Effect of N-acetylcysteine on sympathetic hyperinnervation in post-infarcted rat hearts

Tsung-Ming Lee1,2, Po-Yu Lai3, and Nen-Chung Chang2,4*

1Cardiology Section, Department of Medicine, Chi-Mei Medical Center, Tainan, Taiwan; 2Department of Internal Medicine, School of Medicine, Taipei Medical University, Taipei, Taiwan; 3School of Medicine, Taipei Medical University, and 4Cardiology Section, Department of Internal Medicine, Taipei Medical University Hospital, 252, Wu-Hsing St Taipei, Taiwan

Received 5 May 2009; revised 13 August 2009; accepted 14 August 2009; online publish-ahead-of-print 20 August 2009

Aims
The purpose of this study was to determine whether N-acetylcysteine (NAC) attenuates cardiac sympathetic hyperinnervation through replenishment of glutathione in infarcted rats.

Methods and results
After ligation of the coronary artery, male Wistar rats were randomized to either vehicle, NAC, or vitamins C + E groups for 4 weeks. Post-infarction was associated with increased oxidant release, as measured by tissue isoprostane and myocardial glutathione. Measurement of myocardial norepinephrine levels revealed a significant elevation in vehicle-treated infarcted rats compared with sham-operated rats. Sympathetic hyperinnervation was blunted after administering NAC, as assessed by immunofluorescent analysis of tyrosine hydroxylase and western blotting and real-time quantitative RT–PCR of nerve growth factor. Arrhythmic scores during programmed stimulation in the vehicle-treated infarcted rats were significantly higher than those in animals treated with NAC. Although NAC and vitamins showed similar effects on ventricular remodelling, only NAC demonstrated beneficial effects on sympathetic hyperinnervation. Furthermore, the effects of NAC on nerve growth factor were abolished by administering L-buthionine sulfoximinem, an inhibitor of γ-glutamylcysteine ligase.

Conclusion
Chronic use of NAC, but not vitamins, after infarction is associated with down-regulation of nerve growth factor proteins, probably through a glutathione-dependent pathway, and thus plays a critical role in the beneficial effect on the arrhythmogenic response to programmed electrical stimulation.

Keywords
Glutathione † Myocardial infarction † N-acetylcysteine † Norepinephrine † Sympathetic innervation

1. Introduction
N-acetylcysteine (NAC) is widely used in a variety of branches of medicine with a high safety profile. NAC is readily hydrolysed to cysteine, a precursor of glutathione (GSH). GSH is synthesized by a two-step reaction involving the enzymes γ-glutamylcysteine ligase and γ-glutamylcysteine synthetase. γ-Glutamylcysteine synthetase is the rate-limiting enzyme in the process of GSH synthesis. This mechanism makes NAC function other than as an antioxidant or free radical scavenger. Although NAC can directly scavenge free radicals, the rate constants for their reaction with reactive oxygen species are several orders of magnitude lower than those of antioxidant enzymes such as superoxide dismutase and catalase. Thus, the direct free radical scavenging activity of NAC is not likely to be of great importance for its antioxidant activity in vivo. Several other antioxidants and free radical scavenging agents do not share with NAC the capacity to prevent PC12 pheochromocytoma cell line and sympathetic neuron death. The commonly used antioxidants such as vitamin C can directly neutralize free radicals; however, they cannot replenish the cysteine required for GSH synthesis and replenishment. Although previous studies have demonstrated a cardioprotective effect of NAC on minimization of reperfusion injury in acute myocardial infarction (MI), the observed beneficial effects do not provide information on whether similar effects would be present on ventricular remodelling with long-term administration of NAC. Increased sympathetic nerve density after MI has been shown to be responsible for the occurrence of lethal arrhythmias and sudden cardiac death in humans. During chronic stage of MI, regional increase of sympathetic nerves was commonly observed.

