ORIGINAL PAPER

Expression of Adenomatous Polyposis Coli Protein in Reactive Astrocytes in Hippocampus of Kainic Acid-Induced Rat

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Abstract The adenomatous polyposis coli gene (APC) was initially identified through its link to colon cancer. It is associated with the regulation of cell cycle progression, survival, and differentiation of normal tissues. Recent studies have demonstrated that APC is also expressed in the adult brain at high levels. However, its role in glial cells under pathological progression remains unclear. In this study, we evaluated the expression of APC and its association with β-catenin signaling pathway, following the induction of an excitotoxic lesion by kainic acid (KA) injection, which cause pyramidal cell degeneration. APC was predominantly present in oligodendrocytes in the normal brain, but was specifically associated with activated astrocytes in the KA-treated brain. Our quantitative analysis revealed that APC significantly increased from 1 day post lesion (PI), reached peak values at 3 days PI, and decreased thereafter. The phospho-GSK3β levels also showed similar spatiotemporal patterns while β-catenin expression was reduced at 1 and then increasingly returned to normal levels at 3, 7 days PI. For the first time, our data demonstrate the injury-induced astrocytic changes in the levels of APC, GSK3β, and β-catenin in vivo, which may actively be participate in cell adhesion and in the signaling pathway regulating cell survivals during brain insults.

Introduction

The role of the adenomatous polyposis coli (APC) tumor suppressor protein in the initiation and progression of colon cancer has been extensively studied for over a decade. APC's role in tumor suppression occurs through antagonism of the Wnt pathway and it down-regulates β-catenin through an association with the APC/Axin/GSK3β destruction complex. APC loss led to the inappropriate stabilization of β-catenin, which acts as a transcription co-activator with the TCF/LEF family of transcription factors [1, 2]. β-catenin/TCF transcriptional targets included genes such as c-myc and cyclin D1, which are associated with proliferation [3, 4]. This supported a model where misregulation of the Wnt pathway through APC mutations promoted tumorigenesis. APC mutations typically occurred early in the progression of colorectal cancer [5], suggesting that APC is partially responsible for the initiation of colon adenomas that could develop into carcinomas. APC was also important in the maintenance and maturation of colonic epithelial cells [6, 7].

The highly conserved APC ($\sim$312 kDa) contains an N-terminal oligomerization domain and several binding sites, including end-binding protein 1 (EB1), axin, β-catenin, glycogen synthase kinase 3β (GSK3β), APC-stimulated guanine nucleotide exchange factor (Asef), and IQ motif-containing GTPase activating protein 1 (IQGAP1).

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APC interacted with these proteins and participated in proliferation, apoptosis, intracellular signaling, cell adhesion, polarization, and migration [7, 8]. Recent studies have demonstrated that high APC levels were expressed during the development of the rat central nervous system [9], and persisted in the adult rat brain [10]. Especially, the expression of APC in oligodendrocytes is possible that APC mutation contribute to regulation of the transition from proliferating precursor to quiescent oligodendrocyte [10]. It is now generally accepted that APC is an ideal marker for oligodendrocytes in the mammalian brain since APC was preferentially localized in these glial cells. Further, APC is localized in the astrocytic processes to participate in a signal transduction pathway, and also expressed in neurons for regulating of neuronal functions with β-catenin and hDLG [11]. APC localization in astrocytes was reported in some neuropathological conditions in the human brain [12, 13].

In this sense, kainic acid (KA)-induced brain damage may provide a suitable model for evaluating the functions of Akt and GSK3β in reactive gliosis, since the administration KA induces excitotoxic brain injury. Furthermore, KA produces a highly specific pattern of neuronal loss in the hippocampus, which is accompanied by intense astrocyte and microglial reactions [14, 15]. The injury-induced changes of APC, GSK3β and β-catenin, as a target of APC/GSK3β regulation in glial cells may contribute to the mechanism of glial cell protection and adaptation in response to cell damage, and therefore, these may play an important role in the evolution of glial response and excitotoxic lesion outcome.

Experimental Procedure

Experimental Animals and Lesions

Adult male Sprague-Dawley (SD) rats (8 weeks old, 240–270 g) were maintained under 12 h light/12 h dark cycles at 23–25°C. Food and water were available *ad libitum*. Animals were handled in accordance with the guidelines for animal research defined in the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, 1985). Seizures were induced by intraperitoneal (i.p.) injection of rats with 10 mg/kg of KA (Tocris Cookson, Bristol, UK) dissolved in normal saline. Seizures were stopped after 2.5 h with Na phenytoin injections (50 mg/kg i.p.) [16]. KA-injected animals ($n = 24$) and saline-injected control animals ($n = 16$) were allocated to each of four groups which were sacrificed at predetermined times (detailed below) after KA administration. Seizure behavior following KA injection was staged according to the classification system of Zhang et al. [17]. Status epilepticus (SE) rats were injected with KA resulted in development of wet-dog shakes, head nodding, facial clonus, forelimb clonus, continued rearing and falling, and generalized clonic-tonic seizures in all rats at 1–2 h after KA injection. Stage 4 seizures included rearing with forelimb clonus and salivation, and stage 5 seizures included falling and loss of balance. Control rats received equal volumes of saline.

