Effects of oral N-acetylcysteine on walking capacity, leg reactive hyperemia, and inflammatory and angiogenic mediators in patients with intermittent claudication

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NEW & NOTEWORTHY

Our findings indicate that short-term oral treatment with the antioxidant N-acetylcysteine does not improve walking tolerance and leg blood flow and prevents maximal exercise-induced increase in the expression of angiogenic mediators in peripheral blood in patients with intermittent claudication.

PERIPHERAL ARTERIAL DISEASE (PAD) is a common manifestation of atherosclerosis and a significant cause of death and disability in the aging population (51). Recent epidemiological studies indicate that PAD affects 1 in 10 people aged over 70 yr (19). Intermittent claudication (IC) is the main symptom of PAD and is characterized by walking-induced pain, typically in the calf muscles, that subsides with rest (66). Patients with IC have greatly impaired leg functioning and walking capacity (32, 33). Supervised exercise training is the most efficacious therapy to alleviate the symptoms of IC, but, unfortunately, only a small fraction of patients have access to and engage in structured exercise regimens (46). The phosphodiesterase inhibitor cilostazol has also been shown to modestly improve walking capacity in patients with IC, but this drug is costly, has poor tolerability due to side effects, and cannot be used in patients with heart failure (5, 40). A pressing need remains for novel therapeutic strategies that can effectively enhance exercise tolerance in patients with IC (5).

Increased inflammation and oxidative stress are key factors in the development and progression of PAD (6). These patients have higher circulating levels of several inflammatory and oxidative stress markers compared with patients without the disease (20, 39). Of note, inflammatory markers are strong predictors of walking capacity and functional decline in these patients (30, 31). This proinflammatory state is particularly exacerbated by exercise in symptomatic patients. A single bout of maximal exercise in patients with IC triggers robust increases in inflammatory cytokines, vascular adhesion molecules, and markers of oxidative damage (37, 54, 60, 61). Among other consequences, repeated exposure to these injurious events is thought to promote functional and structural abnormalities in skeletal muscle (13, 24, 44) and endothelial dysfunction (57). In addition, increased inflammation and oxidative stress are associated with vascular rarefaction (22) and might impair the angiogenic potential of exercise (2, 62). Thus strategies that prevent and/or minimize exercise-induced oxidative stress and inflammation can potentially restore skeletal
muscle function, improve endothelial function, and enhance the benefits of exercise in patients with IC.

The goal of the present study was to determine the acute effects of oral treatment with the antioxidant N-acetylcysteine (NAC) on walking capacity, postocclusive reactive hyperemia (RH), circulating levels of inflammatory mediators, and whole blood expression of angiogenic factors in patients with IC. Short-term oral treatment with NAC has been shown to mitigate the inflammatory response to exercise in healthy young individuals (36, 58) and increase fatigue resistance in patients with chronic obstructive pulmonary disease (COPD) (26). Furthermore, we have recently shown that treatment with NAC improves fatigue resistance in the soleus muscle in a preclinical model of PAD (49). Based on this evidence, we hypothesized that NAC would abrogate inflammation and enhance angiogenic signaling after a maximal exercise test in patients with IC. We also anticipated that antioxidant treatment would improve walking capacity in these patients.

MATERIALS AND METHODS

Study Population

Male patients with an ankle-brachial index (ABI) ≤ 0.90 and history of stable IC were recruited from the local outpatient vascular clinic. Exclusion criteria included the following: 1) critical limb ischemia; 2) inability to walk on a treadmill at 2 mph; 3) exercise limited by other disease or conditions; and 4) use of drugs to treat claudication (pentoxifylline or cilostazol) and antioxidants. The study was approved by the Human Subject Protection Committee of the University of São Paulo Medical School (189,465). All patients gave written, informed consent to participate in the study.

