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Original Paper

Nitro-Oleic Acid Attenuates OGD/R-Triggered Apoptosis in Renal Tubular Cells via Inhibition of Bax Mitochondrial Translocation in a PPAR-γ-Dependent Manner

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Key Words
OGD/R • Apoptosis • Mitochondria • Bax • Nitro-oleic Acid

Abstract
Background: Nitroalkene derivatives of oleic acid (OA-NO_2) serve as high-affinity ligand for PPAR-γ, which regulates apoptosis, oxidation and inflammation and plays a central role in ischemia-reperfusion injury. In the present study, we elucidated the protective mechanisms of OA-NO_2 against renal ischemia-reperfusion injury. ***Methods:*** HK-2 cells were subjected to oxygen and glucose deprivation followed by re-oxygenation (OGD/R) to mimic renal ischemia-reperfusion injury. Cell apoptosis was analyzed by flow cytometry. Bax mitochondrial translocation, cytochrome c and apoptosis-inducing factor (AIF) cytosolic leakage and Akt/Gsk 3β phosphorylation were evaluated by Western blotting. Bax activation was visualized by immunocytochemistry. GW9662 and siRNA transfection were employed to examine the involvement of PPAR-γ. ***Results:*** OGD/R injury promoted mitochondrial translocation and activation of Bax, leakage of cytochrome c and AIF, subsequent caspase-3 activation, and eventually cell apoptosis. Pre-incubation with OA-NO_2 (1.25 μM, 45min) inhibited Bax activation and blocked apoptotic cascade, while the protective effects were negated by GW9662 or PPAR-γ siRNA. Moreover, OA-NO_2 restored Akt and Gsk 3β phosphorylation in a PPAR-γ-dependent way. ***Conclusion:*** These findings suggest that OA-NO_2 attenuates OGD/R-induced apoptosis by inhibiting Bax translocation and activation and the subsequent mitochondria-dependent apoptotic cascade in a PPAR-γ dependent manner.

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Introduction

Renal ischemia-reperfusion(I/R) injury, which occurs in many clinical settings including renal transplantation, shock, and vascular surgery, is a major cause of acute renal failure. A number of pathologic processes contribute to renal I/R injury, including reduced renal blood flow; tubular obstruction induced by detached epithelium, infiltrated leukocytes and aggregated platelets [1, 2]; activation of pro-inflammatory cytokines, reactive nitrogen species (RNS) and reactive oxygen species (ROS) [2, 3]. These inter-related events cause renal cell injury and lead to eventual cell death. Activation of the apoptotic cell death cascade has been reported to play a key role in post-ischemic tissue damage and organ dysfunction; thus inhibition of apoptosis can ameliorate renal ischemia-reperfusion injury [4, 5].

Peroxisome proliferator-activated receptor γ (PPAR-γ) has emerged as a novel therapeutic target in a wide range of pathologies elicited by ischemia and reperfusion, such as myocardial infarction, ischemic stroke and acute kidney injury. The protective role of PPAR-γ in I/R injury is suggested by data showing that heterozygous PPAR-γ-deficient mice subjected to intestinal I/R suffer more pronounced injury than wild-type control mice [6] and that the PPAR-γ agonist, rosiglitazone, is able to control cell apoptosis and contribute to tissue protection [7-9]. Therefore, agents that regulate PPAR-γ activation are being actively pursued.

Nitrated unsaturated fatty acids (NO_2-FA) are derived from nitric oxide (NO) and NO_2^- dependent redox reactions with unsaturated fatty acids, including nitro-oleic acid (OA-NO_2) and nitro-linoleic acid (LNO_2) [10]. They mediate a broad range of anti-inflammatory, anti-oxidative and vascular-protective actions. NO_2-FA suppress pro-inflammatory gene expression and neutrophil infiltration by either direct nitroalkylation of the nuclear factor κB (NF κB) p65 subunit or inhibition of pro-inflammatory STAT signaling [11-13]. They exert vascular protection by up-regulating HO-1 and endothelial nitric oxide synthase (eNOS) expression in pulmonary epithelium and aortic endothelial cells [14-16]. NO_2-FA also activate heat shock factor (HSF) and nuclear factor-E2-related factor 2(Nrf2), mediators of antioxidant and phase II detoxifying enzymes [17]. Notably, NO_2-FA have been found to be robust endogenous activating ligands for all three PPARs, exhibiting the greatest potency as PPAR-γ agonists [18-20]. They possess a number of PPAR-γ-dependent effects, including promoting adipogenesis in 3T3-L1 pre-adipocytes, inducing CD36 receptor expression in macrophages [18, 19] and inhibiting NF-κB activation in inflammatory disease [12, 21, 22].

The evidence to date suggests that NO_2-FA could reduce cardiac infarct size and enhance cell vitality of cardiomyocytes after ischemia-reperfusion injury both *in vivo* and *in vitro* [23]. We previously showed that OA-NO_2 attenuated functional and histological indices in a mouse model of renal I/R injury [24]. These results suggested protective effects of NO_2-FA against ischemia-reperfusion injury. However, it is unclear whether NO_2-FA can alleviate renal I/R injury via directly regulating cell apoptotic signaling. Therefore, we evaluated whether OA-NO_2 could protect renal tubular cells from mitochondria-mediated apoptosis triggered by combined oxygen and glucose deprivation/re-oxygenation (OGD/R), a widely used *in vitro* model of I/R injury, and determined whether the mechanism was PPAR-γ-dependent.

Materials and Methods

Cell culture and OGD/R protocol, cell treatments

Normal human kidney epithelial (HK-2) cells (CCTCC, Wuhan, China) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, NY, USA), 4 mM glutamine, 100 IU·mL^{-1}penicillin / streptomycin. One day before the assays, $5 * 10^3$ or $2 * 10^5$ cells per well were plated into 96 or 6-well tissue culture plates and cultured at 37°C in a humidified atmosphere with 5% CO_2.

The OGD/R protocol was performed as previously described [25-28] with a slight modification. Confluent HK-2 cells were made quiescent by serum deprivation for 24 hours and then cultured for

the indicated periods of time in DMEM containing no glucose (M&C GENE, Beijing, China) in a hermetic incubator (Hitech biotechnology, Guangzhou, China) with an anaerobic environment (85%N_2, 10%H_2, 5%CO_2). Re-oxygenation was performed in complete medium containing 5.5 mM glucose and a humidified atmosphere with 5% CO_2 at 37°C. Cells maintained at normal culture condition (complete culture medium, 5% CO_2 atmosphere, 37°C) with or without ethanol and/or dimethyl sulfoxide (DMSO) treatment served as controls.(Fig. 1A)

In the pre-treatment assays, cells were treated with OA-NO_2 (Cayman chemical, Ann Arbor, USA) or oleic acid (OA) (Sigma-Aldrich, St. Louis, USA) dissolved in ethanol for 45 min and GW9662 (Sigma-Aldrich, St. Louis, USA), a PPAR-γ inhibitor, dissolved in DMSO for 1 h before the OGD/R injury and persisted for the whole process of OGD/R [18, 19]. The final concentration of ethanol and DMSO did not exceed 0.1% (v/v). OA-NO_2 used in the study was an equimolar distribution of 9- and 10-nitro-octadeca- 9-enoic acid [17].

CCK-8 assay

Cell viability was determined with a cell counting kit (CCK)-8 (Dojindo Laboratories, Kumamoto, Japan), following the manufacturer's instructions. The absorbance was measured at 450 nm using the MULTISKAN MK3 spectrophotometer (Thermo scientific, NY, USA).

Flow cytometry analysis of apoptosis

Cell apoptosis was measured with Annexin V-FITC (Roche Diagnostics, Mannheim, Germany) and 7-AAD (ebio-science, San Diego, USA) in un-transfected cells, and PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, USA) was used to detect cell apoptosis in PPAR-γ siRNA/control siRNA transfected cells. According to the manufacturer's instructions, cells were incubated with 100 μL binding buffer containing 5 μL Annexin V and 5 μL 7-AAD in the dark for 15 min at room temperature. Cell apoptosis was analyzed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, USA).

Hoechst staining

After stress, HK-2 cells were fixed with 4% paraformaldehyde for 30 min at room temperature, and then incubated with Hoechst 33342 (5 $\mu g \cdot mL^{-1}$, AMRESCO, Solon, OH, USA) at 37°C for 20 min. Fluorescent-labelled incisive cells were photographed with a Leica fluorescence microscope.

