研究报告

Dauricine Prevents Bradykinin-induced Alteration of Calcium Homeostasis and tau Hyperphosphorylation in N2a Cells*

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Abstract To study the prevention of dauricine (Dau) on bradykinin (BK) induced alteration of intracellular calcium homeostasis and tau phosphorylation, fluorescence spectrophotometer with dual excitation was utilized to measure the intracellular calcium concentration ($[Ca^{2+}]_i$), MTT to detect cell viability and immuncytochemistry to examine tau phosphorylation. The results showed (1) cells treated with BK 1 μmol/L induced a transit increase in $[Ca^{2+}]_i$ in all the cell lines detected, among them, the sustained increase of $[Ca^{2+}]_i$ level was only seen in PS1Δ9/APPswe cell at 2 h and 24 h after the treatment. Dau (3 μmol/L or 6 μmol/L) prevented BK-induced transit and sustained elevation and fluctuation of $[Ca^{2+}]_i$; (2) BK treatment decreased the cell metabolism detected at 2 h in PS1Δ9/APPswe and Dau antagonized the effect; (3) BK induces Alzheimer-like tau hyperphosphorylation at tau-1 epitope and Dau partially antagonized this effect. In conclusion, Dau inhibits BK-induced disturbance in intracellular calcium homeostasis and tau hyperphosphorylation at tau-1 sites.
Key words dauricine, bradykinin, intracellular calcium, tau protein, phosphorylation, neuroblastoma (N2a) cells

Alzheimer's disease (AD) is characterized by the formation of senile plaques and neurofibrillary tangles in vulnerable brain regions[1,2]. The principle structural components of neurofibrillary lesions are paired helical filaments (PHF), which are made up of microtubule-associated protein tau in a hyperphosphorylation state [3,4]. Hyperphosphorylation of tau has been shown to dissociate tau from microtubules leading to disruption of the neuronal cytoskeleton and interference with cellular transport mechanisms [5,6]. Tau phosphorylation plays a significant role in apoptosis enhancing disruption of microtubules that in turn leads to formation of apoptotic bodies[7].

Calcium channels have been suggested to play a role in mediating the function of tau protein and Aβ in neurons[8,9]. Previous reports have demonstrated that, *in vitro*, acute elevation in the concentration of free cytoplasmic calcium leads to phosphorylation of the microtubule-associated tau protein[10]. Bradykinin (BK) stimulated formation of Ins (1,4,5)P_3 mediated release of Ca^{2+} from endoplasmic reticulum and led to an acute increase in cytosolic free calcium concentration[11,12].

Dauricine (Dau) was isolated from rhizome of memispermum dauricum DC. It is a Ca^{2+} channel blocker. It has been demonstrated that Dau can inhibit L-type calcium current in guinea pig ventricular myocytes and calcium influx in bovine anterior cerebral arterial smooth muscle cells[13].

The purpose of the present study is to determine whether BK alters the level of cytosolic free calcium and induces Alzheimer-like tau hyperphosphorylation at tau-1 epitope, and the possible protective function of Dau on BK's effect.

1 Materials and methods

1.1 Chemicals and drugs

Dulbecco's modified Eagle's (DEM) medium, OPTI-MEM and fetal bovine serum from GIBCO (Grand Island, NY, USA). Bradykinin (BK) and G418 were from Sigma (St. Louis, MO, USA). Fura-2/AM was from Molecular Probes (Eugene, OR, USA). [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] (MTT) was from Boehringer Mannheim (Germany). Dauricin [2-hydroxy-5,4′-bis(2-methyl-6,7-dimethoxy-1,2,3,4-tetra-hydroisoguinolin-1-ylmethyl)

*This work was supported by a grant from The National Natural Science Foundation of China (30430270, 30328007).
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Received: January 13, 2005 Accepted: February 28, 2005

diphenyl ether] [Dau] was supplied by Institute of Clinical Pharmacology, Tongji Medical College, Huazhong University of Science and Technology [13]. Antibodies tau-1 and R134d were kind gifts from Dr. Binder (Northwestern University, Chicago, Illinois, USA) and Dr. Iqbal and Grundke-Iqbal (New York State Institute for basic research, Staten Island, NY, USA).

