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行政院國家科學委員會補助專題研究計畫成果報告

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Advanced Glycosylation End Products - Induce Nitric Oxide Synthase Expression in C6 Glioma Cells: Involvement of p38 MAP kinase Dependent Mechanisms

一、中文摘要：

在許多退行性神經病變及阿滋海默症中，誘導型一氧化氮合成酶(iNOS)的活性扮演著重要的角色。而過度糖化的最終產物(AGEs) 已被證實和在阿滋海默症的類澱粉斑形成有關。本研究主要在探討AGEs是否可以誘發一氧化氮合成的表現，及其蛋白表現調控的訊息傳遞路徑。我們發現在C6神經膠瘤細胞中AGEs可以增加nitrite的產生及iNOS的表現。在C6神經膠瘤細胞中，隨著AGEs劑量的增加或是反應時間的增加，Nitrite的產生都會隨之增加。AGEs誘導產生的一氧化氮，可以被actinomycin D、cycloheximide 以及一氧化氮合成酶抑制劑 l-NAME所抑制。預先以抗AGEs抗體(1:100)處理C6神經膠瘤細胞，也可將由AGEs刺激所產生的NO抑制下來，另一方面，預先以genestein或FPT-II抑制劑，或SB203580處理C6神經膠瘤細胞，發現對於AGEs誘導產生的Nitrite有抑制作用。AGEs可以活化P38 MAP Kinase，同時此反應可以被genestein(20μM)、FPT-II抑制劑(20μM)以及SB203580(10μM)所抑制。綜合以上所述，我們的實驗結果顯示，在C6神經膠瘤細胞中，AGEs誘發iNOS蛋白表現以及NO產生的訊息傳遞路徑，P38 MAP Kinase也包含在其中。

關鍵詞：AGEs, iNOS, C6 glioma cells, MAP kinase。

Abstract：

Induction of iNOS is important in amyloid plagues formation. Given accumulation of AGEs in the brain is linked to Alzheimer's disease, we investigated whether iNOS protein expression is induced by AGEs and whether Ras-MAPK pathway is involved in the AGEs-induced iNOS expression in C6 glioma cells. AGEs caused a dose- and time-dependent increase of nitrite accumulation in C6 glioma cells. The AGEs-stimulated NO production from C6 glioma cells was inhibited by actinomycin D, by cycloheximide and by the NO synthase inhibitor, l-NAME, suggesting the increase of AGEs-induced nitrite release is due to iNOS up-regulation. Consistently, treatment of C6 glioma cells with AGEs stimulated the inducible nitric oxide synthase (iNOS) protein expression. AGEs-stimulated NO production was inhibited by pretreatment of C6 glioma cells with anti-AGEs antibodies (1:100). Pretreatment with genestein (the protein tyrosine kinase inhbitor), or FPT II inhibitor (the Ras-farnesyl transferase inhibitor), or SB203580 (the p38 MAPK inhibitor), prior to the AGEs addition to C6 glioma cells results in partial suppression of AGEs-induced iNOS expression and nitrite release from C6 glioma cells. AGEs activate p38 MAP kinase in C6 glioma cells and the effect was blocked by genesteine (20μM), FPT-II inhibitor (20μM) and SB203580 (10μM). Taken together, our data suggest that p38 MAP kinase is involved in AGEs induced iNOS expression and NO production in C6 glioma cells.