Immunocytochemistry

After transfection and/or stress, cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.3% Triton X-100 for 5 min, blocked with 5% BSA in PBS for 1 h at room temperature and then incubated for 20 h at 4°C with 6A7 Bax monoclonal antibody [29] (1:200, Alexis Biochemicals, San Diego, USA) diluted in PBS. After incubation, the cells were washed three times with PBS and incubated with Rhodamine (TRITC)-conjugated secondary antibody (ZSGB, Beijing, China) for 1 h at 37°C. After being stained with DAPI for 5 min at room temperature, cells were adhered to glass slides in anti-fade mounting medium (Beyotime, Shanghai, China). For detection of Bax co-localization with mitochondria, cells were incubated with Mito-tracker Green (500 nM) (Beyotime, Shanghai, China) for 30 min at 37°C. Cells were then fixed and exposed to 6A7 monoclonal antibody, and routine immunocytochemistry was performed. Fluorescent images were captured using Leica fluorescence microscopy. Quantitation of active Bax immuno-reactivity was performed by determining the percentage of HK-2 cells that showed positive punctate staining with the 6A7 monoclonal antibody.

Subcellular fractionation and Western blotting analysis

After transfection and/or stress, whole cell lysates of HK-2 cells were obtained using total protein extraction kit (DBI Bioscience, Ludwigshafen, Germany) according to the manufacturer's instructions, and the protein concentration was quantified using the Bradford method (Beyotime, Shanghai, China). The samples were separated in 12% SDS-polyacrylamide gels, transferred onto polyvinylidene difluoride membranes (Millipore, MA, USA) and incubated overnight at 4°C with primary antibodies. After incubation with horseradish peroxidase-conjugated secondary antibody (1:5000, ZSGB, Beijing, China) for 2 h at room temperature in 5% skimmed milk/TBST, proteins were detected by enhanced chemiluminescence (Millipore, MA, USA). Primary antibodies used in this study were: rabbit polyclonal antibody to PARP, caspase-3, Cleaved caspase-3, Bcl-2, Akt, Ser473-phospho-Akt (1:1000; CST, Boston, USA); PPAR-γ (1:500; Santa Cruz

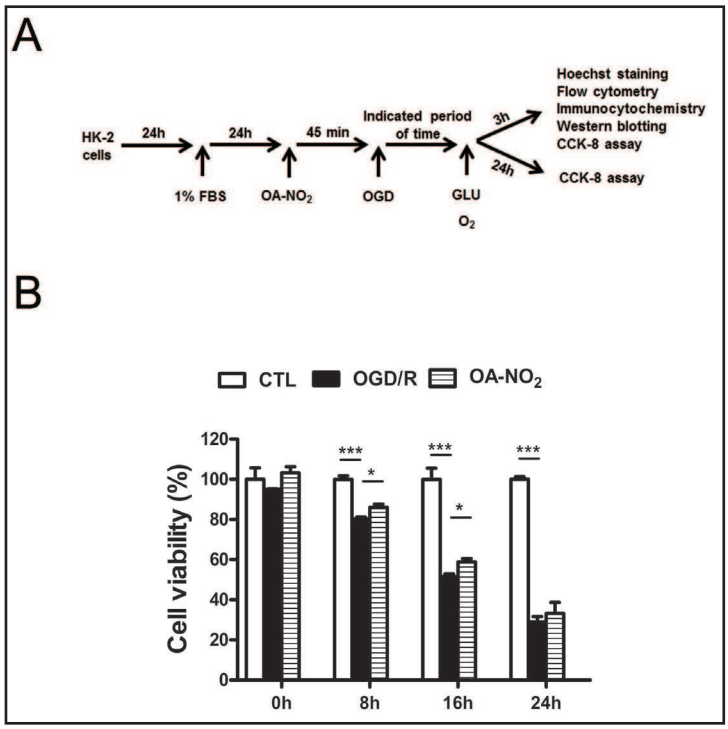

Fig. 1. Oxygen and glucose deprivation/re-oxygenation (OGD/R) gradually decreased viability in HK-2 cells. A: Confluent HK-2 cells were subjected to oxygen-glucose deprivation (OGD) with or without OA-NO_2 pretreatment for the indicated period of time to mimic ischemia. Reperfusion injury was imitated by resupplying glucose and oxygen for 24 h. B: Confluent HK-2 cells were subjected to OGD for 8 h, 16 h or 24 h followed by 24h-recovery with or without OA-NO_2 (1.25 μM) pretreatment. Viability was determined by CCK-8 assay. Each assay was representative of three independent experiments. Data were shown as means ±SEM; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Biotechnology, Dallas, USA); Bax (1:1000; Abcam, Cambridge, UK); cytochrome c (1:400; immunoway, Newark, USA); AIF (1:1000, proteintech, Chicago, USA); β-actin (1:800; Bioworld, Minnesota, USA); rabbit monoclonal antibody to Gsk 3β, Ser9-phospho-Gsk-3β, COX IV (1:1000; CST, Boston, USA).

In some experiments, fractionation of the cell lysates into cytosolic and mitochondrial fractions was performed using a mitochondria isolation kit (Beyotime, Shanghai, China) according to the manufacturer's instructions before the Western blotting.

RNAi transfection

For transfection, the fifth or sixth passage HK-2 cells were planted at a concentration of 4–5 × 10^4/ml. When they reached 30%–50% confluence, the media was replaced with enhanced infection solution (Genechem, Shanghai, China) supplemented with recombinant lentivirus containing PPAR-γ siRNA (CAACAGACAAATCACCATT) or control siRNA (TTCTCCGAACGTGTCACGT) (Genechem, Shanghai, China) at a multiplicity of infection (MOI) of 50. After 10 h transfection, the media was replaced with common media. After being cultured for another 120 h in complete medium, cells were harvested to evaluate the protein contents of PPAR-γ.

Statistical analysis

Data were presented as means ± SEM. Comparisons of results were performed with Student's t-test or one-way ANOVA followed by Dunnett's Multiple Comparison Test. $p < 0.05$ was considered statistically significant. SPSS 17.0 was used for all calculations.

Results

Oxygen and glucose deprivation/re-oxygenation (OGD/R) gradually decreased viability in HK-2 cells

HK-2 cells were subjected to combined deprivation of oxygen and glucose for 8 h, 16 h or 24 h followed by a re-oxygenation period of 24 h to mimic renal ischemia-reperfusion injury *in vitro* (Fig. 1A). The cell viability gradually declined as the OGD time was prolonged. The survival rates were 80.27±0.96%, 51.62±1.37% and 29±2.69% after 8 h, 16 h or 24 h of OGD followed by 24 h of re-oxygenation, respectively (Fig. 1B).