1.2 Cell cultures

Murine N2a cells wild type (N2awt), or transfected with human wild type APP695 (APPwt), or with APP695 bearing Swedish double mutation (APPswe), or with APPswe and wild type PS1 (PS1wt/APPswe), or with APPswe and Δ9 deleted PS1 (PS1Δ9/APPswe) were gifts from Dr. Xu (Burnham Institute, SD, USA). Both APPswe and PS1Δ9/APPswe contain mutated genes seen in patients with familiar Alzheimer disease, and PS1Δ9/APPswe contain mutation in both APP and PS-1. The cells were maintained in medium containing 50% DEME and 50% OPTI-MEM, supplemented with 5% fetal bovine serum (FBS), 200 mg/L G418 in the absence or presence of BK or BK plus Dau. Cells were subcultured weekly, and the medium was replaced every 2 days.

1.3 Measurement of intracellular calcium concentration

Cells were plated at a density of 2×10^4 per well, and grown in 100 μl medium on 96-well plates in a humidified CO_2 incubator. After incubated for 24 h, vehicle, BK 1 μmol/L or BK plus Dau 3 μmol/L were added and intracellular calcium concentration ($[Ca^{2+}]_i$) was determined immediately or after 2 h of further incubation. Briefly, the cells were loaded with 5 μmol/L Fura-2/AM dissolved in culture medium at 37℃ for 40 min, then transferred to the Ca^{2+}-free solution (NaCl 8.182 g/L, KCl 0.335 g/L, $MgCl_2$ 0.200 g/L, glucose 2.000 g/L, HEPES 5.000 g/L, pH 7.2) and intracellular $[Ca^{2+}]_i$ was monitored by alternating excitation wavelengths between 340 nm and 380 nm and emission at 500 nm with F-2000 Fluorescence Spectrophotometer (Instrument Division, Hitachi，underlying Ltd. Tokyo, Japan). The calculation of $[Ca^{2+}]_i$ was carried out by using the formula of $[Ca^{2+}]_i=K_d\cdot(F_D/F_S)\cdot(R-R_{min})/(R_{max}-R)$, in which K_d equals to 224 nmol/L at 37℃, R_{max} is the maximum ratio of fluoresce produced by saturated Fura-2 in 1% Triton X-100, whereas R_{min} is minimum value of free Fura-2 produced in 4 mmol/L EGTA. F_D and F_S represent the fluoresce intensity measured at 380 nm in the absence of Ca^{2+}.

1.4 Measurement of cell viability by MTT

Cells were plated, cultured and treated as above. After addition of vehicle, or the same concentration of BK or BK plus Dau as above for various time periods, MTT with a final concentration of 0.5 g/L was added to each well and incubated for 4 h. Then, 100 μl of DMSO were added to solubilized formazan formed during the reaction. Absorbance of the samples was measured at 570 nm.

1.5 Determination of tau expression and phosphorylation by immunocytochemistry

Cells were seeded onto glass coverslips and grown for 24 h before addition of the drugs. After treatment with vehicle, BK or BK plus Dau for 2 h, the cells were washed with no drug medium and fixed for 10 min in 4% paraformaldehyde in phosphate-buffered saline (PBS) prewarmed at 37℃ for 10 min. Fixed cells were washed 3 times in PBS containing 0.1% Triton X-100 and incubated with 10% goat serum and 3% BSA, incubated with 2% Tween-20 in PBS for 1 h at room temperature to block nonspecific binding of antibodies. The primary antibody was added and incubated at 4℃ overnight. Then washed as above and added to the 2nd and 3rd antibodies and developed with Diaminobenizidine (DAB) system (SP™ kit, ZEMED, South San Francisco, CA, USA). The developed coverslips were mounted with glycerol and visualized under microscope.

1.6 Data analysis

Data were expressed as $\bar{x}\pm s$ and statistical significance was determined by student t-test.