* Corresponding author. Tel: +886 2 2737 2181, Fax: +886 2 2736 4222, Email: ncchang@tmu.edu.tw

Published on behalf of the European Society of Cardiology. All rights reserved. © The Author 2009. For permissions please email: journals.permissions@oxfordjournals.org
at the remote zone.\textsuperscript{9} Nerve growth factor (NGF) is a prototypic member of the neurotrophin family, members of which are critical for the differentiation, survival, and synaptic activity of the peripheral sympathetic and sensory nervous systems.\textsuperscript{10} Levels of NGF expression within innervated tissues roughly correspond to innervation density.\textsuperscript{11} These results demonstrated the importance of NGF in the regulation of sympathetic innervation. The treatment of anti-NGF by administering antisera, target ablation, or gene disruption has been shown to prevent nerve sprouting.\textsuperscript{12}

Cardiac remodelling is an unfavourable evolution associated with myocardial hypertrophy and increased sympathetic nerve density after MI.\textsuperscript{13} Cardiac remodelling is a complex process involving numerous signalling pathways. Post-infarction was associated with decreased GSH activity.\textsuperscript{14} GSH deficiency is thought to contribute to the progression of cardiac remodelling and failure.\textsuperscript{15} GSH exerts a potent neuroprotective effect.\textsuperscript{16} Thus, we assessed whether chronic administration of NAC can result in attenuated heart hyperinnervation after infarction through attenuated expression of NGF and the role of GSH. The purpose of this study was to investigate whether chronic administration of NAC results in attenuated hyperinnervation of the heart through attenuated expression of NGF, and to assess the role of GSH in sympathetic innervation in a rat MI model using \textsuperscript{L}-buthionine sulfoximine (BSO), a specific and transition-state inhibitor of \textsuperscript{\gamma}-glutamylcysteine synthetase.

\section*{2. Methods}

\subsection*{2.1 Animals}

\subsubsection*{2.1.1 Part 1}

Male Wistar rats (300–350 g) were subjected to ligation of the anterior descending artery as previously described\textsuperscript{17} resulting in infarction of the LV free wall. Rats were randomly assigned into either vehicle (saline) group, NAC (250 mg/kg per day, Sigma, St Louis, MO, USA) or vitamin C (150 mg/kg per day, Sigma) and E (200 mg/kg per day, Sigma). The dosages of vitamins C and E were based on previous studies.\textsuperscript{18} To prevent potentially prooxidant actions of vitamin E used alone,\textsuperscript{19} vitamins E and C were both used. Vitamin C is capable of regenerating vitamin E radical after its interaction with reactive oxygen species.\textsuperscript{19} These vitamins were selected because they have been reported to prevent death of neuronal cells induced by oxidative stress.\textsuperscript{20} The doses of NAC used in this study have been shown to effectively modulate cardiac GSH without significantly changing blood pressure.\textsuperscript{21}

The drugs were started 24 h after infarction, at a time when they could produce maximum benefits.\textsuperscript{22} The study duration was designed to be 4 weeks because the majority of the myocardial remodelling process in the rat (70–80\%) is complete within 3 weeks.\textsuperscript{23} The drugs were administered by daily oral gavage. Sham-operated rats served as controls to exclude the possibility that the drugs themselves directly altered sympathetic innervation. In each-treated group, drugs were withdrawn about 24 h before the end of the experiments in order to eliminate their pharmacological actions.

\subsubsection*{2.1.2 Part 2}

Although results of the above study showed that the amount of NGF was significantly attenuated after administering NAC at 4 weeks after infarction (see Results), the involved mechanism remained unclear. To evaluate the importance of GSH in NAC-related NGF expression, we performed an \textit{in vitro} experiment. Four weeks after induction of MI by coronary ligation, infarcted rat hearts were isolated and subjected to no treatment (vehicle), NAC (60 mM), BSO (200 \mu M, Sigma), NAC + BSO. Each heart was perfused with a non-circulating modified Tyrode’s solution as previously described.\textsuperscript{24} Drugs were infused for 120 min. The dose of BSO has been shown to cause the depletion of intracellular GSH.\textsuperscript{25} NAC-promoted survival of sympathetic neurons occurs at concentrations of 20–60 mM.\textsuperscript{26} At the end of the study, all hearts (n = 10 per group) were used for western blot at the remote zone.

