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Research Article

Nephroprotective Effects of N-Acetylcysteine Amide against Contrast-Induced Nephropathy through Upregulating Thioredoxin-1, Inhibiting ASK1/p38MAPK Pathway, and Suppressing Oxidative Stress and Apoptosis in Rats

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Contrast-induced nephropathy (CIN) is a leading cause of hospital-acquired acute kidney injury (AKI) due to apoptosis induced in renal tubular cells. Our previous study demonstrated the novel N-acetylcysteine amide (NACA); the amide form of N-acetyl cysteine (NAC) prevented renal tubular cells from contrast-induced apoptosis through inhibiting p38 MAPK pathway in vitro. In the present study, we aimed to compare the efficacies of NACA and NAC in preventing CIN in a well-established rat model and investigate whether thioredoxin-1 (Trx1) and apoptosis signal-regulating kinase 1 (ASK1) act as the potential activator for p38 MAPK. NACA significantly attenuated elevations of serum creatinine, blood urea nitrogen, and biomarkers of AKI. At equimolar concentration, NACA was more effective than NAC in reducing histological changes of renal tubular injuries. NACA attenuated activation of p38 MAPK signal, reduced oxidative stress, and diminished apoptosis. Furthermore, we demonstrated that contrast exposure resulted in Trx1 downregulation and increased ASK1/p38 MAPK phosphorylation, which could be reversed by NACA and NAC. To our knowledge, this is the first report that Trx1 and ASK1 are involved in CIN. Our study highlights a renal protective role of NACA against CIN through modulating Trx1 and ASK1/p38 MAPK pathway to result in the inhibition of apoptosis among renal cells.

1. Introduction

CIN (contrast-induced nephropathy) has become a leading cause of hospital-acquired acute kidney injury as a result of the increasing use of iodine contrast media and the simultaneous increase in number of at-risk patients, for example, due to diabetes or hypertension [1–4]. The most common clinical course is a transient nonoliguric and asymptomatic decline in renal function with serum creatinine levels peaking at days 3–5, but CIN can also cause long-term adverse events and need for chronic dialysis [1–4]. Thus, it is essential not only to investigate the pathogenesis of CIN but also to develop preventive interventions [5].

There is accumulating evidence that CIN is caused by a combination of a reduction in medullary blood flow resulting in hypoxia and direct tubular damage, including apoptosis [6–8]. Oxidative stress has been identified as an important driver mechanism in the pathogenesis, and this has triggered trials of antioxidants to prevent CIN [6, 9]. Although there is no consensus or standard practice regarding the most effective intervention to prevent CIN besides adequate hydration, the international work group Kidney Disease:

Improving Global Outcomes (KDIGO) suggested using oral administration of the antioxidant N-acetylcysteine (NAC) along with intravenous fluids in patients at increased risk to develop CIN [10, 11]. There is, however, an inconsistency in guidelines regarding the benefit of NAC [3, 4], which highlights the need for more investigation to seek new antioxidants and address the effectiveness of antioxidants in the prevention of the disease.

NACA, also termed AD4, as the amide form of NAC, is a thiol antioxidant with enhanced properties of lipophilicity, membrane permeability, and antioxidant capacity when compared with NAC [12]. Recently emerging evidence confirmed NACA as a protective agent against oxidative stress in vitro and in vivo [13–16]. Our previous study also indicated NACA could protect renal tubular epithelial cell against contrast-induced apoptosis in vitro [6]. We thus hypothesized NACA could be a better renoprotective agent against CIN than NAC in vivo through its prominent antioxidant activity.

We have previously demonstrated that the low-osmolar, nonionic contrast agent iohexol, the most widely used radiocontrast media, induces renal tubular cell apoptosis through activation of the p38 MAPK/iNOS signal pathway in vitro and vivo [6, 7]. This signal pathway has been confirmed by others in a human renal tubular cell line (HK2) and cultured renal tubular cells isolated from CIN patients [17, 18]. We have subsequently identified the Forkhead box O1 transcriptional factor (FoxO1) as a downstream element of the p38 MAPK cascade [7]. However, little is known of the upstream modulators of the p38 MAPK pathway in CIN as well as in kidney disease [19].

One putative candidate for the upstream signal activator of p38 MAPK is Apoptosis Signal-regulating Kinase 1 (ASK1) [20]. As a serine/threonine kinase belonging to the mitogen activated protein kinase kinase kinase (MAP3K) family, ASK1 has been reported to play a critical role in reactive oxygen species (ROS)-induced apoptosis in various cell types and oxidative stress-related diseases such as D-galactosamine/lipopolysaccharide induced hepatotoxicity and cardiovascular disease [21–24]. Meanwhile, as an important redox regulator, thioredoxin-1 (Trx1) could bind to the N terminal non catalytic region of ASK1 and act as an upstream inhibitor of ASK1 [25, 26].

In the present study, we aimed to compare the efficacies of NACA and NAC in preventing CIN and further investigated whether Trx1/ASK1 signal, as the potential upstream modulators of p38 MAPK, were involved in CIN pathogenesis.

2. Materials and Methods

2.1. Reagents. All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. N-Acetylcysteine amide (NACA) was provided by Dr. Glenn Goldstein (New York, NY, USA). The contrast media iohexol (Omnipaque) was purchased from Amersham Health (Princeton, NJ, USA).

2.2. Animals. The study was approved by Medicine Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine. A total of 40 adult 8–10-week-old male Sprague-Dawley rats weighing 200–250 g were purchased from Shanghai Lab Animal Research Center (certificate number: 2016-0021). Rats were housed in an air-conditioned room at 23°C with a cycle of 12 h/12 h light/dark. Food and water were provided ad libitum except for the day of dehydration.