2 Results

2.1 Effect of BK and Dau on $[Ca^{2+}]_i$ homeostasis

Treatment of N2awt, APPwt, APPswe, PS1wt/APPswe and PS1Δ9/APPswe cells with 1 μmol/L BK [14] caused similar immediate elevations of $[Ca^{2+}]_i$ from basal level and the peak values were

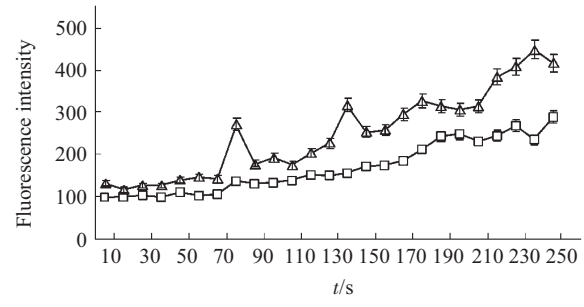

Fig.1 Representative curve showing the effect BK and Dau on $[Ca^{2+}]_i$ level in PS1Δ9/APPswe cells

Similar results were observed with all other cell lines. △—△ :BK; □—□ : Dau+BK.

reached in seconds among various cell lines (Table 1). The data from continuous observations demonstrated that the $[Ca^{2+}]_i$ levels in BK treated samples were fluctuated by multiple brief, lower-amplitude spikes or oscillations that last at least for 3 min, the time pursued in this study (Figure 1). Pretreatment of the cells with 3 μmol/L of Dau for 12 h diminished BK-induced immediate rise in $[Ca^{2+}]_i$ levels (Table 1) as well as sustained elevations in $[Ca^{2+}]_i$ fluctuation in all 5 cell lines (Figure 1). When the concentration of Dau was increased to 6 μmol/L, similar results were obtained (not shown). No obvious effect was seen by using 0.3 μmol/L of Dau (not shown). The data suggested that Dau could arrest BK-induced transit elevation and sustained fluctuation of $[Ca^{2+}]_i$ and there is no obvious difference in the content of $[Ca^{2+}]_i$ alteration within 3 min among various cell lines. When we measured the $[Ca^{2+}]_i$ level at 2 h (Table 2) or 24 h (not shown) after BK 1 μmol/L treatment, we found that a significant increase was only seen in PS1Δ9/APPswe but not in the other cell lines, and pretreatment of cells with Dau also showed significant protective effect. We also noticed that the basal level of $[Ca^{2+}]_i$ was lower in PS1Δ9/APPswe than that measured in other cell lines, the mechanism for this decrease is currently not understood (Table 2).

Table 1 The first peak of $[Ca^{2+}]_i$ level measured immediately after the treatment with BK or Dau plus BK

Treatment	N2awt	APPwt	APPswe	PS1wt/APPswe	PS1Δ9/APPswe
Cont	109.5±15.2	115.0±35.8	127.8±26.0	120.0±20.3	81.9±23.6
BK[1)]	204.3±47.9	211.0±73.3	254.5±29.2	276.3±75.4	222.8±34.7
Dau+BK[2)]	125.7±45.1	123.3±47.8	136.7±52.2	125.3±33.6	137.8±59.2

[1)] $P < 0.01$ compared with control, [2)] $P < 0.01$ compared with BK treated cell. ($\bar{x}\pm s$, n=12)

Table 2 The level of $[Ca^{2+}]_i$ measured at 2 h after the treatments

Treatment	N2awt	APPwt	APPswe	PS1wt/APPswe	PS1Δ9/APPswe
Cont	95.9±22.2	99.3±23.5	109.3±33.0	112.0±24.0	70.6±16.5
BK	98.1±32.0	89.2±19.3	98.0±36.2	117.8±37.2	128.8±38.9[1)]
Dau+BK	104.4±20.2	92.6±29.7	101.1±56.0	113.3±59.2	105.6±54.9[2)]

[1)] $P < 0.01$ compared with control, [2)] $P < 0.01$ compared with BK treated cell. ($\bar{x}\pm s$, n=12)

2.2 Effect of BK and Dau on cell viability

To detect any possible cytotoxicities caused by this different BK-induced increase of $[Ca^{2+}]_i$ in PS1Δ9/APPswe cells, we measured the number of viable PS1Δ9/APPswe cells at different time points after BK treatment by MTT conversion assay, a measurement of functional mitochondria succinate dehydrogenase that correlates with the number of surviving cell. It was shown that the number of viable cell was markedly reduced after exposure of the cells to BK for 2 h and Dau protected the cells from BK-induced decrease in cell viability (Figure 2). However, there was no significant change of cell viability in N2awt cells (data was not shown).

