N-acetylcysteine ameliorates acute kidney injury but not glomerular hemorrhage in an animal model of warfarin-related nephropathy

Kyle Ware,1 Zahida Qamri,1 Ayhan Ozcan,1,3 Anjali A. Satoskar,1 Gyongyi Nadasy,1 Brad H. Rovin,2 Lee A. Hebert,2 Tibor Nadasy,1 and Sergey V. Brodsky1

1Department of Pathology, The Ohio State University, Columbus, Ohio; 2Department of Medicine, The Ohio State University, Columbus, Ohio; and 3Department of Pathology, Gulhane Military Medical Academy, Ankara, Turkey

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Warfarin-related nephropathy (WRN) occurs under conditions of overanticoagulation with warfarin. WRN is characterized by glomerular hemorrhage with occlusive tubular red cell (RBC) casts and acute kidney injury (AKI). Herein we test the hypothesis that oxidative stress plays a role in the AKI of WRN. 5/6 Nephrectomy rats were treated with either warfarin (0.04 mg·kg−1·day−1) alone or with four different doses of the antioxidant N-acetylcysteine (NAC). Also tested was the ability of our NAC regimen to mitigate AKI in a standard ischemia-reperfusion model in the rat. Warfarin resulted in a threefold or greater increase in prothrombin time in each experimental group. Serum creatinine (Scr) increased progressively in animals receiving only warfarin + vehicle. However, in animals receiving warfarin + NAC, the increase in Scr was lessened, starting at 40 mg·kg−1·day−1 NAC, and completely prevented at 80 mg·kg−1·day−1 NAC. NAC did not decrease hematuria or obstructive RBC casts, but mitigated acute tubular injury. Oxidative stress in the kidney was increased in animals with WRN and it was decreased by NAC. The NAC regimen used in the WRN model preserved kidney function in the ischemia-reperfusion model. Treatment with deferoxamine (iron chelator) did not affect WRN. No iron was detected in tubular epithelial cells. In conclusion, this work taken together with our previous works in WRN shows that glomerular hematuria is a necessary but not sufficient explanation for the AKI in WRN. The dominant mechanism of the AKI of WRN is tubular obstruction by RBC casts with increased oxidative stress in the kidney.

oxidative stress; warfarin-related nephropathy; 5/6 nephrectomy

WE SHOWED THAT WARFARIN coagulopathy induces acute kidney injury (AKI) that is associated with severe glomerular hematuria causing widespread tubular obstruction by red blood cell (RBC) casts (5–7). We documented this association in both humans (5–7) and animal models (31, 41). We named this condition warfarin-related nephropathy (WRN). WRN can have dire consequences, particularly in chronic kidney disease (CKD) patients, whose CKD progression can be accelerated (5, 6). In humans, WRN is not an uncommon complication of excessive anticoagulation with warfarin. Our retrospective studies show that when the international normalized ratio (INR) acutely exceeds 3.0, 16% of non-CKD patients (6) and 33–37% of CKD patients (5, 6) may develop AKI within ~1 wk of the INR > 3.0. Those at greatest risk of WRN have CKD or cardiovascular disease (5, 6).

The most conspicuous feature of WRN is the presence of numerous renal tubules obstructed by RBC casts. On this basis, it has been assumed that the tubular obstruction was the principle mechanism of the AKI in WRN. It was recognized, however, that in both patients and animals with WRN and severe reductions in glomerular filtration rate (GFR), only a small percentage of the tubular cross sections in any given slide showed complete obstruction by RBC casts (7, 31). To explain the discrepancy between the degree of tubular obstruction and the degree of GFR decrease, it was assumed that the degree of tubular obstruction shown in any given slide represented only the “tip of the iceberg” of obstructed tubules. On the other hand, the discrepancy between the degree of tubular obstruction and the degree of GFR decrease also raised the possibility that mechanisms, in addition to tubular obstruction, could be involved in the AKI of WRN. For example, warfarin coagulopathy could influence glomerular hemodynamics or the hydraulic conductivity of glomerular filtration barrier (7). Alternatively, the AKI or WRN could be the result of a problem more widespread than complete tubular obstruction by RBC. For example, the AKI could be the result of oxidant damage to tubules by RBC, even though the RBC do not obstruct the tubule.