2.3. Experimental Protocol and Drugs. We used a well-established rat model of CIN [7, 27–29]. Rats were randomly divided into 5 groups of 8 rats each: controls (CON), rats injected with CM (CIN), rats treated with 150 mg/kg/d NAC and injected with CM (CIN+NAC), rats treated with 50 mg/kg/d NACA and injected with CM (CIN+NACA1), and rats treated with 150 mg/kg/d NACA and injected with CM (CIN+NACA2). NAC and NACA were injected intraperitoneally (i.p.) once daily for 4 consecutive days (days 1–4). CON and CIN rats were given the same volume of saline. On day 3, all rats were left without water for 24 h. On day 4, 20 min after injections of saline, NAC or NACA, CIN, CIN+NAC, CIN+NACA1, and CIN+NACA2 rats were injected with a nitric oxide synthase inhibitor (NG-nitro-L-arginine methyl ester, L-NAME, 10 mg/kg, i.p.), followed after 15 and 30 min, respectively, by injection of an inhibitor of prostaglandin synthesis (indomethacin, 10 mg/kg, i.p.) and iohexol (1.5–2 g iodine/kg, i.p.). CON rats received injections of equivalent volume of saline. On day 5, all rats were allowed regular chow and tap water in metabolic cages for 24 h.

Baseline blood samples were collected from the tail vein under ether anaesthesia for analysis of serum creatine (Scr), blood urea nitrogen (BUN), and plasma Cystatin-C (CysC). Urine samples (24-h) were collected on day 1 (baseline) and on day 5 for determination of urinary N-acetyl-β-glucosaminidase (UNAG) and urinary γ-glutamyl transpeptidase (UGGT). At the end of day 5, blood samples were collected from the abdominal aorta under pentobarbital (50 mg/kg) anesthesia for determination of Scr, BUN, and CysC. Subsequently the rats were killed and kidneys removed for biochemical and morphological studies.

2.4. Histopathological Examinations

2.4.1. Light Microscopy. Left kidney samples were fixed in 10% formalin and prepared for examination by light microscopy by hematoxylin and eosin (HE) staining, TUNEL staining, or immunohistochemistry (IHC).

For TUNEL staining, sections were stained using In Situ Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany). TUNEL-positive tubular cell numbers were counted in 20 nonoverlapping random cortical fields under a 400x magnification.

For IHC, sections (4 mm) were immersed in citrate buffer and autoclaved at 120°C for 10 min and then immersed in 3% aqueous hydrogen peroxide (H_2O_2). The sections were incubated with a rabbit polyclonal antibody (phospho-p38 MAPK, #9211, Cell Signaling Technology, Danvers, MA, USA, 1 : 200) for 1 h at room temperature. Immunodetection was performed using biotinylated anti-rabbit IgG and peroxidase-labeled avidin chain working fluid (Beijing Zhong Shan

Golden Bridge Biotechnology Co., China), with diaminobenzidine as the substrate. Finally, the slides were lightly counterstained with hematoxylin for 30 sec. The positive signals were measured using Motic Med 6.0 CMIAS Image Analysis System (Motic China Group Co., Ltd., China). The area density representing the positive staining intensity was calculated as the ratio between the stained area and the total analyzed field. Two blinded examiners independently analyzed all slides.

2.4.2. Transmission Electron Microscopy (TEM). The right renal cortex samples were cut into pieces (2 × 2 mm) on ice and fixed in 2.5% (v/v) glutaraldehyde-polyoxymethylene solution for 6–8 h at 4°C and subsequently embedded in Epon 812. Ultrathin sections (60–70 nm) were stained with uranyl acetate and alkaline lead citrate and visualized under a JEM 100CX transmission electron microscope.

2.4.3. Analysis of Renal Oxidative Stress Indicators. Superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH) levels in renal tissues were determined using SOD kit, MDA kit, and GSH kit, respectively (Sigma, St. Louis, MO, USA). Briefly, tissue blocks of the appropriate size were placed in ice-cold normal saline and homogenated in a ratio of 1 : 9, w (g) : v (ml). After 15 min of centrifugation at 3000 rpm, the supernatant was removed and used for determination of SOD, MDA, and GSH using the respective kits and an UV-visible spectrophotometer.

2.4.4. Quantitative Real-Time PCR (QPCR). Renal cortexes were dissected and total RNA was extracted using Trizol according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). cDNA was synthesized using random primer and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The following primers were used: Trx1 F-GTGGTGTGGACCTTGCAAAA, R-GGAAGGTCGGCATGCATTTG; beta-actin F-CTGTGTGGATTGGTGGCTCT, R-GCAGCTCAGTAACAGTCCGC. QPCR was performed on a 7500 Real-Time PCR System (Applied Biosystems, USA).

2.4.5. Western Blot Analysis. Western blotting was performed as described [6, 7, 30, 31]. Renal cortex samples were lysed in a lysis buffer, separated by electrophoresis in 6–12% polyacrylamide gel, and transferred to polyvinylidene fluoride (PVDF) membranes. Protein expression was quantified by Image J 1.45 software (Wayne Rasband, NIH, Bethesda, MD, USA) after scanning the film. Primary antibodies used included anti-ASK1 (abcam); anti-phospho-ASK1 (abcam, Ser966); anti-Trx1 (abcam); anti-p38 MAPK (Cell Signaling Technology); anti-phospho-p38 MAPK (Cell Signaling Technology, Thr180/Tyr182); anti-cleaved caspase 3 (Cell Signaling Technology); and anti-beta-actin (Cell Signaling Technology). All experiments were performed at least 3 times (i.e., 3 separate protein preparations) under the same conditions.

2.4.6. Statistical Analysis. Results are expressed as means ± SD. One-way analysis of variance (ANOVA) with Tukey's post hoc multiple-comparison test was used to determine the significance of differences in multiple comparisons. Differences were considered significant if $p < 0.05$ and highly significant if $p < 0.01$.

3. Results

3.1. Effects of Induction of CIN (Table 1). At baseline, there was no difference in blood levels of markers of renal functional among the 5 groups. CIN was effectively induced in CIN group as evident from drastic deterioration in renal function (Table 1) and morphological changes, including severe vacuolization of the renal cortex, intratubular cast formation, and medullary congestion (Figure 1(b)).

3.2. Effect of NAC and NACA on Renal Function Parameters. Pretreatment with NAC or NACA preserved renal function as evident from analysis of renal function parameters (Table 1). At the same dose (150 mg/kg/d), NACA was uniformly more effective than NAC as demonstrated by lower Scr, BUN, UNAG, and UGGT (Table 1, $p < 0.05$ versus CIN+NAC). In fact, 50 mg/kg/d NACA had similar effects to 150 mg/kg/d of NAC.