\subsection*{2.1.3 Part 3}

To assess the dose effect of NAC on NGF expression, we performed an additional experiment in which NAC was administered at different doses. Twenty-four hours after ligation of the anterior descending artery, rats (n = 5 in each group) were randomly allocated into control, low-dose NAC (125 mg/kg per day), and high-dose NAC (250 mg/kg per day) groups. The drug was administered as described in Part 1. Western blot of NGF obtained from the remote zone was performed at week 4. The animal experiment was approved and conducted in accordance with local institutional guidelines for the care and use of laboratory animals in the Chi-Mei Medical Center and conformed with the \textit{Guide for the Care and Use of Laboratory Animals} published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).
2.4 **In vivo electrophysiological studies**

To assess the potential arrhythmogenic risk of sympathetic innervation, we performed *in vivo* programmed electrical stimulation after left thoracotomy and artificial respiration. Because the residual neural integrity at the infarct site is one of the determinants of the response to electrical induction of ventricular arrhythmias, only rats with transmural scar were included. Body temperature was maintained at 37°C with a thermostatically controlled heating lamp. Programmed electrical stimulation was performed with electrodes sewn to the epicardial surface of the right ventricular outflow tract. Pacing pulses were generated from a Bloom stimulator (Fischer Imaging Corporation, Denver, CO, USA). To induce ventricular arrhythmias, pacing was performed at a cycle length of 120 ms (S1) for eight beats, followed by one to three extra-stimuli (S2, S3, and S4) at shorter coupling intervals. The endpoint of ventricular pacing was induction of ventricular tachyarrhythmia. Ventricular tachyarrhythmias including ventricular tachycardia and ventricular fibrillation were considered non-sustained when it lasted ≤15 beats and sustained when it lasted >15 beats. An arrhythmia scoring system was modified as previously described. When multiple forms of arrhythmias occurred in one heart, the highest score was used. The experimental protocols were typically completed within 10 min.

2.5 **Real-time reverse transcription–polymerase chain reaction of NGF**

Real-time quantitative reverse transcription–polymerase chain reaction (RT–PCR) was performed from samples obtained from the remote zone (≥2 mm outside the infarct) with the TaqMan system (Prism 7700 Sequence Detection System, PE Biosystems) as previously described. For NGF, the primers were 5’-GGCTACCTGGACACC AATCT-3’ (sense) and 5’-GGCTCCAGACAAAGAACGAC-3’ (antisense). For cyclophilin, the primers were 5’-ATGGTCAACCCACC GTGTTCTTCG-3’ and 5’-CGTGTGAAGTCACCACCCTGAC C-3’. Cyclophilin mRNA was chosen as the internal standard because it is expressed at a relatively constant level in virtually all tissues. For quantification, NGF expression was normalized to the expressed housekeeping gene cyclophilin. Reaction conditions were programmed on a computer linked to the detector for 40 cycles of the amplification step.

2.6 **Western blot analysis of NGF**

Samples obtained from the remote zone at week 4 after infarction. Rabbit polyclonal antibodies to NGF (Chemicon, CA, USA) were used. Western blotting procedures were described previously. Experiments were replicated three times and results expressed as the mean value.