Tissue Preparation

On day, rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and transcardially perfused with heparinized PBS followed by 4% paraformaldehyde in PBS at 1, 3, and 7 days after KA ($n = 4$ at each time point) or saline injections ($n = 2$). Brains were removed, immersed in fixative for 4 h, cryoprotected in 30% sucrose solution, embedded in tissue freezing medium, and frozen rapidly in 2-methyl butane pre-cooled to its freezing point with liquid nitrogen. Frozen coronal sections (35 μm) were obtained with a Leica cryostat. Alternating sections were mounted on gelatin-coated slides or stored free-floating in an antifreeze buffer. Sections were mounted on gelatin-coated slides and stained with cresyl violet for routine histological examination. The stored free-floating sections were processed using immunohistochemical techniques, as detailed below.

Immunohistochemistry

Parallel free-floating sections were processed immunohistochemically to identify the presence of APC (CC-1). Sections were treated with blocking buffer (1% fetal bovine serum in PBS and 0.3% Triton X-100 for 30 min) after endogenous peroxidase blocking with 1% H_2O_2 in PBS, then rinsed thoroughly with PBS. Sections were incubated with the anti-mouse APC primary antibody (CC-1, #OP80, Calbiochem, USA; diluted in PBS + 0.3% Triton X-100 at 1:600) for 24 h at 4°C. Tissues were exposed to the biotinylated anti-mouse IgG and streptoavidin peroxidase complex (Vector, USA) for 1 h at room temperature after rinsing with PBS. Immunostains were visualized with diaminobenzidine (DAB, Sigma, USA) for 3–5 min and mounted using Polymount (Polysciences, USA). Double immunofluorescent experiments for APC were performed using Cy^{TM}2-conjugated anti-mouse IgG (1:400, Amersham, UK). Sections were further processed for anti-rabbit glial fibrillary acid protein (GFAP, 1:1,000, Dako, USA) and anti-rabbit ionized calcium binding adaptor molecule 1 (iba-1, 1:500, generously provided by Dr. Shinichi Kohsaka, National Institute of Neuroscience, Japan) [18]. Tissues were rinsed in PBS and incubated with Cy^{TM}3-conjugated anti-rabbit IgG (1:600, Amersham). All immunoreactions were observed under an Axiophot microscope (Carl Zeiss, Germany).

Western Blot Analysis

On days 1, 3, and 7 after SE onset ($n = 4$, respectively) and after saline injection in control group ($n = 2$), animals were anesthetized. Brains were removed quickly, and the hippocampus was dissected on ice and individually homogenized in lysis buffer (50 mM TrisCl, 150 mM NaCl, 0.02% sodium azide, 100 µl/ml PMSF, 1 µg/ml aprotinin, 1% Triton X-100). Supernatant protein concentrations were determined after centrifugation with Micro BCA protein assay kits. Bovine serum albumin was used as a standard (Pierce Chemical, USA). Aliquots containing 20 µg of protein were boiled in $2\times$ sample buffer (0.8 M TrisCl, 10% glycerol, 20% β-mercaptoethanol, 10% SDS, 0.02% bromophenol blue) for 5 min and loaded onto a polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, UK) after electrophoresis at 250 mA for 1 h. Membranes were incubated in 5% skimmed milk in PBST (0.3% Triton X-100 in PBS) for 1 h to block non-specific binding, and probed with APC (CC-1, 1:1,000), phospho-GSK3β (1:1,000, Cell signaling, USA), β-catenin (1:1,000, BD science, USA), and β-actin (1:5,000, Sigma, USA). Membranes were washed 3 times for 10 min in PBST and incubated for 1 h with peroxidase labeled secondary antibody (Vector) diluted 1:2,000 in PBST. Immunolabeled proteins were detected by chemiluminescence after three additional washes using a Supersignal ECL kit (Pierce Chemical) and Biomax Light-1 films (Kodak, USA). Western blot signals were quantitatively assessed with the NIH image program (ImageJ) for densitometric measurements. Quantitative data were analyzed with the one-way analysis of variance (ANOVA) followed by the Newman–Keuls test. Statistical significance was defined at P values < 0.05.

