

Curcumin prevents lipopolysaccharide-induced matrix metalloproteinase-2 activity via the Ras/MEK1/2 signaling pathway in rat vascular smooth muscle cells

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Abstract. The aim of the present study was to examine the effect of curcumin treatment on lipopolysaccharide (LPS)-induced matrix metalloproteinase-2 (MMP-2) activity, and assess whether the effects are mediated by the Ras/mitogen-activated protein kinase kinase 1/2 (MEK1/2) signaling pathway in vascular smooth muscle cells (VSMCs). VSMCs were isolated from male Sprague-Dawley rats. Protein expression levels were analyzed by western blotting. The activity of MMP-2 was measured with gelatin zymography, and an electrophoretic mobility shift assay was used to detect the DNA binding activity of nuclear factor- κ B (NF- κ B). Curcumin treatment was demonstrated to inhibit LPS-induced MMP-2 activity in rat VSMCs. This inhibitory effect was partially blocked by ammonium pyrrolidinedithiocarbamate, an inhibitor of NF- κ B activation, and farnesylthiosalicylic acid, an inhibitor of Ras. In addition, the results of the present study indicated that LPS-induced phosphorylation of Ras homolog family member A and MEK1/2 was significantly decreased by curcumin. Furthermore, NF- κ B p65 expression in the nucleus and the DNA binding activity of NF- κ B in rat LPS-exposed VSMCs was decreased by curcumin. Taken together, these findings suggest that curcumin prevents of LPS-induced MMP-2 activity through Ras/MEK1/2 and NF- κ B signaling.

Introduction

Vascular remodeling caused by excessive accumulation of lipids in arteries is an important characteristic of the development of atherosclerotic plaques (1). Reconstruction of the vascular wall structure is determined by matrix metalloproteinases

(MMPs), and the majority of the extracellular matrix (ECM) is degraded by MMPs (2). According to a previous study, MMP-2 is involved in normal and pathological alterations in blood vessels (3). This previous study demonstrated that the expression of MMP-2 in vascular smooth muscle cells (VSMCs) was closely associated with certain pathological conditions, in particular atherosclerotic plaques. In vulnerable plaques the expression levels and activation of MMP-2 was significantly increased, indicating a pathogenic involvement of MMP-2 in the progression of atherosclerosis (4-6).

Curcumin, one of the active ingredients obtained from the spice turmeric, has multiple biological activities including anti-inflammatory, antioxidant and anticancer effects (7,8). Previous studies have demonstrated that curcumin inhibits the expression and activity of MMP-2 under various experimental conditions and in different cell types. (9,10). However, the potential molecular mechanisms underlying this remain to be elucidated.

In the present study, the effect of curcumin on lipopolysaccharide (LPS)-induced MMP-2 activity was investigated, as well as the associated underlying mechanisms. The results demonstrated that curcumin prevents LPS-induced MMP-2 activity through the Ras/mitogen-activated protein kinase kinase 1/2 (MEK1/2) and nuclear factor- κ B (NF- κ B) signaling pathways in VSMCs.

Materials and methods

Cell culture and treatment. Rat aortic VSMCs were isolated from male Sprague-Dawley rats, as described previously (11). Rats (age, 50 days; weight, 150-180 g; n=20) were purchased from the Laboratory Animal Center of Southern Medical University (Guangzhou, China) and were kept in the dark at temperature of 20-25°C with free access to food and water. The study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. This study was approved by the Ethics Committee of Southwest Medical University (Luzhou, China). Rats were sacrificed in order to isolate the VSMCs by injection with a fatal dose of 4% pentobarbital (Beyotime Institute of Biotechnology, Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo

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Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere.

