17-Dimethylaminoethylamino-17-demethoxygeldanamycin Attenuates Inflammatory Responses in Experimental Stroke

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Heat shock protein 90 (HSP90) is a ubiquitous molecular chaperone involved in the proper conformation of many proteins. HSP90 inhibitors (17-dimethyl aminoethylamino-17-demethoxygeldanamycin hydrochloride [17-DMAG]) bind to and inactivate HSP90, suppressing some key signaling pathways involved in the inflammatory process. Since considerable evidence suggests that inflammation accounts for the progression of cerebral ischemic injury, we investigated whether 17-DMAG can modulate inflammatory responses in middle cerebral artery occluded (MCAO) mice. Male C57/BL6 mice were pretreated with 17-DMAG or vehicle for 7 d before being subjected to transient occlusion of middle cerebral artery and reperfusion. Mice were evaluated at 24 h after MCAO for neurological deficit scoring. Moreover, the mechanism of the anti-inflammatory effect of 17-DMAG was investigated with a focus on nuclear factor kappa B (NF-κB) pathway. 17-DMAG significantly reduced cerebral infarction and improved neurological outcome. 17-DMAG suppressed activation of microglia and decreased phosphorylation of inhibitory (IκB) and subsequent nuclear translocation of p65, which eventually downregulated expression of NF-κB-regulated genes. These results suggest that 17-DMAG has a promising therapeutic effect in ischemic stroke treatment through an anti-inflammatory mechanism.

Key words heat shock protein 90 (HSP90); stroke; inflammation

Acute ischemic stroke is one of the most frequent causes of death and permanent disability in adults worldwide. Although various mechanisms are involved in the pathogenesis of stroke, recent work has demonstrated that inflammatory responses accompanying necrotic brain injury contribute to ischemic pathology, and anti-inflammatory strategies have become popular. Nuclear factor-kappa B (NF-κB) is a central regulator of inflammatory response in ischemic stroke, whose activation is required for the transcriptional induction of many proinflammatory mediators involved in innate immunity, such as cellular adhesion molecules, cytokines, and growth factors. Indeed, it has been evidenced that factors that modulate the activity of NF-κB could potentially regulate inflammatory processes in ischemic stroke.

Heat shock protein 90 (HSP90) is a ubiquitous molecular chaperone which plays essential roles in the folding, activation, and assembly of client proteins. Many key proteins involved in signal transduction of inflammatory responses are either direct client protein or indirect client protein. Among the different client proteins modified by HSP90 inhibitors, proteins involved in NF-κB signalling pathway play important roles in mediating inflammatory and immune responses. Blocking the ATP-binding site of HSP90 by inhibitors, results in degradation of some client proteins (e.g. inhibitory (IκB) kinase (IKK)) via an ubiquitin–proteasome-dependent pathway. Previous studies showed that inhibition of HSP90 by injections of geldanamycin (GA, an HSP90 inhibitor) into the cerebral ventricles protects ischemic stroke through stimulation of heat shock gene transcription (such as HSP70 and HSF1). 17-Dimethylaminoethylamino-17-demethoxygeldanamycin hydrochloride (17-DMAG) is a more water soluble HSP90 inhibitor and can be administered orally. However little is known about anti-inflammatory effect of HSP90 inhibitor during ischemic stroke and whether 17-DMAG, a brain-permeable small-molecule inhibitors of Hsp90, can protect against ischemic stroke.

Since there is accumulating evidence showing that inflammation accounts for the progression of ischemic stroke, at least acutely, we hypothesize that HSP90 inhibition by 17-DMAG modulates inflammatory responses in a mice model of transient middle cerebral artery occlusion (tMCAO).

MATERIALS AND METHODS

Animals Animal research protocols were approved by Shanghai Jiaotong University animal care committee and conformed to the National institutes of health guide for care and use of laboratory animals. Adult male C57/BL6 mice (20 to 25 g) were housed individually and maintained on a 14:10 light/dark cycle. The animals were allowed ad libitum access to tap water and rodent chow throughout the study.

Therapeutic Agents and Protocol for Administration 17-DMAG was obtained from Sigma. An optimal dose of 17-DMAG was determined based on a previous study in mice. All the mice were divided into 3 groups randomly: sham-operated group (Sham); vehicle-treated MCAO group (Vehicle); stroke groups treated with 5mg/kg 17-DMAG orally every other day from 7 d before ischemia.

