



## Apolipoprotein D modulates F<sub>2</sub>-isoprostane and 7-ketocholesterol formation and has a neuroprotective effect on organotypic hippocampal cultures after kainate-induced excitotoxic injury

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### ABSTRACT

Apolipoprotein D (apoD), a member of the lipocalin family of transporter proteins binds a number of small lipophilic molecules including arachidonic acid and cholesterol. Recent studies showed a protective function of mammalian apoD as well as its insect and plant homologs against oxidative stress. In this study we investigated the effect of direct addition of exogenous human apoD protein purified from breast cystic fluid to rat hippocampal slice cultures after excitotoxic injury induced by the glutamate analog kainate. ApoD at a concentration of 10 µg/ml partially prevented loss of MAP2 immunostaining and LDH release from injured hippocampal neurons after kainate injury. ApoD also attenuated the increase in oxidative products of arachidonic acid and cholesterol, F<sub>2</sub>-isoprostanes and 7-ketocholesterol, respectively, after kainate treatment. In view of the molecular structure of apoD which consists of an eight stranded β barrel that forms a binding pocket for a number of small hydrophobic molecules, we propose that apoD promotes its neuroprotective effects by binding to arachidonic acid and cholesterol thus preventing their oxidation to neurotoxic products such as 4-hydroxynonenal (4-HNE) and 7-ketocholesterol.

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Apolipoprotein D (apoD), a 24–29 kDa glycoprotein, was first identified as an apoprotein component of plasma HDLs, and is therefore designated as an apolipoprotein [14]. It however, does not share sequence similarity with other apolipoproteins, but instead is a member of the lipocalin superfamily of proteins which share a conserved tertiary structure and function in the transport of small hydrophobic ligands. The molecular structure of apoD consists of an eight-stranded β barrel (β-clam) that forms a binding pocket suitable for a number of small hydrophobic molecules including arachidonic acid, cholesterol and progesterone [8,15,20,21]. Apolipoprotein D is the major protein component in cyst fluid from

women with human breast gross cystic disease [1]. Its expression has been shown to be increased in the brain in patients with Alzheimer's disease, Parkinson's disease or schizophrenia [19,26,28], and animal models of Niemann–Pick type C disease, transmissible spongiform encephalopathy, ischemic stroke and excitotoxic injury [5,18,22,25].

Recent studies indicate that apoD has an important function as a neuroprotective and antioxidant protein. In a screen to identify genes that protect *Drosophila* against acute oxidative stress, a fly homolog of apoD, glial lazaro (Glaz) was identified. Overexpression of Glaz resulted in increased resistance to hyperoxia as well as a 29% extension of lifespan under normoxia [30]. In contrast, the absence of Glaz reduces the organism's resistance to oxidative stress and starvation and shortens male lifespan. Glaz mutants also have a higher concentration of lipid peroxidation products, pointing to a role of lipid peroxidation protection or scavenging as the mechanism of action of this lipocalin [23]. Furthermore, flies that express the human form of apoD are long lived and protected against stress conditions associated with aging and neurodegeneration, including hyperoxia, dietary paraquat and heat stress [16].

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Similar findings to those observed in *Drosophila* have been noted in vertebrates and even plants. Loss of mouse apoD function increases the sensitivity to oxidation stress and the levels of brain lipid peroxidation, and impairs locomotor and learning abilities [9]. In contrast, human apoD overexpression in the mouse brain produces opposite effects, increasing survival and preventing the rise of brain lipid peroxides after oxidant treatment [9]. ApoD has also been shown to provide resistance against coronavirus infections in mice [6]. Plants that overexpress apoD, likewise, show increased resistance to oxidative stress [4]. Cellular stresses that either cause extended growth arrest, such as hydrogen peroxide and UV light, or increase in proliferation, such as lipopolysaccharide treatment were found to increase apoD production *in vitro*. At the promoter level, NFkB, AP-1 and APRE-3 proved to be the elements implicated in the lipopolysaccharide response [7].