Protocol

Before commencing the experimental protocol, participants were asked to complete two familiarization sessions, at least 1 wk apart. In each session, subjects underwent a graded treadmill exercise test to determine the test-retest reliability of pain-free and maximal walking times. Next, using a randomized double-blinded crossover design, patients received NAC (1,800 mg/day) or placebo (PLA) for 4 consecutive days. On the 5th day, the patients came to the laboratory for the experimental session and received a final dose of NAC (2,700 mg) or PLA 1 h before assessment of physiological outcomes. This regimen was based on the study by Koechlin and coworkers (26), which showed that oral treatment with NAC improved quadriceps endurance time and prevented exercise-induced oxidative stress in patients with COPD. The last dose was given ~1 h before the treadmill test because prior studies documented that peak plasmatic concentrations of NAC are attained within ~60–120 min following ingestion (3, 4, 42). NAC or the equivalent PLA was given as effervescent tablets (PharmaNAC, 900 mg of NAC per tablet). A washout period of at least 10 days was given between the two arms of the protocol.

Participants were asked to refrain from consuming alcohol during the treatment period and to avoid exercise for 48 h before the experimental sessions. On arrival at the laboratory for the experimental sessions, anthropometric measures of participants were recorded, and a 20-gauge intravenous catheter was placed antiseptically into an antecubital vein by standard techniques. Participants were then given the final dose of NAC/PLA and rested for 1 h sitting on a chair. Next, patients were instrumented for the assessment of leg blood flow, heart rate, and brachial and ankle blood pressures. After completion of these preexercise hemodynamic measurements, the patients underwent a symptom-limited maximal exercise test on the treadmill. Immediately after the test, the patients returned to the examination table, and ankle systolic blood pressure was measured. After 30 min, the second assessment of leg blood flow was performed. Blood samples were taken 10 min after catheter placement, 1 h after ingestion of NAC or PLA, and 5 and 30 min following the completion of the exercise test.

Measurements

ABI. Systolic blood pressure of the brachial artery and dorsalis pedis and posterior tibial arteries were measured bilaterally with, respectively, auscultation and a handheld Doppler (Martec, DV 6000, Ribeirão Preto, Brazil). The ABI was calculated for each leg by the ratio between the highest systolic blood pressure in the leg and highest systolic blood pressure in the brachial artery.

Leg blood flow. Postocclusive RH was determined in the leg with the lowest ABI by venous occlusion plethysmography (64). Peak leg blood flow following circulatory occlusion, as assessed by venous occlusion plethysmography, has been shown to be markedly impaired in patients with IC compared with their healthy counterparts (52). Blood pressure cuffs were firmly wrapped around the lower thigh and the ankle regions, and a mercury-in-Silastic strain gauge (Hokanson, AI-6) was placed around the widest part of the calf. The cuff placed in the ankle region was inflated to 250 mmHg to exclude the influence of the foot circulation. For the baseline measurements, the cuff placed around the thigh was rapidly inflated for 10 s at 40–60 mmHg, followed by 10 s of deflation over a period of 3 min. After the baseline measurements were completed, the cuff was inflated to 200 mmHg for 5 min. Peak RH was defined as the first blood flow measurement obtained immediately following cuff deflation. Blood flow was expressed as milliliters of blood per 100 ml of tissue per minute (38).

Gardner treadmill test. A graded treadmill test (treadmill speed of 2 mph, 0% grade with increments of 2% every 2 min) was performed for determination of pain-free and maximal walking times.Expired gases were continuously measured during the test by means of a metabolic analysis system (Medical Graphics, CPX2D) for assessing the peak oxygen consumption. Blood pressure was measured by auscultation technique by an experienced observer every 2 min, while the heart rate was continuously assessed by an electrocardiograph (Cardio Perfect MD) and registered every minute.

