

## Adiponectin protects rat hippocampal neurons against excitotoxicity

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**Abstract** Adiponectin exerts multiple regulatory functions in the body and in the hypothalamus primarily through activation of its two receptors, adiponectin receptor1 and adiponectin receptor 2. Recent studies have shown that adiponectin receptors are widely expressed in other areas of the brain including the hippocampus. However, the functions of adiponectin in brain regions other than the hypothalamus are not clear. Here, we report that adiponectin can protect cultured hippocampal neurons against kainic acid-induced (KA) cytotoxicity. Adiponectin

reduced the level of reactive oxygen species, attenuated apoptotic cell death, and also suppressed activation of caspase-3 induced by KA. Pretreatment of hippocampal primary neurons with an AMPK inhibitor, compound C, abolished adiponectin-induced neuronal protection. The AMPK activator, 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside, attenuated KA-induced caspase-3 activity. These findings suggest that the AMPK pathway is critically involved in adiponectin-induced neuroprotection and may mediate the antioxidative and anti-apoptotic properties of adiponectin.

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### Abbreviations

AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
KA	Kainic acid
AMPK	AMP-activated protein kinase
AICAR	5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside
ROS	Reactive oxygen species
CR	Calorie restriction
AD	Alzheimer's disease
PD	Parkinson's disease
DR	Dietary restriction

### Introduction

Adiponectin is a 30-KD protein primarily produced by adipose tissues prior to its release into circulation. Adiponectin has multiple functions in the peripheral and central nervous systems. It acts as an insulin-sensitizing factor as evidenced by its ability to reduce glucose production by increasing hepatic insulin sensitivity. Adiponectin also increases glucose uptake in adipocytes and myocytes, and enhances fatty acid oxidation in muscle (Kadowaki and Yamauchi 2005; Dyck 2009). In addition, adiponectin acts to inhibit inflammatory processes and stimulate endothelium-dependent nitric oxide-mediated vasorelaxation (Takemura et al. 2007; Beltowski et al. 2008). Adiponectin appears to exert its biologic actions through its two known receptors, which are prevalently expressed in skeletal muscle (adiponectin receptor 1, AdipoR1) and liver (adiponectin receptor 2, AdipoR2). Adiponectin can transduce signals through the activation of adenosine monophosphate-activated protein kinase (AMPK), and peroxisome proliferator-activated receptor  $\gamma$ , leading to increased fatty acid oxidation in skeletal muscle and decreased glucose synthesis in liver (Kadowaki and Yamauchi 2005).

In addition to its actions in peripheral tissues, adiponectin reportedly contributes to the central regulation of food intake and energy homeostasis (Kubota et al. 2007) as well as reproduction (Budak et al. 2006; Michalakis and Segars 2010). Metabolic state and

reproductive capacity are known to interact. Extreme states such as severe calorie restriction (CR) or obesity often interfere with reproduction. Intracerebroventricular administration of adiponectin has been found to decrease body weight, ostensibly by stimulating energy expenditure (Qi et al. 2004). Related to reproductive ability, addition of globular adiponectin to GT1-7 cells derived from hypothalamic neurons reduced gonadotropin-releasing hormone by activating AMP-activated protein kinase (Wen et al. 2008).

In addition to effects on energy homeostasis and reproduction, adiponectin may have other functions in brain. Expression of AdipoR1 and AdipoR2 was observed in hippocampal neurons in mice, GT1-7 cell line derived from mouse hypothalamic neurons in rat, and the pituitary, nucleus basalis and hypothalamus in humans (Jeon et al. 2009; Wen et al. 2008; Guillod-Maximin et al. 2009; Psilopanagiotti et al. 2009). Adiponectin-knockout mice exhibit enlarged brain infarction and increased neurological deficits after ischemia reperfusion compared to wild-type mice, while adenovirus-mediated supplementation of adiponectin significantly reduces cerebral infarct size in both wild-type and adiponectin-deficient mice (Nishimura et al. 2008), suggesting that adiponectin may have neuroprotective activity. Jeon et al. (2009) observed that preloading adiponectin centrally via lateral ventricular injection in mice attenuated subsequent neuronal damage from kainic acid-induced seizure activity in hippocampal neurons. They further demonstrated that this attenuated damage was likely due to less blood brain barrier leakage and lower expression of endothelial nitric oxide synthase induced by the adiponectin pretreatment. In addition, intracerebroventricularly injected adiponectin has been shown to induce phosphorylation of AMPK in the rat hypothalamus suggesting a role of adiponectin in modulating energy homeostasis (Guillod-Maximin et al. 2009). However, it is far from clear whether adiponectin acts directly on neuronal cells to protect them from cytotoxic insults or protects through other as yet identified, indirect mechanisms.

Excessive release of the excitatory neurotransmitter glutamate can induce neuronal damage and death, and this excitotoxicity appears to be involved in age-related neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (Fan and Raymond 2007; O'Neill and Witkin 2007; Palop et al. 2007).