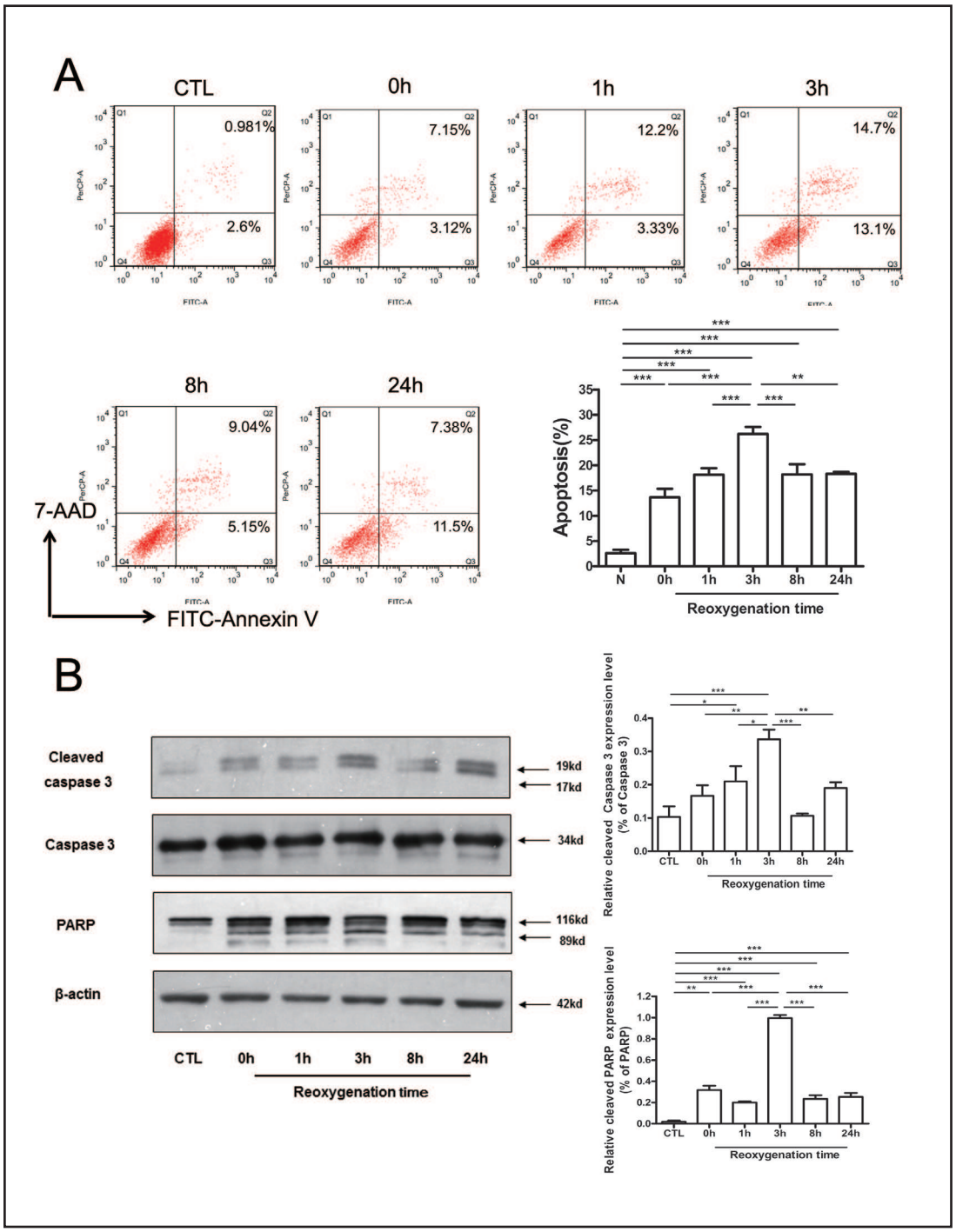

Fig. 2. OGD/R induced cell apoptosis. Confluent HK-2 cells were subjected to OGD for 16 h followed by an aerobic recovery period of 0 h, 1 h, 3 h, 8 h, or 24 h, respectively. A: Apoptotic cells were analyzed by flow cytometry. B: Cleaved caspase-3 and PARP expressions were examined by western blotting. Each assay was representative of three independent experiments. Data were shown as means ±SEM; *p < 0.05, **p < 0.01 and ***p < 0.001.

Apoptosis of HK-2 cells was then analyzed by flow cytometry after 16 h of OGD followed by 0 h, 1 h, 3 h, 8 h and 24 h of re-oxygenation. The apoptotic rate was only 13.67±1.70% without re-oxygenation, but increased in the following hours and peaked at 26.2±1.42%

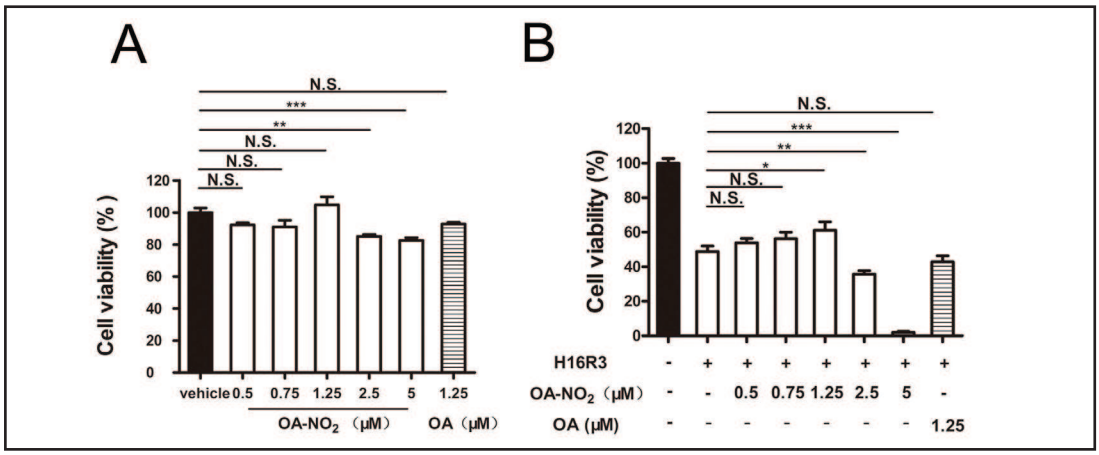

Fig. 3. OA-NO_2 but not OA improved cell viability after OGD/R injury. A: Confluent HK-2 cells were treated with OA-NO_2 (0.5- 5 μM) or OA (1.25 μM) for 20 h. Cytotoxicity of OA-NO_2 was determined by CCK-8 assay. B: Confluent HK-2 cells were subjected to OGD for 16 h followed by 3 h re-oxygenation period (H16R3) with or without OA-NO_2 (0.5-5 μM) or OA (1.25 μM) pretreatment. Viability was determined by CCK-8 assay. Each assay was representative of three independent experiments. Data were shown as means ±SEM; *p<0.05, **p<0.01 and ***p<0.001, N.S. meant no significance.

at 3 h of re-oxygenation. The rate subsequently declined to 18.20±2.00%, and remained at 18.31±0.40% by the end of the 24h-long recovery (Fig. 2A).

The time course of active caspase-3 and cleaved PARP during reperfusion was further investigated (Fig. 2B). Cleaved caspase-3 appeared instantly after OGD injury, reached peak levels by 3 h of re-oxygenation and was detectable for several hours afterwards. Accordingly, the expression of 89 kDa PARP fragment followed a profile similar to that of the cleaved caspase-3.

Overall, these data indicated that cell viability declined as duration of OGD increased and cell apoptosis occurred during the whole re-oxygenation period, peaking at 3 h. Thus 16 h of OGD followed by 3 h of re-oxygenation was selected as the standard assay condition for studying the OGD/R-induced apoptosis.

OA-NO_2 but not OA improved cell viability after OGD/R injury

The CCK-8 assay showed that proliferation was significantly inhibited in HK-2 cells treated with OA-NO_2 (2.5 or 5 μM), but was not affected at lower concentrations (0.5, 0.75 or 1.25 μM) (Fig. 3A).

Pretreatment with OA-NO_2 (1.25 μM) for 45 min dramatically increased cell viability from 48.88±3.13% to 61.17±4.90% (p<0.05) after 16h-OGD followed by 3h-re-oxygenation. In contrast, the native fatty acid precursor of OA-NO_2, oleic acid (OA), did not show a protective effect (Fig. 3B).

This finding suggested that the protective effect of OA-NO_2 in OGD/R injury was attributable to the nitration of the fatty acid. Based on this, we selected OA-NO_2 (1.25 μM) to examine its protective effect in the following assays.

OA-NO_2 protected HK-2 cells against OGD/R-induced apoptosis

The potential for OA-NO_2 to protect against OGD/R-induced apoptosis was tested with flow cytometry. Pretreatment with OA-NO_2 for 45 min reduced cell apoptosis from 27.11±1.29% to 9.73±0.59% (p<0.001) after 16h-OGD followed by 3h-re-oxygenation (Fig. 4A).

The nuclei morphology was also visualized by Hoechst 33342 staining. Under normoxia, the nuclei appeared relatively large and faintly stained. After 16h-OGD followed by 3h-re-oxygenation, the nuclei appeared to be condensed, fragmented or heterogeneous. OA-NO_2 restored the nuclei shape after OGD/R injury (Fig. 4B).