Blood sampling. Fifteen milliliters of blood were collected in standard anticoagulant EDTA-treated tubes for the determination of plasma levels of soluble vascular cell adhesion protein-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), endothelin-1 (ET-1), monocyte chemotactic protein-1 (MCP-1), and glutathione (GSH). Samples were centrifuged within 30 min of collection, divided into aliquots, and stored at −80°C until analysis. Another 3 ml of blood were collected in special tubes containing an RNA-stabilization solution (PAXgene blood RNA tube, Becton Dickinson) for the determination of whole blood expression of microRNA (miRNA)-126, sprouty-related EVH1 domain containing 1 (SPRED1), phosphatidylinositol 3-kinase regulatory β-subunit (PI3KR2), vascular endothelial growth factor (VEGF), and endothelial nitric oxide synthase (eNOS).

Inflammatory markers, ET-1, and redox balance. sVCAM-1, sICAM-1, MCP-1, and ET-1 were determined by enzyme-linked immunosorbent assay, according to the manufacturer’s instructions (Quantikine, R&D Systems). The levels of GSH and oxidized glutathione (GSSG) were determined using a fluorescent detection kit following the manufacturer’s instructions (Arbor Assays, Ann Arbor, MI). The samples used in this assay were not treated with butylated hydroxytoluene.

Whole blood RNA isolation. Total RNA, including miRNA, was isolated using the PAXgene Blood miRNA Kit (Qiagen, Hilden, Germany), following the manufacturer’s recommendations. Isolated RNA was stored at −80°C. After extraction, the total RNA concentration was quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and checked for integrity by EtBr-agarose gel electrophoresis.
RESULTS

Thirteen patients were initially enrolled in the study and completed the initial familiarization sessions. Of these, two patients withdrew consent after the first experimental visit, and one patient developed an ulcer in the foot and was not able to complete the protocol. The clinical characteristics of the remaining 10 patients are presented in Table 1. There were no differences in demographic and clinical characteristics between excluded patients and the remaining cohort. Treatment with NAC was well tolerated with no adverse effects.

Reproducibility of Walking Test

Comparison of walking times in the two familiarization sessions revealed that determination of pain-free (test 1: 128 ± 23 s and test 2: 242 ± 35 s, intraclass correlation coefficient of 0.76, and coefficient of variation of 14.39) and especially maximal walking times (test 1: 558 ± 86 s and test 2: 527 ± 83 s, intraclass correlation coefficient of 0.98, and coefficient of variation of 7.6) are reliable in these patients.

Redox Status

As shown in Fig. 1, GSH-to-GSSG ratio (GSH/GSSG) increased markedly 1 h following the ingestion of NAC and remained elevated until the end of the experimental session. There were no changes in this variable after the ingestion of PLA (Fig. 1).

Walking Capacity and Responses to Exercise Test

Pain-free and maximal walking times were not different between experimental sessions (Fig. 2). Likewise, peak oxygen consumption, rest and peak heart rate, systolic blood pressure, diastolic blood pressure, and rest and postexercise ankle blood pressure were similar after treatment with NAC or PLA (Table 2).

Postocclusive RH

Baseline and postocclusive leg blood flow determined before and after the exercise bout are shown on Table 3. Baseline blood flows were slightly increased in the postexercise assessment.

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Table 1. Demographic and clinical characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n</th>
<th>SE</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>72</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26.3</td>
<td>1.2</td>
<td></td>
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<tr>
<td>ABI most affected leg</td>
<td>0.49</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>ABI least affected leg</td>
<td>0.65</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Race, %white</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smoking, %</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medication, %</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statins</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antihypertensive agents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-blocker</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diuretics</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiotensin II receptor antagonist</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthine oxidase inhibitor</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metformin</td>
<td>40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Values are means ± SE or percentages; n, no. of subjects. ABI, ankle-brachial index; ACE, angiotensin converting enzyme inhibitor.