In this investigation, we studied the effect of direct addition of exogenous human apoD protein purified from human breast cystic fluid to rat hippocampal slice cultures after excitotoxic injury induced by the glutamate analog kainate (KA). Our results reveal a neuroprotective effect of apoD against kainate induced excitotoxicity, and suggest that this effect may be due to the ability of apoD to sequester and reduce the levels of arachidonic acid and cholesterol oxidation products, F<sub>2</sub>-isoprostanes and 7-ketocholesterol, respectively.

Organotypic hippocampal slice cultures were prepared as previously described [24], with minor modifications [13]. In brief, 10-day-old Wistar rat pups were anesthetized with intraperitoneal injections of 3.5% chloral hydrate, decapitated, and the brain removed. The hippocampi were dissected out, and sectioned transversely at 350  $\mu$ m thickness using a tissue chopper. The slices were transferred to 30 mm Millicell CM culture plate inserts with 0.4  $\mu$ m polytetrafluoroethylene membranes (Millipore, Bedford, MA, USA), and placed in 6-well culture plates containing culture medium (50% minimum essential medium, 25% horse serum, 25% Hanks balanced salt solution, supplemented with D-glucose [6.5 mg/ml], glutamine [2 mM] penicillin G [1 unit/ml] and streptomycin sulfate [1  $\mu$ g/ml], pH 7.15). The slices were maintained at 37 °C, 100% humidity and 95% air and 5% CO<sub>2</sub>. The medium was changed to fresh medium every 3 days in culture. The effects of kainate and other agents were tested in hippocampal cultures after 14 days *in vitro*. The cultures were treated with KA (100  $\mu$ M) for 3 h in serum free culture medium. The above dose and duration of kainate treatment has been shown to be toxic to neurons in hippocampal slices [13].

After kainate treatment, cultures were incubated with 1–10  $\mu$ g/ml of apoD, diluted in fresh serum-free medium for 24 h. ApoD is present at 1–2  $\mu$ g/ml concentration in the cerebrospinal fluid (CSF) of healthy humans, but levels in brain and CSF are several-fold increased in Alzheimer's disease [26]. An 8–16 fold increase in apoD protein concentration is present in the hippocampus after entorhinal cortex lesions [27], and increased apoD immunoreactivity is found in hippocampal pyramidal neurons after kainate induced excitotoxicity in rats [18]. In view of the range of brain and CSF apoD concentrations in health and disease, we chose 1–10  $\mu$ g/ml as the concentration to test in our experiments. Human apoD protein in these experiments was prepared as previously described [20]. Human breast cystic fluid was centrifuged at 35,000  $\times$  g for 2 h, followed by dialysis of the aqueous phase against K<sub>2</sub>HPO<sub>4</sub> 10 mM pH 7.4, and hydroxyapatite in same buffer. The protein elutes in the flow through and is concentrated on DEAE-agarose and eluted with 400 mM NaCl. Stock apoD was dissolved in serum-free medium and sterilized through a 0.22- $\mu$ m filter. Controls consisted of untreated slices in serum free media, or slices incubated with 10  $\mu$ g/ml of bovine serum albumin (BSA).

