**Abstract**

**Background.** Rhabdomyolysis-induced acute kidney injury (AKI) accounts for about 10 to 40% of all cases of AKI. It is known that N-acetylcysteine (NAC) is effective in various experimental renal injury models; however, little information is available about the rat model of glycerol-induced rhabdomyolysis. In this study, we hypothesize that NAC plays a renoprotective role via the anti-apoptotic pathway.

**Methods.** Male Sprague-Dawley rats were divided into four groups: (i) saline control group, (ii) NAC-treated group (N-acetylcysteine) (150 mg/kg), (iii) glycerol-treated group (50%, 8ml/kg, IM) and (iv) NAC plus glycerol-treated group. Rats were sacrificed at 24 h after glycerol injection, and the blood and renal tissues were harvested.

**Results.** Glycerol administration caused severe renal dysfunction, which included marked renal oxidative stress, significantly increased blood urea nitrogen (BUN) and serum creatinine levels. Histopathological findings, such as cast formation and tubular necrosis, confirmed renal impairment. We noted a marked activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), but not p-38, in the glycerol-treated group. We also observed high expression of Bax and Bad but only weak expression of Bcl-2 and Bcl-xL in the glycerol-treated group. However, NAC pretreatment significantly improved renal function and decreased the activation of ERK, JNK, Bax and Bad, whereas it increased Bcl-2 and Bcl-xL.

**Conclusion.** These results demonstrate that NAC protects against renal dysfunction, morphological damage and biochemical changes via the anti-apoptotic pathway in the glycerol-induced rhabdomyolysis model in rats.

**Keywords:** acute kidney injury; Bel-2 family proteins; MAPKs; N-acetylcysteine; rhabdomyolysis

**Introduction**

Rhabdomyolysis is a syndrome involving the breakdown of skeletal muscle, which causes myoglobin and other intracellular proteins and electrolytes to leak into the circulation [1]. It is often complicated by acute kidney injury (AKI), electrolyte imbalance and disseminated intravascular coagulation. About 10 to 50% of patients suffering from significant rhabdomyolysis develop some degree of AKI [2]. Although the treatment has been much improved, the mortality rate may still be as high as 8% [1,3,4]. The experimental model for rhabdomyolysis is easily acquired by injecting glycerol intramuscularly into rats or mice [5].

AKI by rhabdomyolysis has three pathogenic mechanisms: tubular obstruction, renal vasoconstriction and oxidative stress. Oxidative stress has been an important target in the prevention of myoglobin-induced renal injury [6]. The administration of antioxidants has been shown to provide partial protection against myoglobinuric-induced AKI [7–11]. N-acetylcysteine (NAC), one of these antioxidants, is a source of sulfhydryl and glutathione (GSH) groups in cells and, due to its interaction with reactive oxygen species, is a scavenger of free radicals [12]. The protective effect of NAC with respect to renal injury has been proven in various models, such as cisplatin [13], ischemia–reperfusion injury [14,15] and chronic kidney disease [16]. However, there is little data for administering NAC in the rhabdomyolysis model, and the results are controversial [17,18].
There is conclusive evidence that renal tubular cells die by apoptosis as well as necrosis in experimental models [19–21]. The c-Jun N-terminal kinase (JNK) and p38 pathways are activated in response to environmental stress, and this activation is frequently associated with the induction of apoptosis [22,23]. Additionally, it is also known that the balance between cell survival and apoptosis is delicate, and the direction taken by the cell can be settled by activation of the extracellular signal-regulated kinase (ERK) and JNK/p38 kinase pathways [22]. These mitogen-activated protein kinases (MAPKs) play an important role in determining the fate of renal tubular cells [24,25]. Rodent models of renal ischaemia–reperfusion were associated with the activation of renal JNK [26] and p38 [27] but not ERK [28]. Toxic renal injury, induced by mercuric chloride administration, was associated with two temporal peaks of renal ERK activation [29], whereas both renal ERK and JNK activation were increased in the glycerol model of myoglobinuric AKI [17]. Thus, the evaluation of these pathways is considered essential for the therapeutic intervention in AKI. Moreover, it has not been studied how NAC affects the activation of MAPKs in the rhabdomyolysis.