2.7 **Immunofluorescent studies of tyrosine hydroxylase, growth-associated factor 43, and neurofilament**

In order to investigate the spatial distribution and quantification of sympathetic nerve fibres, analysis of immunofluorescent staining was performed on LV muscle from the remote zone. Papillary muscles were excluded from the study because a variable sympathetic innervation has been reported. Paraffin-embedded tissues were sectioned at a thickness of 5 μm. Tissues were incubated with anti-tyrosine hydroxylase (1:200; Chemicon, CA, USA), anti-growth associated protein 43 (a marker of nerve sprouting, 1:400; Chemicon), and anti-neurofilament antibodies (a marker of sympathetic nerves, 1:1000; Chemicon) in 0.5% BSA in PBS overnight at 37°C. Rhodamine-conjugated anti-rabbit IgG from goat was used as secondary antibody. Isotopy-identical directly conjugated antibodies served as a negative control.

The slides were coded so that the investigator was blinded to the identification of the rat sections. The nerve density was measured on the tracings by computerized planimetry (Image Pro Plus, Media Cybernetics, Silver Spring, MD) as described previously. The density of nerve fibres was qualitatively estimated from 10 randomly selected fields at a magnification of 400× and expressed as the ratio of labelled nerve fibre area to total area.

2.8 **Laboratory measurements**

GSH activity of homogenized heart tissue from the remote zone was measured by using a commercially available kit (Cayman chemical, Ann Arbor, USA) and following the manufacturer’s instructions. Heart tissue was homogenized in cold 50 mM MES buffer (pH 6.7, provided by the assay kit manufacturer) containing 1 mM EDTA per gram tissue and centrifuged at 10 000 g for 15 min at 4°C to obtain supernatant for GSH analysis. The absorbance was read at 405 nm. Levels of GSH activity were expressed as micromoles per gram of protein.

Myocardial tissue free 15-F2t-isoprostane, a reliable index for *in vivo* oxidative stress-induced lipid peroxidation, was measured by using an EIA kit (Cayman chemical, Ann Arbor, USA). Homogenized heart tissue (in PBS) was purified using Affinity Sorbent/Column (Cayman chemical) in the presence of 0.01% butylated hydroxytoluene and then processed for analysis of 15-F2t-isoprostane as previously described. The values of heart tissue 15-F2t-isoprostane were expressed as pg/g tissue.

Although cardiac innervation was detected by immunofluorescence staining of tyrosine hydroxylase, growth-associated factor 43, and neurofilament, it did not imply that the nerves are functional. Thus, to examine the sympathetic nerve function after administering NAC, we measured LV norepinephrine levels from the remote zone. The myocardium were minced and suspended in a 0.4 N perchloric acid with 5 mmol/L reduced GSH (pH 7.4), homogenized with a polytron homogenizer for 60 s in 10 vol. Total norepinephrine was measured using a commercial ELISA kit (Noradrenalin ELISA, IBL Immuno-Biological Laboratories Co., Hamburg, Germany).

2.9 **Statistical analysis**

Results were presented as mean ± SD. Statistical analysis was performed using the SPSS statistical package (SPSS, version 11.0, Chicago, IL). Differences among the groups of rats were tested by ANOVA. In case of a significant effect, the measurements between the groups were compared with Bonferroni’s correction. Electrophysiological data (scoring of programmed electrical stimulation-induced arrhythmias) were compared by a Kruskal–Wallis test followed by a Mann–Whitney test. The significant level was assumed at value of *P* < 0.05.

3. **Results**

Differences in mortality among the infarcted groups were not found throughout the study. Either NAC, or vitamins had little effect on cardiac gross morphology in the sham-operated rats. Four weeks after infarction, the infarcted area of the LV was very thin and was totally replaced by fully differentiated scar tissue. The vehicle- and vitamin-treated infarcted groups had an increase in right-ventricular weight/body weight ratio and lung weight/body weight ratio, compared with NAC-treated infarcted group. The weight of the LV inclusive of the septum remained
essentially constant for 4 weeks among the infarcted groups (Table 1). LV end-systolic pressure, LV end-diastolic pressure, \( +\text{dp/dt} \), \( -\text{dp/dt} \), and infarct size did not differ among the infarcted groups.