Experimental Ischemic Stroke After 1 week respective treatment, the MCAO surgery was conducted as previously described. Briefly, mice were anesthetized by face mask using 3% to 4% isoflurane followed by 1.5% to 2% for maintenance. A monofilament was introduced into the internal carotid artery through an incision of the left common carotid artery. In this position, the middle cerebral artery was occluded for 60 min. After occlusion, the suture was retracted to allow reperfusion.

The authors declare no conflict of interest.
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Measurement of Physiological Parameters  Regional cerebral blood flow (rCBF) was measured by the probe of Laser-Doppler flowmetry (MoorLDI2, Moor Co., Ltd., U.K.) which was attached to the skull at 3 mm lateral and 1 mm caudal to the bregma. In randomly selected animals, mean arterial pressure was monitored, and arterial blood pH, pCO₂, and pO₂ were measured before MCAO operation, during (10 min after MCAO), and 30 min after reperfusion (Rapid Lab 248, Bayer, Wuppertal, Germany). Core temperature was maintained at about 37°C±5°C during the surgery.

Determination of Infarct Volume  Twenty four hours after MCAO, mice were deeply anesthetized with isoflurane and killed. The brains were removed and frozen in 20% sucrose. Coronal sections (20 µm) were cut in a cryostat (−20°C), collected and stained with cresyl violet. Areas of infarction were measured using an image analysis system, as previously described. ¹⁰

Evaluation of Neurological Deficits  Neurological examinations were performed at 24 h after MCAO. The neurological findings were scored on a 4-point scale: 0 (no neurological sure); 1 (Horner’s syndrome); 2 (forelimb flexion); 3 (circling to right). Cumulative scores were calculated for each group.

Measurement of Microglia/Macrophages Activation  After 24h occlusion and immersed in 4% paraformaldehyde (PF) for 24 h at 4°C. Brain sections (5 µm-thick) were blocked in 3% H₂O₂, 3% normal goat serum, and incubated with primary antibodies: mouse anti-IBa1, 1/500; anti-MHC Class II, 1/500 (Abcam, Oxford, U.K.). Secondary antibodies were incubated for 2 h at room temperature (from Jackson; 1:200). Fluorescently stained sections were then washed, and coverslipped with Gel/Mount (Biomeda, Foster City, CA, U.S.A.). Negative controls were prepared by omitting the primary antibodies. The immunoreactive cells were counted in five visual fields of ischemic cortex region around the infarct under a 400× light microscope.

Quantitation of mRNA Expression  Total RNA was extracted from cells or aortic tissue using TRIZOL (Invitrogen). One micro-gram total RNA was used to perform the reverse transcription with High Capacity cDNA Archive Kit (Applied Biosystem). Real-time quantitative polymerase chain reaction (PCR) analysis for tumor necrosis factor (TNF)-α, interleukin (IL)-1β, intercellular cell adhesion molecule-1 (ICAM-1), and inducible nitric oxide synthase (iNOS) was performed using TaqMan gene expression assays and the ΔΔCt method with housekeeping gene 18S as the endogenous control. The DNA sequences of the primers and probes used were as follows: (1) TNF-α: Fwd: 5′-GCC ACC AGG CTC TCT TGT-3′, Rev: 5′-GGG GGC CTT TTG GGA ACT-3′; Intern: 5′-6FAM TAC TGA ACT CCG GGT GGA TCGG TCT CTA TMA R-3′; (2) ICAM-1: Fwd: 5′-GC CCG CT TTT TGC TCT GC-3′, Rev: 5′-CC AAC GC AG TCG TCT CG-3′, Intern: 5′-6FAM GAG TG CCG GGA AG TCT CTT GCT CTA TMA R-3′; (3) iNOS: Fwd: 5′-CCC TTG TTT GTG CGA GT-3′, Rev: 5′-CCCAA TAT GAT CG AA GG-3′, Intern: 5′-6FAM AGG TG GGA GGA AGT TCT CGA CT TMA R-3′; (4) IL-1β: Fwd: 5′-AGG TCG CTC AGG GT CAC A-3′, Rev: 5′-G TT GGT GCC CAT CAG AGG-3′; Intern: 5′-6FAM TGG CAC ATT CG TGC AAC AA GAG AG C TA M A R-3′.

