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The Status of Nitric Oxide and its Backup, Heme Oxygenase 1, in Thromboangiitis Obliterans

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Abstract

Background: Until recently, a gene polymorphism in the promoter region of endothelial nitric oxide synthase has been suggested as a risk factor for thromboangiitis obliterans (TAO) development. The aim of this study was to compare the metabolites of nitric oxide (NO) and its backup, heme-oxygenase-1 (HMOX1), between TAO patients and those of a smoking control group matched by race, age, sex, and smoking habits.

Methods: Twenty-four male Caucasian TAO patients and 20 male Caucasian controls enrolled in the study. Their smoking habits were matched based on the serum cotinine levels of 17 of the TAO patients and the 20 controls. A colorimetric kit was used to measure NO, and an enzyme-linked immunosorbent assay kit was used to measure cotinine and HMOX1 levels.

Results: The mean serum level of NO metabolites in the TAO group was significantly less than in the controls ($p = 0.03$) and also significantly less in the patients with below-knee amputations than in non-amputees ($p= 0.018$). Also, HMOX1 was significantly greater in the TAO patients than in the controls ($p= 0.01$). No significant correlation was found between NO and HMOX1 ($p = 0.054$).

Conclusions: Nitric oxide may play a pivotal role in TAO development and its outcome. However, the intact HMOX1 pathway may demonstrate the unique role of NO, which cannot be compensated for by HMOX1 and whose absence may make patients susceptible to developing TAO. In addition, another pathway besides NO, with influence on vascular tone and hemostasis, might be involved in TAO development, such as the autonomic nervous system. Further studies are suggested regarding these issues.

Keywords: Cotinine, Heme oxygenase 1, Nitric oxide, Peripheral arterial disease, Smoking, Thromboangiitis obliterans

Introduction

Thromboangiitis obliterans (TAO), also known as Buerger's disease, is an inflammatory and thrombotic occlusive peripheral arterial disease (PAD) usually seen in male smokers from the Middle East, the Far East, southeast Asia, eastern Europe, and South America (1, 2). The etiology and pathophysiology of the disease are not well understood (3); however, patients are almost always cigarette smokers, and better outcomes are seen in patients who stop smoking than in those who do not (4). Given the millions of smokers worldwide, it is not known why only a small number develop TAO. It has been demonstrated that some gene polymorphisms, such as the polymorphism in the promoter region of endothelial nitric oxide synthase, may increase the risk of TAO development (5). Because nitric oxide (NO) is responsible for vascular tone and inhibition

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of platelet activation and aggregation, as well as inhibition of vascular oxidative stress (6, 7), it has been suggested that NO may play a role in the pathophysiology of TAO (5). Due to the critical role of NO in the body, it is little wonder that NO has a backup system: the so-called heme oxygenase (HMOX) system (8). Heme oxygenase 1 (HMOX1) is an enzyme that degrades heme into carbon monoxide (CO), biliverdin, and ferrous iron (9). It has been demonstrated that the isoform HMOX1 has anti-inflammatory, anti-apoptotic, and antioxidant functions (10-12), and the CO produced by this enzyme can induce vasodilation by activating cGMP synthesis (13-18).

Because NO is relatively unstable, in this study, the serum levels of the NO metabolites nitrate and nitrite, and its backup, HMOX1, were compared between TAO patients and controls matched for race, age, sex, and smoking habits.