Kainic acid (KA), an analog of glutamate, can cause neuronal damage by inducing excessive calcium influx, which in turn stimulates elevated levels of reactive oxygen species (ROS) and reactive nitrogen species. As a final result of the excitotoxic insult, there is generally damage to intracellular membranes that triggers apoptotic pathways leading to cell death (Wang et al. 2005). Here, we report results of an investigation to determine the direct role of adiponectin to protect hippocampal neurons against KA-induced excitotoxic insult and potential underlying mechanisms related to this neuroprotection.

## Materials and methods

### Primary hippocampal neuron culture

Primary hippocampal cell cultures were established from day 18 prenatal Sprague Dawley rats according to methods previously described (Mattson et al. 1989; Gleichmann et al. 2009). Briefly, cells were seeded into polyethyleneimine-coated plastic culture dishes containing Eagle's minimum essential medium supplemented with 26 mM NaHCO<sub>3</sub>, 40 mM glucose, 20 mM KCl, 1 mM sodium pyruvate, 10% (v/v) heat-inactivated fetal bovine serum and 0.001% gentamycin sulfate. The medium volume for cells in 35-mm dishes was approximately 1.0 ml. After 5-h incubation to allow for cell attachment, medium was replaced with 1 mL of neurobasal medium with B27 supplements (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine, 25 µg/ml gentamycin, and 1 µM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid with 0.001% gentamycin sulfate. The experimental treatments with rat adiponectin (Alpco, USA), 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR; Calbiochem, USA), and compound C (Calbiochem, USA) were performed on 7–8-day-old cultures, in which the cultures contained ~90–95% neurons and 5–10% astrocytes according to methods previously described for human fibroblast cells (Tang et al. 2007). The duration and time points of these treatments are specified in the “Results” section.

### Quantification of neuronal survival

Neuronal survival was quantified by a previously described method (Mattson et al. 1989). Before any treatment, several fields were marked on the bottom of

the culture dishes. The viable neurons in pre-marked fields were counted under a microscope with a 20× objective before and after the experimental treatment at different time points. In each field, neurons that died during the intervals between examination time points had usually disappeared. The viability of the remaining neurons was assessed by the following morphological criteria: neurons with intact neurites of uniform diameter and a smooth round soma were considered viable; neurons with fragmented neurites and vacuolated cell bodies were considered nonviable. Five randomly chosen microscope fields from ten different dishes in each treatment were examined.

### Analysis of gene expression

Total RNA was isolated from cultured rat hippocampal neurons and hippocampal tissue using the Mini RNA Isolation I Kit (Zymo Research Corporation, USA). Single-stranded complementary DNA (cDNA) was synthesized from DNase-treated RNA samples using Malooney murine leukemia virus reverse transcriptase with a mixture of an oligo (dT) primer and random hexanucleotide primer. Expression of adiponectin receptors, AdipoR1 and AdipoR2, was assessed by quantitative polymerase chain reaction (qPCR) using the following gene-specific primers: 5' AGATGGGCTGGTTCTTCCTCAT3' and 5' CAGAC AACTCAGACTCTTCCTC3' for AdipoR1; 5' ATGTTTGCCACCCCTCAGTA3' and 5' CAGATGT CACATTTGCCAGG3' for AdipoR2. PCR was performed in 50 µl with 150 ng cDNA, 0.2 µM reverse and forward primers, 200 µM dNTPs, and 1 unit of Taq DNA Polymerase (Promega, USA) using the DNA Engine (PTC-200) Peltier Thermocycler (Bio-rad Laboratories, USA) as follows: 94°C, 3 min for denature, 94°C 30 s, 55°C, 30 s, and 72°C, 45 s for 30 cycles, 72°C, 10 min for final extension. PCR products were separated in a 2% agarose gel containing ethidium bromide and visualized under UV light. The samples were also tested without reverse transcriptase to ensure that there was no contamination with genomic DNA.

### Immunostaining

We used a double immunostaining method to determine whether there was protein expression of adiponectin receptors in the cultured hippocampal cells. Cultured hippocampal cells on glass cover-

slips were used for processing double-labeled immunofluorescence for AdipoR1 and neuronal cell-type specific marker  $\beta$ -tubulin (Tuj-1, a marker of immature and mature neurons) or AdipoR2 and neuronal marker NeuN (Neuronal Nuclei). Cells were washed with phosphate-buffered saline (PBS), and then incubated with 10% goat serum for 1 h. For detecting the expression of AdipoR1, the cells were then incubated with primary antibody-Tuj-1 (1:500, rabbit polyclonal antibody, Covance, USA) and anti-AdipoR1 antibody (1:1,000, Abcam, USA). Alexa Fluor 568 anti-mouse secondary antibodies and Alexa Fluor 488 anti-rabbit secondary antibodies (1:250, Vector, USA) were applied for 1 h (Molecular Probes, USA). For detecting the expression of AdipoR2, cells were incubated in primary antibody anti-NeuN (1:1,000, Sigma Aldrich, USA) and anti-adiponectin receptor 2 (1:1,000, Abcam, USA) at 4°C overnight. Alexa Fluor 568 anti-rabbit secondary antibodies and Alexa Fluor 488 anti-mouse secondary antibodies (1:250, Vector, USA) were applied for 1 h (Molecular Probes, USA). Immunostained cells were visualized and images were acquired using a Zeiss 510 confocal microscope.