Western Blots  Total proteins were extracted from cortex. Cytoplasmic and nuclear protein subfractions were prepared as described previously. ¹¹ Protein concentration was measured by BCA kit (Thermo Scientific). Equal amounts of protein lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane and then immunoblotted with antibodies against p65 (1:1000 dilution; Cell Signaling Technology), p-IκBα (1:1000 dilution; Cell Signaling Technology), and GAPDH (1:1000 dilution; Cell Signaling Technology). Protein bands were visualized by Odyssey imaging system (LICOR).

Microwell Colorimetric NF-κB p65 Assay  DNA-binding activity of NF-κB was analyzed with the TransAM NF-κB p65 Transcription Factor Assay Kits (Active Motif, Carlsbad, CA, U.S.A.) according to the instructions with minor alterations for brain tissue. The optical density (OD) of the samples was quantified on a spectrophotometer at 450 nm with a reference wavelength of 655 nm. NF-κB activity was expressed as a percentage by using the formula: sample OD value blank OD value/positive control OD value.

Statistical Analysis  GraphPad InStat (GraphPAD Software) was used to analyze data. For the ischemia models, differences between groups were analyzed by ANOVA with Bonferroni’s post-hoc test for continuous data, and Mann–Whitney for non-continuous data. Results are expressed as mean±S.E.M. Statistical significance was defined at the p<0.05.

Table 1. Physiological Parameters Before, During, and After 1-h Ischemia

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Before</th>
<th>During</th>
<th>After</th>
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<tbody>
<tr>
<td>rCBF (%)</td>
<td>Vehicle</td>
<td>100</td>
<td>8.1±1.4</td>
<td>33.7±6.6</td>
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<tr>
<td></td>
<td>DM</td>
<td>100</td>
<td>10.4±3.9</td>
<td>35.2±7.4</td>
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<tr>
<td>MABP (mmHg)</td>
<td>Vehicle</td>
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<td></td>
<td>DM</td>
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<td>95.5±7.2</td>
<td>93.2±9.1</td>
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<tr>
<td>pH</td>
<td>Vehicle</td>
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<td>7.3±0.07</td>
<td>7.4±0.09</td>
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<tr>
<td></td>
<td>DM</td>
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<td>7.3±0.1</td>
<td>7.3±0.06</td>
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<tr>
<td>PCO₂ (mmHg)</td>
<td>Vehicle</td>
<td>47.8±5.4</td>
<td>45.4±4.9</td>
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<tr>
<td></td>
<td>DM</td>
<td>49.2±6.3</td>
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<td>46.5±5.4</td>
</tr>
<tr>
<td>PO₂ (mmHg)</td>
<td>Vehicle</td>
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<td>113.5±15.4</td>
<td>132.4±15.9</td>
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<td></td>
<td>DM</td>
<td>122.6±19.8</td>
<td>114.8±17.9</td>
<td>141.9±18.9</td>
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<tr>
<td>Temperature (°C)</td>
<td>Vehicle</td>
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<td>36.8±0.1</td>
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<td></td>
<td>DM</td>
<td>37.0±0.1</td>
<td>36.7±0.2</td>
<td>37.1±0.2</td>
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</tbody>
</table>

Data are expressed as the mean±S.D. MABP, mean arterial blood pressure. No statistically significant differences were seen between vehicle treated and 17-DMAG treated mice at any time point.
RESULTS

Effects of 17-DMAG on Physiological Parameters The effects of 17-DMAG on regional cerebral blood flow, arterial blood pressure, pH, pO$_2$, and pCO$_2$ were evaluated in rats after MCAO. No significant difference was observed between 17-DMAG- and vehicle-treated groups in any of the respiratory and cardiovascular parameters tested (Table 1).

17-DMAG Reduces Infarct Size and Improves the Neurological Deficits We first determined whether 17-DMAG protects against ischemia stroke. As shown in Fig. 1A, representative brain section from a 17-DMAG-treated mouse has smaller infarct size than that from a vehicle-treated littermate. Compared with vehicle-treated group, the infarct volume of 17-DMAG-treated group was significantly reduced by about 45% (Fig. 1B). 17-DMAG also improved the neurological scores compared with vehicle-treated mice at 24 h after MCAO (Fig. 1C).