### 3.1 Tissue GSH, 15-F_{2t}-isoprostane, and norepinephrine levels

Ventricular remodelling was associated with a significant reduction in GSH content (4.2 ± 1.1 vs. 15.3 ± 2.8 \( \mu \text{mol/g} \) protein in sham, \( P < 0.001 \), Table 2). Treatment with vitamins had a significant effect on GSH depletion. Administration of NAC induced a significantly increased GSH content compared with vitamins.

Myocardial free 15-F_{2t}-isoprostane in vehicle-treated infarcted rats significantly increased as compared to sham (\( P < 0.001 \), Table 2). Myocardial free 15-F_{2t}-isoprostane in NAC-treated infarcted rats can be reduced to the levels similar to those in the vitamin-treated infarcted rats.

To investigate the possible role of cardiac norepinephrine synthesis, we determined the LV norepinephrine levels. Either NAC or vitamin administration did not affect tissue norepinephrine concentrations in sham-operated rats. LV norepinephrine levels were significantly upregulated 1.9-fold in the vehicle-treated infarcted rats in comparison with sham (2.28 ± 0.25 vs. 1.18 ± 0.22 \( \mu \text{g/g} \) protein, \( P < 0.001 \)). When compared with vehicle- and vitamin-treated infarcted rats, NAC-treated infarcted rats had significantly lower LV norepinephrine.

### 3.2 Echocardiography

Compared with sham, MI hearts showed structural changes such as increased LV diastolic and systolic diameters (Table 3), consistent with LV remodelling. Echocardiography showed a significant decrease in LV end-systolic dimension and LV end-diastolic dimension in NAC- or vitamin-treated infarcted rats in comparison with

### Table 1 Cardiac morphology and haemodynamics at the end of study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham</th>
<th>Infarction treated with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>NAC</td>
</tr>
<tr>
<td>Number of rats</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>402 ± 16</td>
<td>418 ± 18</td>
</tr>
<tr>
<td>Heart rate, b.p.m.</td>
<td>378 ± 21</td>
<td>398 ± 18</td>
</tr>
<tr>
<td>LVESP, mmHg</td>
<td>108 ± 7</td>
<td>104 ± 6</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>6 ± 1</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>+dp/dt, mmHg/s</td>
<td>7425 ± 464</td>
<td>7382 ± 363</td>
</tr>
<tr>
<td>−dp/dt, mmHg/s</td>
<td>6782 ± 372</td>
<td>6092 ± 382</td>
</tr>
<tr>
<td>Infarct size, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVW/BW, mg/g</td>
<td>2.18 ± 0.19</td>
<td>2.08 ± 0.21</td>
</tr>
<tr>
<td>RVW/BW, mg/g</td>
<td>0.54 ± 0.07</td>
<td>0.53 ± 0.09</td>
</tr>
<tr>
<td>LungW/BW, mg/g</td>
<td>4.16 ± 0.59</td>
<td>4.19 ± 0.43</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

BW, body weight; LungW, lung weight; LVEDP, left-ventricular end-diastolic pressure; LVESP, left-ventricular end-systolic pressure; LVW, left-ventricular weight; NAC, N-acetylcysteine; RVW, right-ventricular weight; Vitamins, vitamins C + E.

†P < 0.005 compared with sham.

‡P < 0.01 compared with respective sham-operated rats.

### Table 2 Tissue GSH, 15-F_{2t}-isoprostane, and norepinephrine concentration at the end of study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham</th>
<th>Infarction treated with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>NAC</td>
</tr>
<tr>
<td>Number of rats</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>GSH, ( \mu \text{mol/g protein} )</td>
<td>15.3 ± 2.8</td>
<td>18.4 ± 3.1</td>
</tr>
<tr>
<td>15-F_{2t}-isoprostane, ( \mu \text{g/g} ) tissue</td>
<td>562 ± 72</td>
<td>524 ± 73</td>
</tr>
<tr>
<td>NE, ( \mu \text{g/g} ) protein</td>
<td>1.18 ± 0.22</td>
<td>1.13 ± 0.21</td>
</tr>
</tbody>
</table>

Values are mean ± SD. Abbreviations as in Table 1. NE, norepinephrine.