We hypothesized that (i) glycerol-induced renal dysfunction and histopathological changes are due to a decrease in the renal anti-oxidant reserves, and that these changes are correlated with activation of the MAPKs signaling leading to renal tubular apoptosis and (ii) NAC plays major renoprotective roles through controlling the signaling pathway which leads to the prevention of renal tubular apoptosis.

Materials and methods

Experimental designs
Male Sprague-Dawley rats (220–230g, Central Lab. Animals Inc., Seoul, Korea) were housed in temperature-controlled conditions under a light/dark photocycle with food and water supplied ad libitum. Animal studies were conducted according to the Gyeongsang National University of Health Guide for Care and Use of Laboratory Animals. Rats were maintained on standard chow and were dehydrated for 16 h before glycerol injection. Rats were divided randomly into four different groups. The first group (Con, n = 7) was injected intramuscularly with normal saline (8 ml/kg); the second group of rats (NAC, n = 7) was injected intravenously (IV) with NAC into the tail vein (150 mg/kg) (ASIA PHARM. IND. CO., Seoul, Korea); the third group (Gly, n = 7) was injected intramuscularly with 50% glycerol (8 ml/kg) (Amresco, Solon, OH, USA) into a hind limb; the fourth group (NAC Gly, n = 7) was injected intravenously with 150 mg/kg of NAC 30 min prior to glycerol injection. Rats were followed up for 24 h, blood was collected by cardiac puncture, and a bilateral nephrectomy was performed.

Assessment of renal function
Serum samples were examined for blood urea nitrogen (BUN; Urea nitrogen Reagents, Bayer, USA) and creatinine (Creatinine Reagents, Bayer, USA) using standard diagnostic kits in an autoanalyser (ADIVA 1650, Bayer, Japan).

Renal histology
Five μm sections were stained with haematoxylin and eosin. Tubular injury was defined as tubular epithelial necrosis, cast formation, intratubular debris and loss of the brush border. Tubular injury has been scored by grading the percentage of affected tubules under a high-powered field (>400): 0, 0%; 0.5, <10%; 1, 10 to 25%; 2, 26 to 50%; 3, 51 to 75%; 4, 75 to 100%. To score injured tubules, whole tubular numbers per field were considered as standard under ×400 magnification. The grading percentage was calculated in each field as follows; injury score (%) = (number of injured tubules / number of whole tubules) × 100. At least 10 areas in the cortex per slide were randomly selected.

Renal GSH level
Renal GSH content was measured using Glutathione Assay kits (Sigma, St. Louis, MO). One hundred milligrams of each renal tissue was homogenized in 0.5 ml of GSH reaction buffer. To generate nicotinamide adenine dinucleotide phosphate diaphorase (NADPH), 20 μl of NADPH Generation Mix and 140 μl of GSH reaction buffer were mixed and incubated at room temperature for 10 min. Then, 20 μl of the sample solution was added, followed by incubation at room temperature for 5 min and a further addition of 20 μl of substrate solution. A microplate reader was used to measure the absorbance at 405 nm (Molecular Devices Corp., Sunnyvale, CA, USA).

TUNEL assay
The degree of apoptosis was assessed using a TUNEL assay. Detection of DNA fragmentation was performed using a kit from Roche Applied Sciences (Indianapolis, IN, USA). A semiquantitative analysis was performed by counting the number of TUNEL-positive cells per field, in the renal tissue, at ×400 magnification. At least 10 areas in the cortex per slide were randomly selected. The mean number of brown colored cells in these selected fields was expressed as the number of TUNEL-positive cells.

