N-Acetylcysteine Treatment Normalizes Serum Tumor Necrosis Factor-α Level and Hinders the Progression of Cardiac Injury in Hypertensive Rats

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**Background**—Studies in isolated cardiomyocytes showed that replenishment in cellular glutathione, achieved with the glutathione precursor N-acetylcysteine (NAC), abrogated deleterious effects of tumor necrosis factor-α (TNF-α).

**Methods and Results**—We examined the ability of NAC to limit the progression of cardiac injury in the rat model of hypertension, induced by the nitric oxide synthase inhibitor N^6^-nitro-L-arginine methyl ester (L-NAME) (50 mg/kg per day SC) and high-salt diet (HS) (8% NaCl). Four-week HS/L-NAME administration induced hypertension (193±8 versus 122±4 mm Hg for low-salt diet [LS] group) and left ventricular (LV) dysfunction, revealed by echocardiography and characterized by decreased LV shortening fraction (38±2% versus 49±4% for LS group; P<0.05) and decreased LV posterior wall thickening (49±3% versus 70±4% for LS group; P<0.05). LV dysfunction worsened further after 6-week HS/L-NAME administration. Importantly, increase in serum TNF-α level was strongly correlated with shortening fraction decrease and cardiac glutathione depletion. NAC (75 mg/d) was given as a therapeutic treatment in a subgroup of HS/L-NAME animals during weeks 5 and 6 of HS/L-NAME administration. NAC treatment, which replenished cardiac glutathione, had no effect on hypertension but reduced LV remodeling and dysfunction, normalized serum TNF-α level, and limited activation of matrix metalloproteinases -2 and -9 and collagen deposition in LV tissues.

**Conclusions**—These findings suggest that glutathione status determines the adverse effects of TNF-α in cardiac failure and that TNF-α antagonism may be achieved by glutathione supplementation. (Circulation. 2004;110:2003-2009.)

**Key Words:** tumor necrosis factor □ acetylcysteine □ glutathione □ heart failure □ N^6^-nitroarginine methyl ester

During the past decade, β-adrenergic antagonists and angiotensin-converting enzyme inhibitors have considerably improved therapy of heart failure. Nevertheless, heart failure remains a major health problem in developed countries with an aging population.1 In concert with neurohormones, the proinflammatory cytokine tumor necrosis factor-α (TNF-α) contributes to cardiac remodeling and heart failure progression.2 Accordingly, TNF-α antagonism constitutes an important target of heart failure therapy.1,2 However, compounds that trap TNF-α, comprising infliximab, an antibody directed to TNF-α, and etanercept, a soluble recombinant receptor of TNF-α, gave disappointing outcomes in clinical trials.2,3 Several explanations for why those therapies have failed have been proposed. On the one hand, infliximab, through complement fixation, lys TNF-α—expressing cells. Those include cardiomyocytes in failing hearts. On the other hand, by stabilizing biologically active (homotrimeric) TNF-α, etanercept acts, in the long term, as a TNF-α agonist. Finally, physiological levels of TNF-α are necessary for cardiovascular homeostasis, and sustained lowering of TNF-α may contribute to loss of beneficial effects of the cytokine.2,4

In cardiomyocytes, activation of the neutral sphingomyelinas mediates TNF-α–induced apoptosis and negative contractile effect.5–8 Glutathione is the physiological inhibitor of the neutral sphingomyelinas.9 We recently showed that administration to rats of the glutathione precursor N-acetylcysteine (NAC) abrogates TNF-α–induced neutral sphingomyelinas activation, oxidative stress, and negative effects on contraction in isolated cardiomyocytes.8 These results prompted us to examine the effects of NAC treatment in an experimental model of cardiac dysfunction and remodeling.

Chronic administration to rats of the NO synthase inhibitor N^6^-nitro-L-arginine methyl ester (L-NAME) produces systemic arterial hypertension that leads to cardiac remodeling.