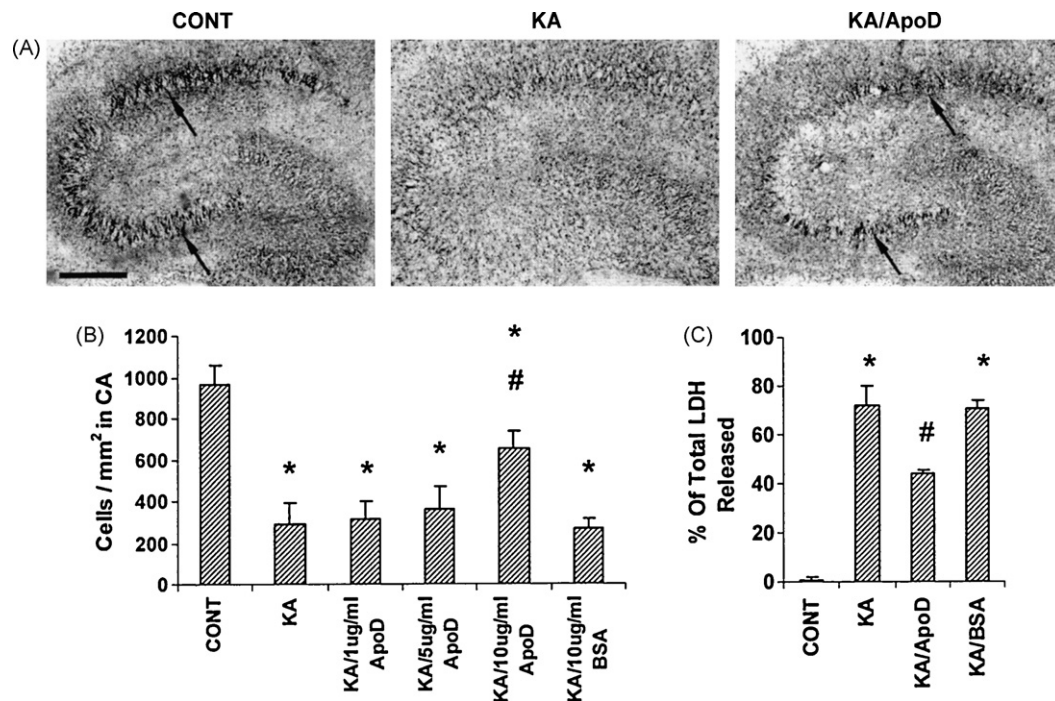
Assessment of neuronal damage was carried out by immunostaining and lactate dehydrogenase (LDH) assay. Immunostaining was carried out by fixing the slices in a fixative containing 4%

paraformaldehyde in phosphate buffer (pH 7.4) for 30 min, followed by washing in phosphate buffered saline (PBS, pH 7.4) for 3 h to remove traces of fixative. The slices were then detached from the membranes and incubated with monoclonal antibody to microtubule-associated protein 2 (MAP2) (Sigma, 1:1000 dilution). The slices were washed in three changes of PBS and incubated for 1 h at room temperature in a 1:200 dilution of biotinylated horse anti-mouse IgG (Vector, Burlingame, USA). The slices were then reacted at room temperature for 1 h with an avidin-biotinylated horseradish peroxidase complex, and the reaction was visualized by treatment for 5 min in 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) solution in Tris buffer containing 0.05% H<sub>2</sub>O<sub>2</sub>. The color reaction was stopped with several washes of Tris buffer, followed by PBS. The slices were mounted on gelatin-coated glass slides and counterstained with methyl green before coverslipping with Permount. The number of MAP2 positive neuronal cells in the CA fields of the hippocampal slices was counted in a "blind" manner on coded slides at 200 $\times$  magnification with the help of a grid. At least six slices were quantified for each treatment. The mean number of stained cells/mm<sup>2</sup> was then calculated for each treatment, and the results were subjected to statistical analyses, using one-way ANOVA with Bonferroni's multiple comparison post-hoc test.  $P < 0.05$  was considered significant.

LDH assay was carried out by collecting the culture media after various treatments and analysis using a LDH cytotoxicity detection kit (Roche, Basel, Switzerland) as follows: neuronal death = [(A – Min)/(Max – Min)]  $\times$  100, in which A is LDH activity measured in media of test condition, Max is maximum LDH release after 3 h treatment with Triton X-100, defined as 100% of cell death, Min is the LDH activity in media of untreated slices. Media from three culture dishes in each treatment group was collected for a single experiment. The mean and standard deviation from three separate experiments were then calculated. The results were subjected to statistical analyses, using one-way ANOVA with Bonferroni's multiple comparison post-hoc test.  $P < 0.05$  was considered significant.