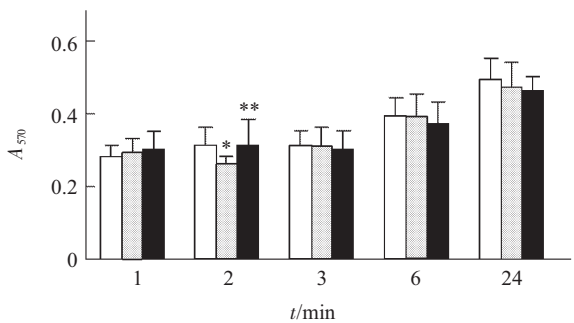

Fig.2 MTT assay of PS1Δ9/APPswe cell treated with BK or Dau plus BK

*: $P < 0.05$ compared with normal group, **: P<0.05 compared with BK group. □: Cont; ▨: BK; ■: Dau+BK.

2.3 Effect of BK and Dau on tau expression and phosphorylation

Further study was carried out to investigate the possible influence of BK and Dau on tau expression and phosphorylation and the difference between N2awt and PS1Δ9/APPswe cells. A panel of antibodies, including R134d, a polyclonal antibody reacting with total tau, tau-1, PHF-1 and 12e8, monoclonal antibodies recognize respectively tau not phosphorylated at Ser199/202 (tau-1), tau phosphorylated at Ser396/404 (PHF-1) or Ser262 (12e8) were used in the present study. It was found that total tau was evenly distributed in cell body and cell processes in N2awt and the staining of tau was significantly enhanced and tended to accumulate to the cell bodies after BK treatment. For PS1Δ9/APPswe cell, total tau was mainly detected in cell body but not in the process. An obviously lighter staining in PS1Δ9/APPswe than N2awt was seen before treatment with BK, and incubation of the cell with BK for 2 h remarkably increased the staining of total tau in the cell body. After pretreatment of the cell with Dau, a decreased total tau staining and a remarkable improvement in cell morphology were achieved both in N2awt and PS1Δ9/APPswe cells. With tau-1, the staining in the two cell lines was only seen in cell surface but not in the process, and lighter staining was induced by BK treatment, treatment with Dau restored slightly the tau-1 stinging (Figure 3). Further study in determining the phosphorylation sites with PHF-1 and 12e8 was not successful because of an unstable staining with these antibodies.

