Acta Pharmacologica Sinica (2011) 32: 1100–1108
 1671-4083/11 $32.00
www.nature.com/aps

Original Article

T33, a novel peroxisome proliferator-activated receptor γ/α agonist, exerts neuroprotective action via its anti-inflammatory activities

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Aim: To examine the neuroprotective effects of T33, a peroxisome proliferator-activated receptor gamma/alpha (PPARγ/α) agonist, in acute ischemic models *in vitro* and *in vivo.*
Methods: Primary astrocytes subjected to oxygen-glucose deprivation/reperfusion (O/R) and BV-2 cells subjected to hypoxia were used as a model simulating the ischemic core and penumbra, respectively. The mRNA levels of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were measured using qPCR. The levels of TNF-α secreted by BV-2 cells were measured using ELISA. Protein levels of cyclooxygenase-2 (COX-2), p65, phosphorylated I-κBα/I-κBα, phosphorylated I-κB kinase (pIKK), phosphorylated eukaryote initiation factor 2α (p-eIF-2α)/eIF-2α and p-p38/p38 were detected using Western blot. PPARγ activity was measured using EMSA. The neuroprotection *in vivo* was examined in rat middle cerebral artery occlusion (MCAO) model with neurological scoring and TTC staining.
Results: Addition of T33 (0.5 μmol/L) increased the level of I-κBα protein in primary astrocytes subjected to O/R, which was due to promoting protein synthesis without affecting degradation. In primary astrocytes subjected to O/R, addition of T33 amplified I-κBα gene transcription and mRNA translation, thus suppressing the nuclear factor-kappa B (NF-κB) pathway and reducing inflammatory mediators (TNF-α, IL-1β, and COX-2). In BV-2 cells subjected to hypoxia, T33 (0.5 μmol/L) reduced TNF-α, COX-2, and p-P38 production, which was antagonized by pre-administration of the specific PPARγ antagonist GW9662 (30 μmol/L). T33 (2 mg/kg, ip) attenuated MCAO-induced inflammatory responses and brain infarction, which was antagonized by pre-administered GW9662 (4 mg/kg, ip).
Conclusion: T33 exerted anti-inflammatory effects in the ischemic core and penumbra via PPARγ activation, which contributed to its neuroprotective action.

Keywords: stroke; middle cerebral artery occlusion; oxygen-glucose deprivation; astrocytes; BV-2 cells; inflammation; nuclear factor-kappa B; T33; peroxisome proliferator-activated receptor γ

Acta Pharmacologica Sinica (2011) 32: 1100–1108; doi: 10.1038/aps.2011.69; published online 1 Aug 2011

Introduction

Acute brain ischemia leads to a damaged ischemic core and salvageable surrounding tissue (penumbra). In addition to treatments targeting the ischemic core, interventions preventing the progression of the penumbra to infarction help to reduce the final infarct area[1, 2]. Although different mechanisms are involved in the pathogenesis of strokes, appreciable evidence supports the fact that massive inflammation accounts for the progression of strokes, at least in the acute phase[3, 4]. Obstruction of blood flow to the brain elicits the activation of glial cells and infiltration of peripheral leukocytes facilitated by pro-inflammatory factors. Reperfusion leads to the over-production of reactive oxygen species, further stimulating ischemic glial cells to secrete inflammatory mediators, resulting in subsequent brain infarction. Recent research found compounds able to curtail neuroinflammation were effective in alleviating stroke-induced brain injury[5, 6]. Therefore, simultaneously interfering with the inflammatory responses in both the ischemic core and the penumbra has become an important area of research in the treatment of ischemic stroke, whose therapeutic management is limited to thrombolysis[7, 8].

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear transcription factors regulating cell proliferation, differentiation, insulin sensitivity and inflammatory responses[9]. To date, three PPAR isoforms, designated α, β, and γ, have been identified. The PPARγ isoform is involved in controlling immune reactions and reduces

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Received 2011-02-14 Accepted 2011-04-22

inflammatory mediators upon activation[10, 11]. Most of the anti-inflammatory activities of PPARγ have been suggested to arise through the inhibition of NF-κB, a transcription factor controlling the expression of multiple inflammatory genes during ischemia[10]. Moreover, emerging evidence implies that PPARγ activation is protective against brain damage after stroke, and PPARγ deficiency promotes brain damage after stroke[12–14]. Synthesized PPARγ agonists, including the well-known thiazolidinediones (TZDs), exhibit potent neuroprotective effects in stroke models mainly by blocking the production of inflammatory mediators[15, 16]. PPARγ/α dual agonists, expected to prevent weight gain and exert cardioprotective effects due to PPARα activation, are currently being developed[17, 18]. Whereas some PPARγ/α agonists such as aleglitazar have entered clinical trials for the treatment of diabetes, little research concerning the potency of dual agonists toward brain ischemia-induced inflammation has been reported.

T33, a benzopyran derivative, is a novel dual PPARγ/α agonist that has been demonstrated to alleviate diabetic injuries[19, 20]. The present study is the first to investigate the effects of T33 on inflammation and brain injury in the CNS, as well as the underlying mechanisms, by employing both *in vitro* and *in vivo* ischemic models.

Materials and methods

T33 preparation

T33 was provided by Prof Yu-she YANG of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences. For *in vitro* experiments, T33 was dissolved in DMSO and diluted with culture medium before it was added to cell cultures. For *in vivo* administration, T33 was dissolved in DMSO and Cremaphor (Sigma-Aldrich) and diluted with saline.