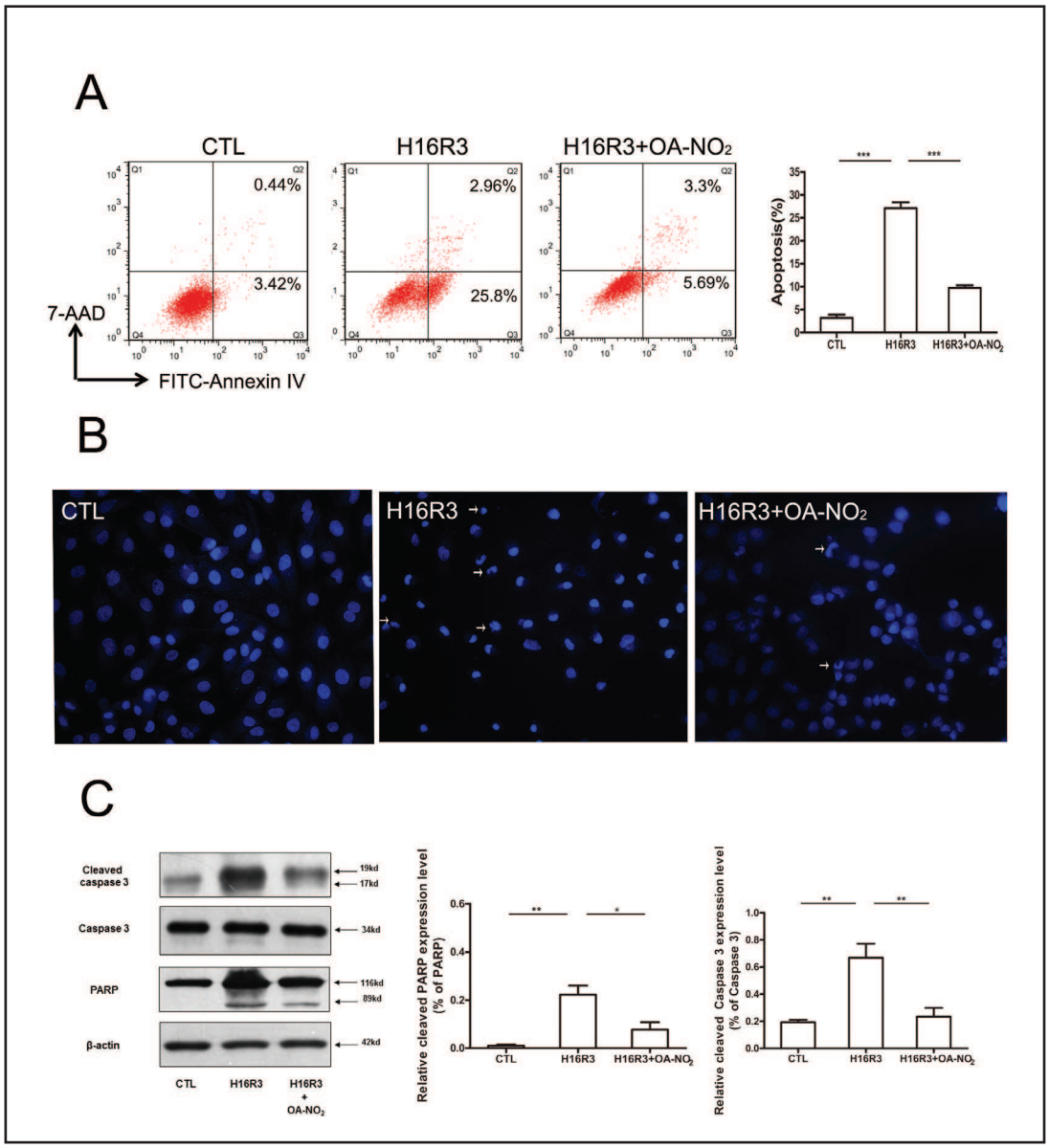

Fig. 4. OA-NO_2 protected HK-2 cells against OGD/R-induced apoptosis. Confluent HK-2 cells were subjected to OGD for 16 h followed by 3 h re-oxygenation period (H16R3) with or without OA-NO_2 (1.25 μM) pretreatment. A: Apoptotic cells were analyzed by flow cytometry. B: Nuclei morphological changes were visualized by Hoechst 33342 staining. The arrows indicated the condensed or fragmented nuclei. Magnification: x 200. C: Cleaved caspase-3 and PARP expressions were examined by western blotting. Each assay was representative of three independent experiments. Data were shown as means ±SEM; *p<0.05, **p<0.01 and ***p<0.001, N.S. meant no significance.

In accordance with the results of flow cytometry and Hoechst staining, Western blotting showed that OA-NO_2 reduced the expression of cleaved caspase-3 and cleaved PARP induced by 16h-OGD and 3h-re-oxygenation (Fig. 4C). Above all, these results indicated that OA-NO_2 could exert its renal protection via inhibiting apoptosis induced by OGD/R injury.

OA-NO_2 inhibited OGD/R-induced Bax translocation, activation and subsequent Mitochondrial Outer Membrane Permeabilization (MOMP)

Bax is a key pro-apoptotic factor. It permeabilizes the mitochondrial outer membrane and induces leakage of cytochrome c and AIF into the cytosol upon activation [30]. Here,

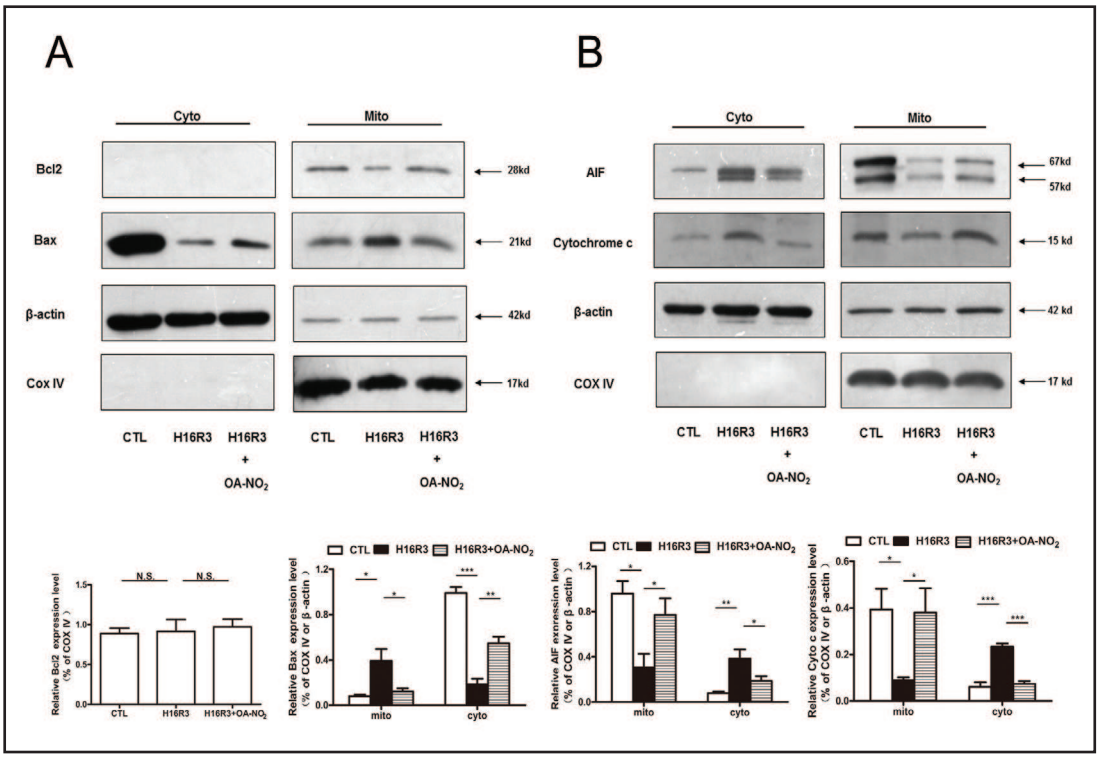

Fig. 5. OA-NO_2 inhibited OGD/R induced Bax translocation and subsequent Mitochondrial Outer Membrane Permeabilization (MOMP). Confluent HK-2 cells were subjected to OGD for 16 h followed by 3 h re-oxygenation period (H16R3) with or without OA-NO_2 (1.25 μM) pretreatment. Expressions of Bax, Bcl-2, cytochrome c and AIF in mitochondrial (Mito) and cytosolic (Cyto) fractions were examined by western blotting. Each assay was representative of three independent experiments. Data were shown as means ±SEM; *p < 0.05, **p < 0.01 and ***p < 0.001, N.S. meant no significance.

we analyzed Bax and Bcl-2 expression in cytosolic and mitochondrial fractions. OGD/R injury markedly promoted the translocation of Bax from cytosol to mitochondria. OA-NO_2 pretreatment inhibited this process with no discernible change in the protein level of Bcl-2 (Fig. 5A). OA-NO_2 significantly decreased the subsequent mitochondrial cytochrome c and AIF leakage caused by OGD/R injury (Fig. 5B).