*P < 0.025 compared with respective sham-operated rats.

†P < 0.005 compared with the vehicle-treated infarcted group.

‡P < 0.01 compared with the NAC-treated infarcted group.

‡P < 0.005 compared with the NAC-treated infarcted group.
those in vehicle-treated infarcted rats. LV FS was significantly higher in NAC- or vitamin-treated infarcted rats in comparison with those in vehicle-treated infarcted rats (19 ± 4% in vehicle vs. 23 ± 3%, 25 ± 3%, both P < 0.0001, respectively). These findings suggest the improvements in LV remodelling and function by NAC or vitamin treatment.

### 3.3 Immunofluorescent analyses

The tyrosine hydroxylase-immunostained nerve fibres appeared to be oriented in the longitudinal axis of adjacent myofibres (Figure 1, upper panel). Tyrosine hydroxylase-positive nerve density was significantly increased in the vehicle-treated infarcted rats than that in sham group (Figure 1, lower panel). NAC-treated rats show lower nerve density at the remote regions than vehicle- and vitamin-treated rats (0.32 ± 0.14%, 0.28 ± 0.15% vs. 0.09 ± 0.20% in NAC group, both P < 0.0001, respectively). Similar to tyrosine hydroxylase results, densities of growth associated protein 43-(Figure 2) and neurofilament-positive (data not shown) nerves were significantly attenuated in the NAC-treated infarcted rats compared with those in vehicle- and vitamin-treated infarcted groups. These morphometric results mirrored those of norepinephrine contents.

### 3.4 NGF protein and mRNA expression

Western blot shows that NGF levels were significantly upregulated 5.3-fold at the remote zone in the vehicle-treated infarcted rats than in sham-operated rats (P < 0.0001, Figure 3 upper panel). When compared with vehicle- and vitamin-treated infarcted rats, NAC-treated infarcted rats had significantly lower NGF levels at the remote zone. To elucidate the role of GSH in modulating NGF, BSO was assessed in an in vitro model. Figure 3 middle panel shows that BSO significantly increased expression of NGF compared with NAC alone, confirming the role of GSH in mediating NGF expression. Furthermore, NAC treatment significantly attenuated NGF expression in a dose-dependent manner (Figure 3, lower panel).

PCR amplification of the cDNA revealed that the NGF mRNA levels showed a 4.4-fold upregulation at the remote zone in the vehicle-treated infarcted rats compared with sham-operated rats (P < 0.0001, Figure 4). In NAC-treated infarcted rats, the NGF mRNA levels were significantly decreased compared with those in the vehicle- and vitamin-treated infarcted rats.

### 3.5 Electrophysiological stimulation

To further elucidate the physiological effect of attenuated sympathetic hyperinnervation, ventricular pacing was performed. Arrhythmia score in sham-operated rats was very low (0.2 ± 0.6) (Figure 5). In contrast, ventricular tachyarrhythmias consisting of ventricular tachycardia and ventricular fibrillation were inducible by programmed stimulation in vehicle-treated infarcted rats. NAC treatment significantly decreased the inducibility of ventricular tachyarrhythmias compared with those in the vehicle- and vitamin-treated infarcted groups.

### 4. Discussions

Our present study shows for the first time that chronic treatment for 4 weeks with NAC leads to attenuated sympathetic innervation after MI. Further, our results, which show the inability of alternative antioxidative agents such as vitamins C + E to mimic the NAC effect on norepinephrine, suggest that the effect of NAC on sympathetic innervation is independent of its antioxidative property. These results were consistent with the beneficial effects of NAC, as documented structurally by reduction in cardiac nerve sprouting, molecularly by myocardial NGF protein and mRNA levels, biochemically by tissue GSH and norepinephrine levels, and functionally by improvement of ventricular remodelling and fatal ventricular tachyarrhythmias. Taken together, in spite of similar ventricular remodelling after infarction in rats treated with either NAC or vitamins, NAC, but not vitamins, can attenuate ventricular arrhythmias. The results were consistent with the findings of Belichard et al., showing that the improvement of adverse ventricular remodelling after MI did not imply the beneficial effect on arrhythmias and the relation between ventricular remodelling and arrhythmias was more complex than previously thought.