Immunoblot analysis
Kidneys were removed and homogenized in lysis buffer. Sixty micrograms of proteins was loaded. Blots were probed with primary antibodies to polyclonal anti-cleaved caspase-3, pERK, p-p38 and pJNK (Cell Signaling Tech, Beverly, MA) and polyclonal anti-Bax, Bad, Bcl-2 and Bcl-xl (Santa Cruz Biotech, CA, USA) at 4°C overnight. The primary antibody was visualized using secondary antibodies with a chemiluminescent leagent kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Intensity analysis was carried out using Sigma Gel 1.0 (Jandel Scientific, Germany) and Sigma Plot 7.0 (SPSS Inc, Chicago, IL).

Renal heme oxygenase (HO) activity
Kidney microsomes that were prepared by ultracentrifugation were suspended in 100mM potassium phosphate buffer. Supernatant was added to the reaction mixture, which contained 2 mg of liver, 10 μM hemin, 0.2 mM glucose-6-phosphate, 0.2 U of glucose-6-phosphate dehydrogenase and 0.8 mM NAPDH. The formed bilirubin was extracted with same volume of chloroform and was calculated by the difference in absorbance between 464 and 530 nm. HO activity was expressed as nanomoles of bilirubin formed per milligram of protein per hour.

Statistical analysis
Data are expressed as the mean ± standard error (SE) (n = 7). Statistical analysis was conducted using the Sigma Plot 7.0 (SPSS Inc, Chicago, IL, USA). Statistical differences between two groups were determined by the Student’s t-test. *P < 0.05 was considered statistically significant.

Results

NAC-pretreated rats have marked protection from glycerol-induced renal dysfunction
The administration of glycerol led to a rise in BUN from 16 mg/dl (baseline) to 145 mg/dl and a rise in serum creatinine from 0.5 mg/dl (baseline) to 4.0 mg/dl at 24 h after glycerol injection compared with controls and the NAC-only group. In contrast, NAC-pretreated rats that received glycerol showed significant attenuation in BUN and serum creatinine elevation (140 mg/dl vs 100 mg/dl, **P < 0.005 for BUN; 4.0 mg/dl vs 2.2 mg/dl, *P < 0.05 for creatinine) (Figure 1) at 24 h when compared with glycerol alone. The
results indicate that NAC pretreatment ameliorated glycerol-induced renal dysfunction.

**NAC protects rats from renal tubular injury by glycerol**

The renoprotective effects of NAC were confirmed by the preservation of renal histology. The controls and the NAC-only group did not show any histological changes (Figure 2A-a, b). Rats that received glycerol alone developed extensive tubular damage (Figure 2A-c). In comparison, NAC-pretreated rats had significantly reduced tubular injury (Gly vs NAC Gly, for tubular necrosis and intratubular debris, **P < 0.005, for cast formation and loss of brush border, *P < 0.05) (Figure 2B). These findings were consistent with the differences in renal function. This result shows that renal structural injury after glycerol administration was clearly prevented by NAC.

**NAC inhibits the reduction in the renal GSH content by glycerol**

NAC had antioxidant and protective effects against oxidative damage [13,15,30,31]. To determine whether the previous effects of NAC could be reproduced in the current model, the tissue levels of GSH were measured. There were no significant differences in baseline renal GSH levels between the control and NAC-only group. Glycerol-only injection induced significant depletion of GSH levels compared with the control and NAC-only groups (Con vs Gly, **P < 0.005) (Figure 3). NAC pretreatment attenuated this reduction in GSH levels (Gly vs NAC Gly, *P < 0.05) (Figure 3). This result suggests that NAC is linked to an improvement in the oxidative stress induced by glycerol administration.