#### Quantification of caspase-3 activity

Caspase-3 activity was determined using a colorimetric assay with Caspase-Glo<sup>®</sup> 3/7 Reagent according to the manufacturer's protocol (Promega, USA). The assay was used to estimate the ability of cellular caspase-3 to cleave the labeled substrate N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline, which can be spectrophotometrically measured. Briefly, hippocampal neurons were seeded in 96-well plates at a density of  $1 \times 10^4$  cells/well. After 7–8 days, hippocampal neurons received treatments as follows: cells were treated with either 100  $\mu$ M KA (Sigma Aldrich, USA) for the duration indicated or physiological saline as a control, and then 100  $\mu$ l Caspase-Glo 3/7 reagent was added. The plate was then incubated at room temperature for 1 h, and the luminescence of each sample was measured with a HTS 7000 Plus Bio Assay Reader (Perkin-Elmer, USA).

#### Western blot analysis

Protein (50  $\mu$ g) in hippocampal cell lysates was separated on a 10% or 15% Tris-glycine gel and

transferred to nitrocellulose membranes (0.45  $\mu$ m, Invitrogen, USA). The membranes were blocked with 5% nonfat milk (Sigma Aldrich, USA) in PBS buffer containing 1% Tween-20 for 1 h at room temperature, then incubated with one of the following primary antibodies: anti- $\beta$ -actin (1:5,000, Sigma-Aldrich, USA), p-AMPK or AMPK (1:1,000, Cell Signaling, USA) overnight at 4°C. The membranes were then washed three times in PBS buffer containing 1% Tween-20 and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (1:3,000, Jackson ImmunoResearch, USA) for 1 h at 20–25°C. Bound antibodies were visualized by ECL<sup>™</sup> Western Blotting Detection kit as recommended by the manufacturer (GE Healthcare, USA). Western blot analysis was replicated at least three times for each treatment. The expression level of each protein was quantified by densitometric analysis.

#### Assessment of reactive oxygen species

Levels of intracellular ROS were quantified using a fluorescence probe with 2, 7-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA, Invitrogen, USA). Oxidation of H<sub>2</sub>DCFDA occurs almost exclusively in the cytosol, generating a fluorescent response proportional to ROS generation (Wardman 2007). Briefly, hippocampal neurons cultured in the 96-well plate were pretreated with 5 or 20  $\mu$ g adiponectin or saline for 48 h. After washing cells with Dulbecco's phosphate buffered saline two to three times, 100  $\mu$ l culture medium with 5  $\mu$ M H<sub>2</sub>DCF-DA was added to each well and cells were incubated at 37°C for 30–60 min. Cells were then exposed to 100  $\mu$ M KA in 100  $\mu$ l culture medium for 30 min or 2 h. Cells were subsequently washed twice with Locke's buffer. Fluorescence was quantified using a fluorescence plate reader (485 nm excitation and 538 nm emission).

#### Statistical analysis

Data are presented as means  $\pm$  SEM. The systematic morphometric quantifications were performed with coded dishes and the investigator remained blinded as to treatment until all analyses were completed. One-way and two-way analysis of variance (ANOVA) followed by Student–Newman–Keuls or Fisher post-hoc comparisons to determine group differences were

used for statistical analysis;  $p < 0.05$  was accepted as statistically significant.