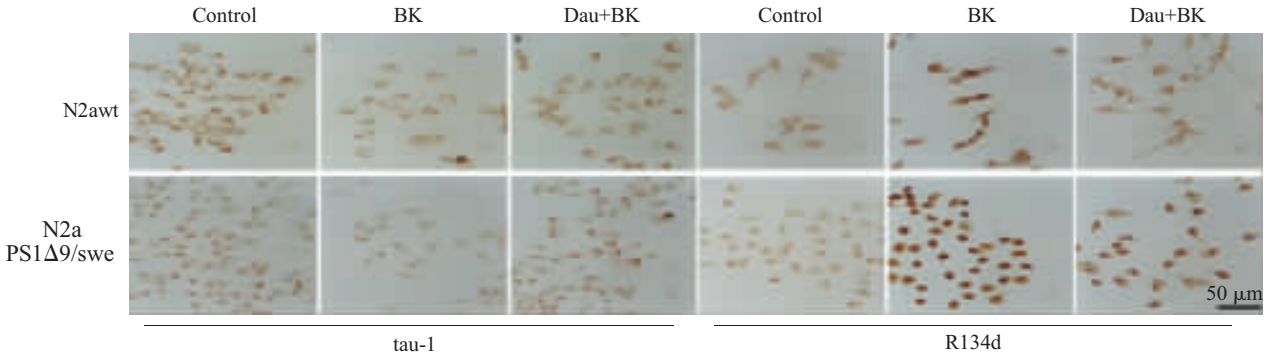

Fig.3 Effect of BK and Dau plus BK on tau expression and phosphorylation

3 Dissusion

Alzheimer disease (AD) is one of the most common cause of dementia [15], affecting severely the life quality in old populations. Numerous studies have demonstrated that tau hyperphosphorylation and amyloid β overproduction are the major pathological processes in the development of the disease. However, the upstream factor (s) leading to tau hyperphosphorylation is not understood, and there is still no effective cure for the disease. In the present study, we have found that BK, an activator of calcium/calmodulin-dependent protein kinase Ⅱ (CaMK Ⅱ) [14], increases intracellular calcium and Alzheimer-like tau hyperphosphorylation as well as cell vulnerability. Dauricine, a calcium blocker [13], attenuates BK-induced lesions. Therefore, we propose that an imbalanced calcium homeostasis might be at least one of the upstream stimulator for Alzheimer-like tau hyperphosphorylation, and dauricine is one of the candidates in inhibiting the pathological processes. The involvement of calcium homeostasis in tau hyperphosphorylation and amyloid β precipitation [16] was also studied previously, however, this is the first report about the prevention of this imbalance by dauricine.

Tau can be phosphorylated by various kinases [17]. Studies have shown that BK can stimulate the formation of Ins (1,4,5)P3 through the membrane-bound BK receptor. The increased Ins (1,4,5)P3 induces Ca^{2+} release from endoplasmic reticulum and leads to a greatly transient increase of intracellular $[Ca^{2+}]_i$ which can activate cyclin-dependent protein kinase-5 (cdk-5) in addition to CaMK Ⅱ [18]. The activation of protein kinases will then lead to hyperphosphorylation of tau protein. In the present study, we observed that treatment of the cells with BK decreased tau-1 (recognizing Ser198/ser199/Ser202 unphosphorylated tau) staining in N2awt and N2aPS1Δ9/APPswe cell lines even when the

immunoreaction to the total tau (R134d) was significantly enhanced at the same condition (Figure 3), representing hyperphosphorylation of tau at tau-1 sites.

BK only induces a transit elevation of calcium in N2awt and no significant change in cell viability, but in N2aPS1Δ9/APPswe, a sustained (determined at 2 h and 24 h after BK treatment) high level of calcium with a concurrent decreased cell viability was observed at 2 h. However, no obvious alteration in cell viability was seen at 24 h after BK treatment, when the calcium level was still much higher than that of control (data not shown), suggesting that in addition to the increased level of $[Ca^{2+}]_i$, other factors, maybe the increased production of β-amyloid [19] would also contribute to the cell death observed in this study. Obvious accumulation of total tau (R134d) into the cell body in N2aPS1Δ9/APPswe even without any treatment but not N2awt was another evidence for such a speculation. These data together also indicate that the cells bearing multiple Alzheimer-like mutations are more vulnerable to the pathological attack. Further study is needed to explore the underlying mechanism.

Dauricine is a calcium blocker, which has been widely used in cardiovascular system. In this study, we have found that pretreatment of the cells with dauricine arrests BK-induced imbalance in calcium homeostasis as well as Alzheimer-like tau hyperphosphorylation and cell death in N2aPS1Δ9/APPswe. These results indicate the potential therapeutic value of dauricine in AD.

References

1 Avila J, Lim F, Moreno F, *et al*. Tau function and dysfunction in neurons: its role in neurodegenerative disorders. Mol Neurobiol, 2002, **25** (3): 213~231

2 Guillozet A L, Weintraub S, Mash D C, *et al*. Neurofibrillary tangles, amyloid, and memory in aging and mild cognitive impairment. Arch Neurol, 2003, **60** (5): 729~736

3 Iqbal K, Alonso Adel C, El-Akkad E, *et al*. Significance and mechanism of Alzheimer neurofibrillary degeneration and therapeutic targets to inhibit this lesion. J Mol Neurosci, 2002, **19** (1-2): 95~99

4 Delacourte A. Biochemical and molecular characterization of neurofibrillary degeneration in frontotemporal dementias. Dement Geriatr Cogn Disord, 1999, **10** (Suppl 1): 75~79

5 Terwel D, Dewachter L, Van Leuven F. Axonal transport, tau protein, and neurodegeneration in Alzheimer's disease. Neuromolecular Med, 2002, **2** (2): 151~165

6 Gong C X, Lidsky T, Wegiel J, *et al*. Phosphorylation of microtubule-associated protein Tau is regulated by protein phosphatase 2A in mammalian brain. J Biol Chem, 2000, **275** (8): 5535~5544