二、緣由與目的：

Advanced glycosylation end products (AGEs) are fluorescent substances formed by nonenzymatic "Maillard reaction". Several lines of evidence indicate that AGEs contribute to the pathogeneses of diabetic complications and aging (Brownlee, 1991). Accumulation of AGEs in the brain is the biochemical basis of many neuropathological features of Alzheimer's disease including extensive protein cross linking (Smith et al., 1996; Munch et al., 1997a). Toxic action of advanced glycation end products has also been demonstrated in gingiva (Schimidt et al., 1996), in human penis (Seftel et al., 1997), and in murine macrophages (Rojas et al., 1996; Ramirez et al., 1997),

Nitric oxide is a diffusible gas that generated enzymatically from L-arginine and molecular oxygen by NO synthase. To date, at least three different types of nitric oxide synthase have been characterized. The endothelial type, eNOS, and the neuronal type, nNOS, are constitutively expressed whereas the inducible type, iNOS is induced by a variety of signals in many cell lines (Knowles and Moncada. 1994). Nitric oxide plays important roles in both physiological and pathological conditions. Over production of nitric oxide in the brain is the biochemical basis of many neuropathological features, oxidative stress (Li and Dickson, 1997) and neuronal cell death (Zimmerman et al., 1995; Horie et al., 1997). Microglial cell derived NO could contribute to oligodendrocyte degeneration and neuronal cell death. In Alzheimer's disease, neurons are subjected to the deleterious cytotoxic effects of activated microglia (Munch et al., 1997b).

In the present study, we demonstrated that AGEs may increase nitrite accumulation and iNOS expression. The mechanism of the signal transduction cascade involved in the induction of iNOS in response to AGEs was mediated through tyrosin kinase, Ras and p38MAPK in C6 glioma cells.

三、結果

Exposure of C6 glioma cells to AGEs stimulates nitrite production in dose and time dependent manners (Fig. 1). The EC_{50} of AGEs-stimulated nitrite production was about 30 μg/ml, with the maximum at about 1000 μg/ml of AGEs at 24 h. The nitrite accumulation was apparent at 6hrs and increase there after. The maximum response was at 24hrs. AGEs-stimulated NO release from C6 glioma cells can be blocked by AGEs-specific antibody (Fig. 2A), by pre-treatment of *l*-NAME (Fig. 2B), actinomycin D (Fig. 2C), and by cycloheximide (Fig 2D) suggesting the AGEs-specificity transcription and de novo protein synthesis are required for the AGEs-stimulated NO accumulation in C6 glioma cells. AGEs-induced NO production is accompanied by the increased expression of the 130-kDa iNOS expression but not the α-tubulin in C6 glioma cells.

To investigating whether the induction of iNOS is mediated through the Ras-MAPK signaling pathway, the protein tyrosine kinase inhbitor, genestein, the Ras-farnesyl trans-ferase inhibitor, FPT II inhibitor, or the p38 MAPK inhibitor, SB203580 were used to pretreated the cells and the AGEs-stimulated nitrite accumulation was investigated. Table I shows that genestein; FPT-II inhibitor and SB203580 all attenuated the AGEs-stimulated NO release. Thus, the protein tyrosine kinase-Ras-p38 MAPK signaling pathway seems to be involved in the AGEs-stimulated NO release.

The data in Tab.1 suggested that the p38 MAPK activated pathway might contribute to the signaling mechanism for AGEs-induced iNOS expression in C6 glioma cells. This notion was further supported by the facts that p38 MAPK activity was stimulated by BSA-AGEs in C6 glioma cells. As shown in Fig. 4A, addition of BSA-AGEs to C6 glioma cells stimulated p38 MAPK activity as determined by immuno-complex kinase assay using ATF-2 as substrate. The p38 MAPK was transiently activated within 15 minutes, maximum at sbout 1 hr and decrease these after. Western blot analysis using anti-p38 MAPK indicated that the total protein

expression of p38 MAPK was unaffected by BSA-AGEs induction (Fig. 4B). The activation of p38 MAPK by BSA-AGEs was inhibited by SB203580, genestein and FPT-II inhibitor (Fig. 5,6). These data confirm that activation of p38 MAPK plays a crucial role which promotes iNOS expression and nitrite accumulation in C6 glioma cells.