3.3. Effects of NAC and NACA on Histopathology and Ultrastructures (Figure 1). Histopathological examinations of renal samples from CIN rats revealed normal glomerulus structure but severe renal tubular interstitial injury (Figure 1(b)). Pretreatment with NAC or NACA markedly attenuated the development of these lesions (Figures 1(c), 1(d), and 1(e)).

Renal tubular epithelial cell apoptosis in CIN rats was evident by TEM. Cells undergoing apoptosis were characterized by injuries to ultrastructures (Figure 1(g)), for example, condensation of the nuclear chromatin, wrinkling of nuclear membranes, swelling of mitochondria, fracture of cristae, and shedding of microvilli of cell cavity. In comparison, both the apoptotic cell number and injuries to ultrastructures were obviously reduced in CIN+NAC, CIN+NACA1, and CIN+NACA2 rats compared to CIN rats (Figures 1(h), 1(i), and 1(j)). Consistent with the findings under light microscopy, no obvious glomerular lesions were observed by TEM in CIN rats (Figures 1(k) and 1(l)).

3.4. Effects of NAC and NACA on Renal Tubular Apoptosis as Assessed by TUNEL Staining and Analysis of Cleaved Caspase 3 (Figure 2). In addition to TEM, apoptosis was assessed with two independent methods: TUNEL staining in kidney sections and analysis of cleaved caspase 3 by western blotting. Compared to CON rats, CIN rats exhibited markedly increased numbers of TUNEL-positive tubular cells (Figures 2(a), 2(b), and 2(f), $p < 0.01$ versus CON) and also increased cleavage of caspase 3 (Figures 2(g) and 2(h), $p < 0.01$ versus CON). Pretreatment with NAC or NACA significantly decreased both apoptotic cell numbers (Figures 2(c)–2(f), $p < 0.01$ versus CIN) and caspase 3 cleavage (Figures 2(g) and 2(h), $p < 0.01$ versus CIN). At the same dose (150 mg/kg/d), NACA exhibited better protection than NAC

Table 1: Effects of NACA on renal function parameters. The baseline levels of Scr, BUN, CysC, UNAG, and UGGT did not differ among the groups. In CIN rats, BUN, Scr, CysC, UNAG, and UGGT were markedly increased on day 5 as compared to CON rats ($p < 0.01$). Pretreatment with NAC or NACA attenuated the effect of CM, and the increases in Scr, BUN, CysC, UGGT, and UNAG were significantly lower than in CIN group ($p < 0.05$ or 0.01). At the same dose (150 mg/kg/d), NACA was significantly more effective than NAC (CIN+NACA2 versus CIN+NAC).

	CON ($n = 8$)	CIN ($n = 8$)	CIN+NAC ($n = 8$)	CIN+NACA1 ($n = 8$)	CIN+NACA2 ($n = 8$)
Scr (μmol/l)					
Baseline	23.30 ± 3.12	22.38 ± 3.07	23.35 ± 4.52	23.45 ± 2.72	24.75 ± 3.28
Day 8	22.49 ± 2.65	82.23 ± 13.08**	52.89 ± 11.33$^{**\#}$	50.71 ± 7.03$^{**\#}$	37.12 ± 5.69$^{*\#\#\&}$
Serum BUN (mmol/l)					
Baseline	5.83 ± 0.82	5.91 ± 0.84	5.52 ± 1.03	5.70 ± 0.29	5.62 ± 0.78
Day 8	5.77 ± 0.44	44.54 ± 6.54**	16.45 ± 2.42$^{**\#\#}$	15.79 ± 2.87$^{**\#\#}$	10.65 ± 2.08$^{*\#\#\&}$
Serum Cystatin-C (U/l)					
Baseline	1.04 ± 0.05	1.15 ± 0.20	1.19 ± 0.31	1.13 ± 0.16	1.11 ± 0.19
Day 8	1.28 ± 0.25	6.71 ± 1.30**	3.55 ± 0.89$^{**\#}$	3.25 ± 0.91$^{**\#}$	2.92 ± 0.61$^{**\#\#}$
UNAG (U/l)					
Baseline	33.71 ± 5.20	35.25 ± 5.38	35.60 ± 3.20	35.95 ± 6.12	33.12 ± 5.50
Day 8	35.55 ± 6.18	69.27 ± 10.28**	51.25 ± 7.37$^{**\#}$	48.69 ± 8.27$^{**\#}$	41.50 ± 9.50$^{*\#\#\&}$
UGGT (IU/l)					
Baseline	667.74 ± 89.10	648.26 ± 112.08	650.38 ± 117.90	640.73 ± 59.07	639.41 ± 137.55
Day 8	673.28 ± 26.69	6690.15 ± 257.48**	3236.77 ± 536.80$^{**\#\#}$	3064.73 ± 350.29$^{**\#\#}$	2537.17 ± 400.50$^{**\#\#\&}$

Data are means ± SD. $^{*}p < 0.05$ versus CON, $^{**}p < 0.01$ versus CON, $^{\#}p < 0.05$ versus CIN, $^{\#\#}p < 0.01$ versus CIN, and $^{\&}p < 0.05$ versus CIN+NAC.