7 Guise S, Braguer D, Caries G, *et al*. Hyperphosphorylation of tau is mediated by ERK activation during anticancer drug-induced apoptosis in neroblastoma cells. J Neurosci Res, 2001, **63** (3): 257~267

8 Furukawa K, Wang Y, Yao P J, *et al*. Alteration in calcium channel properties is responsible for the neurotoxic action of a familial frontotemporal dementia tau mutation. J Neurochem, 2003, **87** (2): 427~436

9 Sennvik K, Benedikz E, Fastbom J, *et al*. Calcium ionophore A23187 specifically decreases the secretion of beta-secretase cleaved amyloid precursor protein duringapoptosis in primary rat cortical culture. J Neurosci Res, 2001, **63** (5): 429~637

10 Mattson M P. Oxidative stress, perturbed calcium homeostasis, and immune dysfunction in Alzheimer's disease. J Neurovirol, 2002, **8** (6): 539~550

11 El-Bizri N, Bkaily G, Wang S, *et al*. Bradykinin induced a positive chronotropic effect via stimulation of T- and L-type calcium currents in heart cells. Can J Physiol Pharmacol, 2003, **81** (3): 247~258

12 Linhart O, Obreja O, Kress M. The inflammatory mediators serotonin, prostaglandin E2 and bradykinin evoke calcium influx in rat sensory neurons. Neuroscience, 2003, **118** (1): 69~74

13 Guo D L, Zhou Z N, Zeng F D, *et al*. Dauricine inhibited L-type calcium current in single cardiomyocyte of ginea pig. Acta Pharmacol Sin, 1997, **18** (5): 419~421

14 Wang Q B, Wang J Z. Injection of bradykinin or cyclosporine A to hippocampus induces Alzheimer-like phosphorylation of tau and abnormal behavior in rats. Chinese Med J, 2002, **115** (6): 884~887

15 Kumari S, Ram V J. Future trends in the treatment of cognitive disorders. Drugs Today (Barc), 2001, **37** (10): 675~689

16 O'Neill C, Cowburn R F, Bonkale W L, *et al*. Dysfunctional intracellular calcium homoeostasis: a central cause of neurodegeneration in Alzheimer's disease. Biochem Soc Symp, 2001, (67):177~194

17 Pei J J, Gong C X, An W L, *et al*. Okadaic-acid-induced inhibition of protein phosphatase 2A produces activation of mitogen-activated protein kinases ERK1/2, MEK1/2, and p70 S6, similar to that in Alzheimer's disease. Am J Pathol, 2003, **163** (3): 845~858

18 Town T, Zolton J, Shaffner R, *et al*. p35/Cdk5 pathway mediates soluble amyloid-beta peptide-induced tau phosphorylation *in vitro*. J Neurosci Res, 2002, **69** (3): 362~372

19 Zhang Y C, Wang Z F, Wang Q, *et al*. Melatonin attenuates beta-amyloid-induced inhibition of neurofilament expression. Acta Pharmacol Sin, 2004, **25** (4): 447~451

蝙蝠葛碱拮抗缓激肽引起的钙稳态改变及细胞骨架蛋白的异常磷酸化 *

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摘要 为研究蝙蝠葛碱 (dauricine，Dau) 拮抗缓激肽 (bradykinin，BK) 诱导的 Alzheimer 样钙稳态失衡及细胞骨架蛋白异常磷酸化的作用，采用双波长荧光分光光度计测定细胞内钙离子浓度($[Ca^{2+}]_i$)，用 MTT 法检测细胞代谢水平，用免疫组织化学方法观察 tau 蛋白表达和磷酸化. 结果表明，Dau (3 μmol/L，6 μmol/L) 可抑制 BK 诱导的$[Ca^{2+}]_i$升高，保护 BK 引起的神经元代谢降低，拮抗 BK 引起的 tau 蛋白异常磷酸化和聚集. 结果提示：Dau 可拮抗 BK 诱导的 Alzheimer 样钙稳态失衡及细胞骨架蛋白异常磷酸化的作用.

关键词 蝙蝠葛碱，缓激肽，细胞内钙离子浓度，tau 蛋白，磷酸化，鼠成神经瘤细胞

学科分类号 R745.7，Q513

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*国家自然科学基金资助项目(30430270, 30328007).

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收稿日期: 2005-01-13, 接受日期: 2005-02-28