Results

Histology of KA-Induced Hippocampus

Cresyl violet stains revealed a selective lesion in the hippocampal region of KA-treated rats. Pyramidal cell degeneration was not evident in saline-treated control rats (Fig. 1a) but was apparent in the CA3 region from 1 day post-injection (PI) rats (Fig. 1b), and glial reactivity were evident in the CA3 region (Fig. 1c, d) at 3 and 7 days PI. Degeneration varied among animals but was most prominent in the CA3 bend; however, there was some cell sparing in these regions. Pyramidal cell death was also
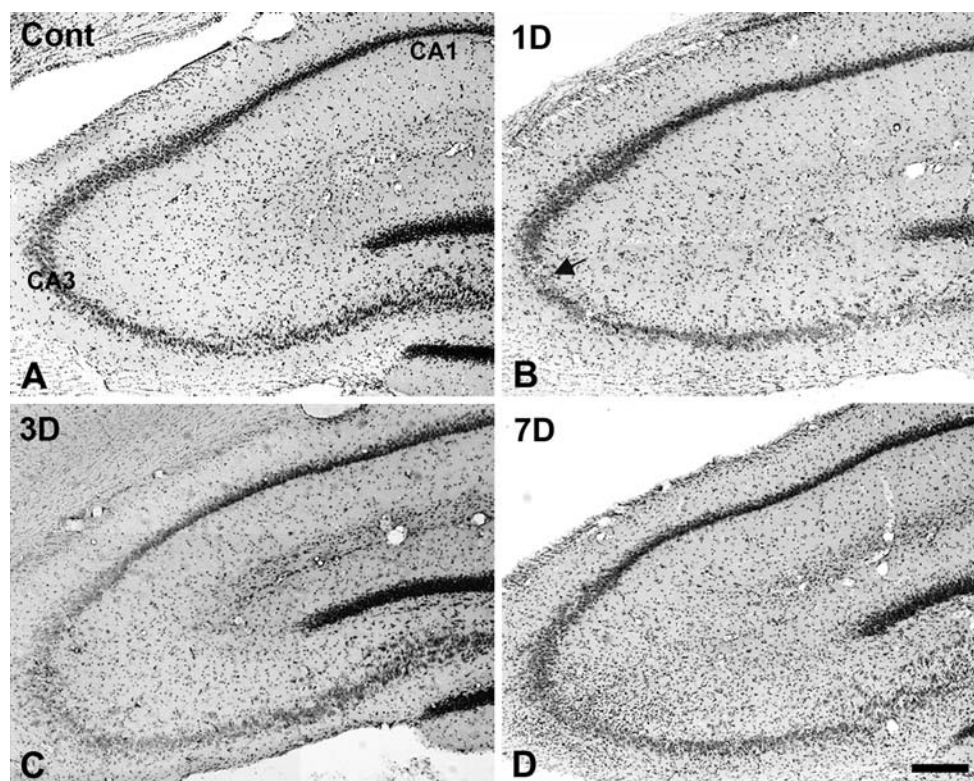
Fig. 1 Cresyl violet staining in hippocampal sections of KA-treated rat hippocampi. In contrast to normal rat tissue (**a**), pyramidal cell degeneration was apparent in the CA3 region (*arrow*) at 1 day post-injection (**b**). Pyramidal cell loss and glial reactivity were evident (**c**, **d**) at 3 and 7 days post-injection. *Scale bar* 100 µm

observed to a lesser extent in the CA1 regions and was excluded from this study. These findings confirmed that the i.p. KA injections resulted in specific losses of CA3 pyramidal cells, as seen in previous studies [19, 20].

Immunohistochemistry

Adenomatous polyposis coli immunoreactivity was seen in small hippocampal control cells (Fig. 2a, e). These cells resembled oligodendrocytes because of their small size and distribution, as demonstrated by previous studies in the normal brain [10]. We performed APC immunohisto-chemistry for the rat brain treated with Na Phenytoin alone, and obtained a similar staining pattern to the normal brain (data not shown). APC immunoreactivity was observed also in small glial cells in KA-treated rat hippocampi, and small numbers of APC immunoreactive cells were