## Results

AdipoR1 and AdipoR2 are expressed in cultured hippocampal neurons

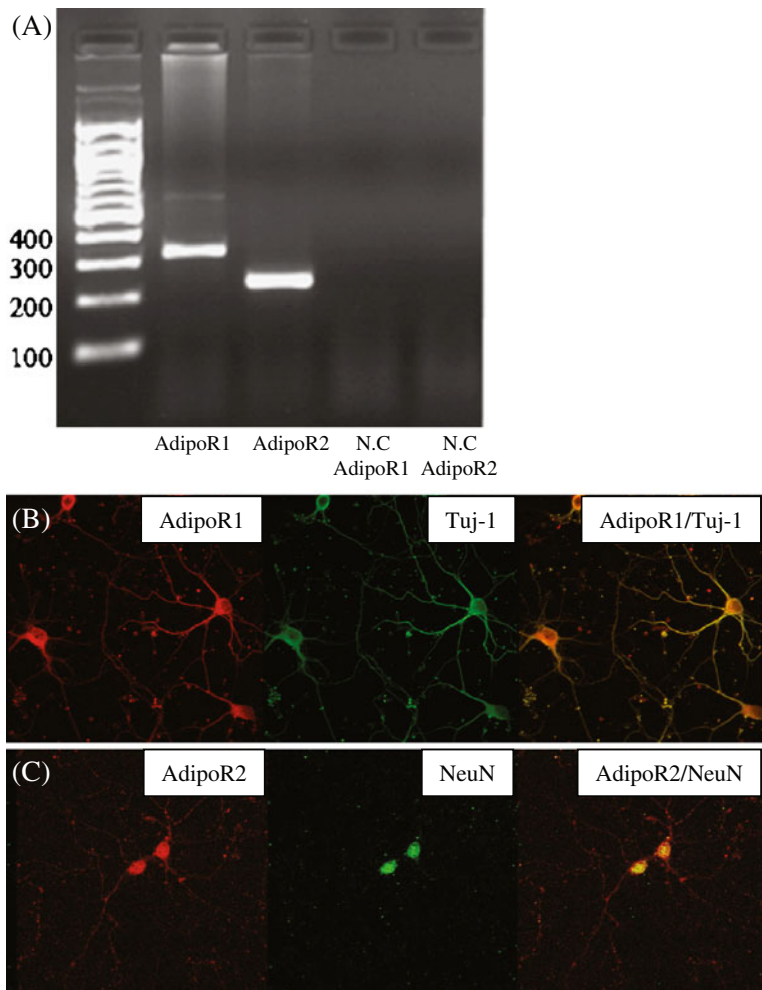
We first tested whether adiponectin receptors, AdipoR1 and AdipoR2, were expressed by cells in our hippocampal cultures by performing reverse transcription (RT)-PCR and immunohistochemical analysis. We detected the expression of mRNA s for both AdipoR1 and AdipoR2 (Fig. 1a). AdipoR1 immunoreactivity was present in hippocampal neurons where

it was present in the cell body and in the neurites (Fig. 1b). Similarly, AdipoR2 immunoreactivity was observed in the cell body and neurites of hippocampal neurons (Fig. 1c). All neurons examined exhibited AdipoR1 and AdipoR2 immunoreactivities, suggesting that both receptors were widely expressed in primary hippocampal neurons.

Adiponectin protects neurons against excitotoxic neuronal death

Previous studies have shown that AMPK protects hippocampal neurons against metabolic and excitotoxic insults and promotes neuronal survival (Culmsee et al. 2001), and binding of adiponectin to its receptors results in activation of AMPK signaling pathways (Kubota et

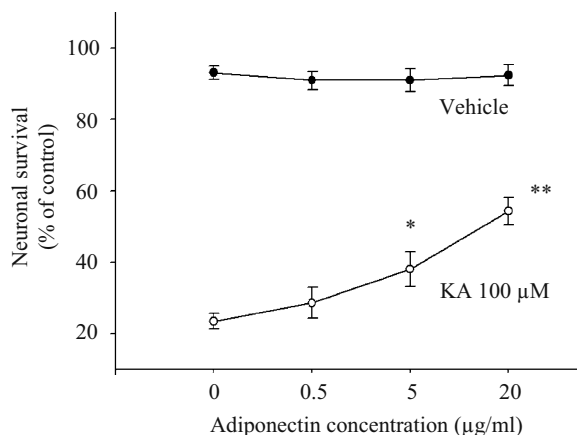
**Fig. 1** Adiponectin receptors are expressed in hippocampal neurons. **a** The agarose gel image shows RT-PCR analysis of hippocampal mRNA using primers specific for the receptors AdipoR1 and AdipoR2 (lanes 1 and 2). No PCR products were observed in the two RT negative controls in which reverse transcriptase enzyme were omitted from the cDNA synthesis reaction: lane 3 marked RT used primers for AdipoR1; lane 4 used primers for AdipoR2. **b** Representative microphotographs illustrating cells positive for both adiponectin receptor 1 (red, left) and neuronal cell marker Tuj-1 (green, middle). Merged image is shown to the right. **c** Representative microphotographs illustrating cells positive for both adiponectin receptor 2 (red, left) and neuronal cell marker NeuN (green, middle). Merged image is shown to the right



