.

2. Materials and methods

2.1. Materials

Recombinant human gAd (gAcrp30/Adipolean, cat# 450-21) was purchased from PeproTech Canada (Ottawa, ON, Canada). MCP-1 (cat#DY279) and IL-6 (cat#DY206) enzyme-linked immunosorbent assay (ELISA) kits (Duoset) were from R&D system (Minneapoli, MN). The following reagents were obtained from Thermo Fisher Scientific (Ottawa, ON, Canada): bovine serum albumin, Dulbecco's modified Eagle medium nutrient mixture F-12 Ham (DMEM-F12) and trypsin/ethylenediaminetetraacetic acid (EDTA) solution. Specific inhibitors of intracellular signaling molecules SP600125 (cat#10010466), SB202190 (cat#10010399), LY294002 (cat# 70920), PD98059 (cat# 10006726) and BAY-110–7082 (cat# 10010266) were obtained from Cayman Chemicals (Burlington, ON, Canada). Aurum RNA extraction kit, iScript cDNA synthesis kit and SSOfast quantitative PCR (qPCR) reaction mix were purchased from Bio-Rad (Mississauga, ON, Canada).

2.2. Cell culture

The human astrocytic U373 MG cell line was used as an established model of human astrocytes [32]. Cells were maintained in T75 flasks in DMEM-F12 supplemented with 10% fetal bovine serum (Life Technologies, Burlington, ON, Canada). Adherent U-373MG were detached for use in experiments by addition of 2 ml of 0.25% trypsin/EDTA solution for 5–10 min. The flask was washed with 10 ml of F10 medium and cells were counted, centrifuged at 450*g* for 7 min, and re-suspended in F5 media to a final concentration of 0.2 million cells/ml. Subsequently, 0.4 ml of U373 MG cell suspension were plated per well in sterile 24-well plates and incubated for 24 h to allow cells to adhere. On the morning of the experiment, cells were supplemented with fresh F5 for 2 h first. For the time course experiment, cells were then treated with gAd (1 μg/ml) or vehicle control [DMEM-F12 containing sterile-filtered 0.1% BSA in PBS; equivalent to the solvent for gAd] for 6, 12, 24 and 48 h. For the dose response experiment, cells were treated with gAd (1 and 3 μg/ml) for 12 h. Similarly, for the pharmacological inhibitors experiment, the specific inhibitors of p38MAPK (SB202190; 20 μM), JNK (SP600125; 5 μM), PI3 K (LY294002; 20 μM), ERK1/2 (PD98059; 25 μM), and NF-κB (BAY-110–7082; 10 μM) were administered to the cells 1 h prior to the addition of gAd (1 μg/ml) and subsequently cells were incubated for 12 h. At the end of the experiment, supernatants were collected and stored at −80 °C for measurement of cytokine secretion. Cells were washed twice with cold sterile phosphate-buffered saline and then collected for RNA extraction.

2.3. Cytokine measurement

The concentration of MCP-1 and IL-6 were measured by ELISA according to the manufacturer's instructions.

2.4. Reverse transcription (RT)-PCR analysis of adiponectin receptor expression

The expression of adiponectin receptors adipoR1 and adipoR2 in U373 MG cells and human primary astrocytes isolated from epilepsy surgical samples [32] was measured by RT-PCR. The primers used for human adipoR1 were: forward, GAG CAT CTT CCG CAT TCA TA and reverse AAG AGC CAG GAG AAG CTG AG [31]. The primers used for human adipoR2 were: forward GAC TTC CTC TTG CAT GGA CA and reverse AAA GGA GAT ATT TGG GCG AA [31].

2.5. qPCR

Astrocyte total RNA was extracted using the Aurum mini-kit. RNA (1 μg) was converted to cDNA using the iScript cDNA synthesis kit. qPCR was performed using a CFX96 Real-Time System (Bio-Rad). Primers were custom-designed for SYBR chemistry using the NCBI Primer-BLAST tool and are shown in Table 1. Relative differences in gene expression between groups were determined using the $2^{-\Delta\Delta CT}$ method. The amplification efficiencies of the gene of interest and the housekeeping gene (18S) were equivalent (between 90%–110%). Experimental manipulations had no effect on the expression of housekeeping genes (<0.5 cycle threshold difference).

2.6. Statistical analysis

Treatments were performed in duplicate or triplicate with at least three independent experiments conducted. All values are

Table 1
Primers used for analysis of gene expression.