In support of the hypothesis that oxidative stress may be involved in WRN pathogenesis are the multiple lines of evidence that oxidative stress is involved in the progression of kidney injury. For example, CKD patients have reduced plasma antioxidant enzyme activities, such as glutathione peroxidase and catalase (12). Growing evidence indicates that glomerular hemmorhage may lead to tubular epithelial cell injury (3, 25). The pathogenesis of tubular injury by RBC includes heme toxicity and iron-associated cellular damage (19, 32, 39). N-acetylcysteine (NAC), a thiol-containing antioxidant, was shown to ameliorate AKI by reducing oxidative stress (14, 29).

The aim of this study is to test the hypothesis that the AKI of WRN is related to increased oxidative stress in the kidney.

MATERIALS AND METHODS

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) (11). Male Sprague-Dawley rats (140–160 g) were allowed food and water ad libitum.

Protocol for induction of WRN. The 5/6 nephrectomy surgery was performed under a ketamine/xylazine (6.0 mg/0.77 mg/100 g) anesthesia by a nephrectomy of the right kidney and resection of two-thirds of the left kidney (31, 41). Animals were allowed to recover from the surgery for 3 wk before the start of warfarin treatment.
Warfarin and NAC were given per os in drinking water in the concentrations described below. Daily water consumption was measured. Deferoxamine (DFO), an iron chelator, was dissolved in 0.9% normal saline and administered by intraperitoneal injections daily (2, 28). Warfarin, NAC, and DFO dosage was based on the animal weight. Serum creatinine (Scr) and hematuria were measured daily, as described below.

Protocol for ischemia-reperfusion AKI. Ischemia-reperfusion was created after unilateral nephrectomy of the right kidney and clamping of the left renal artery for 30 min as we described earlier (8). Treatment with NAC was started 24 h before the ischemia was initiated to achieve the pharmacologic NAC concentrations in the circulation by the time of ischemia-reperfusion (1).

Measurement of renal function and coagulation parameters. Scr was measured using a creatinine reagent assay (Raichem, San Marcos, CA) according to the manufacturer’s protocol. Briefly, 20 µl of serum were mixed with 200 µl of working reagent at 37°C in a 96-well plate and the absorbance was read at 510 nm at 40 and 100 s using a Molecular Devices Versa Max plate reader (Molecular Devices, Sunnyvale, CA).

Prothrombin time (PT) was measured using an Electra 750 coagulation analyzer (Medical Laboratory Automation, Pleasantville, NY) according to the manufacturer’s protocol. Briefly, blood was collected to a tube containing 3.8% sodium citrate as the anticoagulant in a ratio of 9:1. The blood was centrifuged at 1,000 relative centrifugal force for 15 min. Then, 0.1 ml of plasma was placed in the incubation station for 3 min and 0.2 ml of warm thromboplastin was added. The pipette plunger was pushed down as the test was started. Clotting time was recorded.

Hematuria was measured using DiaScreen (Chronimed, Minnetonka, MN) reagent strips in the urine. Hematuria was graded using a semiquantitative scale of 0 – 3:

- 0 for no hematuria
- 1 for mild hematuria
- 2 for moderate hematuria
- 3 for large hematuria

More prominent effects on warfarin-induced AKI were seen when 40 and 80 mg·kg⁻¹·day⁻¹ of NAC were used (Fig. 1B).

NAC ameliorates AKI associated with warfarin coagulopathy in 5/6 nephrectomy rats. We used 5/6 nephrectomy rats 3 wk after the ablative surgery for these studies. Earlier we reported that the changes in serum creatinine and kidney morphology associated with anticoagulation were more prominent in 5/6 nephrectomy rats at 3 wk than at 9 and 19 wk after the ablative surgery (31).

Treatment with warfarin (0.40 mg·kg⁻¹·day⁻¹) in drinking water resulted in an increase in sINR over fourfold within 7 days in all experimental groups. Treatment with NAC did not affect PT changes induced by warfarin (Fig. 1A).

As we showed earlier, increase in sINR over threefold is accompanied by AKI in 5/6 nephrectomy rats. Scr levels in 5/6 nephrectomy rats treated with warfarin and vehicle were 1.18 ± 0.01 mg/ml after 7 days of treatment compared with 0.77 ± 0.01 mg/ml baseline (P < 0.0001; Fig. 1B). Treatment with NAC ameliorated this increase in Scr in a dose-dependent manner. Hence, Scr levels in 5/6 nephrectomy rats treated with warfarin and NAC (1, 10, 40, and 80 mg·kg⁻¹·day⁻¹) did not increase (Scr levels 1.21 ± 0.01 mg/ml, P = 0.06; 1.16 ± 0.01 mg/ml, P = 0.02; 0.94 ± 0.02 mg/dl, P < 0.001; and 0.78 ± 0.01 mg/dl, P < 0.001, respectively) 7 days after the treatment compared with 5/6 nephrectomy rats treated with warfarin only.