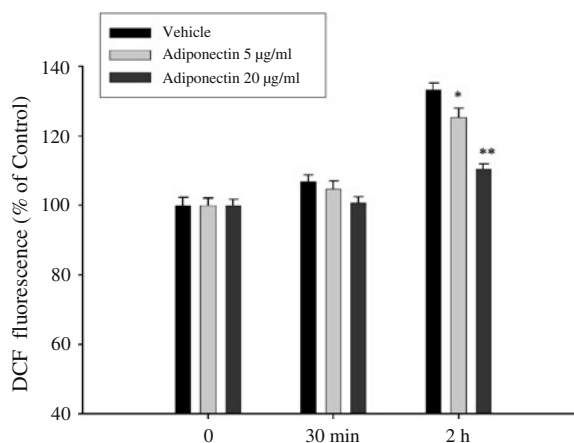
al. 2007). Therefore, we decided to determine whether adiponectin affects neuronal survival under excitotoxic conditions. Hippocampal cultures were pretreated with saline or adiponectin at three different concentrations (0.5, 5, and 20  $\mu\text{g/ml}$ ) for 48 h, and were then exposed to KA at a concentration of 100  $\mu\text{M}$  for 12 h. In cultures not receiving adiponectin, the KA insult killed  $\sim 75\%$  of the neurons (Fig. 2). In contrast, significantly more neurons survived in cultures that were pretreated with either 5 or 20  $\mu\text{g/ml}$  adiponectin (40% and 55% survival, respectively); whereas, the lowest concentration of 0.5  $\mu\text{g/ml}$  adiponectin was ineffective (Fig. 2). The survival of hippocampal cultures pretreated with 20  $\mu\text{g/ml}$  adiponectin for less than 24 h was not significantly different from the control pretreated with saline (data not shown).

Adiponectin reduces the intracellular level of ROS and suppresses apoptosis induced by KA

Since ROS formation plays a pivotal role in excitotoxic neuronal death (Wang et al. 2005), we examined whether adiponectin modified ROS levels by using the fluorescence probe  $\text{H}_2\text{DCF-DA}$  to assess intracellular ROS levels. KA administration induced significant increases in the level of DCF fluorescence in primary hippocampal cultures within 2 h (Fig. 3) compared to the non-treated cultures, indicating



**Fig. 2** Adiponectin protects hippocampal neurons against excitotoxic death. Hippocampal cultures were pretreated with adiponectin at the indicated concentrations (0.5, 5 and 20  $\mu\text{g/ml}$ ) for 48 h and were then exposed to 100  $\mu\text{M}$  KA for 12 h. Neuronal survival was quantified. Values are the mean  $\pm$  SEM from four different cultures; \* $p < 0.05$ , \*\* $p < 0.01$  compared to the corresponding cultures subjected to KA without adiponectin treatment



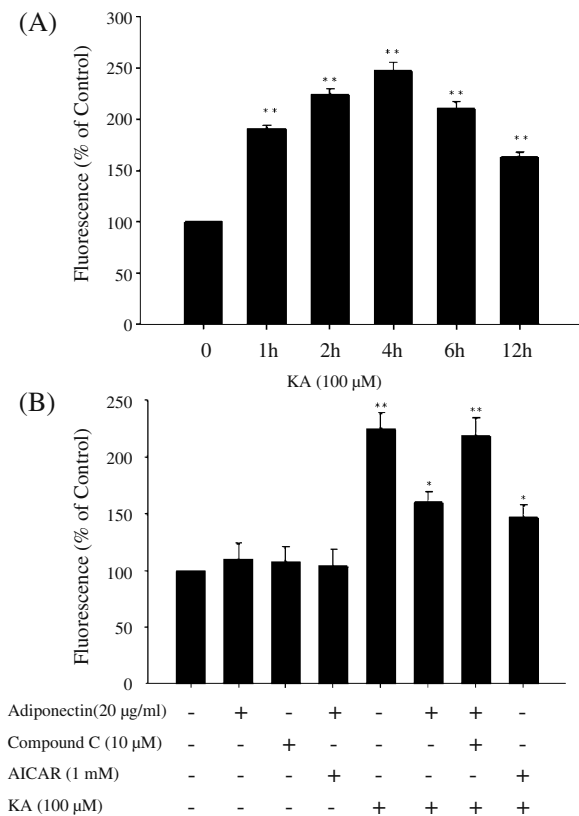
**Fig. 3** Adiponectin suppresses KA-induced oxidative stress in hippocampal neurons. Hippocampal cultures were pretreated with 5 and 20  $\mu\text{g}$  adiponectin or saline for 48 h and were then exposed to 100  $\mu\text{M}$  KA for 30 min and 2 h. The level of DCF fluorescence was quantified. Values are the means  $\pm$  SEM from at least six cultures; \* $p < 0.05$ , \*\* $p < 0.01$  compared to the corresponding vehicle-treated control cultures

that KA increased intracellular levels of ROS. In contrast, KA-induced DCF fluorescence in cultures pretreated with adiponectin was significantly attenuated in a dose-dependent manner. This suggests that adiponectin signaling reduces the level of ROS and, in turn, contributes to the protection of hippocampal neurons against excitotoxic insults.

Elevated levels of intracellular ROS and  $\text{Ca}^{2+}$  induced by KA can activate caspase-3, a marker of apoptosis, in hippocampal neurons (Wang et al. 2005). We examined caspase-3 activity in primary hippocampal cultures at several time points after KA treatment. Rapid activation of caspase-3 was observed in hippocampal neurons in response to KA, emerging within 1 h of KA exposure and peaking at 4 h (Fig. 4a). In contrast, cultures pretreated with adiponectin exhibited a significant attenuation of caspase-3 activation after treatment with KA for 4 h (Fig. 4b). The data suggest that adiponectin can inhibit neuronal apoptosis induced by KA.

