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Effects of Choline, TMA and TMAO on the Expression of Flavin-Containing Monooxygenases 3 and 5 in Mice

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ABSTRACT

Choline and trimethylamine N-oxide (TMAO) have been considered as factors associated with cardiovascular disease (CVD). TMAO is produced from trimethylamine (TMA) oxidation by flavin-containing monooxygenase 3. Flavin-containing monooxygenases (FMOs) are enzymes that catalyze oxygenation of a wide range of compounds and drugs. Human and mouse livers, are included other members of FMO family, FMO4 and FMO5. Expression of FMO5 in our liver is approximately equal to FMO3 expression. FMO3 and FMO5 enzymes are known to be involved in lipids and glucose metabolism. In this study, we investigated the effects of choline, TMA and TMAO on FMO3 and FMO5 expression. For this purpose, these compounds were injected to female NMRI mice as intraperitoneally and then liver tissues were collected to evaluate of FMO3 and FMO5 expression. Trimethylamine administration resulted in 8-fold ($P<0.0001$) increase in FMO3 mRNA levels and an 2.5-fold ($P<0.0001$) increase in FMO3 protein compared to controls (mean different equal to 6.87 for mRNAs and 1.67 for protein). A significant decrease (approximately 45% ($P<0.05$)) in FMO3 mRNA was observed following choline administration (mean difference of FMO3 mRNA levels between treatment and control groups equal to 0.576). FMO5 mRNA levels were decreased to 50% than to controls ($P=0.003$) after TMA treatment. Overall, our data indicate that FMO3 and FMO5 expression is influenced by TMA and choline.

Keywords: Flavin-containing monooxygenase; Gene expression; Mouse; Real-time PCR; Trimethylamine-N-oxide

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INTRODUCTION

Flavin-containing monooxygenases (FMOs) are microsomal enzymes that catalyze the oxygenation of a wide variety of nitrogen- and sulfur-containing compounds [1] as well as drugs and xenobiotics [2]. In mammals, FMOs are expressed in tissue-specific and developmental-dependent manner [3]. In human, five FMO genes encode functional proteins and are on the long arm of chromosome 1 of which FMO1-4 are located in one of cluster at q23-25 and FMO5 is in the region 1q21.1 [3,4]. A second cluster containing 5 pseudogenes was also detected on chromosome1. The mouse genome contains nine FMO genes that FMO1-4 and FMO6 are clustered on chromosome1 and FMO5 is on chromosome1 and FMO5 is on chromosome3 [5]. FMO3 is the most important isoform of FMO enzymes that was expressed in adult human liver [3] and in the liver of adult female mice [6]. The importance of FMO3 is because of its role in the processing of some drugs (e.g. cimetidine), chemicals in cigarette (nicotine) [7] and dietary derived trimethylamine (TMA) [8]. Trimethylamine, as a FMO3 specific substrate, is converted to trimethylamine N-oxide (TMAO) by this enzyme. Most people excrete 95% or more of TMA as TMAO [3]. FMO5 is expressed in the liver and can be detected easily at the protein level in all studied species. Both

in vitro and in vivo studies have shown that liver FMO (FMO3) was affected by exogenous factors such as diet [9] and endogenous factors as hormones [10], obesity and genetic [11]. More recent studies have shown that some members of FMO family play the important physiological roles [12-15]. For example, it is known that FMO5 regulates changes metabolic associated ageing by up-regulation of cholesterol biosynthesis and also promoting the expression of enzymes involved in glycolysis/gluconeogenesis [12]. Recently, it has been found that FMO3 through a gut flora-dependent pathway, TMA/FMO3/TMAO, plays a very important role in the regulation of cholesterol metabolism [15,16]. Following intake of foods containing choline, lecithin, or L-carnitine, these compounds are metabolized by gut entrobacteria to produce TMA. Trimethylamine is readily absorbed from the gastrointestinal tract and subsequently oxidized by liver FMO3 to form TMAO [14,17,18]. It has been suggested that TMAO is associated with atherosclerosis and cardiovascular disease (CVD) [14,16,19]. Trimethylamine N-oxide can result in up-regulation of scavenger receptors such as CD36 and SRA1 which have been linked to atherosclerosis [20,21]. Although TMAO, as a product of FMO3 function, is considered as a potent predictive factor of atherosclerosis in human and is associated with CVD risk [14,17], however the studies have shown that changes of FMO3 expression have profound effects on lipid and glucose metabolism [15,22]. In addition, TMA, as a FMO3 specific substrate, is a compound that likely is involved in the regulation of cholesterol through FMO3 function [15]. Therefore, it is important to study of possible effective factors on FMO3 expression (substrates and products of FMO3) because it contributes to understanding the function of FMO3 in lipid metabolism. In this study, the main objective was to investigate the changes of FMO3 expression in mouse liver after administration TMA (specific substrate), TMAO (FMO3 product) and choline (as a precursor of TMA and a diet component). The addition, FMO5 expression was evaluated as a member of FMO family that is involved in lipid metabolism.