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mRNA and miRNA analysis by real-time polymerase chain reaction. RNA were primed with 0.5 μg/μl oligo(dT) (Fermentas/Thermo Scientific Molecular Biology, Rockford, IL) to generate first-strand DNA. Reverse transcription was performed using Rever-taid M-MuLV Reverse Transcriptase (Fermentas/Thermo Scientific Molecular Biology). cDNA for miRNA analysis was synthesized from total RNA using gene-specific primers, according to the TaqMan miRNA Assay protocol (Applied Biosystems, Carlsbad, CA). Real-time quantification of SPRED1, PI3KR2, VEGF, and eNOS mRNA was performed with a SYBRGreen PCR Master Mix, using ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The expression of cyclophilin was measured as an internal control for sample variation in the reverse transcription reaction. Primers were designed using the Primer3 software (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi). The DNA sequence was obtained from GenBank, and primers were made in separate exons to distinguish PCR products derived from cDNA by size from those derived from genomic DNA contaminants. To accurately detect mature miRNA-126 (INV 2228), the real-time PCR quantification method was performed using TaqMan miRNA Assay Protocol (Applied Biosystems). miRNAs samples were normalized by evaluating U6 expression. Each blood sample was analyzed in duplicate. Relative quantities of target gene expressions were compared after normalization to the values of the reference gene (ΔCt). Fold changes in mRNA and miRNA expression were calculated using the differences in ΔCt values between the two samples (ΔΔCt) and the equation 2−ΔΔCt. Results are expressed as percentage of control.

Statistical Analysis

Data are presented as means ± SE. Paired t-tests were used to compare differences in rest and peak metabolic and hemodynamic variables between the experimental sessions. Two-way ANOVA for repeated measures was used to compare differences before and after PLA or NAC ingestion and after treadmill exercise between experimental sessions. The Tukey post hoc test (STATISTICA software; StatSoft, Tulsa, OK) was used for individual comparisons between means when a significant change was observed with ANOVA. P values < 0.05 were accepted as statistically significant.
ment compared with the preexercise assessment (P < 0.05). Postocclusive RH was not different between experimental sessions before or after maximal exercise (Table 3).

**Inflammatory Markers and ET-1**

Baseline levels of inflammatory markers (sVCAM-1, sICAM-1, MCP-1) and ET-1 were similar between the experimental sessions (Fig. 3). Immediately after exercise, the levels of sVCAM-1 and MCP-1 increased markedly on both sessions. The levels of ET-1 were also significantly higher (P < 0.05) immediately and 30 min after exercise compared with baseline values. The levels of sICAM-1 were not affected by exercise. There were no differences between NAC and PLA for any of these markers before and after exercise.

**Angiogenic Factors**

As shown in Fig. 4, whole blood expression of pro-angiogenic miRNA-126 increased 30 min after maximal exercise in the PLA session. On the other hand, the expression levels of this factor did not change after treatment with NAC. PI3KR2 expression decreased after exercise in the PLA session, while no change was observed in the NAC session. The expression of SPRED1 was not affected by exercise and was similar with PLA and NAC. VEGF and eNOS expression increased after exercise in the PLA, but not in the NAC session (Fig. 4).

**DISCUSSION**

Acute oral NAC improved redox balance in patients with IC as revealed by the increase in plasma GSH/GSSG. Despite this response, NAC had no effect on walking capacity, leg postocclusive RH, and inflammation in these patients. Most importantly, treatment with NAC prevented exercise-induced increases in whole blood expression of miRNA-126, VEGF, and eNOS, important angiogenic mediators. These latter findings suggest that exercise-induced oxidative stress is critical for the systemic angiogenic response to exercise in patients with IC.

