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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