Low concentration *N*-methyl-*N*′-nitro-*N*-nitroguanidine activates p38MAPK signal transduction pathway

SUN Xue-Min，YU Ying-Nian
(*Department of Pathophysiology，Medical School of Zhejiang University，Hangzhou* 310031，*China*)

Abstract：An experimental model in which alkylating agent *N*-methyl-*N*′-nitro-*N*-nitroguanidine (MNNG) with low concentration was employed to induce genetic instability of a monkey kidney vero cell line，it was proved that there were changes in the patterns of protein tyrosine residue phosphorylation and the activation of stress activated kinase (JNK/SAPK). Now with the same experimental conditions，it is discovered that the phosphorylation degree increased in p38MAPK and its upstream kinase MKK3/MKK6 and upstream activator SEK1/MKK4 of JNK/SAPK，suggesting that both stress signaling pathways in mitogen activated protein kinase (MAPK) family be served by MNNG to activate cellular stress response and there may be cross talks between different pathways.

Key words：stress，cells；signal transduction；*N*-methyl-*N*′-nitro-*N*-nitroguanidine

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Researches on cellular stress response to DNA damage have achieved many evidences that there are activation of signal transduction pathways and alteration of gene expression induced by ultraviolet and chemical DNA damaging agents. Although it has been verified on ultraviolet and chemical DNA damaging agents with high concentration that there is signal transduction activated not only by damaged but also by undamaged DNA pathways[1 – 4]，there is less knowledge obtained on details of cellular response to low concentration of chemicals，it is necessary therefore to be explored.

Using the experimental stress model established in our laboratory that a DNA damaging agent with short half-life named *N*-methyl-*N*′-nitro-*N*-nitroguanidine (MNNG) was employed to induce genetic instability in vero cell line[5]，it has been proved that MNNG with low concentration ($0.2\ \mu mol \cdot L^{-1}$) can induce delayed point mutation in the normal sequence unrelated to the damaged nucleotide (non-targeted mutation) which depended on the alteration of gene expression and were enhanced by cycloheximide，the inhibitor of protein synthesis and activator of stress signal transduction pathway mediated by mitogen activated protein kinase (MAPK)[6 – 9]. Besides，in cells exposed to MNNG or MNNG plus cycloheximide，there were changes in the patterns of protein phosphorylation and protein tyrosine residue phosphorylation as well as the activation of stress activated protein kinase (JNK/SAPK)[10 – 12]. All of above demonstrated that chemical DNA damaging agent could activate cellular signaling pathways even at low concentration，though its relationship to non-targeted mutagenesis remained unclear. Among MAPK，both JNK/SAPK and p38MAPK play important roles in cellular stress signal transduction and there are always cross talks between different signaling pathways in cells. In present study the changes of phosphorylation ratio of p38MAPK and its upstream kinase MKK3/MKK6 and upstream activator of JNK/SAPK，the SEK1/MKK4，were observed with the same experimental system so that accumulating knowledge for clarifying the signal transduction pathways activated by low concen-

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Biographies：SUN Xue-Min (1961 –)，female，native of Shaoxing，Zhejiang Province，associate professor，doctor degree，main research field is genetic toxicology；YU Ying-Nian (1932 –)，male，native of Zhenhai，Zhejiang Province，professor，doctor advisor，main research field is molecular mechanism of mutagenesis and cloning and expression of drug metabolism enzymes.

tration of chemical DNA damaging agent and its relationship with nontargeted mutagenesis.

1 MATERIALS AND METHODS

1.1 Agents and cells

Africa green monkey kidney vero cells were provided by our laboratory. Dulbecco's modified eagle medium (DMEM) and calf seras were purchased from Gibco Chemical Co, MNNG and cycloheximide from Sigma. MNNG was dissolved in dimethyl-sulfoxide (DMSO) and cycloheximide in Hanks balanced salt solution (HBSS). The final concentrations in serum free DMEM were regulated to 0.2 $\mu mol \cdot L^{-1}$, 0.2% (V/V) and 1 $mg \cdot L^{-1}$ for MNNG, DMSO and cycloheximide, respectively. Acrylamide and N, N'-mehtylenebisacrylamide was obtained from Promega and the concentration of separating gel was 10% (W/V). The devices of electrophoresis and membrane transfer were the mini-protein series products from Bio-Rad. The Western blotting reagent kits for phospho-p38MAPK, phospho-MKK3/MKK6, phospho-SEK1/MKK4 and for p38MAPK, MKK3/MKK6 were purchased from New England Biolabs Co and anti-SEK1/MKK4 antibody from Santa Cruz.