The effect of NAC on attenuated sympathetic innervation was supported by three lines of evidence: (i) Substantial evidence indicates that the balance between oxidants and antioxidants is severely disturbed in post-infarcted myocardial tissues. Our present study showed that oxidative stress as assessed by myocardial GSH and 15-F_{2α}-isoprostane content is increased in the remote non-infarcted myocardium after MI, consistent with previous studies showing that oxidative stress determined by the level of lipid peroxidation is increased in the remote non-infarcted myocardium after MI in rats; (ii) the beneficial effects of NAC on
attenuated sympathetic innervation might be associated with increased GSH levels. Although both NAC and vitamin treatments displayed comparable reduction in free radical assessed by 15-F_{2\alpha}-isoprostane, tissue GSH levels were significantly increased in infarcted rats treated with NAC compared with vitamins. Vitamins C and E, compounds that are antioxidants but lack GSH-reducing activity, did not attenuate sympathetic innervation. Thus, NAC may affect sympathetic innervation by reducing relevant thiol, but not acting as an antioxidant. It has been suggested that NAC increases intracellular GSH either by being converted to cysteine, a precursor of GSH, or by reducing extracellular cystine to cysteine which is more efficiently transported into cells. These results indicate that the neuronal remodelling after infarction is subject to GSH regulation; (iii) the severity of pacing-induced fatal arrhythmias was associated with the degree of sympathetic innervation. The finding was consistent with the findings of Cao et al., showing that increased post-injury sympathetic nerve density may be responsible for the occurrence of ventricular arrhythmias and sudden cardiac death in animals and patients.

The superior protective effects of NAC on attenuated sympathetic innervation are likely related to its unique antioxidant properties that are not shared by vitamins. First, exogenous oxidants such as vitamins C and E increase gene expression of γ-glutamylcysteine synthetase by activating AT 4 MEK and p38 MAP kinase pathways. Previous studies have demonstrated that there is significant overlap in the functions of GSH and vitamin C in the destruction of free radicals. In newborn rats with GSH depletion by treatment with BSO, administration of vitamin C prevented tissue injury and death. Our data showed that vitamin treatment caused a 1.6-fold increase in myocardial GSH content. NAC administration increases GSH levels three-fold, a significant increase compared with vitamin treatment. GSH has the unusual property that it

Figure 1 Upper: immunofluorescent staining for tyrosine hydroxylase from the remote regions (magnification 400 ×). Tyrosine hydroxylase-positive nerve fibres are located between myofibrils and are oriented longitudinal direction as that of the myofibrils. (A) Sham; (B) infarction treated with vehicle; (C) infarction treated with NAC; (D) infarction treated with vitamins. Bar = 50 μm. Lower: nerve density area fraction (%) at the remote zone. Each column and bar represents mean ± SD. *P < 0.017 compared with vehicle- and vitamin-treated groups.
becomes a more potent reductant and effective antioxidant as its concentration increases. This is a consequence of the formation of a disulfide bridge between two GSHs, when oxidized (GSSG). The reduction potential is dependent upon the Nernst equation and varies as the logarithm of \([\text{GSH}]_2/\text{GSSG}\), becoming more negative with increasing GSH concentration. Therefore, modest increases in GSH concentration result in exceptionally large effects on the antioxidant defense network involving thioredoxin, peroxiredoxins, and glutaredoxins.\(^{37}\) Second, the NGF promoter contains activator protein-1,\(^{38}\) which is subjected to redox regulation through its conserved cysteine residue.\(^{39}\) NAC has been shown to react with cysteine residue on the activator protein-1 molecule, which in turn suppressed the activator protein-1 activation in PC12 cells.\(^{40}\) It is possible that NAC may attenuate the expression of NGF by inhibiting the activator protein-1. Third, previous studies have shown that an increase in the intracellular flux of oxidants activates NF-\(\kappa B\) by initiating dissociation of an inhibitory subunit, I-\(\kappa B\), and the subsequent translocation of the active cytosolic protein complex to the nucleus, with consequent binding to a consensus binding motif in the promoter of various genes.\(^{41}\) NAC, as a thiol-containing reducing agent, can effectively inhibit this activation. Thus, although previous studies have demonstrated that GSH and vitamin C have many actions in common and that they can compensate for each other under conditions where one of them is systemically depleted,\(^{36}\) our present data argue against NAC and vitamins C and E playing equally important roles in sympathetic innervation after infarction. Our study showed that the response of sympathetic innervation after infarction to NAC may be complex, resulting from both direct, NAC-dependent reduction of the proteins and indirect effects via intracellular GSH.

