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Changes of Src-suppressed C kinase substrate expression in cytokine induced reactive C6 glioma cells

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Abstract: Objective To investigate effect of tumor necrosis factor-α (TNF-α) on the Src-suppressed C kinase substrate (SSeCKS) in C6 glioma cells. **Methods** Cultured C6 glioma cells were randomly divided into two groups. In time-dependent group, cells were cultured with TNF-α (2 ng/mL) for 0 h, 1 h, 3 h, 6 h, 12 or 24 h, respectively; in dose-dependent group, cells were cultured with TNF-α (0 ng/mL, 0.02 ng/mL, 0.2 ng/mL, or 2 ng/mL) for 6 h. The expression of SSeCKS was detected by Realtime PCR and Western blot analysis, and immunocytochemistry was used to investigate SSeCKS's subcellular localization. **Results** TNF-α induced rapid phosphorylations of protein kinase C (PKC) substrates in C6 glioma cells, and upregulated SSeCKS expression in a time and concentration dependent manner. Immunocytochemistry suggested that SSeCKS was localized in the cyroplasm and the leading end of podosomal extensions in control groups, while TNF-α induced translocation of SSeCKS perinuclear. This effect could be partly reversed by PKC inhibitor Ro-31-8220. **Conclusion** TNF-α activates PKC and upregulates SSeCKS expression in C6 glioma cells. These effects are associated with PKC activity, suggesting that SSeCKS plays a role in response to glia activation in PKC mediated pathway.
Keywords: tumor necrosis factor-α; Src-suppressed C kinase substrate; protein kinase C

1 Introduction

Glial cells are the major cell population in the central nervous system (CNS). They reside ubiquitously in the brain parenchyma as highly ramified cells and play an important role in maintenance of brain homeostasis by functioning as a source of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interlukin-1 (IL-1), and so on[1,2]. These cytokines act on the CNS to induce expression of multiple genes such as chemokines, other cytokines and adhesion molecules that drive and regulate inflammatory and bactericidal effector pathways, resulting in a shape change (stellation) resembling a reactive gliosis[3]. Thus modulation of the inflammatory response is equally important in order to ensure preservation of immune homeostasis.

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Recent studies comfirmed the critical role of protein kinase C (PKC) in regulating glia cells activation, including cell differentiation, proliferation, migration and biosynthesis[4,5]. These functions are achieved by locally anchoring the kinases to specific organelles or subcellular structures.

It is thought that the proteins such as protein kinase A anchoring proteins (AKAPs) and several classes of PKC targeting proteins serve as linker proteins to position certain kinases to these immobile structures. PKC-targeting proteins such as the substrates interacting with C kinase and receptors of activated C kinase similarly localize PKC. Example is rodent Src-suppressed C kinase substrate (SSeCKS), a major *in vivo* substrate of PKC, which was originally identified as a negative mitotic regulator[6]. As a member of AKAP family, SSeCKS binds to PKC[7], protein kinase A, calmodulin[8] and β_2-adrenergic receptors[9] with the regulatory subuint, suggesting the function of SSeCKS that assemble a multiprotein signaling complex at the sites of its localization. Evidence indicates that PKC-modified phosphorylation affects the intracellular distribution of SSeCKS[10]. Recently, SSeCKS has been shown to be a major

lipopolysaccharide (LPS) response protein which was markedly upregulated in several organs, including lung, heart, kidney, brain etc., indicating a possible role of SSeCKS in the inflammatory process. Such observations have led to the proposition that SSeCKS might be a regulator in PKC-mediated glia cells activation.

Therefore, in the present study, rat C6 cells, which are commonly thought to arise from cells of astrocytic lineage, are used to verify the hypothesis that SSeCKS was associated with the TNF-α-induced PKC signaling in glia cells. Our result indicated a possible role of SSeCKS in inflammatory reactions in the CNS.