Cells were divided into four treatment groups as follows: Control, LPS-exposed, LPS + 20 μ M curcumin and LPS + 40 μ M curcumin. Cells were stimulated with curcumin (20 or 40 μ M) in serum-free DMEM at 37°C for 1 h, and exposed to 1 μ g/ml LPS for an additional 3 h at 37°C. The control cells were subjected to the same treatment, but PBS was used instead of curcumin and LPS. For subsequent experiments, cells were pretreated with 10 μ M ammonium pyrrolidine-dithiocarbamate (PDTC) or 20 μ M farnesylthiosalicylic acid (FTS) for 1 h at 37°C, followed by exposure to 1 μ g/ml LPS for 3 h. The control cells were subjected to the same treatment, but PBS was used instead of PDTC and FTS. In subsequent experiments, cells were pretreated with 20 or 40 μ M curcumin for 1 h, and exposed to 1 μ g/ml LPS for an additional 3 h.

MMP-2 activity assay. The enzymatic activity of MMP-2 was determined by gelatin zymography (12). Proteins were extracted from cells using radioimmunoprecipitation lysis buffer (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 40 min on ice and centrifuged at 15,000 \times g for 15 min at 4°C, following which 50 mg protein was separated by 11% SDS-PAGE at 4°C. Gels were incubated with collagenase buffer and stained with Coomassie brilliant blue, followed by destaining with 30% isopropanol in 10% acetic acid. The images were visualized by Kodak Image Station 2000R (Kodak, Rochester, NY, USA) and densitometric analysis was conducted with Image J software version 1.38 (National Institutes of Health, Bethesda, MD, USA).

Western blotting. Proteins were extracted from cells using radioimmunoprecipitation lysis buffer for 30 min at 4°C. The protein content was determined using a Bicinchoninic Acid Protein Assay kit (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Total proteins (50 μ g per lane) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 8% skimmed milk in TBS buffer, followed by overnight incubation at 4°C with primary antibodies and washing prior to incubation for 2 h at room temperature with a goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (catalog no. SAB5300163; Sigma-Aldrich; Merck Millipore; 1:1,000). The following rabbit primary antibodies were purchased from Santa Cruz Biotechnology, Inc.: anti-Ras homolog family member A (RhoA; catalog no. SAB2102002; 1:2,000), anti-phosphorylated (p)-RhoA (catalog no. SAB4503986; 1:2,000), anti-p-MEK1/2 (catalog no. SAB4504466; 1:2,000) and anti-MEK1/2 (catalog no. MFCD00239713; 1:2,000). Rabbit polyclonal anti-NF- κ B p65 (catalog no. AF0246; 1:1,000) and anti- β -actin (catalog no. AA128; 1:1,000) were purchased from Beyotime Institute of Biotechnology (Haimen, China). The bands were visualized using an Enhanced Chemiluminescence system (GE Healthcare Life Sciences, Chalfont, UK), and the band density was determined by Image J software version 1.38.

Preparation of nuclear proteins. Nuclear proteins were extracted from cells as previously described (9). Protein

content was determined using a Bicinchoninic Acid Protein assay reagent (Beyotime Institute of Biotechnology).

Electrophoretic mobility shift assay (EMSA). EMSA was performed as previously described (13). The nuclear extracts were incubated with a ³²P-end-labeled double-stranded NF- κ B oligonucleotide containing a tandem repeat consensus sequence of 5'-AGTTGAGGGGACTTCCAGGC-3', and were separated by electrophoresis on 4% polyacrylamide gels. The radioactive bands on the dried gels were quantified by a PhosphorImager using ImageQuant software version 5.1 (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

Statistical analysis. All data are presented as the mean \pm standard deviation of three independent experiments. Statistical analysis was performed using one-way analysis of variance followed by Tukey's post hoc test. All statistical analyses were conducted using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Curcumin inhibits MMP-2 activity induced by LPS. MMP-2 activity significantly increased following exposure to LPS compared with the unexposed control (P<0.05; Fig. 1) but pre-treatment with 20 and 40 μ M curcumin significantly decreased MMP-2 activity compared with the LPS-exposed group (P<0.05 and P<0.05, respectively; Fig. 1).