Primary astrocytes

Primary astrocyte cultures were prepared from cerebral hemispheres of neonatal Sprague-Dawley (SD) pups (12–24 h old) according to published procedures with minor modifications[21]. Briefly, brain cortices were digested in 0.25% trypsin before they were dissociated into single cell suspensions in culture medium (DMEM/F12 containing 10% FBS). Cell suspensions were centrifuged at 1000 revolutions per minute for 10 min and pellets were re-suspended in culture medium. Cells were then placed into culture flasks coated with poly-*L*-lysine. Cultures were incubated at 37 °C in a 5% CO_2 incubator. The medium was changed the following day and then twice a week until the cells reached confluence.

Ischemic surgery and drug administration

All surgical procedures used in this study followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No 8023, revised 1978), as well as the guidelines of the Animal Care and Use Committee of Shanghai Institute of Materia Medica. SD rats were randomly divided into four groups: sham-operated, middle cerebral artery occlusion (MCAO), T33-treated MCAO and T33+GW9662-treated MCAO (GW9662 is a PPARγ antagonist).

Rats (male, 200–250 g) were anesthetized with chloral hydrate (400 mg/kg, ip). Focal cerebral ischemia was produced by MCAO as previously described[22]. Briefly, the right external carotid artery (ECA) and internal carotid artery (ICA) were dissected from the surrounding connective tissue through a midline neck incision. A 4–0 monofilament nylon suture with a flame-rounded leading end was advanced from the common carotid artery (CCA) to the ICA until resistance was felt and a slight curving of the advancing suture was observed. MCAO caused a sustained decrease in rat cerebral blood flow (rCBF) levels, which remained stable at approximately 20% of baseline, indicating that the filament was positioned properly to occlude blood flow to the middle cerebral artery (MCA). After 1 h of occlusion, the suture was withdrawn to allow for cerebral reperfusion. Core body temperature was maintained at 37.0±0.5 °C with a heating carpet. T33 (2 mg/kg) was administered intraperitoneally once the filament reached the MCA. GW9662 (4 mg/kg) was injected 30 min before T33 administration. The sham-operated and MCAO groups were administered solvent alone.

Evaluation of neurological deficits

One day after MCAO, neurological deficits were evaluated before euthanization according to a previous report[23]. Four consecutive individual tests were carried out: (a) spontaneous activity (moving and exploring=0, moving without exploring=1, staying still or moving only when pulled by the tail=2); (b) left drifting during displacement (none=0, drifting only when pushed or pulled by the tail=1, spontaneous drifting=2, circling without displacement or spinning=3); (c) parachute reflex (symmetrical=0, asymmetrical=1, contralateral forelimb retracted=2); (d) resistance to left forepaw stretching (resistant to stretching=0, reduced resistance=1, no resistance=2). Neurological scores ranging from 0–9 were calculated as the sum of the scores from the four individual tests described above. Higher scores indicated worse neurological performance.

Cerebral infarction measurement

After 24 h of reperfusion, rats were sacrificed. Brains were sectioned into six 2-mm coronal planes and stained with 2% 2, 3, 5-triphenyl tetrazolium chloride (TTC, Sinopharm Chemical Reagent Co Ltd) at 37 °C for 15 min before they were fixed in 10% formalin overnight. Images of the slices were digitalized, and infarct areas outlined in white were measured using Image-Pro Plus software. Infarct volumes were determined by multiplying the average slice thickness (2 mm) by the sum of the infarct areas in all six brain slices. The percentage of the total infarct area was also calculated for each of the six brain slices.

Oxygen-glucose deprivation (OGD)

For OGD insult, the original medium was removed and primary astrocytes were washed with EBSS. The cultures were then placed in glucose-free DMEM and kept in an incubator containing 95% (*v/v*) N_2 and 5% (*v/v*) CO_2 at 37 °C for 4 h.

T33 was added to the cultures at the beginning of OGD at a final concentration of 0.5 μmol/L. At the end of the exposure period, cells were either collected immediately to be used in experiments (OGD), or glucose was added and the cells were returned to normal conditions for another 24 h before collection (OGD and reperfusion, O/R).

Hypoxia

To simulate hypoxia in the penumbra area, BV-2 cells were transferred to an incubator containing 2.5% O_2 and 5% CO_2, balanced in N_2 at 37 °C. Cells were kept in hypoxic conditions for 10 h before collection. T33 and GW9662 were added to the cell cultures 1 h prior to hypoxia at a final concentration of 0.5 μmol/L and 30 μmol/L, respectively.

TNF-α level analysis

TNF-α secreted in the BV-2 culture medium was measured using a specific enzyme-linked immunosorbent assay (ELISA) kit (Biosource, Camarillo) according to the manufacturer's protocol. Briefly, samples were incubated with biotinylated anti-TNF-α antibody in microtiter wells for 90 min and subsequently with streptavidin-HRP working solution for 30 min. Stabilized chromogen was then added to each well and maintained in the dark for 15 min before termination. Each plate was then read at 450 nm.

RNA extraction and real-time PCR analysis

Total RNA was extracted with TRIzol (Invitrogen) following the manufacturer's protocol and then reverse transcribed into cDNA using the PrimeScript^{TM} RT reagent kit (Takara Biotechnology). Real-time PCR was performed using the SYBR Premix Real-time PCR kit (Takara Biotechnology) according to the manufacturer's instructions. mRNA levels were normalized against β-actin and presented as $2^{-\Delta\Delta CT}$. The primer sequences are listed in Table 1.