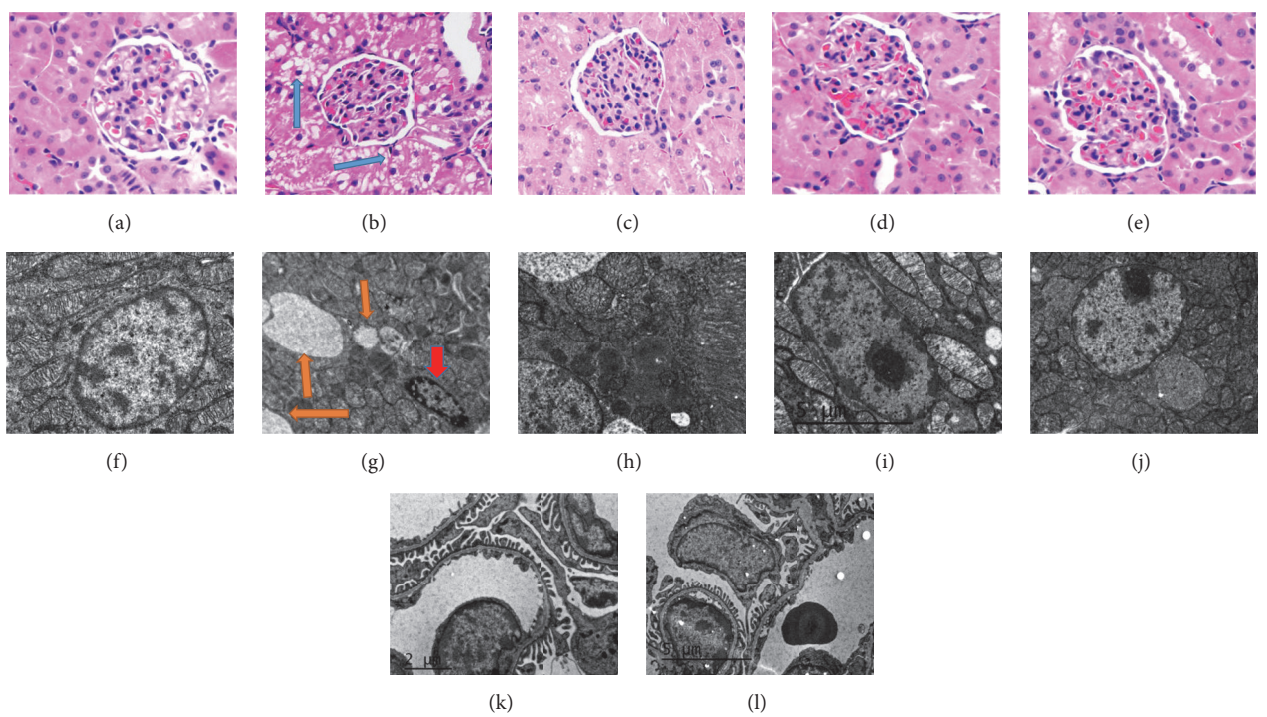

Figure 1: NACA attenuated CM-induced morphological changes. HE staining of kidney sections (magnification ×200) from CON rats (a), CIN rats (b), NAC+CIN rats (c), NACA1+CIN rats (d), and NACA2+CIN rats (e). Representative changes of ultrastructure by TEM (magnifications ×4200) from CON rats (f), CIN rats (g), NAC+CIN rats (h), NACA1+CIN rats (i), NACA2+CIN group (j), normal glomerular basement membrane and podocyte in CON rats (k), and CIN rats (l). Note the condensation of the nuclear chromatin (red arrow) and cytoplasm vacuoles (orange arrow) in CIN rats. Blue arrows refer to severe vacuolization of the renal cortex. Figures are representative of 5 to 8 rats from each group.