1.2 Cultivation and treatment of cells

Vero cells were grown at 37℃ and 5% – 7% CO_2 in DMEM supplemented with 10% calf serum, 100 $kU \cdot L^{-1}$ penicillin, 100 $mg \cdot L^{-1}$ streptomycin, 200 $mg \cdot L^{-1}$ kanamycin and were divided to six groups at 70% – 80% confluence, four of them treated with MNNG (0.2 $\mu mol \cdot L^{-1}$) only for 0.5 h (M0.5), 1 h (M1), 2.5 h (M2.5) and 2.5 h followed by a 3 h interval [M2.5(3)], respectively; one with MNNG for 2.5 h followed by a 3 h cycloheximide treatment (M2.5 C3); and one with cycloheximide for 3 h only (C3). DMSO and HBSS were served as solvent control to MNNG and cycloheximide, respectively.

1.3 Sample preparation and SDS-polyacrylamide gel electrophoresis(PAGE)

After treatment, preparing cell lysates below 0 – 4℃: cells were washed with PBS, detached from culture dishes with versene, and harvested by centrifugation at 3000 × g, then incubated with cell lysis buffer [containing 25 $mmol \cdot L^{-1}$ HEPES, 0.3 $mol \cdot L^{-1}$ NaCl, 1.5 $mmol \cdot L^{-1}$ $MgCl_2$, 0.2 $mmol \cdot L^{-1}$ EDTA, 0.1 $mmol \cdot L^{-1}$ Na_3VO_4, 20 $mmol \cdot L^{-1}$ β-glycerophosphate, 1% Triton X-100 (V/V), 0.5 $mmol \cdot L^{-1}$ dithiothreitol(DTT), 2 $mg \cdot L^{-1}$ leupeptin and 100 $mg \cdot L^{-1}$ phenylmethylsulfonyl fluoride(PMSF)] for 45 min followed by centrifugation at 15 600 × g for 30 min. Supernatants were loaded on 10% acrylamide gels with 40 μg protein per well and per group. Two parallel gels were electrophoresed simultaneously for 90 min under 150 voltages.

1.4 Western blotting and data analysis

Protein bands in two parallel gels were transferred to two nitrocellulose (NC) membranes simultaneously for 90 min under 100 voltages. After then, all performances about the membranes including washing, primary antibody and horseradish peroxidase (HRP) conjugated antibody interactions, enhanced chemiluminescence (ECL) and exposing to films were carried out according the instruction manual provided by the manufacturer. Of two parallel membranes, one was reacted with anti-kinase and the other with anti-phosphokinase primary antibody. Bands emerged on films were scanned with Scanning Densitometer (Pharmacia) and were quantized with Kodak 1 D Analysis 2.0 software. As these enzymes are activated only when their several special amino acid residues are phosphorylated, we employed the P/N ratio as the parameter presenting activation degree of these enzymes, with the "P" served as absorbance values of phosphokinases and the "N" as absorbance values of nonphosphokinases. Both P and N are relative absorbances of treatment groups to controls. Assuming the absorbance of band of control as 1, the P/N ratio of the treatment groups between phospho-band and nonphospho-band of the same kinase on the two parallel films was calculated by comparing the relative absorbances of these two bands.

1.5 Statistical analysis

The P/N ratios in different groups of each ki-

nase were expressed as $\bar{x} \pm s$, and compared by Student's t test with two tails.

2 RESULTS

2.1 Enhancement effect of *N*-methyl-*N'*-nitro-*N*-nitroguanidine (MNNG) on P/N ratios in both p38MAPK and MKK3/MKK6

Fig 1 displays the Western blots of phospho- and nonphospho-p38MAPK and MKK3/MKK6 after treating vero cells with low concentration MNNG at different time point, and Tab 1 displays the P/N ratio values of phospho- to nonphospho-p38MAPK and that of phospho- to nonphospho-MKK3/MKK6. In cells treated with MNNG only, the P/N ratios in p38MAPK and MKK3/MKK6 at group M0.5, M1 and M2.5 were close to 1, among them there were no significant differences. While at group M2.5(3) the ratios went up markedly. There were significant differences between group M2.5(3) and other three groups. The results illustrated that both p38MAPK and its up stream kinase MKK3/MKK6 could be activated at 3 h after 2.5 h MNNG treatment. The activities were as high as 2.056 and 1.699 times than controls, respectively.