**HO-1 expression is highly increased in glycerol kidney injury**

HO-1 induction is indispensable to protect the kidney in an experimental rhabdomyolysis model [15,32,33]. To evaluate HO-1 expression in our experimental model, we measured the HO-1 protein level and HO activity. There was no expression of HO-1 on the saline and NAC group. However, glycerol treatment highly induced HO-1 expression and increased HO activity at 24 h after glycerol injection. NAC pretreatment significantly reduced the increase in HO activity and attenuated the HO-1 protein expression (Figure 4). These results demonstrated that HO-1 played major roles in the pathogenesis of this model. Since NAC pretreatment reduced renal injury in this model, there was less expression of HO-1 protein and decreased enzyme activity in NAC plus glycerol group, compared with only glycerol treatment group.

**NAC protects rats from renal apoptosis by glycerol**

We also examined the effects of NAC on glycerol-induced tubular cell apoptosis by TUNEL staining (Figure 5A). There was no change in the control and NAC groups (Figure 5A-a and b). Glycerol administration resulted in the appearance of TUNEL-positive cells (Figure 5A-c); however, NAC pretreatment significantly decreased the numbers of TUNEL-positive cells (Gly vs NAC Gly, *P < 0.05) (Figure 5A-d). To assess whether NAC could protect against apoptotic death in glycerol-induced renal injury, we also measured cleaved caspase-3 in the protein level. Cleaved caspase-3 was rarely detected in the control and NAC groups but highly expressed in the glycerol-treated group. However, the NAC plus glycerol-treated group showed significant attenuation of this increase, compared with the glycerol group (Figure 5B). This result proves that the protective effect of NAC on renal apoptosis caused by glycerol administration is in accordance with the functional (Figure 1) and histological data (Figure 2).

**NAC attenuates renal ERK and JNK activation but not p38 after glycerol administration**

We analysed the activation of MAPKs which play important roles in the regulation of apoptosis [22,34]. In
the control and NAC-only groups, the activation of these proteins was mildly increased, compared with the glycerol group. Glycerol treatment greatly increased both the levels of pERK and pJNK, although there was no change in p-p38 level. NAC pretreatment decreased the expression of these two molecules (Figure 6). These results suggest that NAC protects the kidney from glycerol injury via the inhibition of the pERK and pJNK signaling pathways.

**NAC changes the expression pattern of Bcl-2 family proteins by glycerol**

It is well known that the Bcl-2 family proteins regulate apoptosis by controlling mitochondrial permeability. Whether mitochondrial control of apoptosis is involved in this rat model, we measured the levels of Bax, Bad, Bcl-2 and Bcl-xL. Figure 7 showed that there was no expression of these four molecules in the control and NAC-only groups but...
highly increased expression of Bax and Bad in the glycerol-treated group. However, NAC pretreatment significantly inhibited the up-regulation of pro-apoptotic proteins Bax and Bad (Figure 7A). In contrast, we observed greatly increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL with NAC pretreatment (Figure 7B). This means that NAC may prevent renal injury by suppression of the pro-apoptotic pathway and promotion of the anti-apoptotic pathway in this rhabdomyolysis rat model.

Discussion and Conclusion

This study revealed that glycerol-induced deterioration of renal function in rat kidneys involved histopathological changes in renal tubules, GSH depletion, increase in apoptotic cells and caspase-3 cleavage, activation of the pro-apoptotic Bcl-2 family and JNK and ERK activation. NAC pretreatment prevented renal dysfunction and its associated structural changes and attenuated GSH depletion due to glycerol administration. These beneficial effects of NAC might originate from the involvement of the Bcl-2 family, since we observed an increase in expression of anti-apoptotic factors and reduced expression of pro-apoptotic factors. Furthermore, reduction of the pro-apoptotic factors might be correlated with a decrease in the activation of ERK and JNK, but not p38.

Two studies have reported that NAC administration prevented renal dysfunction in the myoglobinuric AKI model [18,32]. In one study, NAC (100 mg/kg) was administered intraperitoneally before, or after, glycerol injection. This method did not prevent tissue injury or functional deterioration [18]. The second showed that NAC (1–4 g/kg) given orally to rats 1 h before glycerol injection suppressed an increase in serum creatinine. However, there was no information as to which dose resulted in reno-protection, and they did not show any histological improvement [32]. Dickey et al. [35] mentioned that the protective properties of NAC are affected by the dose and route of administration. Intraperitoneally or orally given NAC (400 mg/kg) produced no renal protection, whereas intravenous NAC reduced nephrotoxicity. Our intravenous administration of NAC (150 mg/kg) led to improvement of renal function and histopathologic findings. These kinds of results suggest that it may be important to select the route and dose when NAC was used for the prevention of the nephrotoxicity model.