Protein extraction and Western blot analysis

For whole cell/tissue protein extraction, samples were lysed in RIPA buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.5% DOC, 1% NP40, 0.1% SDS, 1 mmol/L NaF, 1 mmol/L Na_3VO_4) before they were centrifuged at 10000×*g* for 10 min at 4 °C (an additional sonication step before centrifugation was necessary for rat brain tissue). The supernatants were collected to determine protein concentrations. Nuclear protein extraction was carried out using a kit produced by Beyotimes (China) with minor modifications. Briefly, cells were vortexed vigorously in cytoplasmic protein extraction reagent A for 5 s and left on ice for 10–15 min. Reagent B was then added and the cells were vortexed vigorously for 5 s before being placed in an ice bath for 1 min. The samples were subsequently vortexed for 5 s and centrifuged at 12000–16000×*g* for 5 min at 4 °C. The supernatants containing the cytoplasmic protein were carefully removed. The pellets containing the nuclear protein were resuspended with nuclear protein extraction reagent and sonicated at 25% amplitude (AML). The lysates containing the nuclear protein were placed on ice for 1–2 min before being vortexed for 30 s. After 30 min, the lysates were centrifuged at 12000–16000×*g* for 10 min at 4 °C. Supernatants were collected in pre-chilled tubes for nuclear protein concentration determination. Protein concentrations were determined using a BCA assay kit (Pierce) with bovine serum albumin as the standard. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Blots were blocked in skim milk before being incubated overnight with one of the following primary antibodies: goat anti-COX-2, mouse anti-α-tubulin (Santa Cruz), rabbit anti-IκB, rabbit anti-phospho-IKKβ, rabbit anti-eIF-2α, rabbit anti-phospho-eIF-2α, rabbit anti-p38, rabbit anti-phospho-p38 (Cell signaling), rabbit anti-p65 (Millipore), or mouse anti-TBP (Abcam). Blots were then incubated with secondary antibody (Kangcheng, China) conjugated with horseradish peroxidase for 1 h at room temperature and then developed using the ECL plus (Amersham GE Healthcare) detection system. Immunoreactive bands were visualized by autoradiography and the intensity of each band was quantified with Image J software.

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out using the "Gel Shift" kit closely following the manual (Panomics).

Statistical analysis

Data were expressed as mean±SEM. Neurological deficits were analyzed by the non-parametric Mann-Whitney *U* test. Multiple comparisons were analyzed by one way ANOVA followed by the LSD test unless otherwise specified. At least three independent experiments were carried out. $P<0.05$ was considered to be statistically significant.

Results

T33 alleviates O/R-induced inflammatory responses in astrocytes

Astrocyte activation constitutes one of the major events occur-

Table 1. Primer sequences for real-time PCR analysis.

Gene	Primer sequence	
	Upstream	Downstream
β-actin	5′-GAAGATCAAGATCATTGCTCC-3′	5′-GACTCATCGTACTCCTGCTTG-3′
TNF-α	5′-GACCCTCACACTCAGATCATC-3′	5′-GAACCTGGGAGTAGATAAGG-3′
IL-1β	5′-CAGAAGAATCTAGTTGTCCGTG-3′	5′-CATCAATGAAAGAACTCAGTGC-3′
I-κBα	5′-GGAAGTGATTGGTCAGGTGAAG-3′	5′-GAGTCAAGACTGCTACACTG-3′

ring after focal cerebral ischemia, initiating inflammatory reactions by over-expressing pro-inflammatory factors, thus contributing largely to post-ischemic brain infarction[24–26]. Astrocytes subjected to O/R, a model simulating inflammatory responses in the ischemic core, showed a prominent increase in the mRNA levels of TNF-α and IL-1β and the protein levels of COX-2, which were attenuated by the addition of 0.5 μmol/L T33 (Figure 1, $P<0.05$).

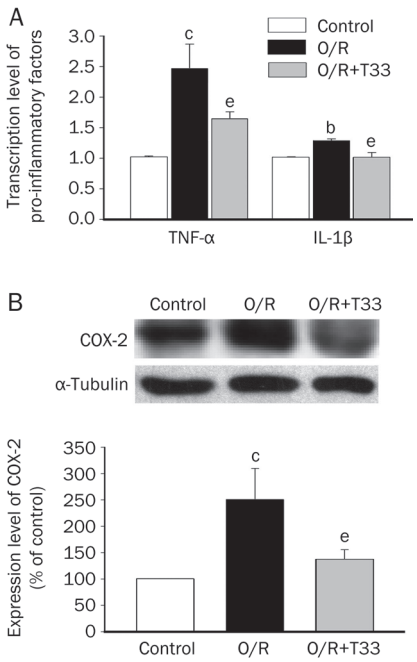

Figure 1. Anti-inflammatory effects of T33 (0.5 μmol/L) on primary astrocytes subjected to O/R. (A) T33 reduced the mRNA levels of TNF-α and IL-1β. $n=4$. (B) T33 reduced the protein level of COX-2 in astrocytes subjected to O/R. $n=6$. $^bP<0.05$, $^cP<0.01$ *vs* control group; $^eP<0.05$ *vs* O/R group.