More prominent effects on warfarin-induced AKI were seen when 40 and 80 mg·kg⁻¹·day⁻¹ of NAC were used (Fig. 1B).

NAC does not change the hematuria or glomerular hemorrhage associated with WRN in 5/6 nephrectomy rats. Treatment with NAC did not significantly change hematuria associated with warfarin treatment in 5/6 nephrectomy rats (Fig. 1C).

We analyzed morphologic findings in kidneys obtained from 5/6 nephrectomy rats treated with warfarin. As we described earlier, excessive anticoagulation with warfarin results in a formation of occlusive RBC casts in 5/6 nephrectomy rats (31, 41). Treatment with NAC did not affect the occlusive RBC casts formation in 5/6 nephrectomy rats treated with warfarin. The occlusive RBC casts were seen in kidneys obtained from animals treated with warfarin and NAC (Fig. 2, A and B).

The percentage of occlusive RBC casts was similar in all experimental groups regardless of the NAC dose (Fig. 1D). Thus, the percentage of tubules containing occlusive RBC casts was 0.91 ± 0.3, 1.02 ± 0.27, 0.83 ± 0.3, 0.92 ± 0.3, and 0.9 ± 0.2% in 5/6 nephrectomy animals treated with warfarin and 0 (vehicle), 1, 10, 40, and 80 mg·kg⁻¹·day⁻¹ of NAC, respectively (P = 0.992). Focal acute tubular injury, seen in 5/6 nephrectomy rats treated with warfarin and vehicle, was not noted in the kidneys obtained from 5/6 nephrectomy rats treated with warfarin and 80 mg·kg⁻¹·day⁻¹ of NAC.

Oxidative stress is increased in the kidney in 5/6 nephrectomy rats treated with warfarin. The most widely used marker of protein oxidation is protein carbonyl content (37). We measured carbonyl content in the renal cortex, because it is more susceptible to oxidative stress compared with the renal cortex.
medulla in a nonischemia-reperfusion model (23). The ablative nephropathy by itself resulted in an increased levels of oxidized proteins in the kidney (protein carbonyl content was 4.0 ± 0.85 au in control and 5.95 ± 1.10 au in 5/6 nephrectomy rats 3 wk after the ablative surgery, \( P_{0.1937} \); Fig. 3A).

Warfarin treatment resulted in significantly increased levels of oxidized proteins in the kidney compared with control rats (protein carbonyl content increased up to 6.57 ± 0.46 au in 5/6 nephrectomy rats treated with warfarin and vehicle, \( P_{0.0135} \)). Treatment with warfarin and NAC resulted in decreased levels of oxidized proteins in the renal cortex in a dose-dependent manner. Thus, protein carbonyl content was 6.89 ± 0.94 au, \( P = 0.7364 \); 6.04 ± 0.83 au, \( P = 0.6058 \); 4.99 ± 0.58 au, \( P = 0.0488 \); and 4.32 ± 0.78 au, \( P = 0.0224 \) in 5/6 nephrectomy rats treated with warfarin and NAC (1, 10, 40, and 80 mg·kg\(^{-1}\)·day\(^{-1}\), respectively; \( P \) compared with 5/6 nephrectomy rats treated with warfarin and vehicle (Fig. 3A).

Iron chelator (DFO) does not affect warfarin-induced AKI. To investigate the role of iron in the pathogenesis of warfarin-induced AKI, 5/6 nephrectomy rats were treated with warfarin and DFO. Cotreatment with DFO did not affect serum creatinine level increase (Fig. 2D), nor hematuria, nor RBC tubular cast formation (data not shown) in 5/6 nephrectomy rats treated with warfarin. Prussian blue stain for iron was negative in the kidneys, even in the tubules with RBC casts (Fig. 2C).