Other slices were pooled (12–16 slices as one sample) and analyzed by GC/MS for F<sub>2</sub>-isoprostanes and cholesterol oxidative products. Lipids were extracted, hydrolyzed and analyzed using GC/MS as described in detail previously [10,11,12]. Essentially lipids were extracted from homogenates using organic solvent (CHCl<sub>3</sub>/methanol 2:1, v/v, +0.005% butylated hydroxytoluene (BHT) and dried under a stream of N<sub>2</sub>. After addition of heavy isotopic standards of F<sub>2</sub>-isoprostanes, and the cholesterol oxidation product 7-ketocholesterol, samples were reconstituted in 1 ml PBS and 1 ml KOH (1 M in methanol) and hydrolyzed overnight at 23 °C in the dark under an argon atmosphere. Solid phase extraction was carried out using 60 mg MAX (mixed ion exchange, Waters, Milford, USA) columns. After elution of F<sub>2</sub>-isoprostanes and 7-ketocholesterol, extracts were dried under N<sub>2</sub> gas, derivatized and analyzed by an Agilent 5975 mass selective detector interfaced with an Agilent 5890II gas chromatograph. Selected-ion monitoring was performed to monitor ions selected from each compound's mass spectrum in order to optimize sensitivity and specificity. Quantification of F<sub>2</sub>-isoprostanes and 7-ketocholesterol was carried out by relating the total peak area of each analyte with their heavy isotopic internal standard. The results were subjected to statistical analyses, using one-way ANOVA with Bonferroni's multiple comparison post-hoc test.  $P < 0.05$  was considered significant.

Treatment of hippocampal slice cultures from rat brain with 100  $\mu$ M kainate for 3 h caused neuronal injury in the hippocampal fields CA1 and CA3. This was shown by the light microscopic observation of decreased MAP2 immunostained pyramidal neurons in hippocampal CA fields. To investigate a possible neuroprotective effect of apoD, the kainate-injured hippocampal slices were treated with 1, 5, and 10  $\mu$ g/ml of apoD. ApoD at a concentra-

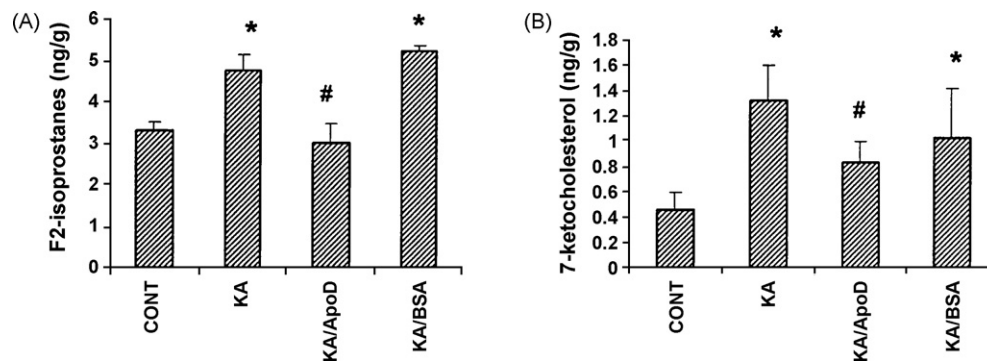


**Fig. 1.** (A) Effect of apoD on neuronal survival after addition of kainate to hippocampal slices. CONT, KA and KA/ApoD indicate untreated, kainate, and kainate plus apoD-treated hippocampal slices as described in the methods. The cultures were fixed in 4% paraformaldehyde and then incubated overnight with monoclonal antibodies to MAP2. Arrows indicate MAP2 immunolabeled neurons. Scale = 300  $\mu$ m. (B) Dose-dependent neuroprotective effect of apoD on cultured hippocampal slices. CONT, KA, KA/1, 5, 10  $\mu$ g/ml apoD, and KA/10  $\mu$ g/ml BSA indicate untreated, kainate treatment only, and kainate treatment followed by incubation for 24 h with 1, 5, 10  $\mu$ g/ml of human apoD or 10  $\mu$ g/ml BSA. (C) Effect of apoD on LDH release in hippocampal slice cultures. CONT, KA, KA/ApoD, and KA/BSA indicate untreated, kainate treatment only, and kainate treatment plus incubation for 24 h with 10  $\mu$ g/ml of human apoD or 10  $\mu$ g/ml BSA. KA-induced cell death quantified by measuring the percentage LDH leakage using a cytotoxicity detection kit (Roche). The values are mean  $\pm$  standard deviation ( $n = 3$ ). Results were analyzed by one-way ANOVA with Bonferroni's multiple comparison post-hoc test.  $P < 0.05$  was considered significant. \*Significant difference compared to CONT group; #significant difference compared to KA group.