Since the expression of inflammatory mediators is regulated by NF-κB in the nucleus[27], the effect of T33 on the levels of nuclear p65, a subunit of NF-κB, was subsequently analyzed. Compared to the vehicle-treated group, the nuclear protein levels of p65 were reduced in T33-treated astrocytes subjected to O/R or OGD (Figure 2A and 2B, $P<0.05$). Since the nuclear protein levels of p65 are modulated by the inhibitory protein I-κBα, further research was conducted on I-κBα in astrocytes subjected to O/R or OGD. The protein levels of I-κBα declined in astrocytes subjected to O/R or OGD ($P<0.01$) and were recovered by treatment with T33 (Figure 2C and 2D, $P<0.05$).

I-κBα, like other proteins, is regulated at multiple levels, including transcription, translation and degradation. The mRNA levels of I-κBα dramatically declined after 4 h of OGD (Figure 3A, $P<0.05$) and self-restored after 24 h of reperfusion to a level higher than that in control astrocytes (Figure 3A, $P<0.05$). T33 rescued I-κBα mRNA levels in astrocytes subjected to 4 h of OGD (Figure 3A, $P<0.01$), but there was no

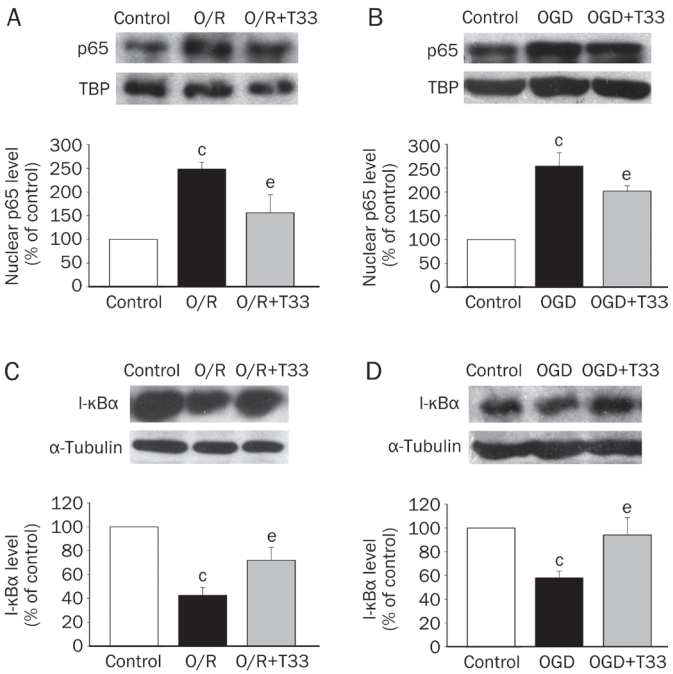

Figure 2. Modulation of the I-κBα/NF-κB pathway by T33 (0.5 μmol/L) in astrocytes subjected to O/R (A, C) ($n=5$) or OGD alone (B, D) ($n=4$). $^cP<0.01$ *vs* control group; $^eP<0.05$ *vs* correspondent OGD or O/R group.

change after reperfusion.

Phospho-IKKβ catalyzes the dual phosphorylation of I-κBα on serines 32 and 36, leading to the degradation of I-κBα. A distinct elevation of phospho-IKKβ levels was observed in astrocytes subjected to 4 h of OGD (Figure 3B, $P<0.05$), but there were no dramatic changes after 24 h of reperfusion (Figure 3B, $P>0.05$). T33 did not influence the phosphorylation of IKKβ in astrocytes subjected to OGD or O/R.

These results suggest that a severe transcriptional deficiency and accelerated degradation account for the reduced I-κBα protein levels in astrocytes subjected to OGD, but neither transcription nor degradation contributes to the drop in I-κBα protein levels in astrocytes subjected to O/R. eIF-2α, a factor controlling mRNA translation in eukaryotes, was found to be over-phosphorylated in astrocytes subjected to O/R (Figure 3C, $P<0.01$), indicating that mRNA translation was disrupted during reperfusion. T33 alleviated phosphorylated eIF-2α levels, restoring mRNA translation during reperfusion (Figure 3C, $P<0.05$).

Given the role of PPARγ in regulating inflammatory reactions, further detection of PPARγ activity was carried out. As indicated by EMSA analysis, PPARγ activity was dramatically impaired after 4 h of OGD in astrocytes (Figure 3D, lane 4; Figure 3E, $P<0.01$), and T33 restored the majority of the activity (Figure 3D, lane 3; Figure 3E, $P<0.01$). There were little changes in PPARγ activity in astrocytes subjected to O/R compared to astrocytes cultured under normal conditions (Figure 3D, lane 5–9).