四、討論：

In the present study, we demonstrated that AGEs increase nitrite production and iNOS expression in C6 glioma cells. Given iNOS upregulation was observed in astrocytes surrounding amyloid plagues (Wallace et al., 1997) and peroxynitrite damage to neurons have been observed in AD's brain (Smith et al., 1997), iNOS induction may play an important role in the pathogeneses of Alzheimer's disease. Further, we presented evidence that the AGEs-stimulated iNOS expression was mediated through as protein-tyrosine kinase-Ras-p38 MAPK pathway. Understanding the signal transduction pathway that ultimately leads to a deleterious effect in the central nervous system is important from a therapeutic standpoint.

In rat pulmonary artery smooth muscle cells, activation of the receptor of AGEs triggers a p21 Ras-dependent mitogen-activated protein kinase pathway. Our results support the notion that AGEs-stimulated iNOS expression is tyrosine phosphorylation and Ras-dependent mitogen-activated protein kinase signaling pathway.

Three parallel protein phosphory-lation cascades in mammalian cells have been described. The extracellular signal regulated kinase (ERK) pathway,the p38 MAPK and SAPK/JNK pathway. Because MAP kinase kinase, ERK-1/ERK-2, p38 MAPK or SAPK/JNK pathways may be activated by the upstream protein tyrosine kinase and Ras, it would be interesting to determine the potential involvement of these kinases in the induction of iNOS by AGEs in C6 glioma cells. In this context, our results showed that AGEs-stimulated nitrite production was inhibited by SB203580; the specific inhibitor of p38 MAPK, suggesting p38 MAPK pathway was involved. In line with this observation, we demonstrated that AGEs-activates p38 MAPK activity and this effect were inhibited by genestein, FPT-II inhibitor and SB203580. The murine iNOS promoter contains 24 transcriptional factor binding sites, including those for NF-kB, AP-1, CREB, and the ets family of transcription factors. Some of these transcription factors are regulated by p38 MAPK. Possibly regulation of the transcription initiation by p38 MAPK mediates AGEs-stimulated iNOS expression.

In conclusion, AGEs-stimulate iNOS expression is mediated through activation of p38 MAP kinase pathway. Although the detail signaling mechanisms remain unclear, distinct signaling pathways appear to be involved. Further work is required to elucidate whether other pathways are involved in mediating the AGEs-mediated inflammation, which subsequently results in neuronal damage.

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Table 1. Effects of tyrosine kinase inhibitor, genestein, the Ras inhibitor, FPT II inhibitor and p38 MAPK inhibitor, SB203580 on AGEs-induced NO release from C6 glioma cells. C6 glioma cells were pretreated with genestein (20 μM), FPT II (20 μM), or SB203580 (10 μM) for 30 min. before the addition of 300 μg/ml BSA-AGEs and incubated for 24 h. The medium was removed and analyzed for nitrite accumulation. Data represents means ± S.E.M. of three independent experiments done in triplicate. *, $p<0.05$ as compare with those of AGEs alone.

	N.A.	+ genestein (20 μM)	+ FPT II inh. (20 μM)	+ SB203580 (10 μM)
Basal	7.10± 0.07	7.48± 0.08	7.74± 0.15	7.82± 0.45
+ AGEs (300 μg/ml)	12.50± 0.71	8.30± 0.16*	7.69± 0.19**	9.03± 0.32*