Genes	5′-sense primer-3′5′-antisense primer-3′
IL-6	GAC CCA ACC ACA AAT GCC A GTC ATG TCC TGC AGC CAC TG
IL-8	CTG GCC GTG GCT CTC TTG CCT TGG CAA AAC TGC ACC TT
MCP-1	CTC TGC CGC CCT TCT GTG TGC ATC TGG CTG AGC GAG
IL-1β	AGC TGA GGA AGA TGC TGG T GTT ATC CCA TGT GTC GAA G
18S	GTA ACC CGT TGA ACC CCA TT CCA TCC AAT CGG TAG TAG CG

expressed as the means ± standard error of the mean (SEM). To control for variability in the absolute values obtained during independent experiments performed on different days, randomized block design analysis of variance (ANOVA) was used. Significant effects were followed by Fisher's least significant differences (LSD) post hoc testing. Effects of gAd versus vehicle control on IL-6 and MCP-1 secretion at the same time point were compared by Student's *t*-test.

3. Results and discussion

3.1. Adiponectin receptor (adipoR) expression in U373 MG cells and primary human astrocytes

RT-PCR results demonstrated that both adipoR1 and adipoR2 are expressed in U373 MG cells and primary human astrocytes (Fig. 1). Adiponectin receptors have been shown to be widely distributed in the CNS of rodents, including hypothalamus, brainstem and endothelial cells, as well as human pituitary gland [27]. Guillard-Maximin et al. [28] have previously reported adipoR1 and adipoR2 expression in rat astrocytes but human data is lacking. A recent study confirmed the existence of adiponectin receptors in human SH-SY5Y neuronal cells [31]. The current identification of both adipoR1 and adipoR2 in human U373 MG cells and primary astrocytes supports the necessity for further determination of the effects of adiponectin on glial cells. Because most of the reported pro-inflammatory effects of adiponectin are attributed to gAd [25,26,33,34], we next focused on the induction of inflammatory cytokines in astrocytes by gAd.

3.2. Effects of gAd on IL-6 and MCP-1 secretion, and IL-6, MCP-1, IL-1β, IL-8 mRNA expression in U373 MG cells

In comparison with vehicle control at the same time point, gAd (1 μg/ml) significantly induced IL-6 and MCP-1 secretion from U373 MG astrocytic cells only at 12 h with no significant effect on IL-6 and MCP-1 secretion at 6, 24 and 48 h (Table 2). gAd at 3 μg/ml promoted similar induction of IL-6 and MCP-1 secretion when compared to 1 μg/ml gAd at 12 h (IL-6: 581 ± 164 vs. 553 ± 110 pg/ml, $p = 0.88$; MCP-1: 2289 ± 124 vs. 2250 ± 243 pg/ml, $p = 0.61$). The release of cytokines appeared attributable to induction of mRNA expression, as gAd (1 μg/ml) increased IL-6 and MCP-1, along with IL-1β and IL-8 mRNA at 12 h assessed by qPCR (Fig. 2). These findings demonstrate that gAd exerts pro-inflammatory effects on U373 MG cells, a cell line that has been shown to closely follow the cytokine release of human primary astrocytes [32,35]. The induction of pro-inflammatory cytokine secretion including IL-6 by gAd has been reported in both human and murine macrophages [25]. Similarly, the induction of pro-inflammatory genes including MCP-1, IL-6, and IL-8 by gAd has also been reported in vascular endothelial cells [26,33,34]. Our current study extends these findings to a cell type of the CNS, indicating that gAd induces pro-inflammatory properties in U373 MG astrocytoma cells.

Physiological levels of adiponectin in human serum have been reported to range from 2 to 17 μg/mL [36], while levels of adiponectin in human cerebrospinal fluid appear approximately 1000-fold lower than serum concentration [37]. Whether physiological levels of gAd may also exert pro-inflammatory properties in brain astrocytes *in vivo* will require further confirmation. However, it is interesting to note that activation of adipoR1 (the primary receptor for gAd) has been shown to exacerbate neuronal cell death in a murine model of ischemic stroke, providing strong evidence that gAd does have functional (detrimental) effects in the brain [38].