MATERIALS AND METHODS

Chemicals and reagents

Choline chloride, Trimethylamine hydrochloride and Trimethylamine N-oxide (TMAO) obtained from Sigma-Aldrich (Germany). Primary antibodies including anti-FMO3, anti-FMO5, goat anti-rabbit IgG-HRP conjugated were from Abcam Biotech Company (Cambridge, UK). Anti-GAPDH and goat anti-mouse secondary antibody were from Novus Biological (Littleton, USA) and R&D Systems Europe (Abingdon, UK), respectively. The cDNA Synthesis kit was from Thermo Scientific (USA) and Real-time PCR kit was obtained from Bio-Rad Laboratories (Hercules, California). All other materials used in this study were of molecular grade.

Animals and treatments

Experiments were performed on 8-9 weeks old female NMRI mice (20-25gr). Thirty five mice were divided into 5 groups. Animals were kept at 12h light: 12 h dark cycle and chow diet as *ad libitum*. In a pilot experiment, 4 groups of animals were injected intraperitoneally with a single dose of choline chloride, TMA, or TMAO and distilled water as a control, and FMO3 mRNA expression was assayed at time intervals 12, 24, 48, 72 and 96 hours. Maximum changes in the FMO3 mRNA were observed at 12h after injection for TMA, and 48h after injection of TMAO or choline. These time periods were therefore selected for subsequent experiments.

One group of animals received choline chloride (1mmol/kg or 139.6mg/kg), the second group was given TMAO (3gr/kg) and the third group was treated with TMA (1mmol/kg) as intra-peritoneal (IP) injection. We used two groups as controls. Both control groups received water as IP injection and were sacrificed at different times.

In mice, the 50% lethal dose (LD50) of choline for ip administration is 225 mg/kg according to "Organization for Economic Cooperation and Development"[23]. We chose 1mmol/kg or 139.6mg/kg for choline, to ensure that the animals do not die and according to previous studies [24]. Also, according to "Acute Exposure Guideline Levels (AEGLS) for trimethylamine", LD50 has been reported as 95-148mg/kg for TMA and 2240-3350mg/kg for TMAO [25]. The selected doses were 1mmol/kg For TMA and 3000mg/kg For TMAO. Twelve hours after injection of TMA and 48h after injection of choline and TMAO, animals were sacrificed by high concentrations of CO2, their livers were removed and snap frozen in liquid nitrogen and stored at -80°C until use. The protocol was approved by the Ethics Committee of Kerman University of Medical Sciences.

RNA extraction

Total RNA from liver tissues, approximately 20-30mg, was extracted using Tripure isolation reagent (Roche applied science, Germany) according to manufacturer's instructions. The quality and purity of extracted RNA were assayed spectrophotometrically using a ND-1000 nanodrop (Thermo Scientific, USA), also the integrity of RNA was evaluated by electrophoresis on 1% agarose gel.