2 Materials and methods

2.1 Chemicals and antibodies TNF-α, phorbol 12-myristate 13-acetate (PMA), Ro-31-8220, polyclonal anti-SSeCKS antibody, monoclonal anti-β-actin antibody, horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG antibody were purchased from Sigma Chemicals (St. Louis, MO, USA). Horseradish peroxidase-conjugated anti-sheep antibody was purchased from Cell Signaling (Beverly, MA, USA). Rhodamin-conjugated anti-sheep antibody was purchased from Jackson laboratory (Bar Harbor, ME, USA). All reagents were obtained from Sigma (St. Louis, MO, USA) unless noted otherwise.

2.2 Cell cultures and cell treatment Rat C6 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured under standard culture condition—DMEM/F12 supplemented with 15% fetal bovine serum and 1% penicillin-streptomycin, and maintained at 37 ºC and 5% CO_2 in a humid environment. At 60% confluence, culture medium was switched to the serum-free DMEM/F12, and experiments were initiated 24 h later. Cultured C6 glioma cells were randomly divided into two groups. In time-dependent group, cells were cultured with TNF-α (2 ng/mL) for 1 h, 3 h, 6 h, 12 or 24 h, respectively; in dose-dependent group, cells were cultured with TNF-α (0.02 ng/mL, 0.2 ng/mL, 2 ng/mL) for 6 h. Non-treated cells were included as control in all experiments.

2.3 Realtime-PCR Total RNA was isolated using the TRIzol method (Invitrogen, Burlington, ON, USA) according to the manufacturer's protocol. Transcript levels of SSeCKS were measured in 36-well microtiter plates using a Roto-gene 2000 Sequence Detector System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The following primers were used: SSeCKS (accession number AY695057, GenBank): forward, 5'-AAG TGC TGG CTT CGG AGA AAG-3'; reverse, 5'-TGA CTT CAG GAA CTT CAA GGC TC-3'. Probes were designed using Primer Express (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). All runs were accompanied by TaqMan β_2-microglobulin (β_2-M) control reagents. Relative differences in the expression between groups were normalized with β-actin. Relative difference between the control and treatment groups was calculated and expressed as relative increase ratio to the control, with the control set as 1. Values were responsible for six independent reactions.

2.4 Western blot analysis Cells were thoroughly scraped from the culture dishes with a cell scraper. Lysates were homogenized for 10 s at 6 000 r/min in a homogenizer (Brinkman). Protein content was normalized using protein assay kits (Bio-Rad Laboratories). Samples were subjected to SDS-polyacrylamide gel electrophoresis, followed by transferring onto a polyvinylidine difluoride membrane filter (Immobilon, Millipore). Filters were incubated in PBS containing 0.5% Tween 20 (PBS-T) and 5% non-fat milk, and then with anti-SSeCKS antibody (1:1 000) at 4 ºC overnight. To measure PKC kinase activity, filters were incubated with anti-phospho-Ser PKC substrates antibody (1:1 000). After being incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5 000), blots were washed and immunoreactive proteins were visualized on a film with an enhanced chemiluminescence kit (NEN Life Science Products, Boston, MA, USA). Optical density on the film was measured with a computer imaging system (Imaging Technology, Ontario, Canada). The relative difference between the control and treatment groups was calculated and expressed as relative increase ratio to the control, with the control set as 1. Values were responsible for three independent reactions.

2.5 Immunofluorescence Cells growing on glass coverslips were fixed in 4% paraformaldehyde (PFA) for 15 min. After washed with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min. For SSeCKS staining, 0.01% Triton X-100 in PBS was used. Cells were incubated with diluted sheep antibody against SSeCKS (1:200) overnight at 4 ºC. After washed, cells were incubated with a tetramethyl rhodamine iso-thiocyanate (TRITC) fluorochrome conjugated secondary antibody (1:250 dilution in PBS) and a fluorescein isothiocyanate (FITC) conjugated phalloidin (1:800 dilution in PBS). Hoechst was used as a counterstain for DNA. Stained cells were examined with a fluorescence

microscope (BX 60; Olympus, Tokyo, Japan).

2.6 Statistical analysis Data were presented as mean ± SEM of at least 3 independent determinations. Comparisons were analyzed by using one-way analysis of variance (ANOVA) followed by the posteriori Student-Newman-Keuls' *t*-test. $P < 0.05$ was considered significant.