Neuroprotection by adiponectin involves activation of AMPK signaling

Binding of adiponectin to its adiponectin receptors can result in activation of AMPK, and it has been reported that AMPK activation reduces the intracellular level of ROS in endothelial cells (Ouedraogo et



**Fig. 4** Adiponectin suppresses activation of the excitotoxic apoptosis cascade. **a** The effects of KA on activation of caspase-3. Hippocampal cultures were subjected to 100 μM KA for the duration indicated. Luminescence analysis showed that caspase-3 activity significantly increased after 1 h KA treatment and reached a peak at 4 h. **b** The effect of adiponectin on activation of caspase-3 induced by KA. Hippocampal cultures were pretreated with compound C or vehicle, and exposed to 20 μg/ml adiponectin or vehicle (control) or AICAR for 48 h, and then exposed to 100 μM KA or vehicle for 4 h. Levels of luminescence were quantified. Values are the means ± SEM from at least six cultures; \* $p < 0.05$  compared to the cultures treated with KA alone, \*\* $p < 0.01$  compared to the cultures untreated with KA. Two-way ANOVA was used to find the main effects of KA and five treatments, the latter of which refer to the control, adiponectin, compound C AICAR and combination of adiponectin and compound C groups (data not shown for the last group; KA,  $F = 281.2$ ,  $p < 0.001$ ; Five treatments,  $F = 5.0$ ,  $p < 0.01$ ; interaction between KA × five treatment,  $F = 6.0$ ,  $p < 0.001$ ). Further post hoc test (Student–Newman–Keuls) indicated that the adiponectin and AICAR groups were significantly different from three other groups ( $p < 0.01$ )

al. 2006). Our immunoblot analysis revealed a rapid activation of AMPK, as indicated by the level of phosphorylation of AMPK, in hippocampal neurons within 30 min of exposure to adiponectin (Fig. 5a and

b). Treatment of neurons with the AMPK activator AICAR, as a positive control, also increased phosphorylation of AMPK. However, when neurons were treated with compound C (10 μM) for 30 min, a specific inhibitor of AMPK phosphorylation, AMPK phosphorylation induced by adiponectin was attenuated. This suggests that adiponectin activates the AMPK signaling pathway in hippocampal neurons.

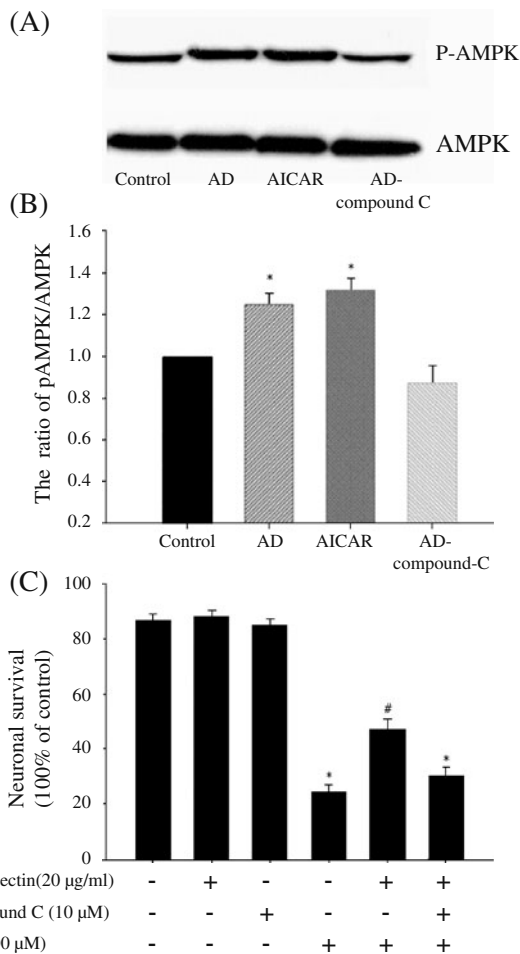
We next evaluated the effects of AMPK inhibition by compound C on neuronal survival in adiponectin-treated and control hippocampal cultures. Neurons were treated with adiponectin alone or in combination with compound C for 48 h and were then exposed to KA for 12 h. Compound C completely blocked the neuroprotective effect of adiponectin (Fig. 5c). These results suggest that AMPK activation is required for adiponectin-mediated cell survival in hippocampal neurons challenged with KA.