observed in the vicinity of the hippocampus at 1 day post-injection (Fig. 2b, f). These immunoreactive cells increased significantly and were scattered throughout the hippocampus at 3 and 7 days after treatment (Fig. 2c, d). APC immunoreactive cells showed morphology of activated glial cells at higher magnification that newly appeared in association with brain injuries (Fig. 2f–h). We performed double labeling experiments with two different glial markers to confirm cells types of APC immunoreactive cells (GFAP for astrocytes and iba-1 for microglia). There were no cells overlapped with two antigens in the control hippocampi. APC was observed in the perikaryon of small round cells while GFAP was noted in larger process-bearing cells (Fig. 3a–c), suggesting that APC-positive cells are oligodendrocytes, not astrocytes. APC immunoreactive cells were largely double-labeled with GFAP in KA-treated hippocampi, except a few APC
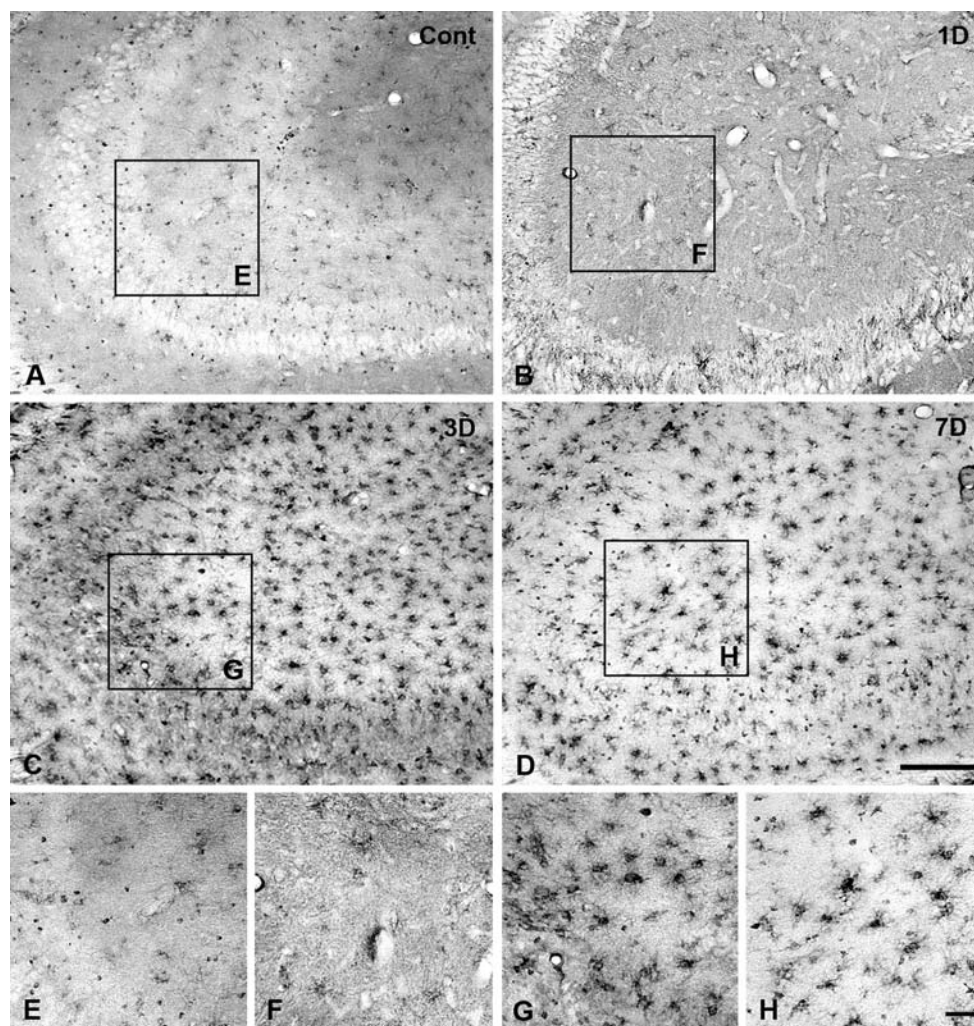