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and dysfunction.10 Because high salt intake aggravates L-NAME–induced cardiac damages,11–13 we combined an 8% NaCl (high-salt) diet (HS) with L-NAME administration. After 4 weeks, HS/L-NAME rats exhibited cardiac dysfunction that we monitored by echocardiography. We then sought to determine whether curative treatment with NAC, given orally (75 mg/d) for weeks 5 and 6 of HS/L-NAME administration, could be effective in replenishing cardiac glutathione content, improving left ventricular function, and reversing elevation of serum TNF-α level in 6-week HS/L-NAME rats.

**Methods**

**Rat Model of Chronic Inhibition of NO Synthesis Associated With High-Salt Diet**

This study was performed in accordance with the Helsinki Recommendations for Humane Treatment of Animals During Experimentation. Male Wistar rats (weight, 300 to 360 g; Janvier, LeGenest St Isle, France) were used. Animals had free access to either standard 0.8% NaCl or 8% NaCl rat chow (UAR) and tap water. L-NAME (Sigma; 50 mg/kg per day) was administered via Alzet osmotic pumps (model 2ML4, Janvier) that were implanted subcutaneously (Sigma; 50 mg/kg per day) was administered via Alzet osmotic pumps (model 2ML4, Janvier) that were implanted subcutaneously. We combined an 8% NaCl diet with L-NAME administration, could be effective in replenishing cardiac glutathione content, improving left ventricular function, and reversing elevation of serum TNF-α level in 6-week HS/L-NAME rats.

**Echocardiography**

Echocardiographic examinations were performed with the use of a VINGMED CFM750 echocardiograph with a 9-MHz probe. After intraperitoneal anesthesia with xylazine (20 mg/kg) and ketamine (50 mg/kg) and bidimensional short-axis examination, a time-motion line was drawn between papillary muscles to record instantaneous elevation of serum TNF-α one content, improving left ventricular function, and reversing elevation of serum TNF-α level in 6-week HS/L-NAME rats.

**Measurement of Serum TNF-α Levels**

Serum TNF-α levels were determined with a double-sandwich ELISA specific for rat TNF-α (Pierce Endogen-Perbio).

**Measurement of Cardiac Glutathione Content**

Glutathione was measured in the cardiac tissues according to a modification of Tietze.14

**Measurement of Matrix Metalloproteinase Activities**

Left ventricles, stored in liquid nitrogen after rat euthanasia, were homogenized at 4°C with an Ultra-Turrax T25 (Janke-Kunkel) in 50 mmol/L HEPES buffer, pH 7.4, containing 1 mmol/L phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 3000g for 10 minutes.

Matrix metalloproteinase (MMP)-2 and -9 activities in supernatant fractions were assayed by gelatin zymography on the basis of their molecular weights (58 to 62 kDa and 92 kDa in nonreducing conditions, respectively), as previously described.15 Activities were quantified by scanning densitometry (NIH Image 1.61; David Chow and Jai Evans) and calculated from a standard curve established with varying recombinant MMP-9 samples, with 1 U of MMP-9 taken as 100%.

**Histological Analysis and Collagen Quantification**

Hearts, stored in formaldehyde solution, were embedded in paraffin and cut into 4-μm sections that were mounted onto slides (Superfrost Plus, Menzel-Glaser) and stained with Sirius red F3BA (0.1% solution in saturated aqueous picric acid) to color collagen. Intersitial fibrosis was analyzed by capture with Image-Pro Plus 5.0 (Jai Evans) and calculated from a standard curve established with varying recombinant MMP-9 samples, with 1 U of MMP-9 taken as 100%.

**Systolic diameter; LV shortening fraction (LV SF), defined as (LV EDD–LV end-systolic diameter)/LV EDD×100; LV indexed end-diastolic diameter (LV iEDD), defined as LV EDD/body weight (BW); LV posterior end-diastolic wall thickness (LV EDWT) and LV posterior end-systolic wall thickness; LV mass, calculated according to the formula LV mass=1.05×([LV EDD+(LV EDWT)×2–LV EDD])/LV EDWT×100; and LV posterior indexed end-diastolic wall thickness (LV iEDWT), defined as LV EDWT/BW.**

Echocardiographic examinations were performed at baseline, before implantation of minipumps, and after 4- and 6-week treatments. Baseline data were compared for all treatment groups and did not show differences (not shown).