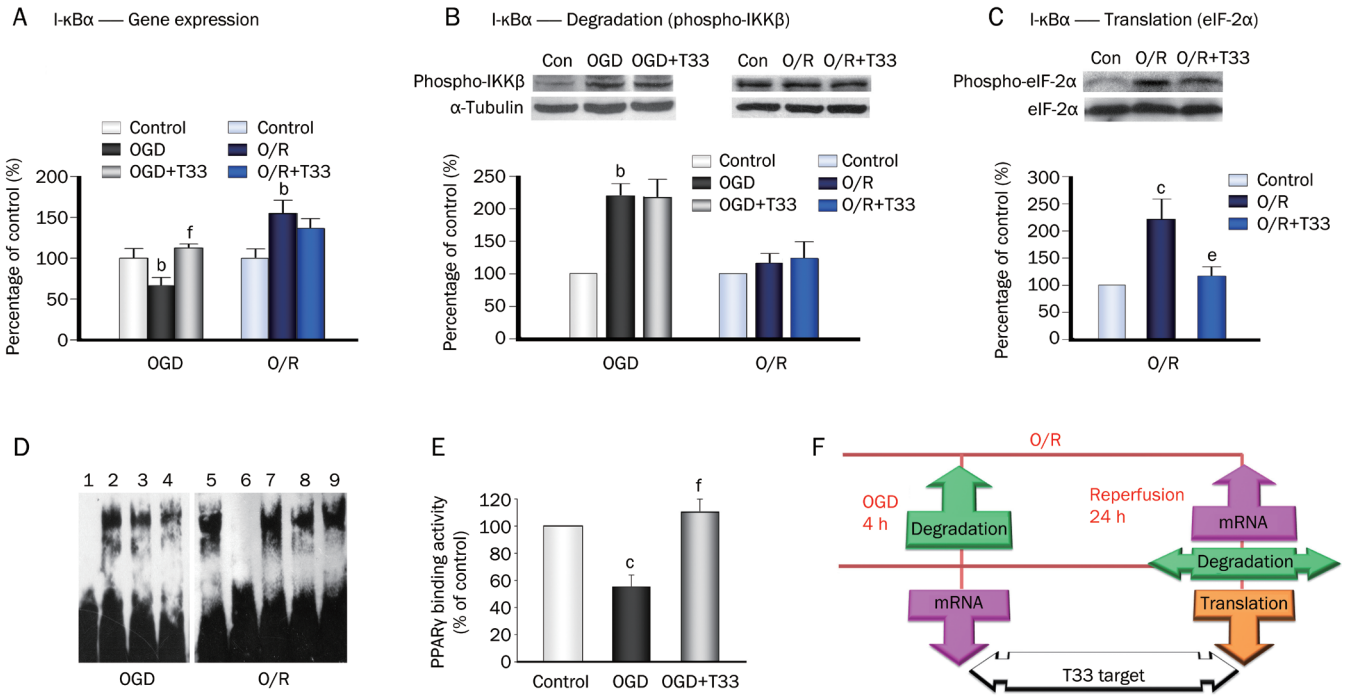

Figure 3. Effects of T33 (0.5 μmol/L) on the gene transcription (A), degradation (B), and translation (C) of I-κBα in primary atrocytes subjected to OGD or O/R (n=4). (D) A representative picture shows the effect of T33 (0.5 μmol/L) on PPARγ binding activity in primary atrocytes subjected to OGD or O/R. [Lane 1) positive control+200-fold cold probe; 2) control sample; 3) OGD+T33 sample; 4) OGD sample; 5) positive control; 6) positive control+200-fold cold probe; 7) control sample; 8) O/R sample; 9) O/R+T33 sample]. (E) Statistical analysis of PPARγ binding activity is shown (n=3). (F) A generalization of the multi-level regulation of I-κBα in ischemic core models and the T33 target is shown. ^{b}P<0.05, ^{c}P<0.01 *vs* control group; ^{e}P<0.05, ^{f}P<0.01 *vs* correspondent OGD or O/R group.

T33 alleviates hypoxia-induced inflammatory responses in BV-2 cells subjected to hypoxia

Since inflammation cascades in the penumbra facilitate its progression into infarction, the anti-inflammatory effects of T33 in models simulating the inflammatory responses in the penumbra were evaluated. We detected a marked increase in TNF-α levels (73.86 pg/mL) in the culture medium from BV-2 cells subjected to hypoxia compared to normoxia treated cells (6.87 pg/mL). Pre-treatment with T33 reduced the level of TNF-α to 25 pg/mL (Figure 4A, P<0.05), and GW9662 restored the content to 85.86 pg/mL (Figure 4A, P<0.05). Similar results were observed for the protein levels of COX-2, which were elevated in hypoxia-treated BV-2 cells compared to the normoxia treated group (Figure 4B and 4C, P<0.05) and were alleviated by treatment with T33 (Figure 4B and 4C, P<0.05). P38 phosphorylation, which mediates inflammatory responses in BV-2 cells subjected to hypoxia, was reduced by T33 pre-treatment (Figure 4B and 4D, P<0.01). The ameliorating effects of T33 on COX-2 and p-p38 levels were antagonized by pre-administration with GW9662 (COX-2: Figure 4C, P<0.05; p-p38: Figure 4D, P<0.01).

Anti-inflammatory and neuroprotective effects of T33 in rats subjected to MCAO

Given the strong anti-inflammatory activities of T33 in *in vitro* models, further *in vivo* evaluation of the compound in the MCAO model was necessary. The infarct area in all six consecutive coronal sections was reduced by the administration of T33 (Figure 5A, 5C, and 5D, P<0.05). Correspondingly, the neurological score was improved by T33 treatment (Figure 5B, P<0.01). In addition, inflammatory responses were investigated in ipsilateral (ischemic)/contralateral striatum and cortex. The transcriptional levels of TNF-α and IL-1β and the protein level of COX-2 were reduced in the ischemic cortex and striatum of rats treated with T33 (P<0.05, P<0.01), whereas the expression of these inflammatory mediators remained the same in the contralateral side (Figure 6). These neuroprotective and anti-inflammatory effects of T33 were reversed by pre-administration with GW9662 (P<0.05, P<0.01).