**NAC and Walking Capacity**

In a seminal report, Arosio and coworkers (1) showed that intravenous infusion of GSH twice a day for 5 consecutive days improved pain-free walking time and leg blood flow recovery after exercise in patients with symptomatic PAD. These findings suggested that enhancing GSH, a major endogenous antioxidant, is a promising therapeutic strategy for PAD (1). Unfortunately, direct administration of GSH results in poor tissue availability (53), and, most importantly, repeated intravenous administration is not amenable for long-term clinical treatment. One attractive alternative is the use of NAC, which acts as a cysteine donor and supports the biosynthesis of GSH (12). As shown in the present study (Fig. 1) and previous studies (10, 15, 36), oral treatment with NAC elevates blood GSH levels. We hypothesized that the improvements in GSH redox status would lead to enhanced exercise performance in patients with IC. Contrary to our predictions, pain-free and maximal walking times were not altered by antioxidant treatment in the present study (Fig. 2). This observation at odds with the findings of Koechlin and coworkers (26), which reported that an oral NAC regimen similar to the one employed in this study improved quadriceps endurance in patients with COPD. The exact reasons behind these discrepant findings are not known, but it is conceivable that, in addition to differences in the pathophysiology of PAD and COPD, differences in the exercise modality and intensity between the two studies might play a role. Indeed, while Koechlin and coworkers (26) used submaximal, small-muscle mass exercise (dynamic knee extensions at 40% of the maximal voluntary contraction), the patients in the present study underwent an incremental, symptom-limited treadmill exercise test. These protocol differences are important because the effects of NAC on muscle fatigue appear to be task and intensity specific (16, 29). In fact, marked improvements in muscle performance after NAC are particularly evident during small-muscle mass exercise at submaximal intensities (26, 29). Nonetheless, we chose treadmill testing

**Table 2. Cardiopulmonary and hemodynamic responses to treadmill exercise following treatment with NAC and PLA**

<table>
<thead>
<tr>
<th></th>
<th>PLA</th>
<th>NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak oxygen uptake, ml·kg⁻¹·min⁻¹</td>
<td>14.8 ± 1.1</td>
<td>15.1 ± 1.1</td>
</tr>
<tr>
<td>Peak heart rate, beats/min</td>
<td>112 ± 6</td>
<td>113 ± 6</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>127 ± 5</td>
<td>129 ± 7</td>
</tr>
<tr>
<td>Peak</td>
<td>174 ± 9</td>
<td>171 ± 11</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>73 ± 4</td>
<td>74 ± 4</td>
</tr>
<tr>
<td>Peak</td>
<td>80 ± 6</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>Ankle blood pressure, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>78 ± 6</td>
<td>83 ± 6</td>
</tr>
<tr>
<td>5 min postexercise</td>
<td>62 ± 5</td>
<td>61 ± 11</td>
</tr>
</tbody>
</table>

Values are mean ± SE. PLA, placebo; NAC, N-acetylcysteine.

**Table 3. Leg blood flow measured at baseline and after 5 min of circulatory arrest before and 30 min following the completion of the exercise test after treatment with NAC and PLA**

<table>
<thead>
<tr>
<th></th>
<th>PLA</th>
<th>NAC</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline, ml·100 ml tissue⁻¹·min⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>1.23 ± 0.14</td>
<td>1.19 ± 0.15</td>
<td>Treatment: 0.90</td>
</tr>
<tr>
<td>Post</td>
<td>1.74 ± 0.41*</td>
<td>1.81 ± 0.55*</td>
<td>Time: 0.00</td>
</tr>
<tr>
<td>Peak, ml blood·100 ml tissue⁻¹·min⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>4.01 ± 0.75</td>
<td>4.81 ± 0.99</td>
<td>Treatment: 0.33</td>
</tr>
<tr>
<td>Post</td>
<td>5.52 ± 1.03</td>
<td>4.84 ± 1.53</td>
<td>Time: 0.61</td>
</tr>
</tbody>
</table>

Values are mean ± SE. Pre and Post, before and 30 min after completion of the exercise test. *Significantly different from Pre (P < 0.05).
instead of small-muscle mass exercise because treadmill walking mimics more closely what these patients experience in daily life (25).