Materials and Methods

Subjects

Because the storage duration can affect the serum NO concentrations, we could not use samples from our TAO patient biobank; hence, we sampled both the TAO patients and controls over a six-month period to reduce the storage duration effect on NO metabolites. In this study, a total of 26 male Caucasian TAO patients reported to the Buerger's disease clinic from August 2016 to January 2017. The diagnosis of TAO was based on Shionoya's clinical criteria with angiography confirmation. The inclusion criterion for TAO patients was having received no antiplatelet or vasodilator treatments for at least six months before coming to the clinic. For the control group, the inclusion criteria were normal fasting blood sugar, normal lipid profile, normal blood pressure, the absence of a history of cardiovascular disease, and the absence of antiplatelet or vasodilator medications. Based on the primary evaluations, 24 TAO patients were initially included in the study. Twenty-four control smokers matched to the TAO patients by race, age, sex, and smoking habits, based on self-reports, were also initially included. However, because the self-reported data regarding smoking habits were not reliable, we decided to match the smoking habit based on serum cotinine, a major nicotine metabolite with a long half-life (19, 20). For seven TAO patients with very high serum cotinine levels, we could not find proper matched controls, although we evaluated more than 50 healthy smokers. Therefore, the 17 TAO patients were matched with 20 controls by race, sex, and age, as well as serum cotinine level. Written consent forms were signed by all participants (ethical code: 950482).

Collection of serum samples

A blood sample was collected from each participant between 9:00 and 11:00 a.m., and the serum was isolated and immediately stored at –80°C.

Biochemical examination

Serum cotinine, NO metabolite, and HMOX1 concentrations were determined by ELISA using the following kits from ZellBio GmbH-Germany: Human Cotinine ELISA kit, cat. no. ZB-12043S-H9648, Total Nitric Oxide (NO), cat. no. ZB-NO-96A, and Human HMOX1, cat. no. ZB-10932S-H9648, respectively.

Statistical analysis

Statistics were analyzed with SPSS for Windows, version 11.5. The data were described as mean ± SD. According to the Kolmogorov-Smirnov test, a parametric independent T-test was performed to evaluate the serum levels of cotinine and NO metabolites in the two groups, and a nonparametric Mann-Whitney test was used to evaluate HMOX1 between the groups. To compare the levels of cotinine between the TAO and control groups, an independent T-test was used. Spearman's test was used to calculate the correlation between NO and HMOX1. A p value less than 0.05 was considered significant.

Results

Twenty-four male Caucasian TAO patients and 20 male Caucasian controls were enrolled. Seven of the 24 TAO patients had undergone below-knee amputations (BKAs). The mean ages in the TAO and control groups were 41.4 ± 1.3, and 42.7 ± 1.2 years, respectively. The age difference between the two groups was not significant ($p = 0.91$).

To match the cotinine levels between the patients and controls, we omitted the outliers;

thus, seven TAO cases were omitted from the comparison of the TAO patients and controls. The mean serum cotinine concentrations in the 17 TAO patients and 20 controls were 182 ± 18.4 and 151.9 ± 8.2 pg/ml, respectively. This difference was not significant (p = 0.12) (not shown).

The mean serum NO nitrate and nitrite metabolite concentrations in the TAO and control groups were 29.3 ± 1.3 and 35.7 ± 2.3 µM. This difference was significant (p = 0.031) (Fig. 1A). The mean serum HMOX1 concentrations in the TAO and control groups were approximately 2 and 1 ng/ml. This difference was also significant (p = 0.01) (Fig. 1B).

Additionally, of the 24 TAO patients, the mean concentrations of the NO metabolites in the 17 non-amputee and seven BKA patients were 31 ± 1.3 and 23.9 ± 1.6 µM, respectively. This difference was significant (p = 0.018) (Fig. 1C). The mean HMOX1 concentrations in the non-amputee and BKA patients were approximately 2 and 3 ng/ml, respectively. This difference was not statistically significant (p = 0.4) (Fig. 1D).

The correlation between NO metabolites and HMOX1 was not quite significant (p = 0.054, CC = –0.3).