#### AMPK pathway induction mediates the anti-apoptotic effects of adiponectin

Since AMPK was found to inhibit apoptosis in neurons (Culmsee et al. 2001), we examined whether AMPK pathways are involved in the anti-apoptotic effect of adiponectin. Pretreatment of cells with compound C (10 μM) markedly attenuated the adiponectin-induced AMPK kinase activity (Fig. 5a and b), and reversed the inhibitory effect of adiponectin on caspase-3 activity induced by KA (Fig. 4b). Treatment with the AMPK activator AICAR protected neurons against KA-induced toxicity. These findings suggest that AMPK pathway is involved in the anti-apoptotic effects of adiponectin.

## Discussion

Adiponectin is secreted from adipose tissue and released into circulation at high concentrations. The concentration of adiponectin in serum and cerebrospinal fluid (CSF) ranges from 9 to 18 μg/ml and 100 to 330 ng/ml, respectively in mice (Qi et al. 2004). A critical function of adiponectin appears to be its regulation of energy intake and expenditure ranging from appetite, insulin sensitivity, and glucose and lipid homeostasis in skeletal muscle and liver (Kadowaki and Yamauchi 2005; Dyck 2009). In addition, adiponectin has been reported to be



**Fig. 5** AMPK phosphorylation modulates the neuroprotective effect of adiponectin. **a** Hippocampal cultures were pretreated with compound C or vehicle, and exposed to 20 µg/ml adiponectin or vehicle (control) or AICAR for 30 min. Cell lysates were subjected to Western blot analysis using antibodies against p-AMPK and AMPK. **b** Densitometric analysis of ratios of p-AMPK/AMPK in the hippocampal cultures for four groups; \* $p < 0.05$  compared to the corresponding vehicle-treated controls or the cultures treated with adiponectin and compound C. **(c)** Hippocampal cultures were pretreated with compound C or vehicle for 2 h and were then treated with adiponectin for 48 h, followed by exposure to 100 µM KA. Neuronal survival was quantified. The values are the means  $\pm$  SEM from four cultures; \* $p < 0.01$  compared to the hippocampal cultures without any treatment; # $p < 0.01$  compared to the hippocampal cultures subjected to KA without adiponectin pretreatment or hippocampal cultures subjected to KA with adiponectin and compound C pretreatment. Two-way ANOVA was used to find the main effects of KA and four treatments, the latter of which refer to the control, adiponectin, compound C and combination of adiponectin and compound C groups (data not shown for the last group) (KA,  $F = 431.9$ ,  $p < 0.001$ ; four treatments,  $F = 6.9$ ,  $p < 0.001$ ; interaction between KA  $\times$  four treatment,  $F = 3.1$ ,  $p < 0.05$ ). Further post hoc test (Student–Newman–Keuls) indicated that the adiponectin group was significantly different from other three groups ( $p < 0.01$ )

involved in control of inflammation and atherogenesis (Guzik et al. 2006; Fantuzzi 2009), induction of the immune response, suppression of cancers and maintenance of vascular homeostasis (Guzik et al. 2006; Kelesidis et al. 2006). The majority of these effects are due to its function in peripheral tissues. Abnormally low levels of adiponectin in human and animal models have also been linked to obesity, insulin resistance, and type two diabetes (Whitehead et al. 2006). In contrast, plasma levels of adiponectin are elevated in animal studies of calorie restriction, which usually results in an increase in insulin sensitivity and glucose metabolic efficiency (Kemnitz et al. 1994; Wan et al. 2009). Adiponectin also increases when people diet (Yang et al. 2001) or obese individuals undergo gastric bypass surgery (Faraj et al. 2003), which results in improved glucose levels and insulin sensitivity. Building on the findings of Jeon et al. (2009), who observed neuroprotection following adiponectin injection into the brain of mice, our findings confirm a direct action of adiponectin on hippocampal neurons that promotes their survival under excitotoxic conditions.

The peripheral effects of adiponectin are mediated by two types of receptors, AdipoR1, originally cloned from skeletal muscle, and AdipoR2, cloned from the liver. We found that both AdipoR1 and AdipoR2 are expressed in primary hippocampal neurons, consistent with one or both of these receptors mediating the neuroprotective action of adiponectin. Using the same antibodies against AdipoR1 and AdipoR2 used in our study, Miller et al. (2009) found that AdipoR1 but not AdipoR2 is expressed in airway epithelial cells by immunohistochemistry staining of lung sections in a mouse model of chronic obstructive pulmonary disease. By immunohistochemistry staining of the hippocampal sections of adult Sprague Dawley rats, we found that AdipoR1 is co-localized with NeuN (mature neurons), glial fibrillary acidic protein (GFAP; astrocyte) and BrdU (proliferating cells) positive cells, while AdipoR2 is co-localized with NeuN- and BrdU-positive cells, but not GFAP-positive cells (unpublished results by Yau and So). Recent research points to an important role for adiponectin in the central nervous system. Both AdipoR1 and AdipoR2 have been identified in human hypothalamus (Kos et al. 2007). Related evidence demonstrates that central adiponectin administration induces weight loss, by stimulating energy expenditure and increasing thermogenesis. Central adminis-



tration of adiponectin also reduces serum glucose and lipid levels (Qi et al. 2004). Existing data on blood brain barrier permeability to peripheral adiponectin remains equivocal, but it is clear that adiponectin is present in the brain parenchyma and cerebrospinal fluid (Ebinuma et al. 2007; Neumeier et al. 2007; Pan and Kastin 2007).