Ras/MEK1/2 signaling pathway mediates the increase of MMP-2 activity induced by LPS. To further investigate whether the Ras/MEK1/2 signaling pathway mediates the LPS-induced increase in MMP-2 activity, cells were pretreated with the inhibitors of NF- κ B and Ras, PDTC and FTS. Compared with exposure to LPS alone, PDTC and FTS pretreatment significantly reduced LPS-induced MMP-2 activity (P<0.05 and P<0.05, respectively; Fig. 2).

Curcumin prevents the LPS-induced increase in MMP-2 activity via the Ras/MEK1/2 pathway. Various lines of evidence have suggested that the Ras/MEK1 pathway is involved in the activation of MMPs (14). To elucidate the possible underlying mechanisms, this signaling pathway was investigated in the present study. The results revealed that 20 and 40 μ M curcumin treatment significantly inhibited LPS-induced activation of p-RhoA compared with the LPS-exposed group (P<0.05 and P<0.05, respectively; Fig. 3A). In addition, MEK1, a key effector of Ras in the regulation of MMPs, was examined. Treatment with 20 and 40 μ M curcumin suppressed LPS-induced activation of p-MEK1/2 compared with the LPS-exposed group (P<0.05 and P<0.05, respectively; Fig. 3B).

Curcumin inhibits LPS-induced NF- κ B activity. The Ras/MEK1 signaling pathway is involved in the activity of NF- κ B in MMP transcription (15). In addition, a previous study demonstrated that NF- κ B is involved in regulating MMP-2 at the transcriptional level (16). Therefore, it was hypothesized that curcumin prevents LPS-induced increases

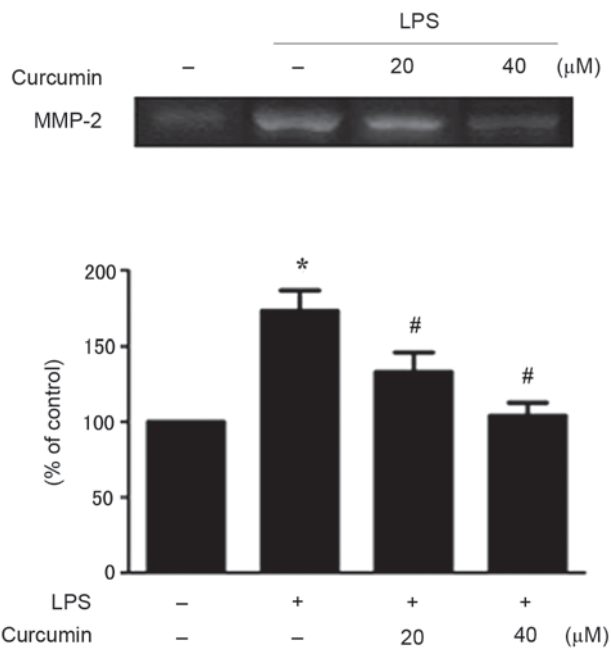


Figure 1. Curcumin inhibits LPS-induced MMP-2 activity in rat vascular smooth muscle cells, as measured by gelatin zymography. * $P < 0.05$ vs. control; # $P < 0.05$ vs. LPS only. Data are expressed as the mean \pm standard deviation of three independent experiments. LPS, lipopolysaccharide; MMP-2, matrix metalloproteinase-2.