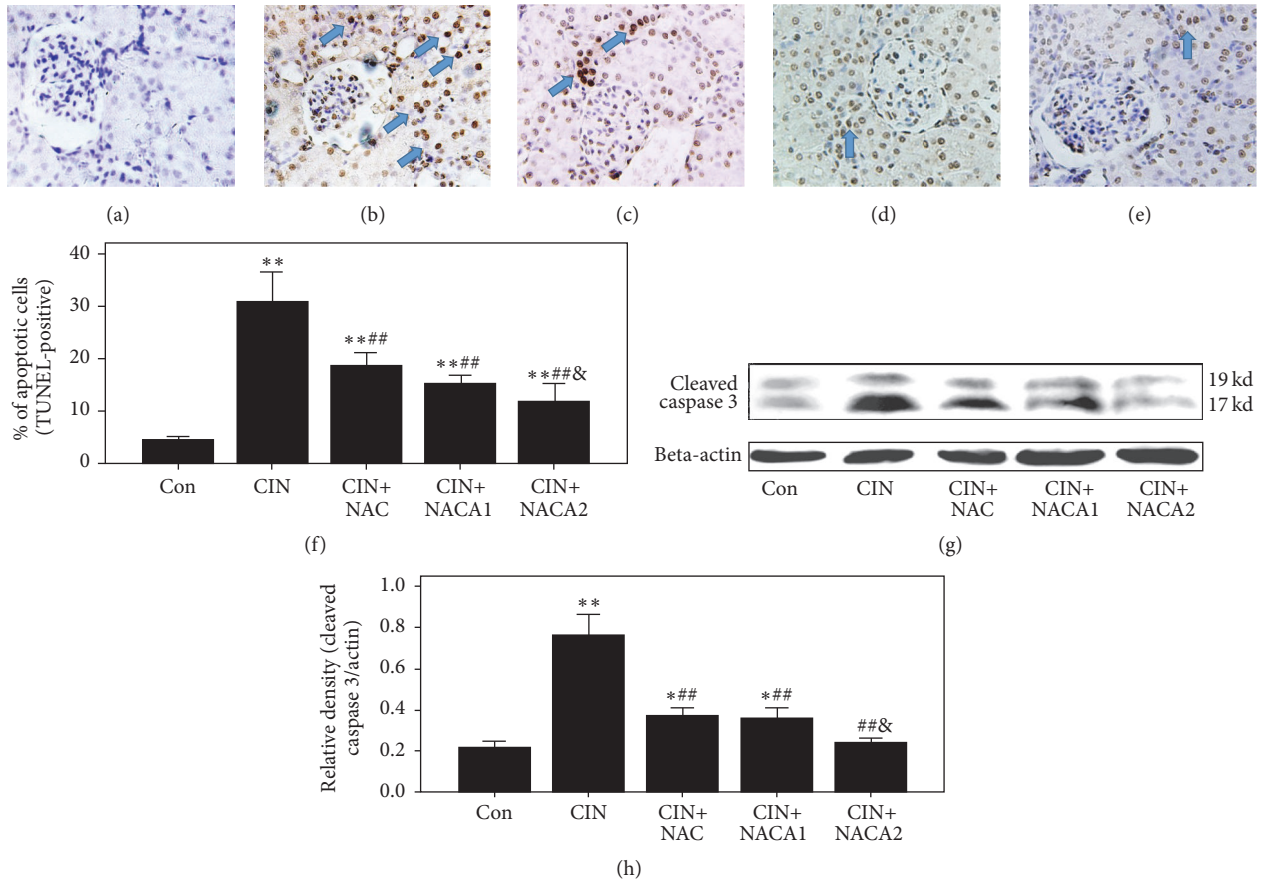

Figure 2: NACA inhibited CM-induced renal tubular cell apoptosis as detected by TUNEL staining and western blot analyses of cleaved caspase 3. CM increased the number of TUNEL-positive renal tubular cells (blue arrows), and pretreatment with NAC or NACA blocked this effect. TUNEL-stained kidney sections (magnifications ×200) from CON rats (a), CIN rats (b), NAC+CIN rats (c), NACA1+CIN rats (d), and NACA2+CIN rats (e). TUNEL-positive cells are marked by blue arrows. (f) Quantitative analysis of TUNEL-positive cell number. (g) Western blot analyses of cleaved capsase-3. (h) Relative densitometry analysis of the ratio of cleaved caspase 3 to beta-actin. Figures are representative of 5 to 8 rats from each group. The values are means ± SD ($n = 5$). $^{*}p < 0.05$ versus CON, $^{**}p < 0.01$ versus CON, $^{\#\#}p < 0.01$ versus CIN, and $^{\&}p < 0.05$ versus CIN+NAC.

(Figure 2(f), $p < 0.05$ versus CIN+NAC, and Figures 2(g) and 2(h), $p < 0.05$ versus CIN+NAC).

3.5. Effects of NAC and NACA on Indicators of Oxidative Stress in Renal Tissue. Induction of CIN attenuated renal SOD (Figure 3(a)) and GSH (Figure 3(c)) and increased MDA (Figure 3(b)) levels ($p < 0.01$ versus CON). Pretreatment with NAC or NACA significantly prevented this (Figure 3, $p < 0.05$ and $p < 0.01$ versus CIN, resp.). At equimolar concentrations, NACA was more effective than NAC at preserving SOD, MDA, and GSH levels ($p < 0.05$ versus CIN+NAC, Figures 3(a)–3(c)).

3.6. Effects of NAC and NACA on P38 MAPK Phosphorylation. Activation of p38 MAPK was confirmed by a significant increase in phospho-p38 MAPK levels as detected by western blotting and IHC (Figures 4(b) and 4(g), $p < 0.01$ versus CON). Pretreatment with NAC or NACA significantly prevented the CIN-induced p38 MAPK activation in kidney (Figures 4(c), 4(d), 4(e), and 4(g), $p < 0.01$ versus CIN). Again, NACA was more effective than NAC at the same dose (Figures 4(f) and 4(h), $p < 0.05$ versus CIN+NAC).

3.7. Effects of NAC and NACA on ASK1 Phosphorylation. We next examined whether ASK1, a potential upstream signal of p38 MAPK, was involved in CIN pathogenesis. As shown in Figures 5(a) and 5(b), phospho-ASK1 level was upregulated in CIN rats ($p < 0.01$ versus CON). Pretreatment with NAC ($p < 0.01$ versus CIN) or, more effectively, NACA (Figures 5(a) and 5(b), $p < 0.05$ versus CIN+NAC) inhibited this activation.

3.8. Effects of NAC and NACA on Trx1 mRNA and Protein Expressions. In order to deduce the role of Trx1 in CIN, Trx1 mRNA and protein expressions were evaluated by QPCR and western blotting, respectively. As shown in Figure 5, Trx1 protein expression (c, d) and mRNA level (e) were decreased in renal cortex following CIN induction ($p < 0.01$ CON