Our finding that adiponectin acts directly on hippocampal neurons, resulting in AMPK activation and protection against apoptosis, suggests an important role for adiponectin in protecting neurons in acute and chronic neurodegenerative conditions. The present results are consistent with other recent studies suggesting a neuroprotective role of adiponectin. Adiponectin-deficient mice exhibit enlarged brain infarction and increased neurological deficits after ischemia reperfusion compared to wild-type mice, and adenovirus-mediated supplementation of adiponectin reverses the deficit in adiponectin deficient mice and improves protection in wild-type mice (Nishimura et al. 2008). Elevated plasma adiponectin attenuates cardiac ischemia/reperfusion damage in mice (Shinmura et al. 2007; Gonon et al. 2008). In addition, in our previous experiments, we found that the hippocampal cells treated with serum from dietary restriction (DR) rats for 48 h were more resistant to KA-induced toxicity compared to those treated with serum from ad libitum rats (Qiu, unpublished observation). The concentration of adiponectin in serum from DR rats can be as high as 20  $\mu\text{g/ml}$  (Wan et al. 2009), which is the concentration of adiponectin required to protect neurons from excitotoxic neuronal death observed in our *in vitro* cell culture study. However, the concentration of adiponectin used in our *in vitro* study is much higher than that expected in CSF, which ranges from 1% to 4% of that in serum (Qi et al. 2004). This suggests that other factors in CSF sensitize the action of adiponectin in brain. Nevertheless, our findings suggest that adiponectin plays an important role in the neuroprotective effect of CR.

Although the mechanisms of excitotoxic apoptosis are not completely understood, excessive levels of ROS, increased mitochondrial membrane permeability, and activation of caspase-3 are believed to play roles. AMPK activation has previously been linked to the beneficial effects of adiponectin (Kukidome et al. 2006). Our study demonstrates that adiponectin and the AMPK activator AICAR can reduce excitotoxic apoptosis and activate AMPK as indicated by

threonine-172 phosphorylation of AMPK. On the other hand, compound C, an AMPK specific inhibitor, decreases adiponectin-induced AMPK phosphorylation. Furthermore, compound C blocks the inhibitory effect of adiponectin on suppression of apoptosis. Our results demonstrate that both adiponectin and AICAR protect hippocampal neurons against excitotoxicity; whereas, compound C abolishes the protective effect induced by adiponectin. These observations suggest that the inhibitory effect of adiponectin on apoptosis depends on AMPK activation. The activation of AMPK has been found to protect hippocampal cell death induced by glucose deprivation, chemical hypoxia, and exposure to glutamate and amyloid beta-peptide *in vitro* (Culmsee et al. 2001).

It has been reported previously that adiponectin protects neuroblastoma SH-SY5Y cells against cytotoxicity induced by 1-methyl-4-phenyl-2,3,6-tetrahydropyridine acetaldehyde or 1-Methyl-4-phenylpyridinium ion (MPP+), and that adiponectin also exerts a cerebroprotective action attenuating cerebral ischemic injury (Jung et al. 2006; Nishimura et al. 2008). Excessive glutamate receptor signaling has been linked to neuronal death in epilepsy, stroke, AD, PD, and amyotrophic lateral sclerosis (Fan and Raymond 2007; O'Neill and Witkin 2007; Palop et al. 2007). Results of the present study suggest that the beneficial effects of adiponectin may offer protection from and possible therapeutic applications to age-related brain disorders such as AD and PD.

Interestingly, obesity which suppresses circulating adiponectin levels has now been identified as a risk factor for AD (Luchsinger 2008). Indeed, several lines of evidence suggest that adiponectin may contribute to brain health via several pathways. Firstly, adiponectin increases insulin sensitivity and inhibits gonadotropin-releasing hormone by regulating AMPK (Tomas et al. 2002; Wen et al. 2008), which is a sensor of cellular energy status in almost all eukaryotic cells. The activation of AMPK also protects neurons against stroke (McCullough et al. 2005), decreases glutamate toxicity in hippocampus and increases hippocampal neurogenesis (Dagon et al. 2005). Secondly, adiponectin may improve endothelial cell function of brain vasculature which can benefit neural function (Jeon et al. 2009). More importantly, DR, a nongenetic intervention that increases lifespan in a wide range of species, increases adiponectin levels in mammals, suggesting

that this adipose-derived hormone may have an important regulatory role in mediating the beneficial effects of DR including neuroprotection (Shimura et al. 2007; Zhu et al. 2007). Whether adiponectin plays a role in DR-induced beneficial effects and how adiponectin affects other important pathways in DR, remain important topics for investigation.

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