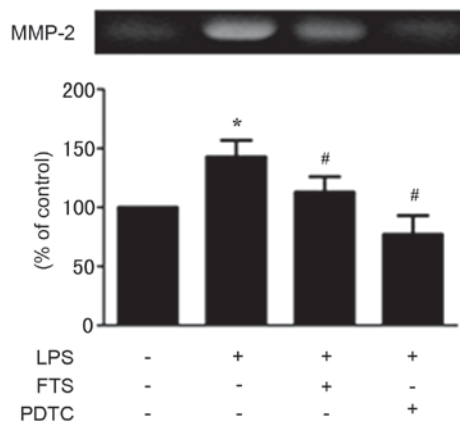


Figure 2. Ras/MEK1/2 signaling pathway is involved in the LPS-induced increase in MMP-2 activity. MMP-2 activity was assessed by gelatin zymography following treatment with LPS and the nuclear factor- κ B inhibitor PDTC or the Ras inhibitor FTS. * $P < 0.05$ vs. control; # $P < 0.05$ vs. LPS only. Data are expressed as the mean \pm standard deviation of three independent experiments. MEK1/2, mitogen-activated protein kinase kinase 1/2; LPS, lipopolysaccharide; MMP-2, matrix metalloproteinase-2; FTS, farnesylthiosalicylic acid; PDTC, ammonium pyrrolidinedithiocarbamate.

in MMP-2 activity via the suppression of NF- κ B activation. The results of the present study indicated that LPS exposure significantly increased NF- κ B p65 protein expression levels compared with the control group ($P < 0.05$; Fig. 4A); however, this increase was significantly inhibited by treatment with 20 and 40 μ M curcumin compared with the LPS-exposed group ($P < 0.05$ and $P < 0.05$, respectively; Fig. 4A).

In addition, LPS exposure significantly increased the DNA binding activity of NF- κ B compared with the control group ($P < 0.05$; Fig. 4B); however, this effect was significantly

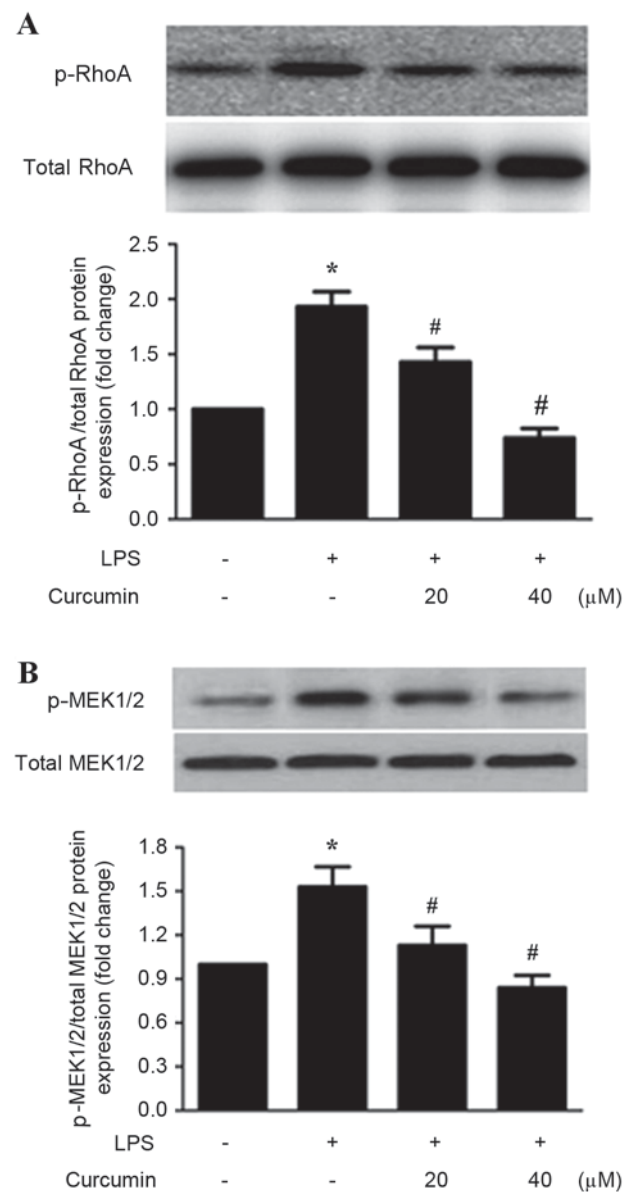


Figure 3. Curcumin prevents the LPS-induced increase of matrix metalloproteinase-2 activity via the Ras/MEK1/2 signaling pathway. Cells were pretreated with 20 or 40 μ M curcumin for 1 h, and exposed to 1 μ g/ml LPS for an additional 3 h. (A) LPS-induced phosphorylation of RhoA was significantly inhibited by curcumin. (B) LPS-induced phosphorylation of MEK1/2 was significantly inhibited by curcumin. * $P < 0.05$ vs. control; # $P < 0.05$ vs. LPS only. Data are expressed as the mean \pm standard deviation of three independent experiments. LPS, lipopolysaccharide; MEK1/2, mitogen-activated protein kinase kinase 1/2; RhoA, Ras homolog family member A; p, phosphorylated.