Fig. 2 APC immunoreactivity in the normal (a) and KA-treated rat hippocampi at 1 day (b), 3 days (c), and 7 days (d) after treatment. APC immunoreactivity was identified in the small cells of the hippocampus (a, e) in control samples. APC immunoreactivity was also observed in glia-like cells in the KA-treated hippocampi. These cells appeared 1 day after treatment, increased in number (b–d) and size (f–h) at 3 and 7 days after treatment. e–h Higher magnification of rectangular area in a–d. Scale bar a–d = 100 μm, e–h = 20 μm
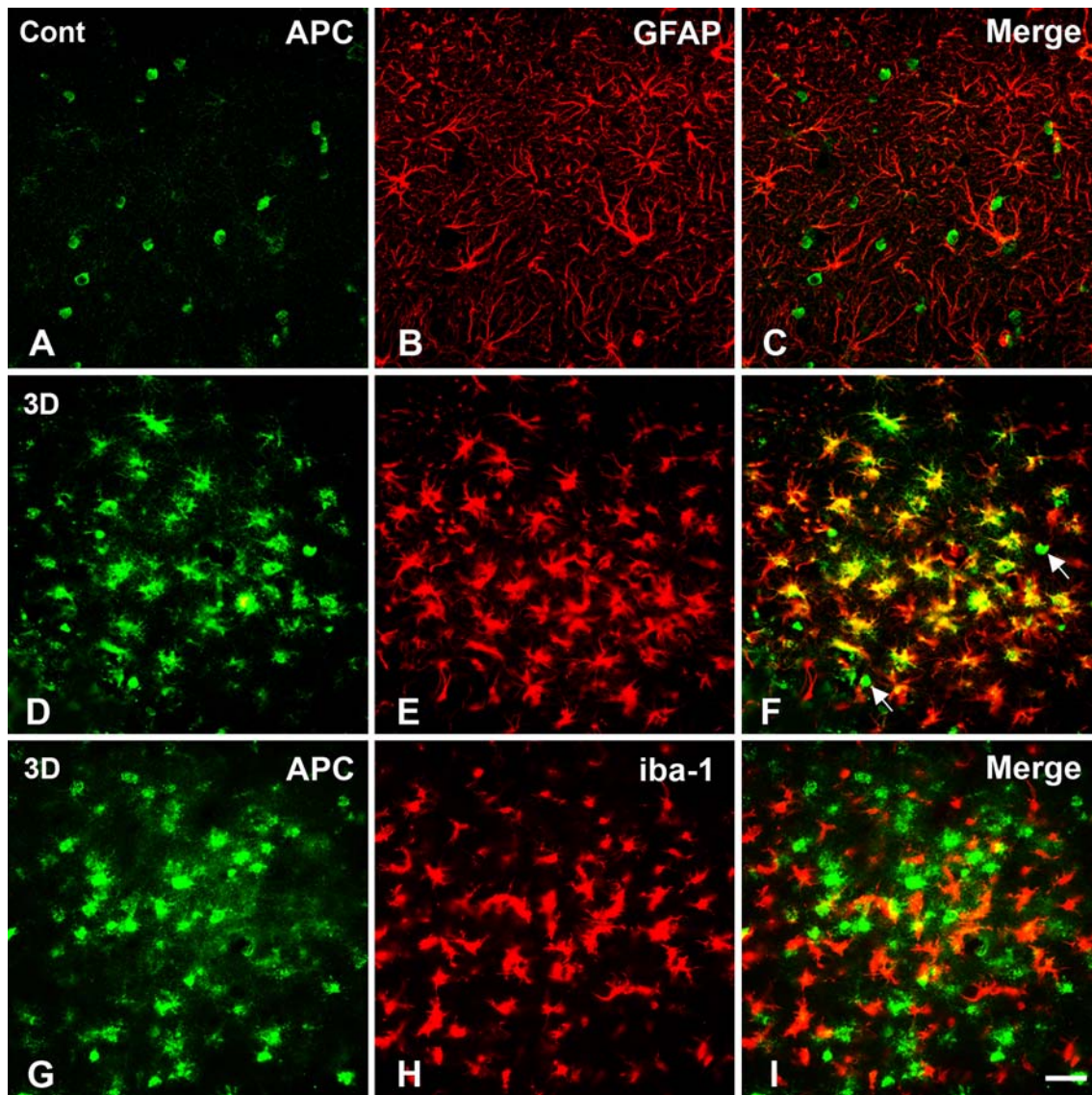
Fig. 3 Double immunofluorescent staining to identify APC-positive cells in control (**a**–**c**) and KA-treated hippocampi (**d**–**i**). APC was localized in the perikarya of small round cells in the control samples while GFAP was localized in the larger process-bearing cells. No overlaps were identified between them (**a**–**c**), suggesting that APC is expressed in oligodendrocytes but not GFAP-positive astrocytes in the normal brain. APC largely overlapped with GFAP (**d**–**f**) in the KA-treated hippocampi except for a few cells that APC-positive only and GFAP negative (*arrows*). APC/iba-1 double labeling revealed that the antigens were separately localized and no cells overlapped (**g**–**i**). *Scale bar* **a**–**i** = 20 μm