3 Results

3.1 Effect of TNF-α on SSeCKS mRNA expression in C6 cells Recently, SSeCKS has been shown as a major LPS response protein. To examine whether SSeCKS was involved in TNF-α induced cell responses in glia cells, we first observed the SSeCKS expression after TNF-α stimulation. According to the results of Realtime-PCR, TNF-α stimulation resulted in remarkable SSeCKS mRNA upregulation at the concentration of 0.2 ng/mL and 2 ng/mL (Fig. 1).

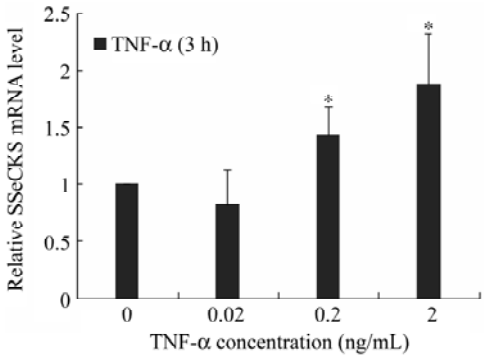

Fig. 1 Concentration dependence of SSeCKS mRNA expression by TNF-α in C6 cells. For quantitative analysis, the SSeCKS mRNA level was normalized by those of β_2-M mRNA. Values were responsible for 6 independent experiments. * $P < 0.05$ *vs* untreated group.

3.2 Effect of TNF-α on SSeCKS protein expression in C6 cells To determine whether the protein levels of SSeCKS changed simultaneously with its mRNA levels, two sets of experiments were conducted. We challenged serum-starved astrocytes with TNF-α (2 ng/mL) for varying lengths of time, ranging from 0 h to 24 h. The amount of SSeCKS in C6 cells was determined by Western blot analysis and was normalized with β-actin. As shown in Fig. 2, TNF-α can time-dependently induce SSeCKS expression in C6 cells. The strongest activation was obtained at 6–12 h. This increase persisted for up to 24 h.

When C6 cells were treated with increasing concentrations of TNF-α (0.2–20 ng/mL) for 6 h, SSeCKS was concentration-dependently upregulated with the most pronounced effect at the concentration of 2 ng/mL (Fig. 3). Taken together, these results suggest that activation of TNF-α may stimulate SSeCKS protein expression in C6 cells.

3.3 TNF-α stimulation resulted in increased PKC activity in C6 glioma cells Signaling from TNF-α to glia cells activation can be transmitted by several distinct pathways. To verify whether PKC mediated signaling pathway was involved in TNF-α treated C6 cells, an antibody specific to

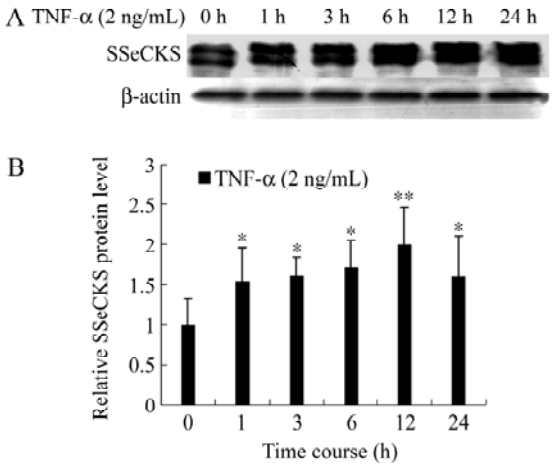

Fig. 2 Time course of SSeCKS protein expression by TNF-α in C6 cells. A: Representative blot from one of the individual experiments. Upper blots show SSeCKS induced by TNF-α; lower blots demonstrate equal loading of protein by detecting β-actin. B: Bands were quantified by densitometer. Amount of SSeCKS was normalized by referring to amount of β-actin. Values were responsible for 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ *vs* untreated group.