inhibited by treatment with 20 and 40 μ M curcumin compared with the LPS-exposed group ($P < 0.05$ and $P < 0.05$, respectively; Fig. 4B).

Discussion

VSMC migration to the vascular intima and ECM remodeling are important steps in the development of atherosclerosis (17). In this process, the abnormal secretion and activation of MMPs (in particular, MMP-2) and tissue inhibitors of metalloproteinases (TIMPs; in particular TIMP-2) in rat VSMCs leads to an altered balance between the two, resulting in the

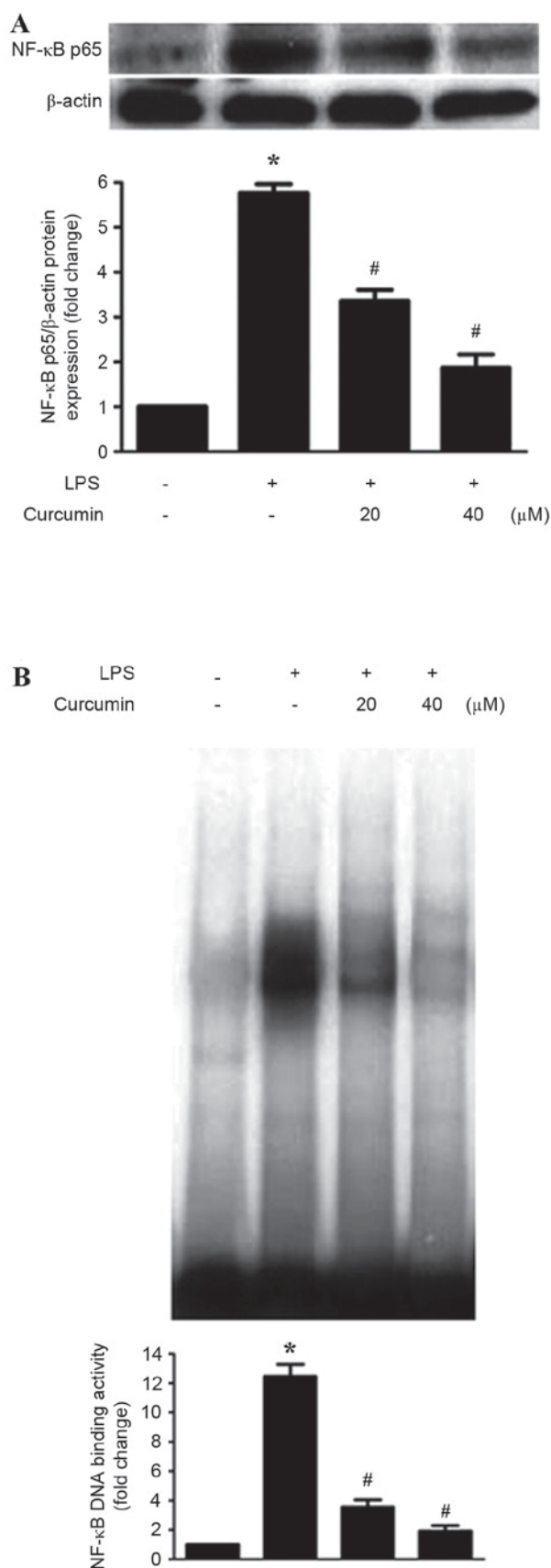


Figure 4. Curcumin suppresses NF- κ B DNA binding activity. Cells were pretreated with 20 or 40 μ M curcumin for 1 h, and exposed to 1 μ g/ml LPS for an additional 3 h. (A) Curcumin inhibited the LPS-induced increase of NF- κ B p65 subunit protein expression levels in the nucleus. (B) Curcumin inhibits NF- κ B DNA binding activity. * $P < 0.05$ vs. control; # $P < 0.05$ vs. LPS only. Data are expressed as the mean \pm standard deviation of three independent experiments. NF- κ B, nuclear factor- κ B; LPS, lipopolysaccharide.

remodeling of the ECM (18). Thus, it is important to reduce the activation and expression of MMP-2 in rat VSMCs.