It is true that most of the action of NAC has been focused on the role of antioxidants or scavenger of radicals. However, some in vitro studies exhibited that NAC is directly associated with inhibition of apoptosis and promotion of cell growth [36,37]. They showed that NAC can protect cells from apoptotic death through a mechanism other than the scavenging of radicals. These groups also suggested that apoptotic death of neuronal cells by depletion of the trophic factor is prevented by affecting the cell cycle of NAC, not by their antioxidant role [38]. Zafarullah et al. proposed that NAC may have beneficial effects through multiple mechanisms including its anti-apoptotic capacity according to the cell types [12]. Our study proved that NAC ameliorates glycerol-induced kidney injury through inhibition of apoptotic cell death by regulating...
Fig. 5. NAC reduces the apoptotic renal injury by glycerol. Glycerol-induced renal apoptosis is illustrated by the TUNEL assay (A). TUNEL-positive cells were stained brown (arrow). To distinguish the nucleus, TUNEL-stained tissue sections were stained with hematoxylin (original magnification, ×400). TUNEL-positive cells were counted as described in ‘Materials and methods’. Levels of cleaved caspase-3 (B). Immunoblot analysis was carried out with a specific antibody against cleaved caspase-3. Rats were treated with saline (Con), N-acetylcysteine (NAC), glycerol (Gly) and NAC plus glycerol (NAC Gly). The blots shown in this figure are representative of three separate experiments. The data were normalized against the density of α-tubulin. Data shown represent the average of three independent experiments (± SE), *P < 0.05 versus Gly group.
Bcl-2 family proteins. We think that these beneficial effects of NAC may be partially or mainly derived from its direct effect on cell in our in vivo model.

MAPKs play a key role in determining the fate of renal tubular cells after kidney injury [39,40]. The activation of ERK may be involved in the induction of apoptosis in renal cells as reported previously [41,42]. Recent studies have demonstrated that JNK contributes to the activation of the intrinsic apoptotic pathway in response to cellular stress [23, 43–45]. Our results also showed that ERK and JNK activation were increased in the glycerol-induced kidney, and that NAC pretreatment attenuated this level

Fig. 6. Activation of ERK and JNK by glycerol was decreased by NAC. Immunoblot analysis with phosphor-specific antibodies was done against MAPKs (pERK, pJNK and p-p38). The blots were reprobed for α-tubulin to monitor comparable protein loading and transfer in each lane. The blots shown in this figure were representative of three separate experiments. Rats were treated with saline (Con), N-acetylcysteine (NAC), glycerol (Gly) and NAC plus glycerol (NAC Gly). The data were normalized against the density of α-tubulin (B, C and D). Data shown represent the average of three independent experiments (± SE), *P < 0.05, **P < 0.005 versus Gly group, respectively.

Fig. 7. NAC pretreatment activates the anti-apoptotic and suppresses pro-apoptotic pathway by glycerol. Immunoblot analysis for Bcl-2 family proteins was carried out with a specific antibody against Bax, Bad (A) and Bcl-2, Bcl-xL (B). α-Tubulin was used as a loading control. The blots shown in this figure are representative of three separate experiments. The data were normalized against the density of α-tubulin (A and B). Data shown represent the average of three independent experiments (± SE). *P < 0.05 versus Gly group.
with diminution of apoptosis as shown by the decrease in cleaved caspase-3. Recent studies have provided evidence to support the involvement of p38 in AKI models [46–48]. Luo et al. [13] reported that the activation of p38 caused apoptosis through tumor necrosis factor-α and/or nuclear factor-κB (NF-κB) activation in cisplatin-induced AKI. However, the activation of p38 and NF-κB was not involved in our model (data not shown). These results demonstrate that protective mechanisms of NAC against glycerol-induced AKI are clearly different from that against AKI induced by other causes.

