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Article

Trimethylamine-N-Oxide, a Metabolite Associated with Atherosclerosis, Exhibits Complex Genetic and Dietary Regulation

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SUMMARY

Circulating trimethylamine-N-oxide (TMAO) levels are strongly associated with atherosclerosis. We now examine genetic, dietary, and hormonal factors regulating TMAO levels. We demonstrate that two flavin mono-oxygenase family members, FMO1 and FMO3, oxidize trimethylamine (TMA), derived from gut flora metabolism of choline, to TMAO. Further, we show that FMO3 exhibits 10-fold higher specific activity than FMO1. FMO3 overexpression in mice significantly increases plasma TMAO levels while silencing FMO3 decreases TMAO levels. In both humans and mice, hepatic FMO3 expression is reduced in males compared to females. In mice, this reduction in FMO3 expression is due primarily to downregulation by androgens. FMO3 expression is induced by dietary bile acids by a mechanism that involves the farnesoid X receptor (FXR), a bile acid-activated nuclear receptor. Analysis of natural genetic variation among inbred strains of mice indicates that FMO3 and TMAO are significantly correlated, and TMAO levels explain 11% of the variation in atherosclerosis.

INTRODUCTION

Recently, plasma trimethylamine-N-oxide (TMAO) was identified as a metabolite strongly associated with atherosclerosis in a large case-control cohort for cardiovascular disease (CVD), and studies in mice indicated a causal relationship (Wang et al., 2011). TMAO is derived from dietary choline through the action of gut flora, which metabolize choline to trimethylamine (TMA), a gas that is then absorbed into the circulation and further metabolized to TMAO. Likely candidates for the conversion of TMA to TMAO are members of the flavin mono-oxygenase (FMO) family. In particular, FMO3 has been implicated in the oxidation of TMA, since individuals with mutations in FMO3 present with accumulation of TMA levels, causing fish malodor syndrome (Treacy et al., 1998). TMAO appears to contribute to the development of atherosclerosis in part by promoting cholesterol accumulation within macrophages, perhaps by inducing scavenger receptors such as CD36 and SRA1, both of which are involved in the uptake of modified lipoproteins (Wang et al., 2011). One crucial question is how TMAO influences cellular metabolism and whether this is direct or indirect. Another important question relates to the nature of the variations in plasma TMAO levels in human populations and whether modulating TMAO levels can result in reduced risk of atherosclerosis.

We now report studies of the metabolism of TMA and TMAO in mice and humans. We examine the activities of the FMO family members and show that FMO3 is the most active in metabolizing TMA to TMAO. Using transgenic and adenoviral approaches, we show that upregulation of hepatic FMO3 decreases TMA and increases TMAO levels in the circulation, while antisense-mediated silencing of FMO3 increases TMA and decreases TMAO levels. We further show that FMO3 is dramatically downregulated by testosterone in mice, suggesting a mechanism for the greater susceptibility of female mice to atherosclerosis as compared to males, and suggesting that FMO3 expression is modestly decreased in males as compared to females in human populations. We also find that FMO3 is dramatically upregulated by bile acids and that this is mediated by the action of the nuclear receptor FXR (NR1H4). Finally, we have examined