Discussion

Inflammatory mediators synthesized in astrocytes contribute largely to inflammatory reactions in the ischemic core. Consistent with previous findings[5, 28], we found that O/R induced an overexpression of TNF-α, IL-1β, and COX-2 in primary astrocytes. The ability of T33 to alleviate the increased expression of these inflammatory mediators could be ascribed at least in part to a reduction in the activation of NF-κB, a principal mediator of post-ischemic inflammatory responses[27, 29]. Inhibiting NF-κB activation by T33 was achieved by restoring the protein levels of I-κBα, which binds to NF-κB in the cytosol and masks the nuclear localization signal within the p65 sub-

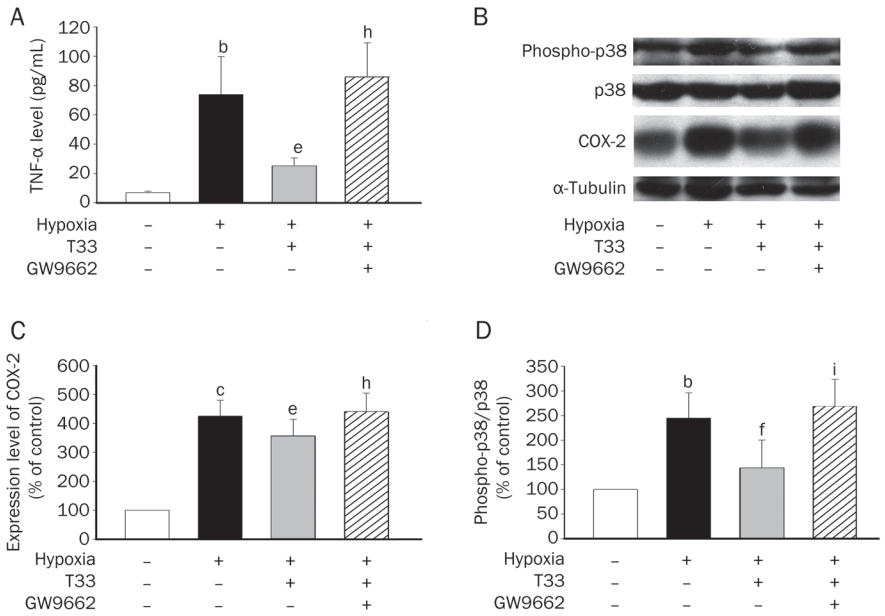

Figure 4. Anti-inflammatory effects of T33 (0.5 μmol/L) in BV-2 cells subjected to hypoxia. (A) The effects of T33 on TNF-α levels in culture medium are shown. (B, C, D) The effects of T33 on the protein levels of COX-2 and p38 phosphorylation are shown (B: representative Western blots; C, D: statistical results). n=4. Comparisons were made using a paired sample t-test. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control group; $^{e}P<0.05$, $^{f}P<0.01$ *vs* hypoxia group; $^{h}P<0.05$, $^{i}P<0.01$ *vs* hypoxia+T33 group.

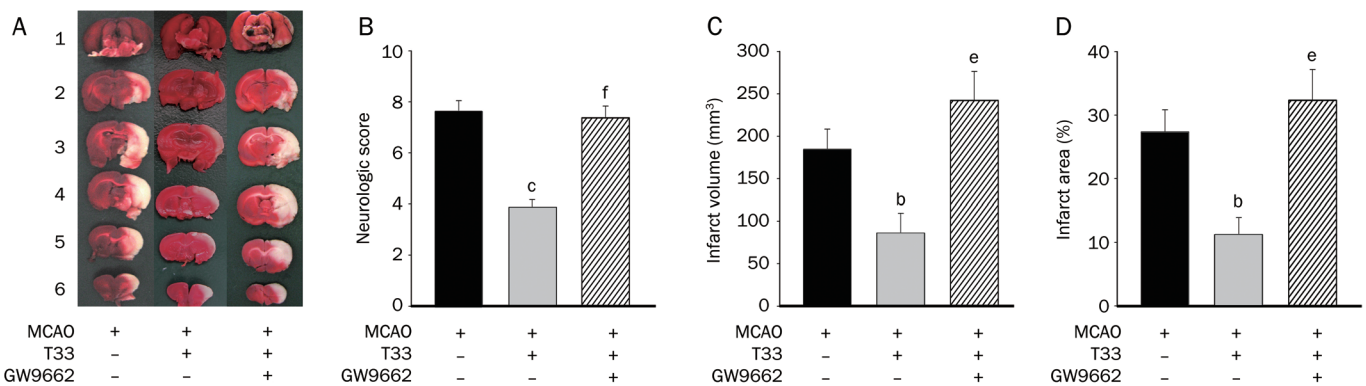

Figure 5. Neuroprotective effects of T33 (2 mg/kg) in rats subjected to MCAO. (A) T33 reduces cerebral infarct size. Six consecutive TTC-stained coronal brain slices are arranged in cranial to caudal order. (B) T33 alleviates the neurological deficits of rats subjected to MCAO. (C) T33 reduces total infarct volume (statistical results). (D) Infarct percentages are shown. n=8. $^{b}P<0.05$, $^{c}P<0.01$ *vs* MCAO group; $^{e}P<0.05$, $^{f}P<0.01$ *vs* MCAO+T33 group.