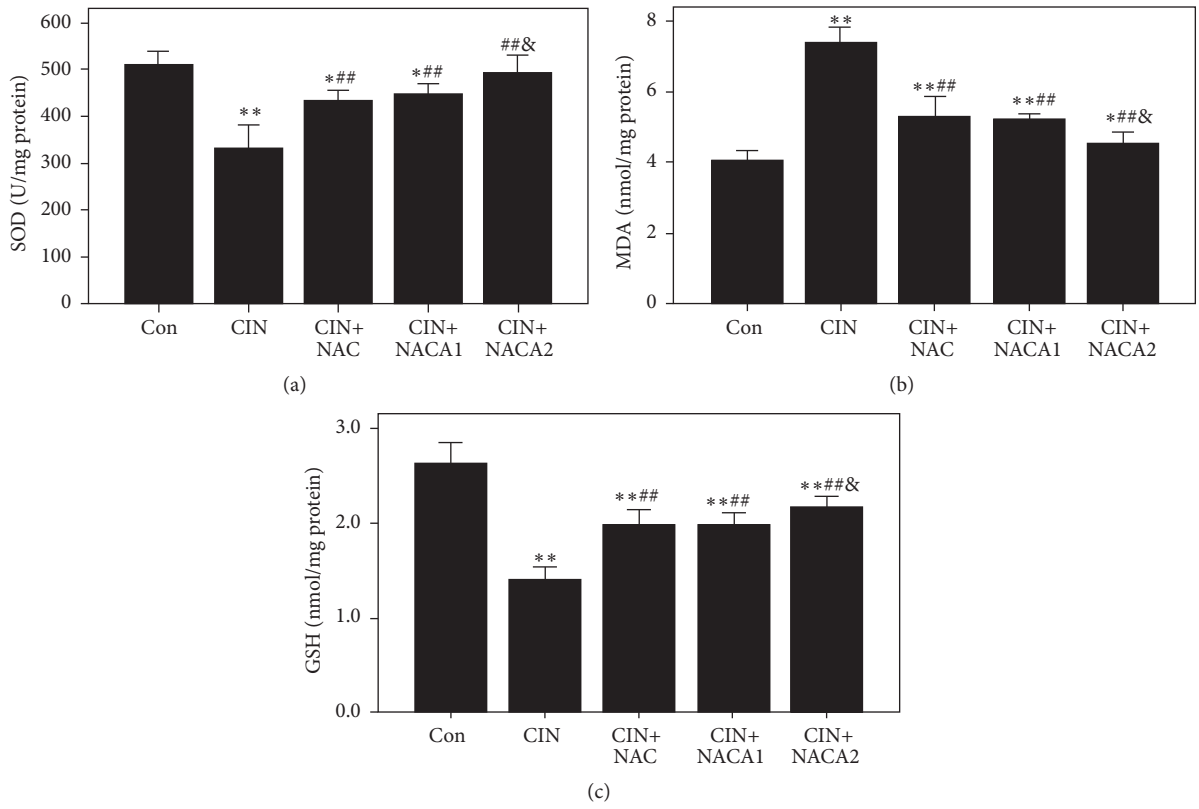

Figure 3: NACA inhibited CM-induced indicators of oxidative stress in kidney. Exposure to CM significantly attenuated renal SOD (a) and GSH levels and increased MDA (b) levels. Pretreatment with NAC or NACA blocked the CIN-induced changes. Figures are representative of 5 to 8 rats in each group. The values are means ± SD ($n = 5$). $^{*}p < 0.05$ versus CON, $^{**}p < 0.01$ versus CON, $^{\#\#}p < 0.01$ versus CIN, and $^{\&}p < 0.05$ versus CIN+NAC.

versus CIN). However, the downregulated expressions of Trx1 were markedly blocked by NAC and NACA ($p < 0.01$ versus CIN).

4. Discussion

Although the mechanisms underlying CIN have not been fully clarified, several studies have shown that ROS-induced oxidative stress and direct cytotoxicity of contrast media are important in the pathogenesis of the disease [2, 6, 32, 33]. Here, we confirm the importance of oxidative stress and demonstrate that the novel antioxidant NACA, an amide derivate of NAC with better tissue penetration, offers more effective prevention against CIN. Furthermore, we show that Txr1/ASK1 signal act as the upstream modulator of the p38 MAPK and present a potential drug target for prevention. To our knowledge, this is the first report demonstrating that Trx1/ASK1 signal are involved in CIN.

The oxidative stress induced in rats with CIN was evident by drastically decreased levels of SOD and GSH, as well as an increased level of MDA, which is consistent with our previous data [6, 7] and other reports [34, 35]. The findings that NACA consistently exhibited better renoprotection than NAC may be related to its better membrane penetration. However, whether other mechanisms of action are involved is not clear and needs further studies. Overall, the present findings confirmed again that oxidative stress is an important factor in the pathogenesis of CIN.

Since there is significant inconsistency in the guidelines regarding the benefit of NAC to reduce the risk for CIN [1, 35], large-scale, randomized clinical trials that are adequately powered have been proposed to determine the effectiveness of NAC for prevention of CIN. Our present findings suggest that consideration should be given to the use of NACA as a result of its superior renoprotective function relative to NAC.

CIN, also called contrast-induced acute kidney injury (CI-AKI), is typically defined by an increase in serum creatinine after intravascular administration of contrast media, but serum creatinine is a late and insensitive indicator of AKI [7, 36–38]. Thus, several additional biomarkers have been investigated in order to improve both prediction and diagnosis of AKI. Our previous study first reported that urinary γ-glutamyl transpeptidase (UGGT) has good sensitivity in early detection of contrast-induced acute renal injury and thus in early diagnosing AKI [7]. UGGT as a potential early diagnostic biomarker of AKI has been confirmed in AKI patients after liver transplantation [39]. Furthermore, another GSH-dependent enzyme present in large amount in liver, urinary glutathione S-transferases (UGST), has recently been