Effects of NAC on Vascular Function and Exercise-induced Inflammation

In agreement with several other studies (28, 54, 60, 61), we observed that maximal exercise elicited a marked increase in the systemic levels of inflammatory mediators and ET-1 in patients with IC (Fig. 3). One prevailing view is that this abnormal response stems in part from ischemia-induced activation of inflammatory cells in the affected legs (37). As first shown by Neumann and coworkers (37) and later by others (7, 8), exercise in claudicants increases total neutrophil number and the proportion of activated neutrophils in the venous blood draining from the affected leg, whereas no changes are observed in the unaffected leg. We reasoned that NAC would improve leg vascular dysfunction in patients with IC, thereby reducing exercise-induced ischemia and the consequent inflammatory response. Recent studies demonstrated that oral treatment with NAC improved vascular function in patients with chronic kidney disease (50) and cardiorenal syndrome (9). Despite the observed increase in GSH/GSSG, there was no difference in leg postocclusive RH or on MCP-1 or VCAM-1 levels between the sessions in the present study (Fig. 3). It is important to highlight that treatment duration in studies that have reported a positive effect of NAC on vascular function (9, 50) were considerably longer (4–6 wk) than the regimen adopted in the present investigation. Conceivably, a long-term intervention might be required in order for the beneficial effects of NAC on vascular function to manifest.

One potential consequence of exercise-induced increase in systemic oxidative stress and inflammation in patients with IC is a temporary impairment in vascular reactivity. Prior studies have shown that flow-mediated dilation (FMD) of the brachial artery is markedly reduced following maximal exercise in these patients (56, 57). Intravenous infusion of vitamin C prevents the reduction in FMD, which indicates that this abnormal response is mediated, at least in part, by oxidative stress (57). Along these lines, we hypothesized that a maximal exercise test would impair leg postocclusive RH in these patients. However, as shown in Table 3, there were no significant reductions in peak postocclusive RH after exercise in the present study. These findings indicate that the leg vasculature might respond differently to maximal exercise compared with the brachial artery in patients with IC. This is not surprising, as it has been shown that vascular dysfunction is greater in the leg than in the arm in patients with PAD (52). Since leg vascular reactivity is already severely compromised, it is possible that the exercise stimulus might not induce further dysfunction (i.e., “floor effect”). Another possibility is that, if present, the impairment in leg vascular function following exercise is very brief. In the studies of Silvestro and coworkers (56, 57), the postexercise assessment of FMD was performed shortly after the maximal exercise bout, while our measurements of postocclusive hyperemia were conducted 30 min following the completion of the exercise bout. Additional studies are warranted to determine the time course of changes in vascular function in response to exercise in these patients. Lastly, it is worth noting that the mechanisms underlying brachial FMD are likely different than those involved in calf postocclusive hyperemia (11). Therefore, it is conceivable that differences in the method employed for the assessment of vascular function might partially account for the discrepancy between the two studies.

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**Fig. 3.** Plasma levels of soluble vascular cell adhesion protein-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), monocyte chemotactic protein-1 (MCP-1), and endothelin-1 (ET-1) measured before and 1 h following ingestion of NAC or PLA, and 5 and 30 min after the maximal exercise test. Values are means ± SE. *P < 0.05 vs. baseline.
NAC and Angiogenic Signaling