3.3. Effects of pharmacological inhibitors on gAd induced pro-inflammatory cytokine mRNA expression and IL-6 and MCP-1 secretion in U373 MG cells

As shown in Fig. 2A, the p38 MAPK inhibitor SB202190 and the ERK1/2 inhibitor PD98059 partially inhibited gAd induced IL-6 mRNA expression while PD98059 partially inhibited gAd induced MCP-1, IL-1β and IL-8 mRNA expression (Fig. 2B–D). None of the inhibitors affected the viability of astrocytic cells as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [39] (data not shown).

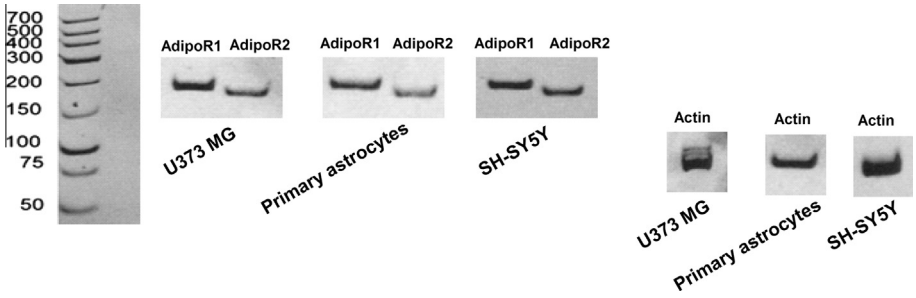

Fig. 1. Human astrocytes express both adiponectin receptor1 (adipoR1) and adipoR2. RT-PCR revealed the presence of ~200 base pair (bp) products corresponding to adipoR1 (192 bp) and R2 (168 bp) in U373 MG astrocytoma cells and primary human astrocytes. SH-SY5Y cells serve as the positive control [30]. Beta-actin was used as an internal control.

Table 2
Effects of gAd on IL-6 and MCP-1 secretion at 6, 12, 24, 48 h.

Cytokines	Groups	6 h (pg/ml)	12 h (pg/ml)	24 h (pg/ml)	48 h (pg/ml)
IL-6	Veh	281.0 ± 50.2	249.5 ± 79.1	743.03 ± 45.1	776.51 ± 37.6
	gAd	482.6 ± 101.7	530.3 ± 131.8*	616.10 ± 46.2	920.66 ± 32.2
MCP-1	Veh	804.3 ± 279.5	1702.4 ± 153.0	1490.62 ± 145.2	2728.08 ± 55.0
	gAd	980.6 ± 324.6	2354.0 ± 102.4*	2626.37 ± 571.6	2868.71 ± 352.0

IL-6 and MCP-1 release into the culture media was measured by ELISA.
* $p < 0.05$ compared to vehicle control (Veh) within the same time point. Data from three independent experiments are presented, $N = 6–8$.