In addition, we co-localized Bax with mitochondria, using active Bax to confirm the effect of OA-NO_2 on Bax activation. The 6A7 monoclonal antibody recognized only the activated/pro-apoptotic form of Bax [29], which was stained red with Rhodamine (TRITC). Mitochondria were labeled with Mito-tracker green. In normal culture conditions, the 6A7 staining was almost undetectable, while OGD/R injury increased active Bax immunoreactivity. Moreover, the 6A7 staining often coincided with the condensed and/or fragmented chromatin. OA-NO_2 significantly reduced the conformational activation of Bax and the apoptotic morphological changes of the nuclei (Fig. 6). These data indicated that OA-NO_2 attenuated OGD/R-induced apoptosis via inhibiting Bax mitochondrial translocation and activation.

OA-NO_2 restored Akt and Gsk 3β phosphorylation in OGD/R injury

We evaluated whether Akt activation was involved in the protection of OA-NO_2. OGD/R injury dramatically decreased Akt phosphorylation ($p<0.001$) without altering total Akt content, whereas OA-NO_2 normalized the Ser473 phosphorylated Akt level. OA-NO_2 also preserved Ser9 phosphorylation of Gsk 3β, an Akt substrate [31, 32] after OGD/R injury (Fig. 7).

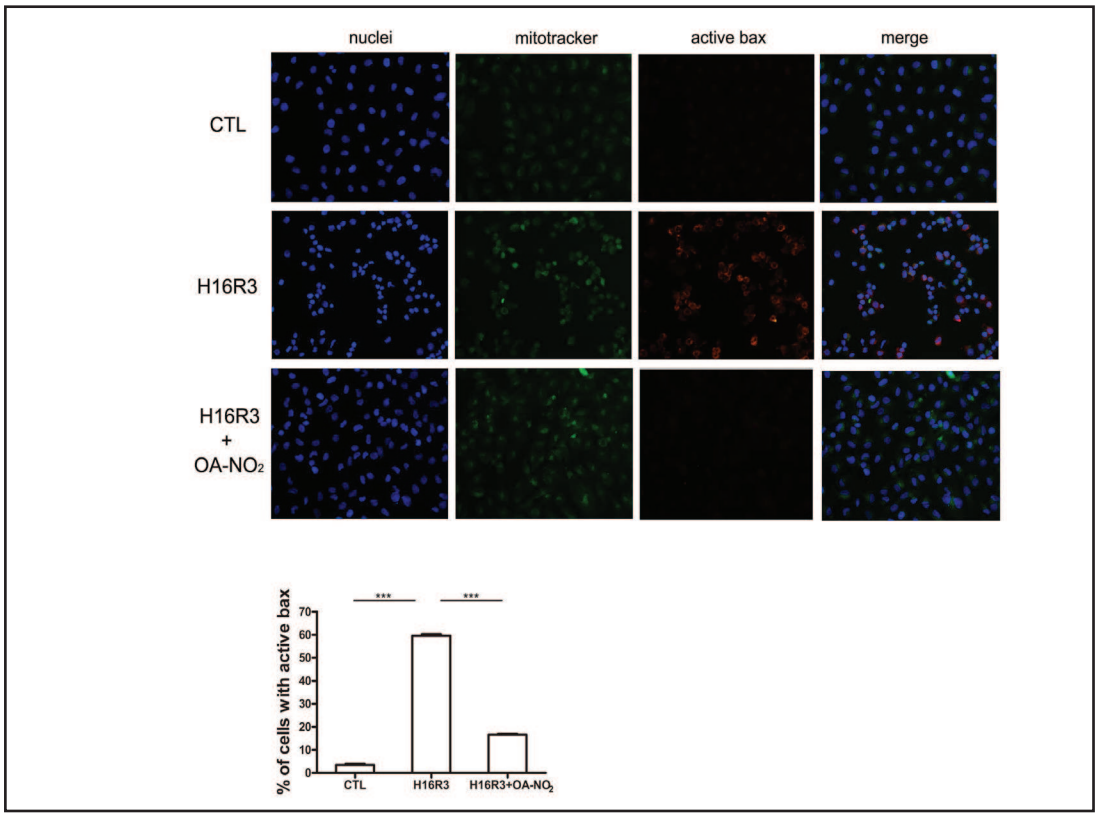

Fig. 6. OA-NO_2 inhibited OGD/R induced Bax activation. Confluent HK-2 cells were subjected to OGD for 16 h followed by 3 h re-oxygenation period (H16R3) with or without OA-NO_2 (1.25 μM) pretreatment. Co-localization of active Bax (red) and Mitochondria (green) was visualized by fluorescence microscope using the 6A7 monoclonal antibody and Mito-tracker Green; nuclei were stained with DAPI (blue). Magnification: x 200. Active Bax immunoreactivity was quantitated in ~300 cells per condition per experiment. Each assay was representative of three independent experiments. Data were shown as means ±SEM; ***p<0.001.

GW9662 and PPAR-γ siRNA abolished the protective effects of OA-NO_2 against OGD/R induced injury

To evaluate the role of PPAR-γ in OA-NO_2-mediated cyto-protection, the specific PPAR-γ antagonist GW9662 (Fig. 8A, B and C) and PPAR-γ siRNA (Fig. 8D, E and F) were studied. The PPAR-γ siRNA successfully suppressed PPAR-γ protein expression by 55% (Fig. 8D) and induced a slight reduction in cell proliferation, compared to the control siRNA. Neither GW9662 nor PPAR-γ siRNA/control siRNA affected the apoptotic rate of cell under normoxia (Fig. 8C, F). However, the protective effects of OA-NO_2 were completely abolished after pretreatment with GW9662 for 1 h or infection with PPAR-γ siRNA before the OGD/R assay, as indicated by declined cell viability (Fig. 8B, E) and increased apoptosis (Fig. 8C, F). Moreover, GW9662 or PPAR-γ siRNA largely eliminated the action of OA-NO_2 on phosphorylated Akt and Gsk 3β. Meanwhile, similar profiles were observed in apoptotic biomarkers (Fig. 9A, B). Taken together, these data suggested that OA-NO_2 exerted its anti-apoptotic effects via a PPAR-γ-dependent mechanism.

GW9662 and PPAR-γ siRNA eliminated the suppression of OA-NO_2 on Bax activation

To confirm that suppression of Bax activation by OA-NO_2 was also mediated via PPAR-γ, HK-2 cells were pre-incubated with GW9662 for 1 h or transfected with PPAR-γ siRNA/control siRNA and then subjected to the standard OGD/R assay. Active Bax was barely detected in OA-NO_2- or/and GW9662-, PPAR-γ siRNA- or control siRNA-treated cells under normal culture conditions. However, Bax was activated intensively upon OGD/R induction in