**Results**

**Echocardiography**

Echocardiographic examinations were performed with the use of a VINGMED CFM750 echocardiograph with a 9-MHz probe. After intraperitoneal anesthesia with xylazine (20 mg/kg) and ketamine (50 mg/kg) and bidimensional short-axis examination, a time-motion line was drawn between papillary muscles to record instantaneous elevation of serum TNF-α one content, improving left ventricular function, and reversing elevation of serum TNF-α level in 6-week HS/L-NAME rats.
Statistical Analysis
All results are given as mean±SEM. Results were analyzed by the Mann-Whitney test or Kruskal-Wallis test and Dunn post test, as appropriate. Differences were considered statistically significant at P<0.05.

Results
Changes in Blood Pressure and LV Parameters After 4 and 6 Weeks of 8% NaCl Diet Combined or Not With L-NAME Administration
The Table displays blood pressure and in vivo LV parameters. Up to 6 weeks, HS diet had no significant effect on SBP. However, LV mass and LV/BW ratio were increased after 4-week HS diet (1.33±0.08 g and 3.2±0.1 mg/g versus 0.92±0.09 g and 2.2±0.2 mg/g for 4-week LS, respectively; both P<0.05). After 6-week HS, the increase in LV mass was associated with modest but significant increase in iEDD and decrease in SF (both P<0.05; Table).

After 4 weeks, SBP in the HS/L-NAME group plateaued at a value approaching 200 mm Hg. Echocardiographic examination showed marked decreases in SF (38±2% versus 49±4% for 4-week LS; P<0.05) and WT (49±3% versus 70±4% for 4-week LS; P<0.05) and an increase in iEDWT (0.54±0.01 versus 0.41±0.01 mm/100 g for 4-week LS; P<0.05). Decreases in SF and WT and increases in iEDWT worsened in 6-week HS/L-NAME rats, which also displayed increases in LV, LV/BW, and iEDD and a decrease in BW (all P<0.05; Table).

TNF-α Level in Serum and Cardiac Glutathione Content
SF decrease in HS/L-NAME rats was strongly correlated with an increase in serum TNF-α level (r=0.61; P=0.0002; Figure 1A). Interestingly, the increase in serum TNF-α level was also strongly correlated with a depletion in cardiac glutathione content (r=0.55; P=0.0074; Figure 1B).

Effects of NAC Treatment on Cardiac Glutathione Content and Serum TNF-α Level
NAC (75 mg/d) was given as a curative treatment during weeks 5 and 6 of HS/L-NAME administration. Cardiac glutathione content, which was decreased by 21% in 6-week HS/L-NAME compared with 6-week LS rats (0.45±0.05 versus 0.57±0.02 μmol/g tissue, respectively; P<0.05; Figure 2A), resumed control values in 6-week HS/L-NAME+NAC rats (0.60±0.02 μmol/g tissue; Figure 2A). Likewise, serum TNF-α level, which was increased by 2.4-fold in 6-week HS/L-NAME compared with 6-week LS rats (341±36 and 145±23 pg/mL, respectively; P<0.05; Figure 2B), was normalized in 6-week HS/L-NAME+NAC rats (194±61 pg/mL; Figure 2B).