unit[30–32]. Both a transcriptional deficiency and an accelerated degradation contributed to the I-κBα protein content decline in astrocytes subjected to OGD, whereas after reperfusion, a decrease in mRNA translation resulted in a sharp reduction in I-κBα protein content. In contrast to other compounds that block I-κBα degradation[33], T33 suppressed the I-κBα/NF-κB pathway by restoring I-κBα synthesis. mRNA translation, found to be severely impaired in ischemic areas, was shown to be related to eIF-2α over-phosphorylation, which blocks the initiation of protein translation and leads to a depression of protein synthesis[34]. In areas vulnerable to ischemia, mRNA translation is persistently inhibited, even after reperfusion, which facilitates excessive toxic reactions and abrogates survival cascades, leading to the formation of the ischemic core[34]. Inhibiting eIF-2α over-phosphorylation restored I-κBα translation after incubation with T33[35]. In addition to ameliorating inflammatory responses, promoting mRNA translation might also rescue pathways that help cells survive and function normally, such as by restoring the expression of growth factors and down-stream molecules. These actions help to reduce the area of ischemic core. Therefore, by rescuing protein translation in the ischemic core model, T33 is potent in protecting areas vulnerable to ischemic injuries.

I-κBα mRNA levels were dramatically reduced in astrocytes subjected to OGD and were restored after reperfusion. Rescuing I-κBα gene transcription enabled T33 to restore I-κBα protein synthesis in astrocytes subjected to OGD and alleviate the activation of the I-κBα/NF-κB pathway. These results suggest that T33 regulates I-κBα protein levels via pleiotropic mechanisms, which involve both the genomic restoration of I-κBα transcription and the non-genomic restoration of I-κBα mRNA translation (Figure 3F).

Although an induction of PPARγ expression has been reported in transient cerebral ischemic models[36], the activ-

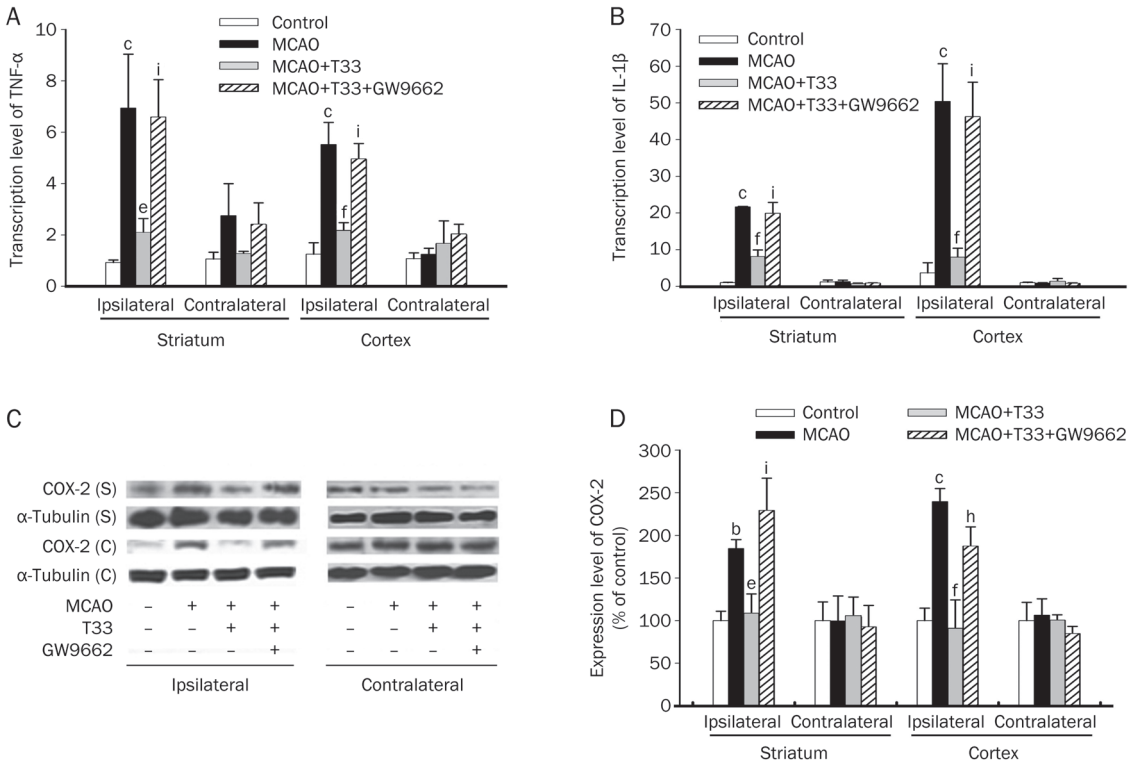

Figure 6. Anti-inflammatory effects of T33 (2 mg/kg) in rats subjected to MCAO. (A) T33 alleviates the mRNA levels of TNF-α in ischemic striatum and cortex, but not in contralateral striatum or cortex. (B) T33 alleviates the mRNA levels of IL-1β in ischemic striatum and cortex, but not in contralateral striatum or cortex. (C, D) T33 reduces the protein levels of COX-2 in ischemic striatum and cortex. n=4. ^{b}P<0.05, ^{c}P<0.01 *vs* control group; ^{e}P<0.05, ^{f}P<0.01 *vs* MCAO group; ^{h}P<0.05, ^{i}P<0.01 *vs* MCAO+T33 group.