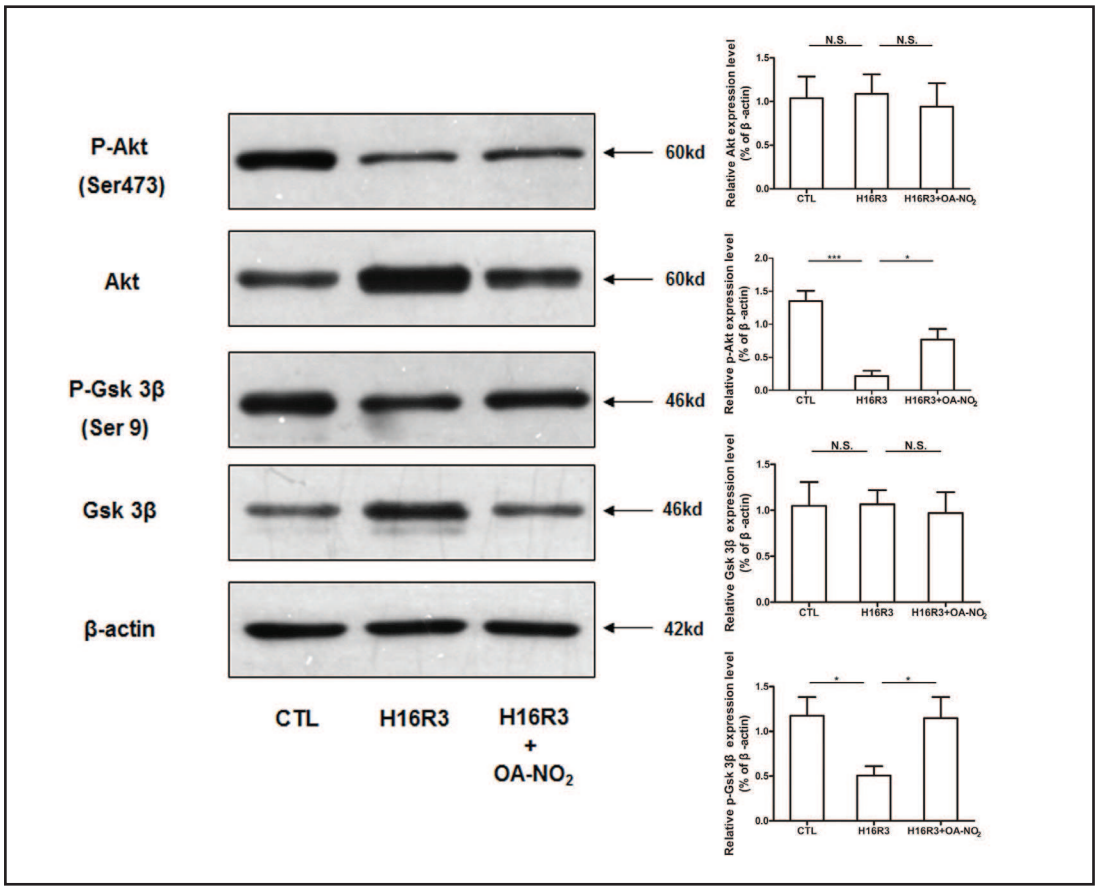

Fig. 7. OA-NO_2 preserved the phosphorylation of Akt and Gsk 3β after OGD/R. Confluent HK-2 cells were subjected to OGD for 16 h followed by 3 h re-oxygenation period (H16R3) with or without OA-NO_2 (1.25 μM) pretreatment. Akt and Gsk 3β phosphorylation were examined by western blotting. Each assay was representative of three independent experiments. *p<0.05 and ***p<0.001, N.S. meant no significance.

cells pre-incubated with GW9662 or transfected with PPAR-γ siRNA in the presence of OA-NO_2 after 16h-OGD followed by 3h-re-oxygenation (Fig. 10). Additionally, the PPAR-γ siRNA itself induced more intense activation of Bax after OGD/R injury, compared to control siRNA ($p<0.05$) (Fig. 10B). This demonstrated that endogenous PPAR-γ played a vital role in OA-NO_2-mediated inhibition of Bax conformational activation.

Discussion

Nitrated unsaturated fatty acids are a novel series of NO•-derived anti-inflammatory and anti-oxidative lipid signaling molecules and have potent therapeutic benefits in acute tubular injury of different etiologies [24, 33, 34], while recent experimental evidence points to their therapeutic potential in directly promoting cell survival in cardiac ischemic infarction [23, 35, 36]. How OA-NO_2 modulates pro-survival signaling against renal ischemia-reperfusion injury is not yet fully understood. In this study, we showed that OA-NO_2 (1.25 μM) ameliorated OGD/R-induced apoptosis in HK-2 cells by inhibiting Bax mitochondrial translocation and thereby attenuating cytochrome c release and the subsequent caspase-3 activation.

Although renal failure after ischemia has traditionally been attributed to acute tubular necrosis, it is now clear that necrosis fails to account for the severity of organ impairment, especially in humans [37]. This implicates cell apoptosis as a potential key participant in

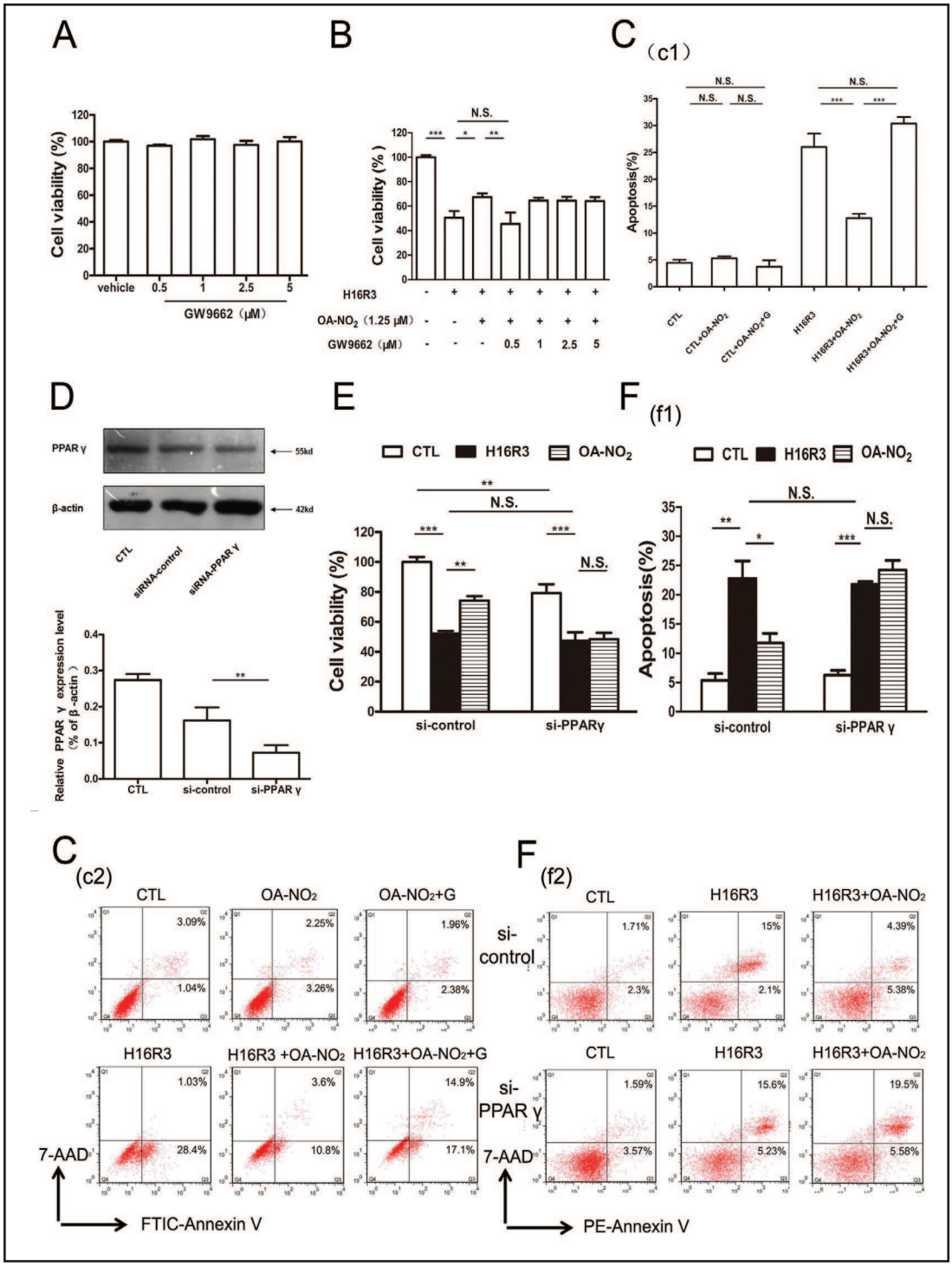

Fig. 8. GW9662 and PPAR-γ siRNA abolished the protective effects of OA-NO_2 against OGD/R induced injury. A: Confluent HK-2 cells were treated with GW9662 (0.5-5 μM) for 48 h. Cytotoxicity of GW9662 was determined by CCK-8 assay. D: Confluent HK-2 cells were exposed to PPAR-γ siRNA/control siRNA. Protein contents were determined by western blotting. Confluent HK-2 cells treated with GW9662 (G) (0.5 μM) or transfected with PPAR-γ siRNA/control siRNA were subjected to OGD for 16 h followed by 3 h re-oxygenation period (H16R3) in the presence of OA-NO_2 (1.25 μM) or not. B, E: Viability was determined by CCK-8 assay. C, F: Apoptotic cells were analyzed by flow cytometry. Each assay was representative of three independent experiments. Data were shown as means ±SEM; *p<0.05, **p<0.01 and ***p<0.001, N.S. meant no significance.