NAC ameliorates AKI in an ischemia-reperfusion model. It is well-established that NAC protects kidney function in an ischemia-reperfusion model in rats (14, 29). The protective...
mechanism is mediated at least in part by the antioxidant effects of NAC. Here, we tested our NAC treatment protocol in a unilateral warm ischemia-reperfusion model. A 30-min ischemia-reperfusion resulted in a significant elevation of serum creatinine 24 and 48 h after ischemia in rats. As shown in Fig. 3B, NAC not only prevented an increase in Scr in a dose-dependent manner, but it also accelerated the recovery of Scr levels by 48-h postischemia. Specifically, Scr levels 24 h after a 30-min ischemia were 1.79 ± 0.15, 1.85 ± 0.03, 1.59 ± 0.02, and 1.05 ± 0.02 mg/dl in animals treated with 0 (vehicle), or NAC at 10, 40, and 80 mg·kg⁻¹·day⁻¹, respectively (P < 0.001). Moreover, 48-h post ischemia Scr levels were 1.65 ± 0.21, 1.12 ± 0.01, 1.01 ± 0.02, and 0.83 ± 0.02 mg/dl in animals treated with 0 (vehicle), or NAC at 10, 40, and 80 mg·kg⁻¹·day⁻¹, respectively (P < 0.001; Fig. 3B). The benefit of NAC in the ischemia-reperfusion model was similar to that of its benefit in WRN. This suggests that the protective effect of NAC in WRN is related to its antioxidant effect.

**DISCUSSION**

The present study is the first to elucidate pathogenetic mechanisms of WRN. We demonstrate herein that treatment with an antioxidant (NAC) does not prevent glomerular hemorrhage but does prevent AKI. The prevention of AKI is demonstrated by stable or nearly stable Scr levels and morphologically by reduced acute tubular injury.

WRN is more prevalent in patients with CKD, and we found that an underlying kidney condition is necessary to develop WRN (7). WRN is reproducible in 5/6 nephrectomy rats (31, 41). An association between oxidative stress and CKD development has been proposed (9, 26). Activity of different antioxidant enzymes, such as glutathione peroxidase and catalase, is reduced in CKD patients (12). The 5/6 nephrectomy model of ablative nephropathy is characterized by increased glomerular hyperfiltration/hyperperfusion injury (20, 21). Levels of angiotensin II (27) and nicotinamide adenine dinucleotide phosphate oxidase (NADPH) activity (38) are increased in 5/6 nephrectomy rats. We demonstrated increased protein carbonyl content in the renal cortex in 5/6 nephrectomy rats 3 wk after the ablative surgery (Fig. 3A), indicating increased oxidative stress. Increased angiotensin II levels are associated with podocyte loss in 5/6 nephrectomy animals (16). Antioxidants prevent increases in transforming growth factor (TGF)-β1, restore neuronal nitric oxide synthase expression in the kidney, and delay progression of glomerular sclerosis in 5/6 nephrectomy rats (18, 38).

Based on these data, we originally hypothesized that oxidative stress plays an important role in the pathogenesis of WRN.

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Fig. 2. Effects of NAC on kidney morphology in 5/6 nephrectomy rats treated with warfarin and effects of treatment with an iron chelator. A and B: animals treated with warfarin and vehicle (A) or warfarin and NAC (B; 80 mg·kg⁻¹·day⁻¹ of NAC is shown) had RBC casts in the tubules. Hematoxylin and eosin stain, ×200. C: stain for iron is negative in the kidney, including tubular epithelial cells surrounding RBC casts. Prussian blue, ×400. D: 5/6 nephrectomy rats were treated with 40 mg·kg⁻¹·day⁻¹ warfarin and deferoxamine (DFO; ip daily injections, doses shown per day). Both 10 and 100 mg·kg⁻¹·day⁻¹ of DFO did not affect serum creatinine increase associated with warfarin treatment.
Our original impression was that warfarin via direct and indirect effects (vitamin K-dependent proteins) may affect the glomerular filtration barrier, which results in glomerular hemorrhage. Subsequent occlusive RBC cast formation results in acute tubular injury and AKI. Based on our original hypothesis, oxidative stress might play a significant role in both glomerular filtration barrier damage and acute tubular injury.