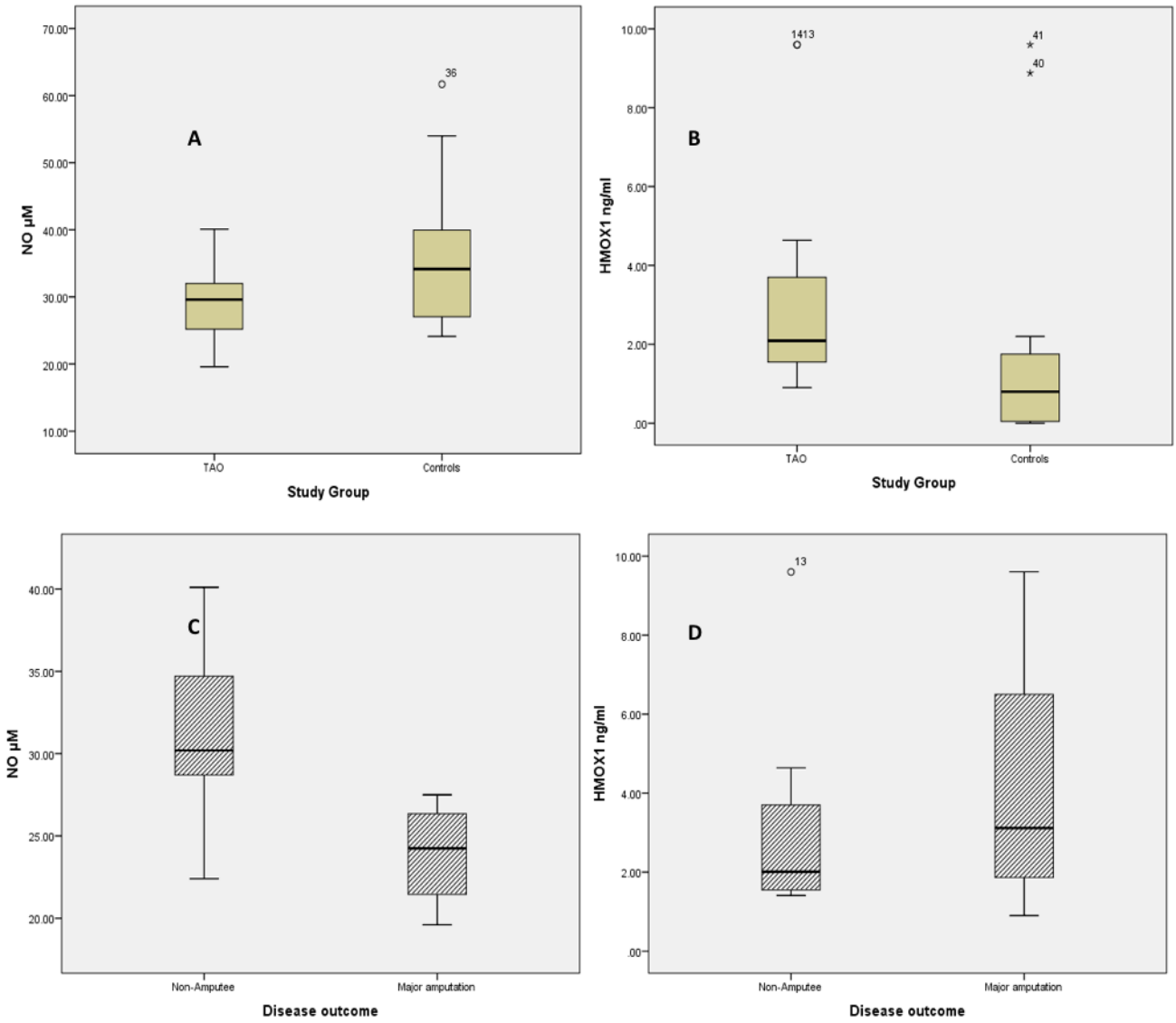

Fig. 1. Serum levels of nitrate and nitrite NO metabolites and HMOX1 in the TAO and control groups and in below-knee and non-amputation patients: NO metabolites were significantly less (A), while HMOX1 was significantly greater (B), in the TAO patients than in controls. Nitric oxide metabolites were significantly greater in major amputation the than in the non-amputation than in the below-knee amputation patients (C), while HMOX1 was not significantly different between the two groups (D).