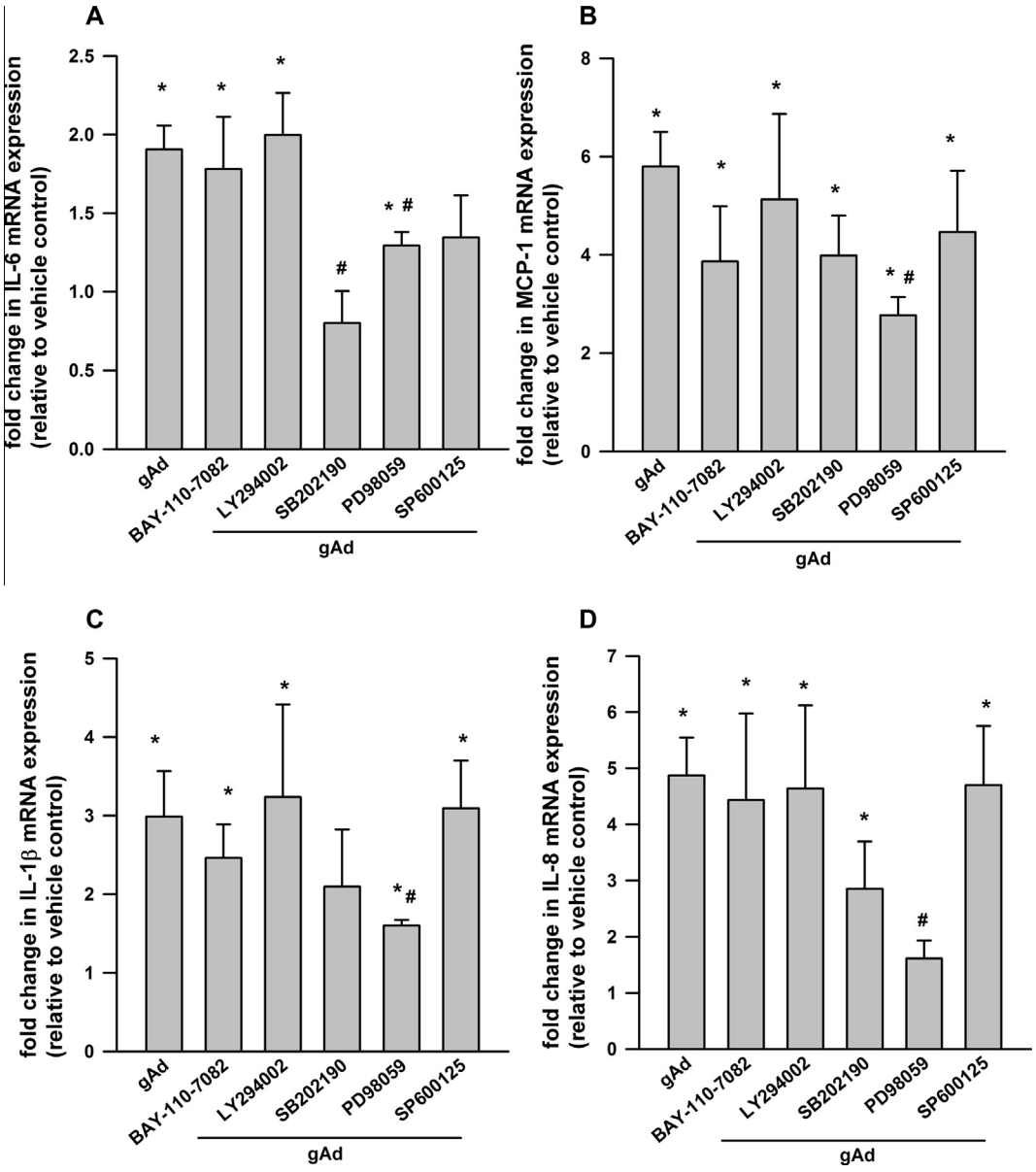

Fig. 2. Induction of IL-6, MCP-1, IL-1β and IL-8 mRNA expression by gAd and inhibition of gAd-induced mRNA expression in U373 MG cells. gAd (1 μg/ml) significantly induced IL-6, MCP-1, IL-1β and IL-8 mRNA expression assessed at 12 h incubation (A–D). SB202190 and PD98059 partially blocked gAd-induced IL-6 mRNA expression in U373 MG cells (A). PD98059 partially blocked gAd induced MCP-1, IL-1β and IL-8 mRNA expression in U373MG cells (B–D). $^{*}p < 0.05$ compared to vehicle control. $^{\#}p < 0.05$ compared to gAd group. Data from three independent experiments are presented, $N = 6–8$.

To confirm the reduction of gAD-induced pro-inflammatory cytokines by the various pharmacological inhibitors, we also assessed IL-6 and MCP-1 secretion. As shown in Fig. 3A, gAd-induced IL-6 secretion was partially blocked by the p38 MAPK inhibitor SB202190 and the ERK1/2 inhibitor PD98059, while gAd induced MCP-1 secretion was partially blocked by the NF-κB inhibitor BAY-110–7082 and PD98059 (Fig. 3B). These data indicate that induction of pro-inflammatory genes in U373 MG astrocytic cells by gAd involves multiple signalling pathways. p38MAPK and ERK1/2 are involved in gAd induced IL-6 secretion and gene expression. Both NF-κB and ERK1/2 are involved in gAd induced MCP-1 secretion, while only inhibition of ERK1/2 blocked gAd-induced MCP-1 gene expression. In addition, ERK1/2 is also involved in gAd-induced IL-1β and IL-8 gene expression in U373 MG cells.