17-DMAG Reduces Number of Active Microglia/Macrophages Activated microglia and/or macrophages were visualized by morphological transformation, increased lectin reactivity, and MHC class II expression. We quantified the number of activated microglia (assessed by IBA1 and MHC class II staining) in the peri-infarct region within the ipsilateral ischemic cortex. Regions of interest were selected based on criteria published previously. Figure 2A shows more intensely stained IBA1-positive cells in a brain section of a vehicle treated mice compared with 17-DMAG treated mice at 24 h after MCAO. Similarly, fewer MHC Class II-positive cells among 17-DMAG-treated mice were observed (Fig. 2A). The number of activated microglia/macrophages as assessed by IBA1 and MHC Class II immunostains were significantly decreased among the 17-DMAG treated mice compared with vehicle treated mice (Fig. 2B).

17-DMAG Inhibits IκB Phosphorylation Activation of NF-κB requires phosphorylation of IκBa by IKK and degradation by the proteasome, allowing NF-κB to transfer to the nucleus to transcriptionally regulate the expression of different proinflammatory genes. Western blots show that MCAO increased the expression of p-IκBa, which was restored by 17-DMAG (Figs. 3A, B).

17-DMAG Reduces NF-κB Nuclear Translocation Western blots of cytosolic and nuclear subcellular fractions show that NF-κB/ p65 subunit (p65) in nucleus is increased in the ischemic hemisphere of vehicle-treated mice compared with the control mice. However, 17-DMAG decreased the nuclear translocation of p65 (Figs. 4A, B).

17-DMAG Decreases NF-κB DNA Binding Capacity To corroborate the inhibitory effects of 17-DMAG on NF-κB activation, we further examined NF-κB DNA binding capacity using a multwell colorimetric technique that was designed to detect the ability of active NF-κB to bind to double-stranded oligonucleotide probes containing consensus binding sequences (5’-GGGACTTCC-3’). Figure 5 shows that NF-κB activity was significantly increased by MCAO which was restored by 17-DMAG treatment (Fig. 5).

17-DMAG Downregulates the Expression of Several NF-κB-Regulated Genes Real-time polymerase chain reaction of four representative pro-inflammatory NF-κB-regulated genes, TNF-α, IL-1β, ICAM-1, and iNOS was performed from brains exposed to tMCAO. Our results show that while ischemic hemispheres from vehicle-treated mice have several-fold greater expression of TNF-α, ICAM-1, iNOS, and IL-1β, at 24 h after tMCAO compared with the control group, the increased expression of these genes was significantly less among the 17-DMAG treated mice (Fig. 6).

DISCUSSION

It is well documented that inhibiting various inflammatory mediators reduces infarct volume induced by cerebral ischemia. Here, we show that inhibition HSP90 by 17-DMAG decreases microglial/macroage activation, downregulates several proinflammatory genes and reduces infarct volume in MCAO mice. Present work provides the first evidence that 17-DMAG inhibits inflammatory responses in MCAO mice. Further, this study shows that 17-DMAG blocks NF-κB activation and expression of several downstream inflammatory genes by inhibiting IκB activation in MCAO mice. To the best of our knowledge, this work is the first time to demonstrate the anti-inflammatory effect of inhibition of HSP90 by 17-DMAG in ischemic stroke. However, as the 17-DMAG also may exert protective effects through other mechanisms, such as inhibition of ROS and MMPs, which subsequently attenuate inflammation and reduce infarct volume in MACO mice, further work is needed to clarify the specific mechanisms.

Although the inhibitors of HSP90 are of therapeutic interest primarily in cancer, previous work has shown that HSP90...
is capable of modulating inflammatory responses and has potential therapy target for the treatment of other inflammatory diseases, such as rheumatoid arthritis.\(^{15}\) However little is known as to whether this is the case in ischemic stroke, although previous study has reported that GA administrated by injections into the cerebral ventricles protects against ischemic stroke via stimulation of heat shock gene transcription.\(^{7}\) Present study found that 17-DMAG administrated orally decreased infarct volume, at least in part, through modulation of inflam-

Fig. 2. 17-DMAG Inhibited Microglia/Macrophage Activation Induced by MCAO
A) Representative I\(\beta\)1 and MHCII staining within the ischemic cortex of mice following tMCAO show less intense staining in 17-DMAG treated mice. B) Fewer I\(\beta\)1 (\(n=8\)/group) and MHC Class II-positive cells (\(n=8\)/group) were detectable in the peri-infarct regions of 17-DMAG treated mice, compared with vehicle treated mice (\(n=7\)/group) (**\(p<0.01\), compared with sham group; ##\(p<0.01\), compared with vehicle treated group).