Discussion

Diseases develop through interactions between host characteristics, triggers, and environmental risk factors (3). Smoking has been suggested as the main risk factor for developing TAO (4); however, the trigger is not yet known (3). In this study, we focused on host characteristics. In TAO, a polymorphism in the promoter region of endothelial NO synthase, eNOS-786, which may consequently reduce NO production, has been suggested as a possible risk factor for developing the disease (5). Because TAO is uncommon and polymorphism studies on TAO are usually conducted on small samples, we started by evaluating NO, which plays a role in vascular tone and hemostasis, as the first step of host characteristics assessment in TAO.

Alterations in NO levels in PAD patients have been investigated in several studies. For instance, in the study by Lofferdo et al., NO was lower in PAD patients than in healthy controls (21), while in the studies by Joaquin et al. and Akkoca et al., NO was greater in the PAD groups than in controls (22). However, the smoking habits of the patients and controls were not matched in these studies. Because smoking influences NO levels, reported differences in NO levels in PAD patients may be due to different smoking habits between patients and controls.

To eliminate age, race, sex, and smoking habit biases on NO levels, we matched the patients and controls based on cotinine levels. In addition to NO, HMOX1, was also measured.

Significantly less NO metabolites in the TAO patients vs. the controls and also in the BKA vs. the non-BKA patients may indicate an important role for NO in both disease development and TAO outcome. However, HMOX1 was significantly greater in the TAO patients than in the controls, and a borderline significant negative correlation was found between NO and HMOX1, which may be explained by HMOX1 attempting to compensate for the low level of NO and an intact HMOX1 pathway in TAO patients.

In contrast to our results, Signorelli et al. showed that HMOX1 was significantly less in PAD patients than in controls (23). However, in their study NO levels and subjects' smoking habits were not reported. Hence, these results may not represent the impaired HMOX1 in PAD patients individually.

However, the question remains regarding why, with intact NO backup in TAO, patients suffer from vasoconstriction, vascular inflammation, and thrombotic events. This may be explained by the unique role of NO, such as that of eliminating intracellular pathogens as an important component of innate immunity (24), which cannot be compensated for by HMOX1, and whose absence makes patients susceptible to developing TAO. Because various infectious pathogens have been suggested as the main trigger for TAO (25, 26), low NO in TAO patients may be a risk factor for the disease. In addition, other pathways besides NO may be responsible for vasoconstriction in TAO, such as neurogenic vasoconstriction and inflammation, and consequent platelet activation. Neurogenic vasoconstriction and inflammation may also explain the considerable sensitivity of the clinical manifestation and outcome of the patients towards smoking and also the favorable response of TAO patients to sympathectomy. Further studies are needed to compare the autonomic nervous systems in TAO patients, patients with other PADs, and healthy controls.

The most significant limitation of this study was its small sample size due to its restricted inclusion criteria. Because TAO is uncommon and because we omitted samples that were more than six months old and samples from patients who took vasodilator medications up to six months before sampling, our patient population was limited. Our approach to matching smoking habits by cotinine levels additionally limited the sample size. However, although our restricted inclusion criteria led to a small sample size, by controlling the confounding factors, the results can be considered reliable and could provide a basis for further multi-center studies on this issue.

This study provides evidence that NO may play a pivotal role in TAO development and its outcome. However, the intact HMOX1 pathway, which backs up NO by controlling vascular tone and hemostasis and reducing vascular oxidative stress, may indicate a unique role for NO that cannot be compensated for

by HMOX1 and whose absence makes patients susceptible to developing TAO. In addition, other pathways besides the NO pathway that influence on vascular tone and hemostasis might be involved in TAO, such as the autonomic nervous system. Further studies are suggested regarding these issues.

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