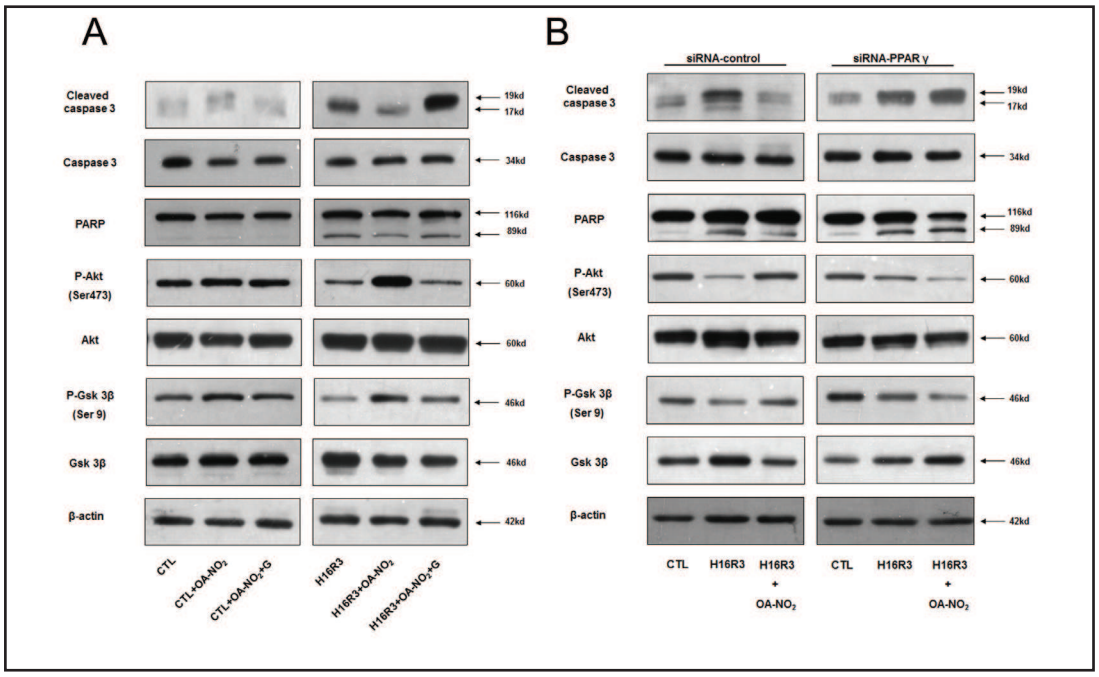

Fig. 9. GW9662 and PPAR-γ siRNA negated the suppressive effects of OA-NO_2 against OGD/R induced Akt inhibition, Gsk 3β and caspase-3 activation. Confluent HK-2 cells treated with GW9662 (G) (0.5 μM) or transfected with PPAR-γ siRNA/control siRNA were subjected to OGD for 16 h followed by 3 h re-oxygenation period (H16R3) in the presence of OA-NO_2 (1.25 μM) or not. Cleaved caspase-3, PARP expressions and Akt, Gsk 3β phosphorylation were examined by western blotting. Each assay was representative of three independent experiments.

organ failure. The contribution of apoptosis to acute organ injury in previous experiments was confounded by the evanescent nature of apoptotic cells and the stochastic nature of the apoptotic process [4]. Despite these limitations, caspases were activated during renal ischemia and reperfusion [38] and caspase inhibitors reduced renal impairment [39], indicating the importance of apoptosis in ischemic renal failure. In this study, we showed that cell apoptosis played a prominent role in OGD/R injury, especially during the re-oxygenation period (Fig. 2), as indicated by decreased cell viability (Fig. 3B), increased cell apoptosis (Fig. 4A), fragmented nuclei (Fig. 4B) and enhanced caspase-3 activity (Fig. 4C), while pretreatment with OA-NO_2 significantly enhanced cell viability and inhibited apoptosis in HK-2 cells with OGD/R injury. Consistent with our observations, NO_2-FA have been reported to induce mild uncoupling of mitochondria and to improve cardiomyocyte viability in isolated post-stimulate ischemia reperfusion cardiomyocytes [35]. Furthermore, long-term pretreatment with OA-NO_2 reduces myocardial infarct size and the density and distribution of TUNEL-positive cells within the area at risk in mice subjected to 30 min of myocardial ischemia and 24 h of *in vivo* reperfusion [23]. NO_2-FA also afford acute protection against I/R injury at the isolated heart level via ANT1-Cys^{57} nitroalkylation [36]. However, others have reported pro-apoptotic actions of NO_2-FA. NO_2-FA were reported to induce rat aortic smooth muscle cell apoptosis via activation of caspase-dependent pathways [40] and to robustly stimulate neutrophil apoptosis in pulmonary allergic inflammation [12]. The basis for these opposite actions of NO_2-FA is unclear, but several sources of variations such as cell types, experimental conditions, and concentrations of the ligands may account for the discrepant observations between the present study and previous reports. In this study, OA-NO_2 at relatively low concentrations (0.5-1.25μM) was protective [35] (Fig. 3), while at higher concentrations (> 5 μM), it was pro-apoptotic in cultured cells [40]. This might be due to the potential toxicity of this compound at high doses. In summary, this study demonstrates for

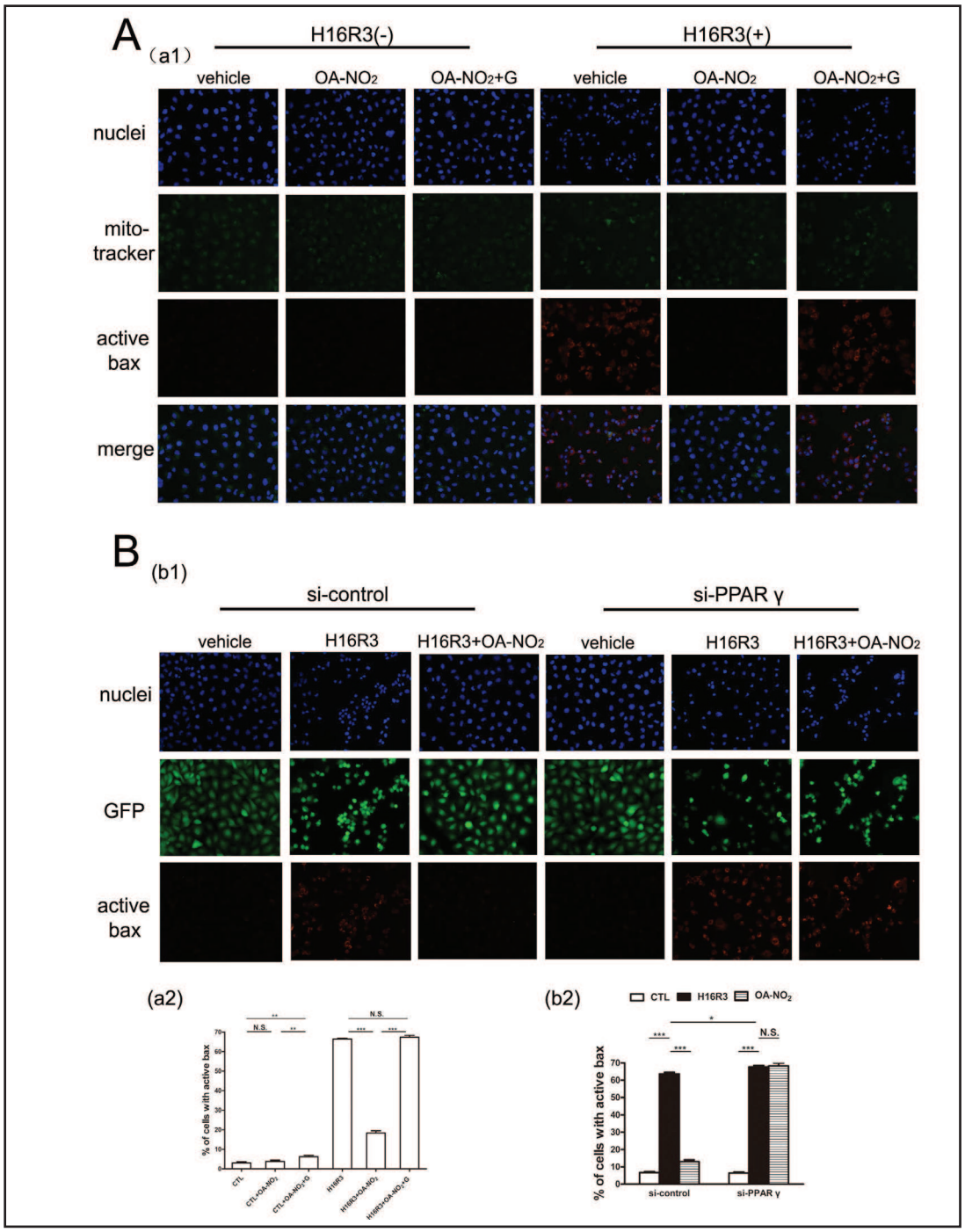

Fig. 10. GW9662 and PPAR-γ siRNA eliminated the suppression of OA-NO_2 on Bax activation. Confluent HK-2 cells treated with GW9662 (G) (0.5 μM) or transfected with PPAR-γ siRNA/control siRNA were subjected to OGD for 16 h followed by 3 h re-oxygenation period (H16R3) in the presence of OA-NO_2 (1.25 μM) or not. A: Co-localization of active Bax (red) and Mitochondria (green) was visualized by fluorescence microscope using the 6A7 monoclonal antibody and Mito-tracker Green in un-transfected cells; nuclei were stained with DAPI (blue). Magnification: x 200. B: Mitochondrial active Bax (red), cellular shrinkage (green) and nuclei (blue) were detected by fluorescence microscope in PPAR-γ siRNA/control siRNA transfected cells. Magnification: x 200. (a2 , b2) showed active Bax immunoreactivity quantitated in ~300 cells per condition per experiment. Each assay was representative of three independent experiments. Data were shown as means ±SEM; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, N.S. meant no significance.