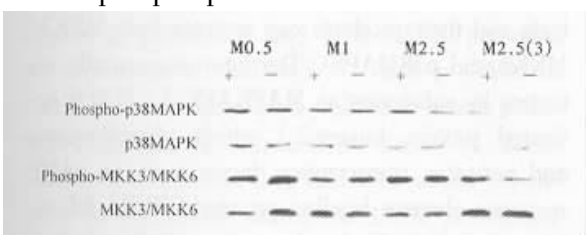

Fig 1. Western blots of phospho- and nonphospho-p38MAPK and MKK3/MKK6 after treatment of vero cells with *N*-methyl-*N'*-nitro-*N*-nitroguanidine (MNNG). + : blots of the kinases from vero cells treated with 0.2 μmol·L^{-1} MNNG; − : blots of the kinases from vero cells treated with 0.2% (*V*/*V*) DMSO; M0.5, M1 and M2.5 : extracts of vero cells prepared immediately after 0.5, 1.0 and 2.5 h of MNNG or DMSO treatment; M2.5(3) : extracts of vero cells prepared at 3 h after 2.5 h MNNG or DMSO treatment. The images shown here were typical of three independent experiments.

2.2 Increase of P/N ratio in SEK1/MKK4 in the group treated with MNNG

In cells treated with low concentration MNNG only, the P/N ratios in SEK1/MKK4 (the upstream kinase of JNK/SAPK) at group M0.5, M1 and M2.5 were close to 1, while in M2.5 (3), it went up to 1.373 (Tab 1). There were no differences among group M0.5, M1 and M2.5, while the P/N ratios in these three groups were different from that in group M2.5(3). The results suggested that low concentration MNNG not only increase activity of JNK/SAPK as reported previously, but also activate its upstream kinase SEK1/MKK4.

Tab 1. P/N ratios of phospho-p38MAPK, MKK3/MKK6, SEK1/MKK4 to nonphospho-p38MAPK, MKK3/MKK6, SEK1/MKK4

Group	P/N Ratio		
	p38MAPK	MKK3/MKK6	SEK1/MKK4
M0.5	$0.89 \pm 0.13^{**\#}$	$0.99 \pm 0.16^{**}$	$0.99 \pm 0.14^{*\#}$
M1	$1.05 \pm 0.16^{**\#}$	$1.00 \pm 0.16^{**}$	$1.08 \pm 0.09^{*}$
M2.5	$0.98 \pm 0.16^{**\#}$	$1.08 \pm 0.13^{**}$	$0.99 \pm 0.16^{*}$
M2.5(3)	2.06 ± 0.25	1.70 ± 0.18	1.37 ± 0.15
M2.5 C3	1.53 ± 0.21	$0.89 \pm 0.21^{**}$	1.43 ± 0.20
C3	$0.98 \pm 0.16^{**\#}$	$1.12 \pm 0.16^{*}$	1.20 ± 0.11

The means of group M0.5, M1.0, M2.5 and M2.5(3) were as the same as Fig 1; M2.5 C3 : extracts of vero cells prepared after 2.5 h MNNG treatment followed by 3 h 1.0 mg·L^{-1} cycloheximide treatment, DMSO and HBSS as solvent control, respectively; C3 : extracts of vero cells prepared immediately after 3 h cycloheximide treatment, HBSS as solvent control. $\bar{x} \pm s$, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with M2.5(3) group; $^{\#}P < 0.05$, compared with M2.5 C3 group.

2.3 P/N ratios of MAPKs in cells treated with both MNNG and cycloheximide

In cells treated with MNNG for 2.5 h followed by cycloheximide for 3 h, the P/N ratios in p38MAPK and SEK1/MKK4 were 1.533 and 1.427, respectively, closing to that in group M2.5 (3) which was treated with MNNG only. The P/N ratio in MKK3/MKK6 had not risen (Tab 1, M2.5C3). In cells treated only with cycloheximide, the P/N ratios in all three kinases were close to 1, suggesting that cycloheximide alone had neither effects on increasing nontargeted mutation frequency as previous report, nor ability to activate MAPKs.

3 DISCUSSION

The MAPK cascades are one of the most intensely studied groups of signal transduction pathways. These signaling pathways are present in all eukaryotes and have been implicated in many physiological and pathophysiological processes, including cell growth, differentiation, oncogenic transformation, immune responses, and apoptosis. Of MAPKs, JNK/SAPK and p38MAPK are regarded as the principal pathways mediating cellular stress signal transduction and share similar upstream activator (MAPK/MKK) with somewhat differences. MKK3/MKK6 is a well-known upstream kinase of p38MAPK while SEK1/MKK4 is a major activator of JNK/SAPK but minor of p38MAPK[13].