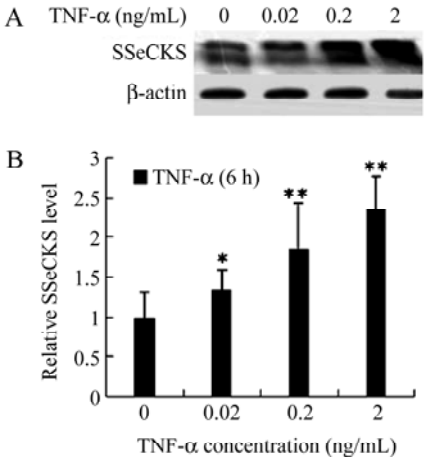

Fig. 3 Concentration dependence of TNF-α-induced SSeCKS protein expression in C6 cells. A: Representative blot from one of the experiment. Upper blots show SSeCKS induced by TNF-α; lower blots demonstrate equal loading of protein by detecting β-actin. B: Normalized SSeCKS expression level by referring to the amount of β-actin. Values were responsible for 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ *vs* untreated group.

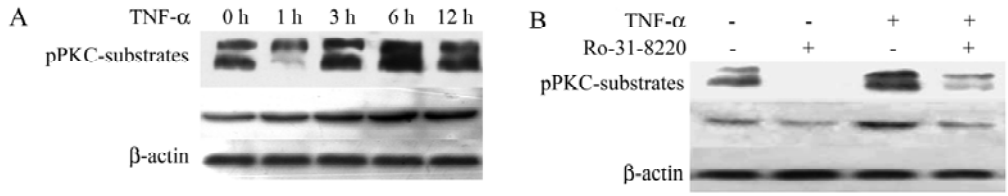

Fig. 4 PKC kinase activity assay in TNF-α-stimulated C6 cells. A: Representative 3 blots show kinetics of TNF-α-induced serine phosphorylation of PKC substrates in C6 cells. B: Representative blots show effects of Ro-31-8220 on TNF-α-evoked serine phosphorylation of PKC substrates. Lower blot demonstrates equal loading of protein by detecting β-actin.