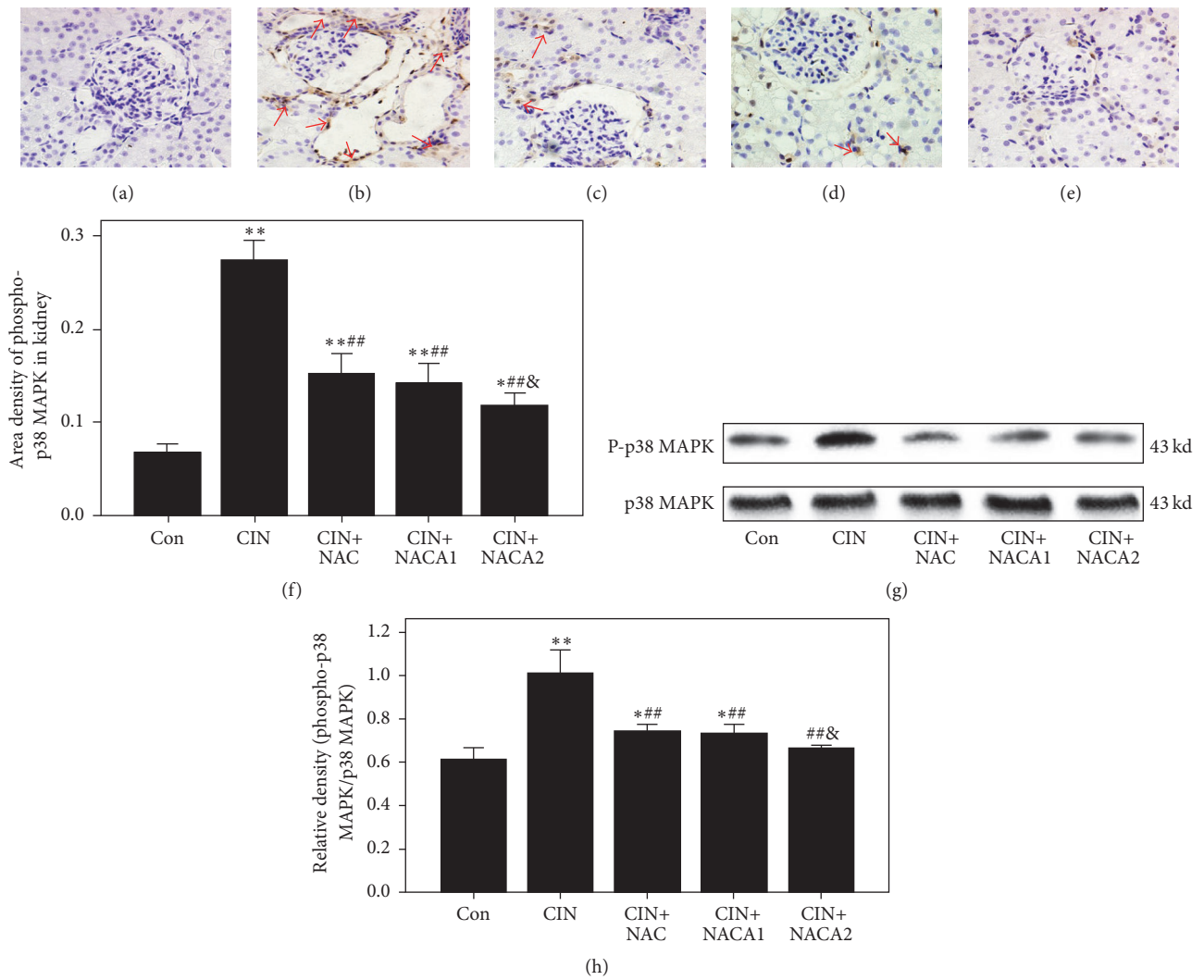

Figure 4: Effects of NACA on p38 MAPK phosphorylation in kidney. IHC staining of phospho-p38 MAPK in CON (a), CIN (b), NAC+CIN (c), NACA1+CIN (d), and NACA2+CIN (e) rats, respectively (figures are representative of 5 to 8 rats in each group). Note positive stained area (yellow) of IHC staining (arrow). (f) Semiquantitative analysis of phosphor-p38 MAPK expression in kidneys with IHC. (g) Phospho-p38 MAPK and total-p38 MAPK expressions by western blotting ($n = 3$ each). (h) Relative densitometry analysis of the ratio of phospho-p38 MAPK to total-p38 MAPK. Data are shown as means ± SD ($n = 3$ each). $^{*}p < 0.05$ versus CON, $^{**}p < 0.01$ versus CON, $^{\#\#}p < 0.01$ versus CIN, and $^{\&}p < 0.05$ versus CIN+NAC.

identified as a biomarker of AKI [40]. Subsequent studies should address usefulness of these and other biomarkers in terms of sensitivity and early detection of kidney injury.

Previous work from our laboratory and others has confirmed that contrast-induced apoptotic cell death via p38 MAPK pathway is an important pathogenic mechanism in CIN [6, 7, 17, 18]. There is evidence that p38 MAPK activation is associated with renal injury, which highlights p38 MAPK pathway as an attractive therapeutic target. However, the potential pathway by which p38 MAPK is activated in CIN is still not well defined so far.

Since MAPKs could be regulated upstream by the MAPK kinase kinase kinase (MAP3K) and p38 MAPK could act as the target of ASK1, we investigated the effect of contrast media on activation of ASK1, a member of MAP3K family [20, 41]. The present data demonstrated increased ASK1 phosphorylation in CIN, which could be blocked by NACA and NAC. Our present data indicated clearly that contrast media could activate the stress/death signaling mitogen-activated protein kinase (MAPK) phosphorylation cascade and thus confirmed that ASK1/p38 MAPK could be a potential drug target for preventing CIN. The study of Ma et al. has also identified ASK1 as a potential therapeutic target in renal fibrosis [20].

Reduced Trx1 could be a direct inhibitor or negative regulator of ASK1, while ROS stimulation could dissociate Trx1 from Trx1/ASK1 complex and lead to ASK1 activation and in turn result in the phosphorylation of its downstream substrate p38 MAPK [22, 26]. As mentioned above, we previously reported that contrast media exposure directly increased cellular oxidation and induced p38 AMPK/iNOS