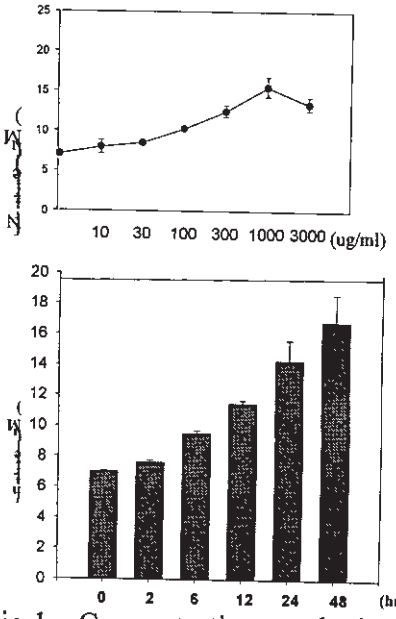
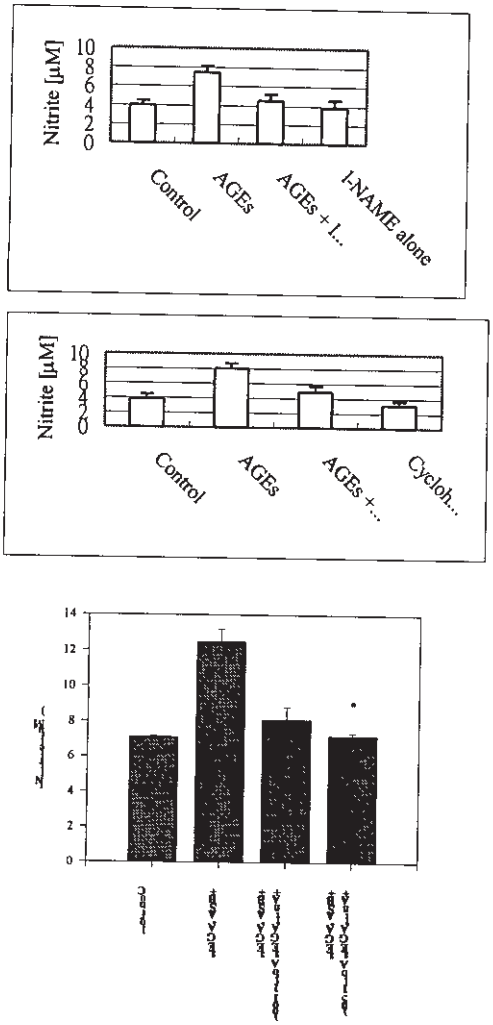


Fig.1. Concentration and time dependent increase of nitrite accumulation caused by BSA-AGEs in C6 glioma cells.



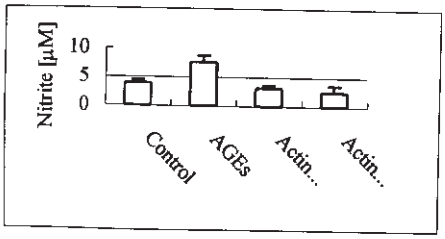


Fig. 2. AGEs stimulated nitrite production from C6 glioma cells can be inhibited by anti-AGEs antibody l-NAME, actinomycin, and cyclohexemide.

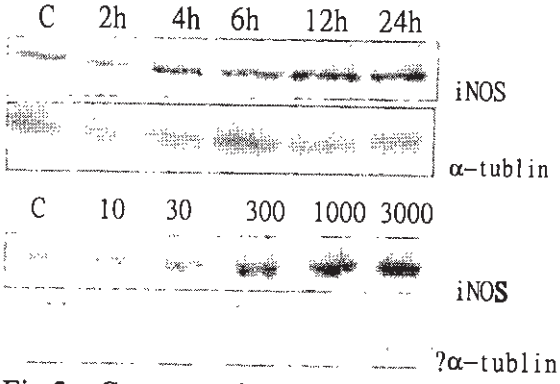


Fig.3. Concentration and time dependent increase of iNOS expression caused by BSA-AGEs in C6 glioma cells.

Fig.4. AGEs activate p38 MAP kinase in C6 glioma cells. C6 glioma cells were incubated with 300μg/ml BSA-AGEs for various time periods. Cells were lysed and determined by immuno-complex kinase assay with ATF-2 as substrate.

Fig. 5. AGEs stimulated p38 MAP kinase activity was inhibited by SB203580 in C6 glioma cells.

Fig. 6 AGEs stimulated p38 MAP kinase activity was inhibited by genestein, and FPT II inhibitor in C6 glioma cells.