immunoreactive cells that were not GFAP-positive. These cells were small and resembled the APC-positive oligodendrocytes which appeared in the control (Fig. 3d–f), suggesting that APC-positive cells are predominantly activated astrocytes. The antigens were separately localized in APC/iba-1 double labeling, and cells did not overlap (Fig. 3g–i). These suggested that APC was specifically expressed in activated astrocytes in the KA-treated rat hippocampi, although oligodendrocytes that remained in the injured brain still expressed APC.

Western Blot Analysis

The first recognized function of APC was its role in Wnt Signaling [21, 22]. APC negatively regulates Wnt signaling by participating in the destruction complex, complex that targets the key effector β-catenin for degradation [23]. So we examined the relation of APC with GSK3β and β-catenin in our KA model. Our western blot analysis showed that APC, phospho-GSK3β, and β-catenin were constitutively expressed in the normal hippocampus. The

APC and phospho-GSK3β levels were increased in the injured brain from 1 day PI, reached peak values at 3 days PI, and decreased thereafter. Conversely, β-catenin, which recognized total protein amounts within cell, was reduced at 1 and then increasingly returned to normal levels at 3, 7 days PI (Fig. 4a, b).

Discussion

This study demonstrated that glial expression of APC was different in control and injured rat brains. APC was primarily expressed in oligodendrocytes in control rat brains, as demonstrated in this study. Our observation was consistent with previous studies which reported that high APC mRNA and protein levels were expressed in oligodendrocytes of the normal adult rat [9, 10], and after spinal cord injuries [24, 25]. However, during the time course of KA-induced neuronal degeneration, APC was specifically expressed in non-oligodendroglia. The majority of these cells corresponded to reactive astrocytes because of their antigenic properties and their morphological appearance. To our knowledge, this is the first report which revealed the induction of APC (CC-1) in activated astrocytes in KA-induced excitotoxic brain injury. There have been several additional studies evaluating the expression of other APC antibodies in neuropathological conditions, such as Alzheimer's disease patients [12, 13].

Adenomatous polyposis coli exerts its tumor suppressor effects as a Wnt pathway antagonist. The central region of the APC protein contains three distinct types of regulation of Wnt signaling [23 for review]. In unstimulated epithelial cells, most of the endogenous β-catenin is found at cell adherens junctions, where it interacts with E-cadherin and -catenin to help mediated cell adhesion. To ensure its rapid turnover, the excess newly synthesized cytoplasmic β-catenin is targeted to a multisubunit destruction complex, which includes Axin, the APC tumor suppressor, protein phosphatase 2A (PP2A), and the protein kinase GSK3β and CK1α. Sequential phosphorylation of β-catenin by first at CK1 and then GSK3β targets it for ubiquitination and subsequent proteolytic destruction by the proteosome
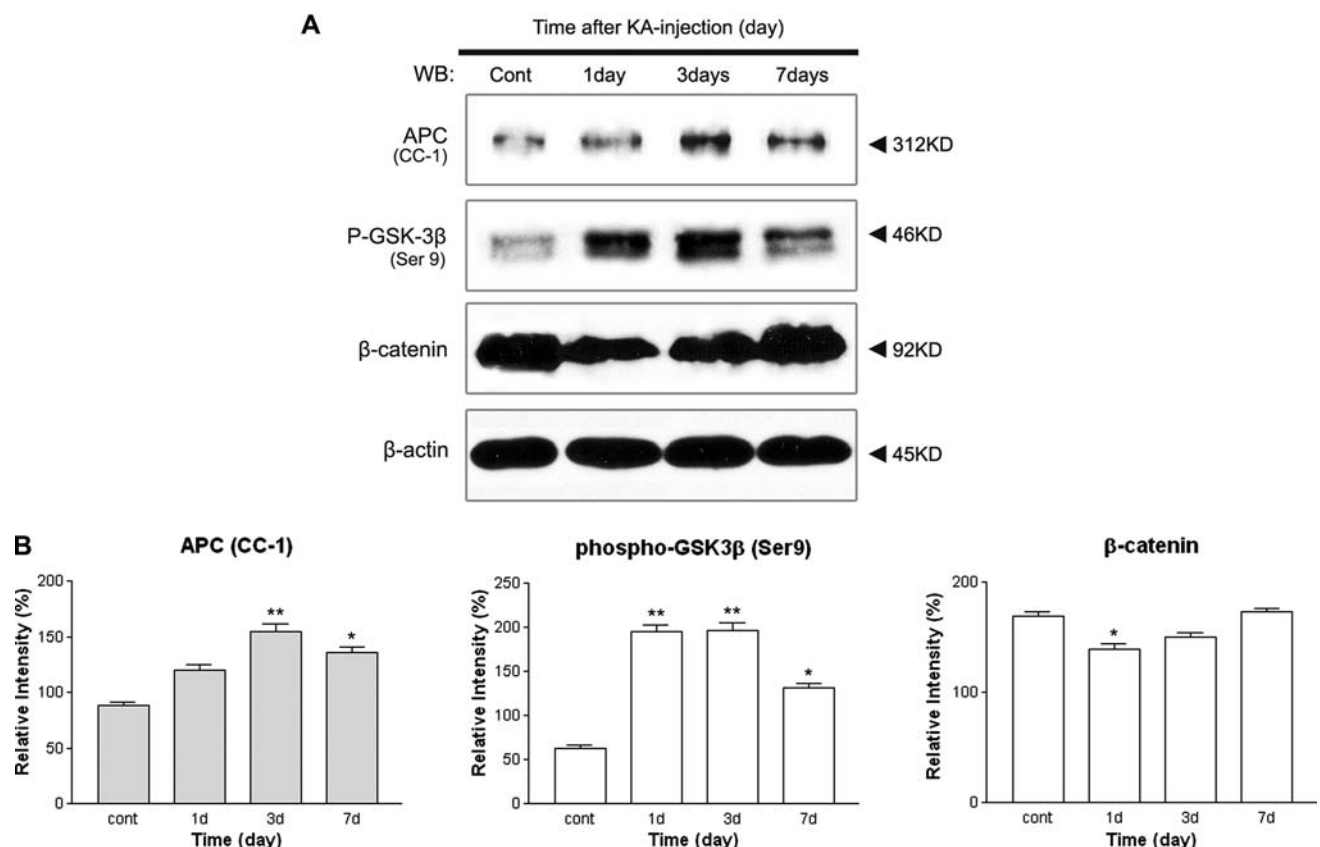
Fig. 4 Western blot analysis displaying the temporal patterns of APC, phospho-GSK3β, and β-catenin expression in control and KA-induced rats. β-actin was used as a loading control (**a**). *Band* densities were analyzed with an image analyzer and were expressed as percentages of controls. *Bars* indicated mean [SD] values (**b**). The differences between the means were analyzed with a one-way analysis of variance followed by the Newman–Keuls test. ** $P < 0.01$, * $P < 0.05$ vs. control. Amounts of APC and phospho-GSK3β increased progressively from 1 day post-KA injection, peaked at 3 days post-injection, and decreased thereafter. In contrast, β-catenin levels decreased at 1–3 days post-injection and returned to normal levels at 7 days post-injection