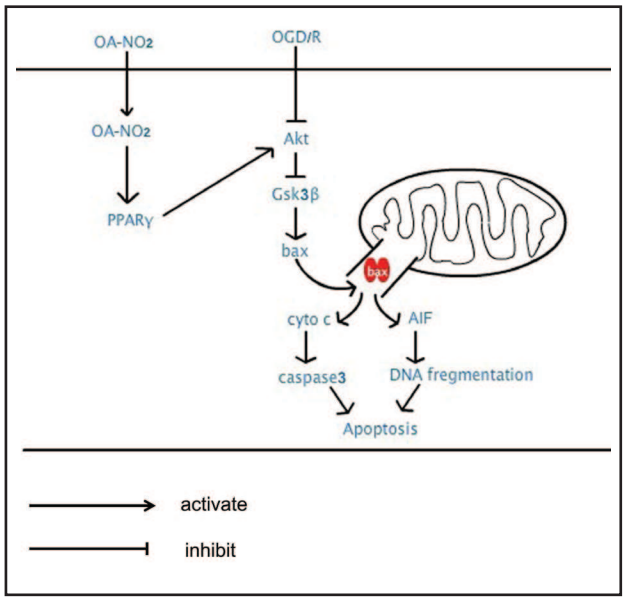

Fig. 11. Possible mechanism of OA-NO_2-mediated cyto-protection against OGD/R induced apoptosis was shown. In the schematic diagram, activation pathways were indicated by (⟶), and the inhibitory pathways were indicated by (⊣).

the first time that OA-NO_2 (1.25 μM) possesses anti-apoptotic effects against OGD/R injury in HK-2 cells.

How does OA-NO_2 ameliorate apoptosis? Mitochondrial Outer Membrane Permeabilization (MOMP) is the decisive event that delimits the frontier between survival and death upon apoptosis induction, and Bax mitochondrial translocation initiates this process [41, 42]. Bax resides predominantly in the cytosol, where it shifts from a cytosol-soluble to membrane-bound form and forms homooligomeric pores or opens existing mitochondrial membrane channels, resulting in MOMP upon apoptosis induction [30, 42, 43]. This leads to the leakage of mitochondrial cytochrome c and AIF and subsequent caspase-dependent or -independent apoptosis [30, 42, 44]. In our study, pre-incubation with OA-NO_2 markedly reduced Bax activation (Fig. 6), leakage of pro-apoptotic factors and subsequent caspase-3 activation caused by OGD/R injury (Fig. 5), and increased cell viability (Fig. 3B) in a pattern similar to that reported previously [45]. In contrast, OA-NO_2 failed to induce a discernible change in the protein levels of Bcl-2 in cytosolic or mitochondrial fraction (Fig. 5A). This suggests that OA-NO_2 may modulate the action of the Bcl-2 family by regulating their post-translational modification rather than influencing protein levels. Bax can be regulated by the altered activity of kinases such as Akt and/or Gsk 3β [29, 41, 46]. Growth factor withdrawal and ATP depletion inactivate Akt, resulting in the activation of Gsk 3β [32], which activates Bax by phosphorylating Ser163 and drives Bax to mitochondria [29, 41]. Interestingly, Gardai et al. proved that Bax could also be phosphorylated at Ser184 by Akt, promoting its heterodimerization with anti-apoptotic Bcl-2 family members and maintaining its localization in the cytosol [46]. Thus, Bax activation during metabolic stress would be favored by the combined inactivation of Akt and activation of Gsk 3β. In support of this concept, Bax activity was found to be regulated by the OA-NO_2-mediated phosphorylation of Akt and Gsk 3β in the present study. First, stress contemporaneously inactivated Akt and activated Gsk 3β (Fig. 7) and Bax (Fig. 6). Second, OA-NO_2 promoted Akt activation and inhibited Gsk 3β and Bax activation. Third, inactivation of the Akt pathway caused by PPAR-γ inhibition completely eliminated the regulatory effects of OA-NO_2 on Akt, Gsk 3β (Fig. 9A, B) and Bax (Fig. 10A, B). All these data further confirm that the OA-NO_2 abolishes Bax activation through regulating Akt and Gsk 3β phosphorylation.

Peroxisome proliferator-activated receptor γ (PPAR-γ) is a member of the nuclear receptor superfamily of transcription factors involved in the regulation of vital processes such as inflammation, proliferation, migration, matrix remodeling, differentiation and metabolic functions [47, 48]. Ligands for PPAR-γ include natural compounds such as eicosanoids, oxidized phospholipids, 15-deoxy-Δ12,14-PGJ2 and drugs such as the thiazolidinedione

derivative rosiglitazone [8], and have been reported to ameliorate ischemia and reperfusion injury in a PPAR-γ dependent manner. The PPAR-γ antagonist, bisphenol A diglycidyl ether (BADGE) abolishes the protection afforded by rosiglitazone and 15d-PGJ2 in a rat model of intestinal I/R [49]. Moreover, rosiglitazone (10 mg/kg) inhibits stomach I/R injury in the heterozygous PPAR-γ-deficient mice, even via activation of reduced levels of PPAR-γ [50]. Since NO_2-FA act as partial agonists of PPAR-γ and covalently modified PPAR-γ by S-nitroalkylation at Cys285 [20, 51], we therefore suggest that the protection observed here was mediated largely via PPAR-γ. Consistent with these findings, PPAR-γ siRNA and GW9662 eliminated the beneficial effects afforded by OA-NO_2 (Fig. 8, 10). Furthermore, they abolished the restoration of Akt and Gsk 3β phosphorylation exerted by OA-NO_2 during OGD/R injury (Fig. 9).

How PPAR-γ regulates Akt activity is presently unclear, even if rosiglitazone has been suggested to activate Akt in a PPAR-γ-dependent way [28, 52, 53]. PPAR-γ might indirectly modulate Akt activation by increasing the expression of HSP27 [54]. HSP27, in turn, promotes Akt activation and inhibits Bax mitochondrial translocation [45]. However, whether the increase of HSP 27 is due to PPAR-γ-activation-induced transcription deserves further study. Alternatively, NO_2-FA have been reported to regulate cell signaling by a covalent and reversible posttranslational modification (nitroalkylation) of key signaling proteins, such as the p65 subunit of nuclear factor κB [10, 11] in a PPAR-γ-independent manner. Thiol residues of proteins are highly susceptible nucleophilic targets of electrophile reactions, with nitroalkylation frequently altering protein structure and function [55]. Protein-tyrosine phosphatases contain an active site motif that includes an invariant Cys with a low pK_a value, a property that promotes nucleophilic reactivity and susceptibility to nitroalkylation [56]. Thus NO_2-FA might regulate Akt activity via nitroalkylation of protein-tyrosine phosphatases, but this remains to be determined and will be the subject of our future investigations.

In conclusion, our study clearly demonstrates that pretreatment of the post-OGD/R renal tubular cells with OA-NO_2 results in the attenuation of ischemia and reperfusion-induced apoptosis. The protective effects of OA-NO_2 were attributed to a combination of Akt activation, Gsk 3β inhibition, Bax inactivation and subsequent suppression of mitochondria-mediated apoptotic cascade in a PPAR-γ-dependent manner (Fig. 11). This study also demonstrated for the first time that OA-NO_2 protects against renal ischemia-reperfusion injury by directly modulating mitochondria-mediated apoptosis. Future investigations will be necessary to determine the mechanism of Akt activation by NO_2-FA. Our findings suggest a novel application for NO_2-FA in the treatment of ischemia and/or reperfusion injury in myocardial infarction, organ transplantation and ischemic stroke in the future.

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Disclosure Statement

None.

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