We tested this hypothesis by treating 5/6 nephrectomy animals with warfarin and different doses of NAC. NAC is a well-characterized antioxidant, which has been used for many years in experimental research and clinical practice, including preventive effects on development of AKI (10, 22, 30, 36). We found that NAC in a dose-dependent manner prevents Scr increase associated with excessive anticoagulation by warfarin (Fig. 1B). However, NAC could not prevent either hematuria or RBC cast formation in 5/6 nephrectomy rats, suggesting that oxidative stress plays little, if any, role in the glomerular filtration barrier injury and glomerular hemorrhage associated with warfarin treatment (31, 41). Nevertheless, NAC in a dose-dependent manner prevented Scr increase in 5/6 nephrectomy rats treated with warfarin, suggesting, that despite RBC cast formation, acute tubular injury seen in WRN is diminished. Indeed, morphologically animals treated with NAC had less acute tubular injury compared with warfarin only-treated 5/6 nephrectomy rats. Beneficial effects of NAC on AKI are well-described in both experimental animals and patients (17, 35). Previous animal studies were based mostly on intraperitoneal administration of NAC (17, 18). We extended those observations and demonstrated that NAC oral treatment also prevented AKI after ischemia-reperfusion injury and accelerated recovery from it in a dose-dependent manner. Therefore, we believe that oxidative stress plays an important role in the deterioration of renal function and acute tubular necrosis in WRN (Fig. 4). It has been demonstrated that oxidative stress plays a significant role in an increased glomerular filtration barrier permeability (15, 33, 34) and development of different kidney diseases, including adriamycin-induced injury (4), podocyte damage, and diabetic nephropathy progression (13, 42). Our findings indicate that glomerular hemorrhage in WRN is independent of oxidative stress. The possible beneficial effects of NAC treatment in WRN may be associated with reduction of oxidative stress in the kidney (3, 25).

Our data suggest the following mechanisms of increased oxidative stress in the kidney in WRN. First, free hemoglobin released by RBC in the tubular lumen affects tubular epithelial cells by generating reactive oxygen species and increased lipid peroxidation (32). Second, free hemoglobin incorporates into the tubular epithelial cells via several surface receptors, such as megalin-cubilin receptors (39). Intracellularly, free hemoglobin activates caspases and induces apoptosis (19). Third, intracellular hemoglobin dissociates into globin and heme, while the latter is also a potent oxidant and activates proinflammatory pathways (39, 40). It is unlikely that free iron plays a signifi-

Fig. 3. Effects of NAC on oxidative stress in warfarin-related nephropathy (WRN) and serum creatinine levels in animals with acute ischemia-reperfusion injury. A: oxidized proteins were measured in the renal cortex by the protein carbonyl assay. Protein carbonyl content was elevated in 5/6 nephrectomy rats 3 wk after the ablative surgery. Treatment with warfarin increased protein carbonyl content in 5/6 nephrectomy rats. NAC in a dose-dependent manner decreased levels of oxidized proteins in 5/6 nephrectomy rats treated with warfarin. Treatments with warfarin, NAC, and ablative surgery are shown by solid lines under the x-axis. NAC doses (when administered) are shown under each column. #P < 0.05 compared with sham-operated rats. *P < 0.05 compared with 5/6 nephrectomy rats treated with warfarin + vehicle. B: control (nonoperated) rats were pretreated with NAC 24 h before ischemia-reperfusion injury. Unilateral ischemia-reperfusion in the left kidney for 30 min was created and unilateral nephrectomy of the right kidney was performed. Treatment with NAC was continued for 2 days after the ischemia-reperfusion injury. NAC in a dose-dependent manner ameliorated serum creatinine increase associated with ischemia-reperfusion injury; n = 6 in each group.

Fig. 4. Proposed role of oxidative stress in the pathogenesis of WRN. Warfarin via direct or vitamin K-dependent mechanisms induces glomerular hemorrhage. Oxidative stress does not play a significant role in the pathogenesis of glomerular hemorrhage, but it is a significant contributor to the acute tubular injury. Tubular occlusion by RBC casts plays a significant role in the development of acute kidney injury by increasing oxidative stress in the kidney.
cant role in the pathogenesis of acute tubular injury, because staining for iron is negative in tubular epithelial cells and treatment with the iron chelator (DFO) does not affect WRN in 5/6 nephrectomy rats (Fig. 2, C and D).

Our study indicates that glomerular hematuria is a necessary but not sufficient mechanism for the AKI in WRN. The dominant mechanism of the AKI of WRN is probably tubular obstruction by RBC casts, which leads to increased oxidative stress in the kidney.

These findings may have important clinical implications, when glomerular hemorrhage may be masked in patients who are treated with antioxidants. Monitoring of hematuria in these patients may provide better information about glomerular hemorrhage, because NAC did not affect hematuria in our experimental animals.

In conclusion, our study indicates that oxidative stress plays an important role in the clinical manifestations of WRN, such as AKI, but not in the cause of it (glomerular hemorrhage). Careful monitoring of hematuria in patients on warfarin treatment and antioxidants (such as vitamin E) is warranted to identify episodes of WRN.

REFERENCES

Author contributions: K.W. and Z.Q. performed experiments; K.W., Z.Q., S. V. Brodsky by the Department of Pathology, The Ohio State University.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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Guru V, Frenses SE. The role of N-acetylcySTEine in preventing radio