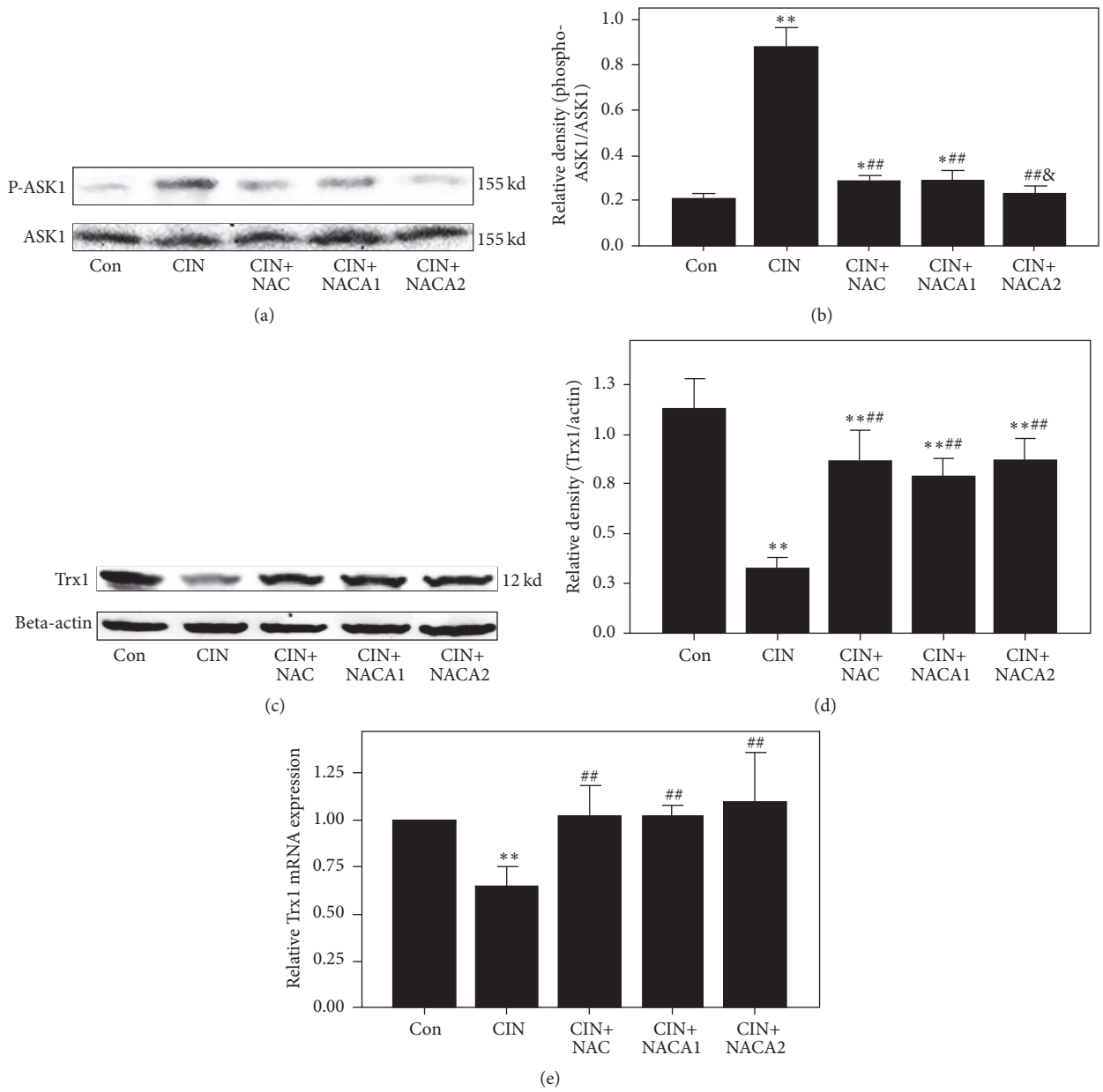

Figure 5: NACA blocked CM-induced p38 MAPK activation by inhibiting ASK1 phosphorylation and upregulating Trx1 mRNA and protein expression. (a) Phospho-ASK1 and ASK1 expressions by western blotting ($n = 3$ each). (b) Relative densitometry analysis of the ratio of phospho-ASK1 to ASK1. (c) Trx1 expression by western blotting ($n = 3$ each). (d) Relative densitometry analysis of the ratio of Trx1 to beta-actin. (e) QPCR analysis for Trx1 mRNA in renal cortex. Data are shown as means ± SD ($n = 3$ each). $^{*}p < 0.05$ versus CON, $^{**}p < 0.01$ versus CON, $^{\#\#}p < 0.01$ versus CIN, and $^{\&}p < 0.05$ versus CIN+NAC.

pathway mediated apoptosis in renal tubular cells in vitro and vivo [6, 7]. Additionally, our present data further clearly indicated that contrast media exposure downregulated Trx1 and increased ASK1 phosphorylation in CIN rat model. Thus we could speculate that the more complete signal mechanism for CIN should include the following key steps (Figure 6); first, contrast medium exposure increased ROS production of kidney, resulting in suppression of Trx1, which lead to Trx1/ASK1 complex dissociation to facilitate the activation of ASK1. This results in the downstream activation of its substrate p38 MAPK and an imbalance of pro- and antiapoptotic members of the Bcl-2 family and finally induced apoptotic cell death. Interestingly, such a pathologic process could be efficiently inhibited by NACA through its antioxidant activity and by upregulating Trx1.

5. Conclusion

Summarily, based on our present and previous studies, we demonstrated that NACA is more effective than NAC in preventing CIN both in vivo and in vitro and identified the

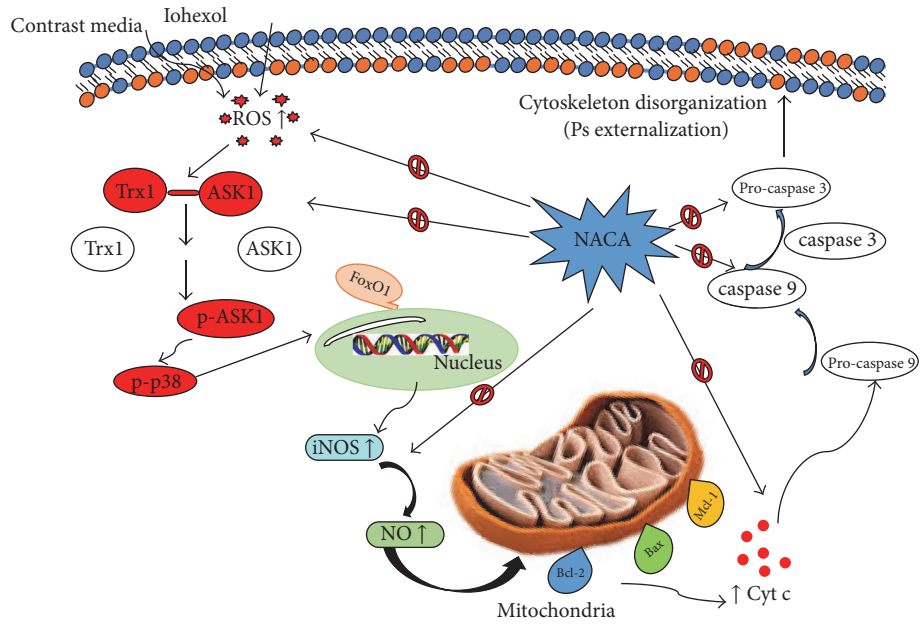

Figure 6: Schematic diagram illustrates the signal mechanism for CIN and the renoprotection of NACA. Contrast media exposure increases ROS production in kidney, resulting in suppression of Trx1 and thus dissociating the Trx1/ASK1 complex to facilitate the activation of ASK1 (phosphorylation). Subsequently, activated ASK1 results in the downstream activation of its substrate p38 MAPK, an imbalance of pro- and antiapoptotic members of the Bcl-2 family, and finally induces apoptosis. NACA efficiently inhibits such a pathologic process through its antioxidant activity and by upregulating Trx1. p, phosphorylation. Ps, phosphatidylserine.

underlying mechanisms including suppression of oxidative stress, upregulating Trx1 and in turn inhibiting ASK1/p38 MAPK pathway, and preventing renal tubular cell apoptosis.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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