Figure 2 Upper: immunofluorescent staining for growth associated protein 43 from the remote regions (magnification 400×). (A) sham; (B) infarction treated with vehicle; (C) infarction treated with NAC; (D) infarction treated with vitamins. Bar = 50 μm. Lower: nerve density area fraction (%) at the remote zone. Each column and bar represents mean ± SD. *\(p < 0.017\) compared with vehicle- and vitamin-treated groups. Growth associated protein 43-positive staining was markedly increased in groups treated with vehicle and vitamins.
4.1 Other mechanisms

Although the present study suggests that the mechanisms of NAC-induced attenuation of sympathetic innervation and arrhythmias may be related to attenuated GSH-dependent NGF expression, other potential mechanisms need to be studied such as TrkA signalling pathway, tumour necrosis factor-α, and electrical remodelling. First, NAC has been shown to attenuate NGF-induced neuronal differentiation by inhibiting TrkA activation and its downstream signalling pathways. Thus, NAC quantitatively and qualitatively suppressed the action of NGF. Second, NAC acting as a tumour necrosis factor-α antagonist, may directly attenuate ventricular arrhythmias. Tumour necrosis factor-α prolonged action potential duration and may be associated with susceptibility to lethal ventricular arrhythmias. No data are available regarding local expression of tumour necrosis factor-α changes after administering NAC and vitamins in infarcted rats and their functional consequences. Further studies are needed to elucidate the role of tumour necrosis factor-α in differential effect of NAC and vitamins on ventricular arrhythmias. Finally, NAC might prevent fatal arrhythmias by directly inhibiting...
excluding automatic ventricular rhythms. Electrophysiological testing is most utilized in humans for determination of arrhythmia vulnerability. In this study, NAC treatment can prevent fatal arrhythmias; however, vitamins C and E did not provide protective effects. Our result may explain in part why large clinical trials dealing with antioxidants, in particular vitamin E and vitamin C, gave contrasting results. The Heart Outcome Prevention Evaluation (HOPE)\(^4\) and HOPE-The Ongoing Outcome\(^5\) trials fail to show beneficial cardiovascular effects in high-risk subjects with vitamin E supplementation. In contrast, the beneficial antioxidant action of NAC has been confirmed in different studies.\(^5\)

### 4.3 Conclusions

These data show that GSH status determines sympathetic hyperinnervation after infarction and that GSH replenishment by administering NAC attenuates sympathetic hyperinnervation. These effects probably are functionally important because they are linked to attenuated severity of fatal arrhythmias.

**Conflict of interest:** None declared.

### Funding

This work was supported by the grants of Chi-Mei Medical Center (CMFHR9744, 98CM-TMU03), National Health Research Institutes (NHRI-EX98-9841S), and Department of Health, Republic of China (DOH98-TD-I-111-TM001).

### References