ity of PPARγ was found to be severely defective upon ischemia[37]. The phenomenon that the severely depressed PPARγ activity levels in astrocytes subjected to OGD is restored to nearly normal levels after reperfusion might result from stress-induced self-protection. The activation of PPARγ by T33 at the beginning of OGD did not lead to a higher level of activity of PPARγ after reperfusion, which could be attributed to mitigated stress by the treatment of T33. Moreover, previous studies have suggested that the activation of PPARγ promotes the repression of the exaggerated expression of inflammatory mediators that occurs in response to transient cerebral ischemia[12, 38]. These actions of PPARγ could be mediated, at least in part, by inhibiting the I-κBα/NF-κB pathway, since I-κBα is a PPARγ target gene[39]. PPARγ activation has been shown to amplify I-κBα expression, negatively interfering with the NF-κB signaling cascade[40]. This notion was further confirmed by the present research, as the alterations in PPARγ activity during OGD and reperfusion paralleled in the changes in the mRNA levels of I-κBα. However, an elevation of gene transcription failed to rescue the protein levels of I-κBα after reperfusion. This phenomenon could be explained by the blunted I-κBα translation function mentioned previously and suggests that the self-recovery of PPARγ activity during reperfusion might be too late to exert further anti-inflammatory effects. Therefore, preserving PPARγ activity by T33 treatment from the start of OGD is crucial in reducing inflammatory responses during ischemia and could also contribute to the late curtailed inflammatory cascades during reperfusion in the current ischemic core model. This result coincides with a recent *in vivo* study demonstrating that the administration of TZDs after reperfusion does not exert a neuroprotective effect toward ischemia[41]. However, we cannot rule out that non-PPARγ dependent pathways may be involved in reserving I-κBα protein levels by T33, because it still remains unknown how PPARγ activation influences eIF-2α phosphorylation. Further studies are needed to clarify the precise mechanisms.

According to existing studies, inflammatory mediators synthesized in microglia upon activation contribute largely to ischemic brain injury, promoting neuronal death in the penumbra and the progression of penumbra into ischemic infarction[42, 43]. The phosphorylation and activation of p38 is of particular importance in regulating the production of inflammatory mediators induced by hypoxia[44, 45]. Treatment with specific inhibitors of p38 could blunt the inflammatory responses[46] and reduce brain injury induced by cerebral focal ischemia[47]. The potency of T33 in suppressing p38-mediated inflammatory reactions dependent on PPARγ provided additional evidence concerning the beneficial effects of this compound in treating hypoxic-ischemic brain injury. Although we focused on the activated inflammatory cascades, it is important to note that p38 over-phosphorylation is involved in other abnormal responses under pathological circumstances

like ischemia. Reactive oxygen species-induced injuries[48] and Aβ-induced neurotoxicity, for example, are also mediated by p38 over-phosphorylation. Since oxidative reactions stimulated soon after ischemia are toxic to neuronal cells, inhibiting p38 phosphorylation might alleviate brain injury by blocking oxidative reactions. Given that post-stroke dementia is associated with the generation of Aβ[49], we predict that the administration of T33 would not only alleviate acute ischemia-induced brain injury but also might reduce post-stroke dementia through the dual reduction of ischemia-induced inflammation and Aβ-induced neurotoxicity.

The above *in vitro* findings provide novel insight into the anti-inflammatory effects and mechanisms of T33 in ischemic core and penumbra models. Because PPARγ agonists are capable of modulating inflammatory responses by reducing the activation of p38 and NF-κB in activated glial cells[50], we hypothesized that PPARγ agonizing might contribute to the anti-inflammatory activity of T33 in an *in vivo* MCAO ischemic model. Since post-ischemic brain inflammation contributes largely to the formation of brain infarction, the neuroprotective effect of T33 was further evaluated. A vigorous neuroprotective effect was observed without affecting the CBF (data not shown). The neuroprotective effect of T33 was shown to be dependent on PPARγ activation and was comparable to the effects of other PPARγ agonists in ischemia-induced brain injuries[51, 52]. In addition, the dose of 2 mg/kg was comparable to that of TZDs in rat ischemic models, indicating that T33 could be neuroprotective at a clinically relevant dosage. The reason that a PPARα antagonist was not used in the present study was that the effects of T33 were completely blocked by a PPARγ antagonist. It seems that the current dosage might not be sufficient for PPARα to exert anti-inflammatory actions in the MCAO model, especially if T33 is administered systemically. These results suggest that T33 could be of therapeutic value for ischemic stroke.

Our study demonstrated for the first time that T33, a novel PPARγ/α agonist with potent neuroprotective activity in a rat transient ischemic model, exerts strong anti-inflammatory effects in ischemic core and penumbra models. These effects occurred via genomic and non-genomic regulation of I-κBα expression and p38 activation. The activation of PPARγ was required for most of the actions of T33, although it is possible that a non-PPARγ pathway might be partially involved. The present study provides a good example of the beneficial effects of T33 and encourages further development of PPARγ/α agonists for the treatment of ischemic stroke.

Acknowledgements

The authors are grateful to Prof Shu-min DUAN for providing the BV-2 cell line.

This research was funded by the National Natural Science Foundation of China (No 30801402), the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry, the National Science & Technology Major Project "Key New Drug Creation and Manufacturing Program" of China (No 2009ZX09301-001; 2009ZX09301-063) and the Shanghai Science and Technology Development Fund (No 10QA1408100).

Author contribution

Hai-yan ZHANG and Ying WANG designed the research; Ying WANG performed the research; Yu-she YANG provided T33; Ying WANG, Xi-can TANG, and Hai-yan ZHANG analyzed the results and wrote the paper.

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