Effects of NAC Treatment on Blood Pressure and LV Alterations
As shown in the Table, NAC treatment had no effect on hypertension (206±3 mm Hg in 6-week HS/L-NAME+NAC rats), nor did it relieve LV enlargement, illustrated by iEDD, which was induced by HS and was similar in the following 3 subgroups: 6-week HS, 6-week HS/L-NAME, and 6-week HS/L-NAME+NAC (2.1±0.1, 2.2±0.1, and 2.1±0.1 mm/100 g, respectively). In contrast, without difference in blood pressure, the decrease in LV SF observed in 6-week HS/L-NAME rats (34±2% versus 49±1% for 6-week LS; P<0.05) was blunted in 6-week HS/L-NAME+NAC rats (41±2%; Table). LV SF value in 4-week HS/L-NAME+NAC rats was 38±2%. NAC treatment also preserved contraction of the LV posterior wall, illustrated by LV WT (50±3% in 6-week HS/L-NAME+NAC versus 39±2% in 6-week HS/L-NAME; P<0.05) and limited hypertrophy of the LV posterior wall, as shown by the lower increase in iEDWT in 6-week HS/L-NAME+NAC rats compared with 6-week HS/L-NAME rats (0.54±0.01 versus 0.41±0.01 mm/100 g for 4-week LS; P<0.05). After 6-week HS, the increase in LV mass was associated with modest but significant increase in iEDD and decrease in SF (both P<0.05; Table).

Figure 1. Increase in serum TNF-α level correlated with decrease in SF (A) and decrease in cardiac glutathione content (B). A, LV SF and serum TNF-α were examined in 6-week LS (n=15; black squares), 6-week HS (n=9; open squares), and 6-week L-NAME (n=8; triangles) rats. r=0.61; P=0.0002. B, Serum TNF-α and glutathione content in cardiac tissue were measured in 6-week LS (n=9; black squares), 6-week HS (n=7; open squares), and 6-week L-NAME (n=8; triangles) rats. r=0.55; P=0.0074; 95% confidence bands of the regression line are represented by dotted lines.
NAME rats (0.50 ± 0.03 and 0.57 ± 0.01 mm/100 g, respectively [P < 0.05], compared with 0.40 ± 0.01 mm/100 g for 6-week LS group). This made iEDWT value in the 6-week HS/L-NAME+NAC group not significantly different from that in the 4-week HS/L-NAME group (0.54 ± 0.01 mm/100 g). WT improvement in the 6-week HS/L-NAME+NAC group, compared with the 6-week HS/L-NAME group, paralleled that of SF (Table). Taken together, these data show that NAC, given as a therapeutic treatment, can prevent the progression of structural and functional cardiac alterations in hypertensive HS/L-NAME rats.

Effect of NAC Treatment on Zymographic Abundance of MMP-2 and MMP-9 and Fibrosis

Gelatin zymographic MMP-2 abundance in cardiac tissue (58- and 62-kDa bands) increased by 2.2-fold in 6-week HS/L-NAME compared with 6-week LS rats (P < 0.05; Figure 3). MMP-9 (92-kDa band), which was undetectable in 6-week LS, was easily measurable in hearts obtained from 6-week HS/L-NAME rats (Figure 3). In 6-week HS/L-NAME+NAC rats, both MMP-2 and MMP-9 zymographic abundance were reduced to approximately 65% and 50% of their values in 6-week HS/L-NAME rats, respectively (both P < 0.05). Collagen accumulation was assessed by Picrosirius red staining of heart sections. Mild perivascular and interstitial fibrosis was observed in 4-week HS/L-NAME rats (Figure 4). Collagen deposits expanded in 6-week HS/L-NAME rats but were considerably reduced in 6-week HS/L-NAME+NAC rats (Figure 4).

Discussion

This in vivo study ensues from our report that NAC treatment protects isolated cardiomyocytes against TNF-α--induced oxidative stress, neutral sphingomyelinase activation, and negative inotropic effect.³ The major findings
of this study are that NAC, given orally as a curative treatment, replenishes cardiac glutathione content, normalizes levels of serum TNF-α, and prevents hypertension-induced morphological and functional injuries in HS/L-NAME rats.