("off" state) [26]. Binding of Wnt ligand to its receptor complex triggers a series of events that ultimately disrupts the APC/Axin/GSK3β complex. Wnt signaling promotes CK1 and GSK3β mediated hyperphophorylation of LRP5/6 and enhances Dsh phosphorylation, which jointly recruit Axin to the receptor complex at the plasma membrane ("on" state), where it undergoes proteolytic degradation. Unphosphorylated β-catenin is no longer rapidly degraded and enters the nucleus. The TCF/LEF family of transcription factors partner with β-catenin in the nucleus to activated Wnt target genes such as c-Myc, cyclin D1, and Axin2 [3, 4].

In summary, since Wnt can inactivate GSK3β and block phosphorylation of β-catenin, this leads to the activation of β-catenin followed by transcription of its target genes. We have previously observed high protein kinase B (Akt)/ GSK3β expression in activated astrocytes at 3 days after treatment in a mouse model of KA-induced hippocampal injury [27]. The Wnt pathway also uses protein kinase to promote cellular differentiation and survival [28]. Akt is necessary in pathways that involve Wnt, since Akt inhibits the activity of GSK3β through phosphorylation of this protein to promote cell survival [29]. In our system, our Western blot analysis revealed that APC and phospho-GSK3β were transiently upregulated at time (3 and 7 days PI) which glial reactivity were evident, while β-catenin was downregulated at 1 day PI and then increasingly returned to normal levels at 3, 7 days PI. The drop of β-catenin at 1 day PI may be attributable to breakdown of cell membrane due to pyramidal cell loss. The combined inhibition of GSK3β activity (i.e. increasement of phospho-GSK3β, which means inactive form of GSK3β) blocks the formation of the protein complex consisting of GSK3β, Axin, and APC. Without, the formation of this protein complex, phosphorylation of β-catenin with its subsequent degradation does not occur and the accumulation of free β-catenin results for translocation to the nucleus. Therefore, this theory well squares with our results that APC, GSK3β and β-catenin were upregulated at the time of gilal activation.

The present study shows the possible signaling pathway from APC/GSK3β to β-catenin in the astrocytes of excitotoxically damaged mouse hippocampi. This finding also means that GSK3β inactivation by Akt-induced phosphorylation may enhance β-catenin level by abrogating the inhibitory effect of GSK3β. The present study shows that expressions of phosphorylated APC, GSK3β and β-catenin play an important role in astrocytic cell death/survival pathways in response to excitotoxins in vivo.