Mitochondrial control of apoptosis has emerged as the major apoptotic pathway in other nephrotoxicity models [49,50]. Bid deficiency ameliorated ischemic acute renal injury and renal tubular apoptosis [51], and survivin, an inhibitor of apoptotic protein, decreased toxin-induced acute renal injury by regulating Bcl-2 family proteins, accompanied by activation of Bcl-xL protein and inhibition of Bad protein [52]. Our study showed that renal injury by glycerol was mediated via the mitochondrial pathway as illustrated by a shift in the balance of the Bcl-2 family proteins into the direction of apoptosis with an increase in Bax and Bad. NAC pretreatment attenuated apoptosis by reversing the direction of the apoptotic balance into the anti-apoptotic pathway with attenuation of Bax and Bad and increase in Bcl-2 and Bcl-xL. These results suggest that NAC may augment the anti-apoptosis of renal tubular cells by regulating Bcl-2 protein family in rhabdomyolysis model, although NAC did not activate these proteins in normal steady state.

In conclusion, we exhibited that acute renal injury by glycerol is mediated by renal apoptosis and is prevented by NAC pretreatment. No pharmacologic treatment has been done to preserve renal function in rhabdomyolysis. Because of safety and widespread use in clinics, NAC can be selected as a potential therapeutic agent of clinical acute renal injury by rhabdomyolysis. Although it is difficult to administer NAC before rhabdomyolysis is clinically diagnosed, it may be beneficial if it is administered at an early phase of rhabdomyolysis, or before overt AKI occurrence.

Acknowledgements. This study was supported by 2007 Gyeong sang National University Hospital Special Clinical Fund (to D.J.P. and J.H.K.).

Conflict of interest statement. None declared.

References
Effects of mycophenolate mofetil on acute ischaemia–reperfusion injury in rats and its consequences in the long term

Massimo Sabbatini1, Francesco Uccello1, Vittorio Serio1, Giancarlo Troncone2, Valeria Varone2, Michele Andreucci3, Teresa Faga3 and Antonio Pisani1

1Department of Systematic Pathology, University Federico II, Naples Italy; 2Department of Bio-morphological Science, University Federico II, Naples Italy and 3Department of Nephrology, University of Magna Graecia, Catanzaro Italy

Correspondence and offprint requests to: Massimo Sabbatini; E-mail: sabbatin@unina.it

Abstract

Background. Renal ischaemia–reperfusion injury (IRI) acutely decreases glomerular filtration rate (GFR) and impairs kidney function in the long term. Pre-treatment with chaetomelicol acid (KM), an inhibitor of membrane-bound Ha-Ras, has demonstrated beneficial effects on acute renal ischaemia.

Methods. We tested whether mycophenolate mofetil (MMF, 20 mg/day for 4 days before IRI), an immunosuppressor with anti-inflammatory properties, improved renal outcome in uninephrectomized rats after IRI (45 min of renal ischaemia), alone or in combination with KM.

Results. One day after ischaemia, GFR was markedly depressed in untreated rats (−75% vs. normal rats, P < 0.001), and pre-treatment with MMF did not modify this fall (−75%, P < 0.001 vs. normal). KM (0.23 μg/kg before IRI) greatly prevented GFR loss (−39% vs. normal, P < 0.05), but its action was not further improved by the combined administration with MMF (GFR, −45% vs. normal, P < 0.05). MMF significantly reduced ICAM-1 expression and monocyte recruitment (P < 0.05 vs. untreated rats);

Received for publication: 19.6.09; Accepted in revised form: 12.11.09

doi: 10.1093/ndt/gfp710
Advance Access publication 22 December 2009