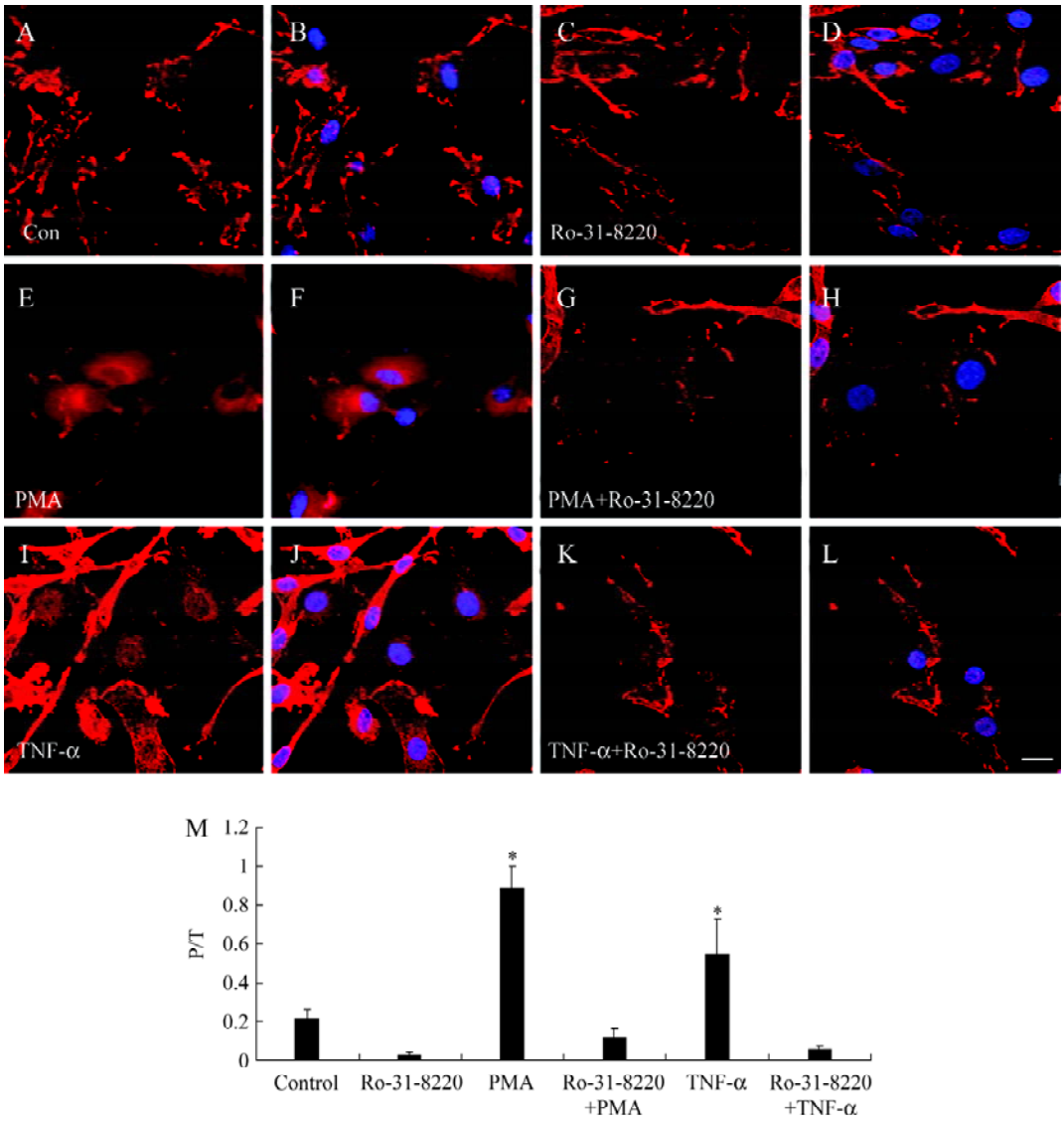

Fig. 5 Representative photomicrographs show the effects of Ro-31-8220, PMA and TNF-α on localization of SSeCKS in C6 cells. A, B: podosome enrichment of SSeCKS in untreated C6 cells. C, D: Ro-31-8220 treatment does not significantly influence the localization of SSeCKS as compared with control. E, F: PMA (30min) induces SSeCKS translocation to the perinucleus in C6 cells. G, H: Pretreatment with the PKC inhibitor, Ro-31-8220, abrogates the stimulatory effect of TNF-α on SSeCKS translocation. I, J: TNF-α (3 h) induces SSeCKS translocation to the perinucleus in astrocytes. These effects are blocked by pretreatment with Ro-31-8220 (K, L). Scale bar: 20 μm. M: SSeCKS-translocation is estimated by cell counting. On average, 3 visual fields were chosen randomly from each coverslip. Number of Cells that displayed significant perinucleus SSeCKS expression was counted (P) and normalized with total cell number (T) per visual field. The rate of SSeCKS-translocation was expressed as P/T. * $P < 0.05$ *vs* untreated group. Data were responsible for 4 independent experiments.

phospho-Ser PKC substrates was applied. As a result of TNF-α stimulation, rapid Ser phosphorylation of PKC substrates was observed at 6 h post-stimulation. The upper three blots of Fig. 4A show the kinetics of serine phosphorylation of three representative PKC substrates in C6 cells. These effects are blocked by pretreatment with Ro-31-8220 for 30 min (Fig. 4B), indicating that PKC specifically plays an important role in TNF-α stimulation induced responses in C6 cells.

3.4 PKC-dependent SSeCKS translocation after TNF-α stimulation As mentioned above, PKC targeting proteins and substrates regulate the function of PKC by locally anchoring the kinases to specific organelles or subcellular structures. SSeCKS, a major substrate of PKC, is phosphorylated as a result of PKC activation in a variety of cell types[11]. Previous studies showed that PMA induced PKC activation in Rat-6 fibroblasts caused pronounced SSeCKS activation, which was characterized by rapid translocation of SSeCKS from plasma membrane and cytoskeletal sites to the perinucleus. Figure 5 showed that by 30 min of PMA treatment (Fig. 5E, F; M, lane 3) or 3 h of TNF-α stimulation (Fig. 5I, J; M, lane 5), SSeCKS translocated to the perinucleus in C6 cells. These effects were blocked by pretreatment with Ro-31-8220 for 30 min (Fig. 5G, H and K, L; M, lane 4 and 6). These data clearly show a link between PKC activation and SSeCKS translocation in TNF-α stimulated C6 cells.