The model of L-NAME rat is related to the cardiovascular disease that renal patients develop because of chronically elevated plasma levels of the endogenous NO synthase inhibitor asymmetrical dimethylarginine. One peculiarity of this model is the limited hypertrophy (Table) that has been previously reported and that is attributable to the negative effect of L-NAME on protein synthesis. Another characteristic of the L-NAME rat model is the onset of LV dysfunction, which depends on the salt regimen. In the present study, within 4 weeks, L-NAME administration combined with an 8% NaCl diet produced LV dysfunction that could be monitored by echocardiography. In addition, postmortem analysis revealed cardiac fibrosis, suggesting that this model was a suitable model of LV remodeling.

An important feature in the present HS/L-NAME rat model is the correlation between LV dysfunction and the increase in serum TNF-α level, which, in humans, is a major hallmark of heart failure. Another advantage of the HS/L-NAME rat model is the rapid onset and progression of the disease. However, its limitation comes from the inhibition of NO synthase, which, in other models of developing heart failure, participates in the oxidative stress and is a component in TNF-α signaling pathways. Ongoing experiments are directed to study the potential long-term beneficial effect of NAC in those other models.

Although the effects of NAC are multiple, it is tempting to confer part of the beneficial effects of NAC on its ability to increase glutathione content in cardiac tissue. In support of this, high cellular glutathione content not only inhibits neutral sphingomyelinase activation, which mediates deleterious effects of TNF-α, but also inhibits nuclear factor-κB activation, which supports TNF-α expression. Furthermore, TNF-α overexpression in transgenic mice leads to adverse cardiac remodeling, characterized by increased total MMP activity and fibrosis. Accordingly, inhibition of MMP activation and reduction in fibrosis observed in hearts of NAC-treated rats would result in part from inhibition of TNF-α pathways. Thus, NAC treatment, through glutathione repletion, would preserve cardiomyocyte contraction and matrix structure, 2 components that are essential for the maintenance of cardiac function.

Oxidative stress is implicated in the pathogenesis of heart failure. Hence, large clinical trials have dealt with...
antioxidants, in particular vitamin E and vitamin C, that gave disappointing results.23 Recent studies focused on glutathione peroxidase, an antioxidant enzyme that uses glutathione to reduce hydrogen peroxide, lipid peroxides, and peroxynitrite. On the one hand, it was shown that erythrocyte glutathione peroxidase I activity is inversely associated with the risk of cardiovascular events in patients.28 On the other hand, in mice, overexpression of glutathione peroxidase prevents LV remodeling and failure after myocardial infarction.29 Furthermore, the beneficial antioxidant action of NAC in acute cardiomyositis has been assessed in different studies.30 Recently, Usui et al reported that, in rats administered L-NAME for 3 days, NAC, given as a preventive treatment, limited inflammatory changes in coronary arteries. Additionally, in hemodialysis patients, treatment with NAC reduces cardiovascular events.31 Taken together, those studies pointed out the antioxidant property of NAC. However, none underscored the possibility that NAC was also a primary glutathione precursor. Our study is the first suggesting that tissue glutathione content determines the severity of heart failure. Glutathione depletion is a common hallmark of chronic diseases, including cancer, HIV infection, and fatigue. Accordingly, the beneficial effects of NAC therapy on immunological function in HIV-positive patients32 and muscular performance in aging individuals33 are attributed to tissular glutathione repletion.

In conclusion, in addition to β-adrenergic receptor blockers and angiotensin-converting enzyme inhibitors,35 TNF-α antagonism remains an important target of heart failure therapy.1,2,36 The decrease in circulating TNF-α was not reached with antagonists of TNF-α, including the soluble recombinant human TNF-α receptor (etanercept) or the monoclonal antibody directed against the cytokine (infliximab). This in part may explain the failure of those therapies.2,3 NAC, given as a curative treatment to rats with cardiac injury, normalizes serum TNF-α and improves heart function. Although direct correlations between the effects of NAC treatment in rats and its possible beneficial effects in patients with heart failure are not appropriate, these findings provide a strong rationale for considering NAC as a possible inexpensive, nontoxic therapy for the management of heart failure.

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