Increased oxidative stress and inflammation have been linked to impaired angiogenic signaling and microvascular rarefaction in skeletal muscle (2, 62). We anticipated that oral treatment with NAC would enhance exercise-induced increase in angiogenic mediators in PAD. We focused on miRNA-126 and its downstream targets (17), which control the expression of VEGF, likely the most important factor involved in exercise-induced angiogenesis (63). miRNA-126 is an endothelial-specific miRNA that promotes VEGF signaling by inhibiting SPRED1 and PIK3R2 (17, 18). The expression of circulating miRNA-126 is known to be markedly reduced in patients with PAD (59). Here, we showed for the first time that miRNA-126 expression in whole blood increases following maximal exercise in patients with IC, while the expression of the VEGF pathway negative regulator PIK3R2 is reduced (Fig. 4). As a result, VEGF and eNOS expression increased after exercise compared with baseline levels in the PLA session. Contrary to our initial hypothesis, treatment with NAC abolished these responses, which indicates that miRNA-126 signaling is redox sensitive in patients with IC. These findings lend support to the increasingly recognized concept that several pivotal pathways involved in exercise-induced adaptations are dependent on oxidative stress (23, 48). For instance, antioxidants have been shown to prevent exercise-induced increased expression of peroxisome proliferator-activated receptor-γ coactivator 1α, a master regulator of angiogenesis in skeletal muscle (48, 55). In a recent study in young trained individuals, Petersen and coworkers (43) showed a high dose of NAC attenuated some of the early adaptive signaling responses in skeletal muscles. Along with our findings, these observations indicate that NAC can potentially have detrimental rather than positive effects for patients with IC.

Clinical Implications

Our laboratory (65) and others (27) have shown that exercise training promotes a substantial improvement in functional capacity in patients with IC. This effect is thought to be mediated in part by an increase in skeletal muscle capillarization, which enhances the surface area for oxygen delivery and

Fig. 4. Expression of circulating microRNA-126 (miRNA-126), phosphatidylinositol 3-kinase regulatory β-subunit (PI3KR2), sprouty-related EVH1 domain containing 1 (SPRED1), vascular endothelial growth factor (VEGF), and endothelial nitric oxide synthase (eNOS) measured before and 1 h following ingestion of NAC or PLA, and 30 min after the end of the maximal exercise test. Values are means ± SE. *P < 0.05 vs. †baseline and ‡PLA.
nutrient exchange (14). Despite this beneficial adaptation, some reports indicate that repeated ischemic exercise can lead to skeletal muscle damage, as revealed by increased tissue fibrosis (24) and activation of proteolytic signaling cascades (13) following exercise training in patients with IC. These abnormalities have been ascribed largely to oxidative damage that occurs during the ischemic exercise bouts (44, 45). Based on this view, minimizing exercise-induced oxidative stress via antioxidant treatment would, in theory, enhance the benefits of training in patients with IC. On the other hand, the findings from the present study suggest that oxidative stress is a requirement for the systemic angiogenic response to exercise in these patients, and inhibiting this signal might lead to impaired skeletal muscle angiogenesis. A growing number of studies have indeed documented that antioxidant treatment hampers exercise training-induced adaptations, including angiogenesis (21) and mitochondrial biogenesis (23, 41, 48). Therefore, the possibility exists that antioxidant treatment during exercise therapy can have both beneficial (e.g., reducing oxidative stress) as well as detrimental (e.g., impaired angiogenesis) consequences for patients with IC. The resulting outcome in terms of walking performance following the combination of these two therapies in patients with IC remains to be determined.

Limitations

This study has several important limitations. First, we utilized a small dose of NAC (final dose of 38 ± 7 mg/kg), and it is unclear whether this regimen was sufficient to increase skeletal muscle GSH bioavailability and thus positively impact muscle function (35). The vast majority of studies that reported meaningful changes in fatigue resistance with NAC treatment in humans used intravenous infusions at doses ranging from 125 to 150 mg/kg (34, 35, 47). Additional studies are needed to determine whether these supraphysiological doses can improve walking tolerance in patients with IC. Second, we studied the impact of NAC on whole blood expression of angiogenic mediators, and it is unclear whether similar observations hold true for skeletal muscle. Finally, since we employed a symptom-limited rather than a fixed-time exercise test, there were minor variations in the total worked performed between experimental sessions. Conceivably, these differences could impact the postexercise measures, including angiogenic and inflammatory markers and postocclusive RH. Nonetheless, the average difference in maximal walking time between the NAC and PLA trials was fairly small (17 s), and it seems unlikely that test duration had a major influence on the postexercise outcomes.

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EFFECTS OF N-ACETYLCYSTEINE IN INTERMITTENT CLAUDICATION


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