MNNG, a chemical stressor to mammalian cells, can activate JNK/SAPK in human 293 cells with concentration as high as 70 $\mu mol \cdot L^{-1}$[4]. In vero cells MNNG with concentration of 20 $\mu mol \cdot L^{-1}$ was enough to kill over 80% of cells while with concentration low as 0.2 $\mu mol \cdot L^{-1}$ would induce highest nontargeted mutation frequency without remarkable mortality[5]. There may be differences in changes of cellular signaling pathways induced by stressors with different degree. It is the fact that cellular response induced by chemical DNA damaging agent with low concentration is more similar to that by environmental chemicals, and is therefore more practically significant. It had been reported from our laboratory previously that the cellular stress model established in this laboratory could increase phosphorylation degree of JNK/SAPK and its kinase activity[12]. With same conditions, the phosphorylation ratio of its upstream activator SEK1/MKK4 also rose in present study, confirming further that there is activation of JNK/SAPK pathways during cellular stress response induced by low concentration MNNG with cooperation of cycloheximide.

Ultraviolet, osmotic shock, cytokines, bacteria and their products can activate both MKK3/MKK6 and p38MAPK. The later sequentially activates its substrates as MAPKAPK-2 (MAPK activated protein kinase-2) which phosphorylates and activates transcription factor such as cAMP response element binding protein (CREB) following expression of related genes mediating desired response to extracellular stimulation[13]. According to reports recently, in neuroblastoma cells, the opioid receptors and opioid receptor-like receptor-1 (ORL1) mediate activation of p38MAPK cascade through cAMP dependent protein kinase (PKA) pathways[14], and in osteoblasts, basic fibroblast growth factor (bFGF) stimulates synthesis of interleukin-6 (IL-6) by PKC activating p38MAPK cascades[15], there are other activators of p38MAPK in addition to MKK3/MKK6. In present study, treatment of cells with low concentration MNNG (0.2 $\mu mol \cdot L^{-1}$) can increase the phosphorylation of p38MAPK and its upstream kinase MKK3/MKK6, suggesting that MNNG activate p38 MAPK stress signaling pathways. In cells treated with MNNG plus cycloheximide, the phosphorylation degree of p38MAPK and SEK1/MKK4 rises while MKK3/MKK6 exhibits no alteration, suggesting that in this case p38MAPK activation be upon pathways other than MKK3/MKK6, for example, SEK1/MKK4. Other experiments from our laboratory proved that under same stress, there were increases in cAMP concentration, PKA activity and CREB phosphorylation (unpublished data from WANG *et al*). All of the results demonstrated that low concentration MNNG may

activate p38MAPK by several signaling pathways including MKK3/MKK6, SEK1/MKK4 and PKA, and thereby mediate cellular stress response. There may be cross talks among different pathways.

Recently, GADD45 was suggested to be involved in p38MAPK and JNK/SAPK activation by interacting with their upstream kinase MTK1 (MEKK4)[16]. However, this suggestion was relied on *in vitro* experiments and ectopic overexpression of GADD45 protein. In fact, in cells exposed to stress and DNA damaging agents, the activation of p38MAPK and JNK/SAPK preceded the expression of GADD45[17], and in GADD45 knockout mouse fibroblasts, p38MAPK and JNK/SAPK can also be activated by stress[18]. These come to a conclusion that GADD45 would not involve in p38MAPK and JNK/SAPK activation, and the pathways by which DNA damage inducing activation of both two kinases have not been identified even expression of GADD45 was certainly induced by DNA damage[19]. While as a well-known DNA damaging agent, ultraviolet light has been proved independent on nuclear signals when inducing cellular stress response through p38MAPK and /or JNK/SAPK pathways[3]. It is suggested that the activation of these two groups of MAPKs in MNNG exposed cells also be triggered by its interaction with cellular macromolecules other than its DNA damaging activity.

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低浓度甲基硝基亚硝胍激活 p38MAPK 信号转导通路

孙雪敏，余应年
（浙江大学医学院病理生理学教研室，浙江 杭州 310031）

摘要 在低浓度甲基硝基亚硝胍（MNNG）诱发猴肾 vero 细胞遗传不稳定的实验模型中，曾经证明受试细胞中酪氨酸磷酸化蛋白谱的改变和 JNK/SAPK 信号通路的激活. 同样条件下，现又发现 p38MAPK 及其上游激酶 MKK3/MKK6，以及 JNK/SAPK 的上游激酶 SEK1/MKK4 的磷酸化程度增高，提示低浓度 MNNG 可通过激活 MAPK 家族的两条应激信号转导通路诱导细胞的应激反应，且不同通路之间可能存在交互作用.

关键词 应激，细胞；信号转导；甲基硝基亚硝胍

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