4 Discussion

TNF-α is closely associated with a variety of neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD). The major immune cells that respond to inflammatory stimuli in the brain are astrocytes and microglia. The inflammatory responses in these cells are coordinated by the subsequent production of more cytokines, chemokines, and reactive oxygen species. These molecules function in a synergistic and/or antagonistic manner, eventually leading to neurodegeneration via inflammatory cascade. Thus, it is important to understand the molecular mechanisms governing immune reactions in the brain.

SSeCKS is originally defined as a PKC substrate *in vitro* and *in vivo*. The coding sequence of SSeCKS contains four domains of overlapping PKC phosphorylation motifs. Each of these sites can bind PKC in a phosphatidylserine-dependent manner[11]. Recent studies confirmed the critical roles of PKC in regulating cell differentiation, proliferation, and transition to a "reactive" phenotype seen in CNS disease[4,5,12,13]. Myristoylated alanine-rich C kinase substrate (MARCKS), another widely studied PKC substrate, sharing biochemical and structural characteristics with SSeCKS, have been demonstrated to be closely linked to glial cells activation processes in both lipopolysaccharide (LPS), amyloid β-induced inflammation[11,14] and kainic acid-induced seizures[15]. Considering that SSeCKS binds PKC in a phosphatidylserine-dependent manner and SSeCKS is also a major PKC substrate *in vitro* and *in vivo*, it is suspected whether SSeCKS could serve as a PKC substrate in C6 cells. Figure 5 shows a PKC-dependent translocation of SSeCKS in C6 cells after TNF-α induction, suggesting that SSeCKS might function downstream of PKC to mediate changes in glia cells functions after TNF-α stimulation.

Expression of SSeCKS has been studied by others. The ability of its regulatory subuint to bind PKC, protein kinase A, calmodulin, and β_2-adrenergic receptors suggests its function to assemble a multiprotein signaling complex at the sites of its localization, and determines the appropriate cell response to stimuli[8,9]. Recent studies indicated a role of SSeCKS in modulating actin-based cytoskeleton and maintaining actin-based stellate MC morphology[10]. More recently, astrocytes expressing SSeCKS was proved to provide stabilizing signals for blood-brain barrier (BBB) integrity under physiological conditions[16]. Since actin based structures regulate cell morphology, motility, metastasis, and cell-to-cell interactions, the role of SSeCKS in astrocytes might be the rearrangement of the actin cytoskeleton, which is essential for the astrocytes activation process under pathological conditions.

Taken together, our results demonstrate that TNF-α induces SSeCKS production and a PKC dependent SSeCKS. Thus, SSeCKS is likely a transducer of PKC-mediated signals in C6 cells. This raises the possibility that specific targeting of such signaling pathways may represent an effective strategy to block the inflammatory cascade of TNF-α.

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Src 抑制的蛋白激酶 C 底物在细胞因子诱导的 C6 胶质瘤细胞中表达的改变

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摘要：目的 研究肿瘤坏死因子(TNF-α)对培养的C6胶质瘤细胞中Src抑制的蛋白激酶C底物(Src-suppressed C Kinase Substrate，SSeCKS)表达的影响。**方法** 根据TNF-α刺激时间与浓度的差异，将培养的C6胶质瘤细胞随机分为TNF-α时间刺激组与浓度刺激组，运用实时荧光定量PCR (Realtime PCR)、免疫印迹和免疫细胞化学法分析SSeCKS的表达变化和亚细胞定位。**结果** 细胞因子TNF-α可引起C6胶质瘤细胞中蛋白激酶C (Protein kinase C，PKC)底物的广泛磷酸化，并以时间及浓度依赖的方式上调PKC底物SSeCKS的表达。免疫细胞化学分析显示，正常情况下，SSeCKS散在分布于细胞质，浓集于细胞伸长的足突中。TNF-α刺激后，SSeCKS向核周迁移。这些改变可被PKC的抑制剂Ro-31-8220部分抑制。**结论** TNF-α可诱导C6胶质瘤细胞中PKC的活性，上调SSeCKS表达，这些改变与PKC的活性相关，提示SSeCKS可能参与胶质细胞中炎症信号的转导。

关键词：肿瘤坏死因子α；SSeCKS；C6 胶质瘤细胞；蛋白激酶C