Fig. 3. 17-DMAG Inhibited I\(\kappa\)B Phosphorylation Induced by MCAO
A) Western blots show stronger I\(\kappa\)B phosphorylation induced by MCAO, which was restored by 17-DMAG. Total-I\(\kappa\)B were decreased by MCAO, which were restored by 17-DMAG. B) Data are presented as the OD of the protein of interest divided by the OD of the GAPDH bands used as a loading control (\(n=5\)/group; **\(p<0.01\), compared with sham group; #\(p<0.05\), ##\(p<0.01\), compared with vehicle group).

Fig. 4. 17-DMAG Inhibits Nuclear Translocation of NF-\(\kappa\)B
A) Western blots of cytosolic and nuclear subcellular fractions show that NF-\(\kappa\)B/p65 subunit (p65) in nucleus is increased in the ischemic hemisphere of vehicle treated mice compared with the sham group, which was restored by 17-DMAG. GAPDH is used as a cytoplasmic marker, and histone 3 is used as a nuclear marker. B) Data are presented as the OD of the protein of interest divided by the OD of the loading control bands (\(n=4–6\)/group, *\(p<0.05\), compared with sham group; #\(p<0.05\), compared with vehicle group).
plexes with HSP90, disruption of these complexes by HSP90 inhibitors in ischemic stroke has been reported. Here we observed up-regulation of anti-inflammatory HSP expression and inflammatory responses could be due to its double activity: degradation of client proteins involved in different inflammatory signalling pathways and up-regulation of anti-inflammatory HSP expression (especially HSP70). Moreover, the neuroprotective effect of HSP90 inhibitors in ischemic stroke has been reported. Here we observed that HSP90 inhibitors could diminish the expression and activation of inflammatory mediators, at least in part, through inhibiting phosphorylation of IκB and subsequent NF-κB nuclear translocation and binding activity. However, further studies are needed to clarify whether the reduced inflammatory response observed with HSP90 inhibitors is mediated through HSP70 up-regulation or not. Among the client proteins of HSP90 involved in inflammatory diseases, IKK is one of the most representative examples. Activation of NF-κB requires phosphorylation of IκB by IKK and degradation by the proteasome, allowing NF-κB to enter the nucleus to transcriptionally regulate the expression of different proinflammatory genes (e.g. MCP-1). As IKK exists in complexes with HSP90, disruption of these complexes by HSP90 inhibitors decrease IKK expression and subsequent NF-κB activation. Previously, it has been reported that NF-κB activation induced by pro-inflammatory cytokines is modulated by HSP90 inhibitors in vascular cells. Herein, we found that HSP90 inhibitors decreased NF-κB activation in MCAO mice. Moreover, expression of some of NF-κB related inflammatory genes was significantly down-regulated by inhibition of HSP90 by 17-DMAG. These results are in agreement with previous papers in which HSP90 inhibitors attenuate NF-κB in other inflammatory diseases.

The clinical significance of our results is related to the emerging evidence that HSP as potential therapeutic targets in different diseases. However, since HSP90 inhibition affects several pathways simultaneously, other pathological processes such as neoangiogenesis, oxidative stress, or apoptosis could also be affected by these drugs. Above all, higher expression of Nox1 and Noxo1 was found in the inflammatory region of human atherosclerotic plaques. But whether this case is also for experimental stroke is still unknown. Therefore, further studies were required to clarify the protective role of HSP90 inhibitors in human diseases. 17-DMAG is a more potent and water soluble derivative than GA and can pass through BBB, which makes it a more feasible long-term therapeutic agent. Our studies demonstrate that HSP90 inhibitors reduce inflammatory responses in ischemic stroke through inhibition of NF-κB pathway. 

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