tion of 10  $\mu$ g/ml partially prevented loss of MAP2 immunostaining from hippocampal neurons from kainate injury (Fig. 1A and B). Furthermore, assessment of LDH from kainate-treated tissues with and without addition of apoD showed significant decrease in LDH release from apoD-treated tissues (Fig. 1C). No protective effect was observed after addition of BSA (Fig. 1B and C). Together, these findings indicate a neuroprotective effect of apoD after kainate induced excitotoxicity that might be related to its ability to prevent the formation of lipid oxidation products. The above results are consistent with recent reports that showed that loss of mouse apoD function increases the sensitivity to oxidation stress and the levels of brain lipid peroxidation. In contrast, human apoD overexpression in the mouse brain produces opposite effects increasing survival and pre-

venting the raise of brain lipid peroxides after oxidant treatment [9]. ApoD has also been shown to provide resistance against coronavirus infections in mice [6].

What may be basis for the neuroprotective effect of apoD? Kainate treatment caused a significant increase in levels of the breakdown product of arachidonic acid peroxidation,  $F_2$ -isoprostanes (Fig. 2A) and the cholesterol oxidation product, 7-ketocholesterol (Fig. 2B) indicating increased oxidative stress, but levels of these oxidation products were attenuated, in slices that were treated with 10  $\mu$ g/ml apoD (Fig. 2A and B). No attenuation of increase in oxidation products after kainate treatment was observed in slices treated with 10  $\mu$ g/ml BSA (Fig. 2A and B). These results extend previous findings which showed decreases in the



**Fig. 2.** Effect of apoD on lipids and lipid peroxidation products in cultured hippocampal slices.  $F_2$ -isoprostanes (A) and 7-ketocholesterol (B) levels were measured by GC-MS in all the samples. CONT, KA, KA/ApoD, and KA/BSA indicate untreated, kainate treatment only, and kainate treatment plus incubation for 24 h with 10  $\mu$ g/ml of human apoD or 10  $\mu$ g/ml BSA. Data was normalized to the weight of the slices and expressed as mean  $\pm$  standard deviation of three experiments. Results were analyzed by one-way ANOVA with Bonferroni's multiple comparison post-hoc test.  $P < 0.05$  was considered significant. \*Significant difference compared to CONT group; #significant difference compared to KA group.



precursor to arachidonic acid, linoleic acid (18:2n6c), arachidonic acid itself (20:4n6) and the elongation product of arachidonic acid, adrenic acid (22:4n6), but increases in LA, eicosadienoic acid and docosahexaenoic acid in apoD knock-out compared to wild-type mice [29]. In view of the molecular structure of apoD which consists of an eight-stranded  $\beta$  barrel ( $\beta$ -clam) that forms a binding pocket for a number of small hydrophobic molecules, we propose that apoD is neuroprotective by binding to arachidonic acid and cholesterol thus preventing their oxidation to neurotoxic products such as 4-hydroxynonenal (4-HNE) [2] and 7-ketocholesterol [3,10,17]. The binding of apoD with arachidonic acid and cholesterol may also facilitate lipid transport mechanisms related to repair processes following KA-mediated toxicity. Further studies are necessary to elucidate factors that regulate the expression of apoD after neuronal injury.

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