Overall, our data indicate that ERK1/2, p38MAPK, and NF-κB pathways are involved in gAd-induced inflammatory response of U373 MG cells with ERK1/2 contributing most. Therefore, the signaling pathways implicated in the pro-inflammatory effects of gAd in peripheral tissues may also be relevant to astrocytes. For example, the activation of NF-κB signaling pathways by adiponectin has been reported in C2C12 murine myoblasts [15], human aortic endothelial cells [40], primary human hepatocytes [41], human umbilical vein endothelial cells [33,34] and human esophageal adenocarcinoma cells [42]. p38 MAPK and ERK1/2 are also involved in adiponectin mediated CXCL8 chemokine secretion in human primary hepatocytes [41]. Although we used pharmacological inhibitors believed to be specific to these signalling pathways, more research is needed to confirm our preliminary results and comprehensively characterize the signalling pathways induced by gAd in astrocytes.

In summary, we demonstrate that human astrocytes express receptors for adiponectin and that gAd induces IL-6 and MCP-1 secretion and gene expression of IL-6, MCP-1, IL-1β and IL-8 in

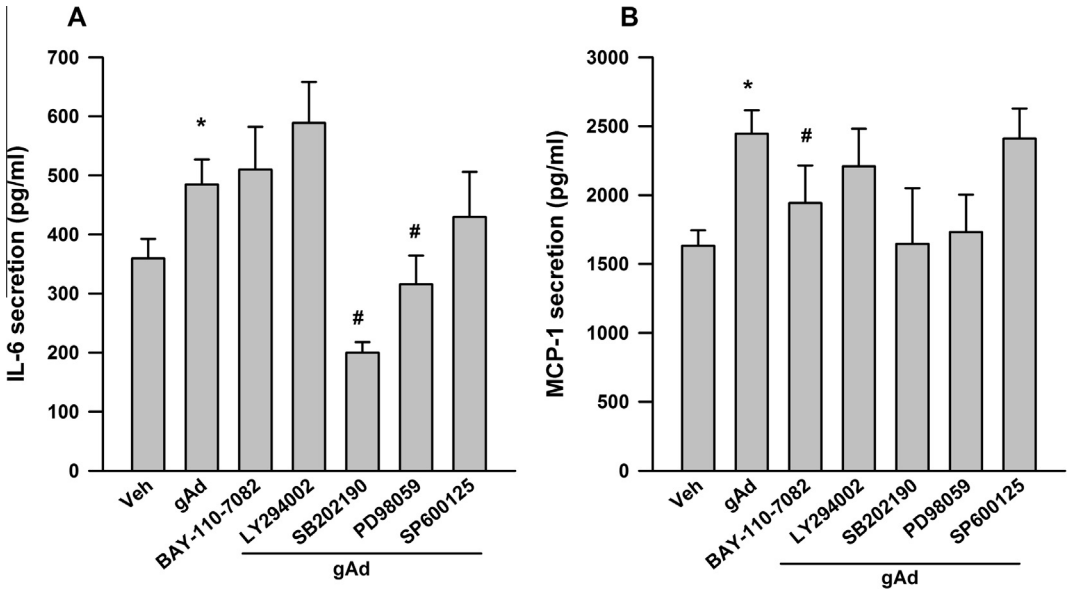

Fig. 3. Inhibition of gAd-induced IL-6 and MCP-1 secretion in U373 MG cells. SB202190 and PD98059 partially blocked gAd (1 μg/ml)-induced IL-6 secretion by U373 MG cells assessed at 12 h (A). BAY1107082 and PD98059 partially blocked gAd-induced MCP-1 secretion from U373 MG cells (B). $^{*}p < 0.05$ compared to vehicle control. $^{\#}p < 0.05$ compared to gAd. Data from three independent experiments are presented, $N = 6–8$.

U373 MG cells. ERK1/2, p38 MAPK, and NF-κB signaling appear to be involved in gAd-induced inflammation. These findings suggest that by activating astrocytes gAd could play a pro-inflammatory role in the CNS.

The effects of adiponectin on neurodegenerative disease mechanisms are largely unexplored. Interestingly, the Framingham Heart Study showed that individuals with higher levels of adiponectin had increased risk of future dementia [29]. Elevated cerebrospinal fluid adiponectin has been reported in older adults with mild cognitive impairment compared to healthy age-matched individuals [30]. A pathogenic role for adiponectin has also been described in ischemic stroke, where adipoR1 expression is increased and gAd enhances neuronal cell death in response to glucose and oxygen deprivation [38]. Our data suggest that induction of astrocyte inflammation may be a potential cellular mechanism linking adiponectin with these previously described neurodegenerative consequences. Further studies are warranted to examine the impact of adiponectin on brain inflammation in neurodegenerative disorders.

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