References

1. Korinek V, Barker N, Morin PJ et al (1997) Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science 275(5307):1784–1787
2. Morin PJ, Sparks AB, Korinek V et al (1997) Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 275(5307):1787–1790
3. He TC, Sparks AB, Rago C et al (1998) Identification of c-MYC as a target of the APC pathway. Science 281(5382):1509–1512
4. Tetsu O, McCormick F (1999) Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398(6726):422–426
5. Powell SM, Zilz N, Beazer-Barclay Y et al (1992) APC mutations occur early during colorectal tumorigenesis. Nature 359(6392):235–237
6. Smith KJ, Johnson KA, Bryan TM et al (1993) The APC gene product in normal and tumor cells. Proc Natl Acad Sci USA 90(7):2846–2850
7. Hanson CA, Miller JR (2005) Non-traditional roles for the adenomatous polyposis coli (APC) tumor suppressor protein. Gene. 361:1–12
8. Näthke IS (2004) The adenomatous polyposis coli protein: the Achilles heel of the gut epithelium. Annu Rev Cell Dev Biol 20:337–366
9. Bhat RV, Baraban JM, Johnson RC et al (1994) High levels of expression of the tumor suppressor gene APC during development of the rat central nervous system. J Neurosci 14(5 Pt 2):3059–3071
10. Bhat RV, Axt KJ, Fosnaugh JS et al (1996) Expression of the APC tumor suppressor protein in oligodendroglia. Glia 17(2):169–174
11. Senda T, Iino S, Matsushita K et al (1998) Localization of the adenomatous polyposis coli tumour suppressor protein in the mouse central nervous system. Neuroscience 83(3):857–866
12. Leroy K, Duyckaerts C, Bovekamp L et al (2001) Increase of adenomatous polyposis coli immunoreactivity is a marker of reactive astrocytes in Alzheimer's disease and in other pathological conditions. Acta Neuropathol 102(1):1–10
13. Cotter D, Honavar M, Lovestone S et al (1999) Disturbance of Notch-1 and Wnt signalling proteins in neuroglial balloon cells and abnormal large neurons in focal cortical dysplasia in human cortex. Acta Neuropathol 98(5):465–472
14. Nadler JV, Perry BW, Gentry C et al (1980) Degeneration of hippocampal CA3 pyramidal cells induced by intraventricular kainic acid. J Comp Neurol 192:333–359
15. Mitchell J, Sundstrom LE, Wheal HV (1993) Microglial and astrocytic cell responses in the rat hippocampus after an intracerebroventricular kainic acid injection. Exp Neurol 121:224–230
16. Kim TY, Yi JS, Chung SJ et al (2007) Pyruvate protects against kainate-induced epileptic brain damage in rats. Exp Neurol 208(1):159–167
17. Zhang X, Le Gal La Salle G, Ridoux V et al (1997) Prevention of kainic acid-induced limbic seizures and Fos expression by the GABA-A receptor agonist muscimol. Eur J Neurosci 9(1):29–40
18. Ito D, Imai Y, Ohsawa K et al (1998) Microglia-specific localisation of a novel calcium binding protein, Iba1. Brain Res Mol Brain Res 57(1):1–9
19. Friedman LK, Pellegrini-Giampietro DE, Sperber EF et al (1994) Kainate-induced status epilepticus alters glutamate and GABAA receptor gene expression in adult rat hippocampus: an in situ hybridization study. J Neurosci 14(5 Pt 1):2697–2707
20. Grooms SY, Opitz T, Bennett MV et al (2000) Status epilepticus decreases glutamate receptor 2 mRNA and protein expression in hippocampal pyramidal cells before neuronal death. Proc Natl Acad Sci USA 97(7):3631–3636

21. Su LK, Vogelstein B, Kinzler KW (1993) Association of the APC tumor suppressor protein with catenins. Science 262(5140):1734–1737

22. Rubinfeld B, Souza B, Albert I et al (1993) Association of the APC gene product with beta-catenin. Science 262(5140):1731–1734

23. McCartney BM, Näthke IS (2008) Cell regulation by the Apc protein Apc as master regulator of epithelia. Curr Opin Cell Biol 20(2):186–193

24. Dougherty KD, Dreyfus CF, Black IB (2000) Brain-derived neurotrophic factor in astrocytes, oligodendrocytes, and microglia/macrophages after spinal cord injury. Neurobiol Dis 7(6 Pt B):574–585

25. Atkinson S, Li YQ, Wong CS (2003) Changes in oligodendrocytes and myelin gene expression after radiation in the rodent spinal cord. Int J Radiat Oncol Biol Phys 57(4):1093–1100

26. Price MA (2006) CKI, there's more than one: casein kinase I family members in Wnt and Hedgehog signaling. Genes Dev 20(4):399–410

27. Kim DW, Lee JH, Park SK et al (2007) Astrocytic expressions of phosphorylated Akt, GSK3beta and CREB following an excitotoxic lesion in the mouse hippocampus. Neurochem Res 32(9):1460–1468

28. Chong ZZ, Li F, Maiese K (2005) Oxidative stress in the brain: novel cellular targets that govern survival during neurodegenerative disease. Prog Neurobiol 75(3):207–246

29. Crowder RJ, Freeman RS (2000) Glycogen synthase kinase-3 beta activity is critical for neuronal death caused by inhibiting phosphatidylinositol 3-kinase or Akt but not for death caused by nerve growth factor withdrawal. J Biol Chem 275(44):34266–34271