In the present study, curcumin was demonstrated to inhibit LPS-induced MMP-2 activity, and the potential underlying mechanisms were identified. LPS is released from Gram-negative bacteria and promotes atherosclerosis in humans by aggravating the inflammatory response in the arterial wall (19). The present study revealed that curcumin inhibited LPS-induced increases in MMP-2 activity, which is consistent with a previous report (9). MMP-2 expression is induced by cytokines, hormones and growth factors and is regulated by the activation or suppression of promoter activity (20-22). A previous study demonstrated that overexpression of mitogen activated protein kinase (MAPK) phosphatases with selective dual specificity suppresses the activation of the MMP promoter, which is a more effective way to inhibit the activation of the transcription factors activator protein-1 (AP-1), E-twenty-six (ETS) and the MMP promoter (23). The DNA binding and transcription ability of the AP-1 and ETS transcription factors is regulated by phosphorylation of MAPKs and serine/threonine kinase, which mediate cell-cell and cell-matrix interactions induced by various stimulating factors. (24-26) Ras activation may further activate ETS-1 and -2, and ERK1/2 and c-Jun N-terminal kinase/stress activated protein kinase signaling pathways (27). Therefore, the involvement of the Ras/MEK1/2 signaling pathway in the LPS-induced increase in MMP-2 activity was investigated. The results indicated that the LPS-induced increase in MMP-2 activity may be suppressed by PDTC and FTS. Similarly, a previous study demonstrated that inhibitors of RhoA and NF- κ B inhibited LPS-induced increase of MMP-2 activity (28). In addition, the results of the present study demonstrated that LPS-induced increased expression levels of p-RhoA and p-MEK1/2 were significantly suppressed by curcumin treatment. These results indicated that curcumin prevents the LPS-induced increased MMP-2 activity via the Ras/MEK1/2 signaling pathway.

The activation of NF- κ B is required for MMP expression (29). Inactive NF- κ B is presented as a heterodimeric complex of the subunits p50 and p65, which combine with inhibitor of κ B (I κ B) in cells. When the cell is stimulated, I κ B is degraded via phosphorylation. The liberated NF- κ B translocates to the nucleus, where the dimer interacts with regulatory κ B elements in promoters and enhancers, thereby controlling gene transcription (30). The activity of NF- κ B in MMP transcription is regulated by the Ras/MEK1 signaling pathway (15). In the present study, NF- κ B p65 expression was inhibited by curcumin treatment in rat VSMCs. The DNA binding activity of NF- κ B was significantly increased by LPS; however, this effect was partially suppressed by treatment with curcumin. These findings are in accordance with another previous study (31).

In conclusion, the results of the present study demonstrated that curcumin treatment significantly inhibited LPS-induced MMP-2 activity in rat VSMCs. Inhibitors of NF- κ B and Ras suppressed LPS-induced increased MMP-2 activity. The results indicated that curcumin significantly inhibited LPS-induced activation of p-RhoA and p-MEK1/2 in rat VSMCs. The expression of NF- κ B p65 was additionally inhibited by curcumin. Furthermore, the LPS-induced increase in NF- κ B

DNA binding activity was abrogated by curcumin treatment. These results suggested that curcumin prevents LPS-induced MMP-2 activity via the Ras/MEK1/2 signaling pathway in rat VSMCs. Therefore, curcumin may be a potential therapeutic agent for the treatment of cardiovascular disease.

Acknowledgements

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