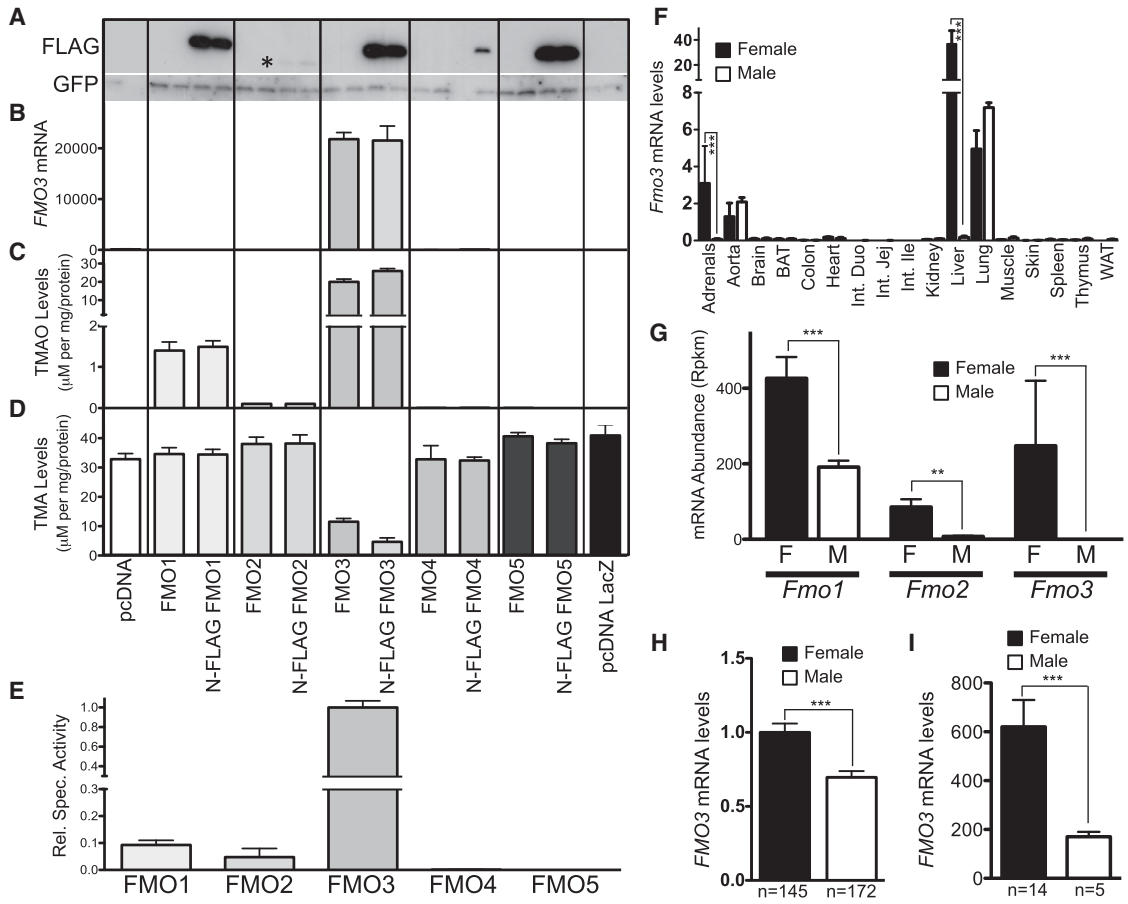

Figure 1. FMO3 Is the Major FMO Family Member Responsible for the Conversion of TMA to TMAO

(A–D) Overexpression of untagged or FLAG-tagged human FMO1–FMO5 in HEK292Ad cells.

(A) Western blotting analysis of FLAG-tagged FMO1–FMO5 transfected into HEK293Ad cells. GFP was cotransfected to normalize transfection efficiency.

(B) FMO3 mRNA levels were determined by RT-qPCR and normalized to GFP mRNA levels.

(C and D) TMAO and TMA levels were determined in the media of transfected cells treated with d9-TMA and then analyzed for d9-TMA and d9-TMAO levels using mass spectrometry (see the Experimental Procedures). TMAO production was determined from triplicate wells for each condition and normalized to the amount of protein per well.

(E) Relative specific activity of FMO1–FMO5 determined by dividing normalized TMAO levels by relative FMO protein levels calculated by densitometry analysis and expressed relative to FLAG-FMO3 activity.

(F) Relative Fmo3 mRNA levels in various mouse tissues from C57BL/6 mice (n = 3 mice/sex). Expression was determined by RT-qPCR and was normalized to 36B4 expression levels.

(G) Relative abundance of FMO1–FMO3 determined by RNA-Seq analysis from male and female C57BL/6 mice.

(H) Relative FMO3 expression in human liver determined by microarray expression profile from 317 Caucasian human individuals segregated by sex.

(I) RT-qPCR analysis of hepatic mRNA levels from human liver biopsies obtained as described in the Experimental Procedures. Data are presented as mean ± SEM. Significance was measured with Student's t test. *$p < 0.05$ and ***$p < 0.001$.

natural variations of FMO3, TMAO, and atherosclerosis in mice. The results indicate that FMO3 contributes significantly to TMAO levels, that other factors must also be involved, and that TMAO explains about 11% of the variation in atherosclerosis susceptibility among common inbred strains of mice.

RESULTS

Expression Levels and Activities of Flavin Mono-oxygenase Family Members

Members of the FMO family are strong candidates for the conversion of TMA to TMAO (Treacy et al., 1998), and the five members of the family, FMO1, FMO2, FMO3, FMO4, and FMO5, exhibit approximately 50% amino acid sequence identity, with high sequence conservation between mouse and human. We were interested in determining which of these related genes can metabolize TMA to TMAO. All five members of the family (human orthologs) were cloned into expression constructs, in either untagged form or tagged with the FLAG sequence at the N terminus, and transfected into the human kidney cell line HEK293AD together with a plasmid expressing green fluorescent protein (GFP). As controls, cells were transfected with pcDNA (empty) or pcDNA-expressing β-galactosidase. We did not detect any significant differences in transfection efficiency (as determined by GFP expression) (Figure 1A; see Figure S1A online). Nonetheless, the relative