Revers transcription-QPCR

First-strand complementary-DNA (cDNA) was synthesized from 1000ng of total RNA using Revert Aid First Strand cDNA synthesis kit (Thermo Scientific) according to the manufacturer's instruction and was used as the template for real-time PCR experiments. Synthesized cDNAs were stored at -20°C until use. Real-time PCR was performed using specific primers (table1) for FMO3, FMO5 and HPRT (hypoxanthine phosphoribosyl transferase) as a reference gene, and iQ-SYBR Green supermix (BioRad) in StepOne Real-time PCR System (Applied Biosystems). Reaction mix was included the SYBR supermix 1X of kit (SYBR® Green I dye, iTaq DNA polymerase; 25U/ml, dNTPmix with 0.2mM each deoxynucleosid-5´ -triphosphates, Tris-HCl; 20mM, MgCl2; 3mM, KCl; 50mM fluorscin; 10mM and stabilizer), Rox dye (500nM), 300-500 nM primers, 2-4µl from cDNA and H2O in final volumes 20µl. Thermal program was as follows: starting point 95°C for 10 min that was followed by 45 cycles at 95°C for 10s, annealing temperature for each primer pair (Table1) for 30s and extension temperature at 72°C for 30s, at the end was used one step collecting melting curve data at 55-95°C with ramping rate of 0.3°C/s. Relative levels of FMO3 and FMO5 mRNAs were determined after data normalization against hypoxanthine phosphoribosyl transferase (HPRT) as a reference and was calculated by delta-delta Ct ($2^{\Delta\Delta Ct}$) method with $\Delta\Delta Ct=(Ct_{target\ gene}-Ct_{HPRT})_{treated}-(Ct_{target\ gene}-Ct_{HPRT})_{control}$ that target genes are FMO3 or FMO5, and control is untreated sample.

Table 1: The primer sequences and conditions used in real-time PCR

Gene	Forward/Revers Primer sequence	Product size (bp)	Accession numbers	Tm[d]	References
mFMO3[a]	5´-CACCACTGAAAAGCACGGTA-3´	151	NM_008030.1	57	[40] and designed
	5´-CTTATAGTCCCTGCTGTGGAAGC-3´				
mFMO5[b]	5´-ATCACACGGATGCTCACCTG-3´	234	NM_001161763.1	58	[40]
	5´-GCTTGCCTACACGGTTCAAG-3´				
mHPRT[c]	5´-AGCTACTGTAATGATCAGTCAAC-3´	198	NM_013556.2	57	[40]
	5´-AGAGGTCCTTTTCACCAGCA-3´				

[a]mouse falvin-containing monooxygenases 3; [b]mouse falvin-containing monooxygenases 5 and [c]mouse hypoxanthine phosphoribosyl transferase.
d Annealing temperature (Tm) for each primer pair.

Western blotting

For protein extraction, approximately 30-40mg of hepatic tissues was homogenized in 750-1000µl ice-cold RIPA buffer (Tris-HCl; 50mM PH=7.5, NaCl; 150mM, SDS; 0.01 %, Na-deoxycholate; 0.5%, NP-40; 1% and EDTA; 1mM) containing 1mM phenylmethylsulfonyl fluoride, PMSF (Sigma Aldrich, Germany), and 1X protease inhibitor cocktail (Santa Cruze, USA). Lysates were centrifuged at 12000g for 15 min at 4°C. Total protein concentration was determined by a bicinchoninic acid assay method using a BCA kit (Santa Cruz) according to manufacturer's protocol. Twenty mg of liver proteins was separated on 10% SDS-acrylamide gels. Separated proteins were transferred to a PVDF (Polyvinylidene Fluoride) membrane (0.2 µm, BIO-RAD, USA) using a Bio-Rad apparatus (USA). The membrane was blocked for 1 hour in %5 skim milk in TBST (Tris-buffered saline with %0.5 tween-20) and incubated for 1-1.5h with primary antibodies as follows: Anti-FMO3 (Abcam-ab126790) was diluted 1/3000 in TBST buffer and anti-FMO5 (Abcam-ab103973) was diluted 1/5000 in TBST buffer. Membranes were then washed 3 times for 20 min each time, with TBST buffer and then were incubated at room temperature for 1.5h with goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Abcam-ab97051) at 1/20000 dilution in TBST buffer. Mouse anti-GAPDH antibody (Novus Biological-NB300-328) was used for normalization of protein levels. Anti-GAPDH antibody was diluted at 1/8000 (in TBST buffer) and membrane was incubated for 1.5 h at room temperature. After washing the membrane 3 times (at 20 min each time) with TBST, PVDF was incubated with goat anti-mouse IgG conjugated to HRP antibody (R&D system-HAF007) diluted 1/5000 (in TBST buffer) for 2 h at room temperature. Finally, the enhanced chemiluminescence substrate (Perkin Elmer, USA) was added to the membranes according to the manufacturer's protocol followed by exposure to X-Ray film. The films were developed and protein bands were measured semi-quantitatively using an Image J software version 1.48.

Statistical analysis

Statistical analysis was performed using SPSS 22 software. The comparison between control(s) and treatment groups was made by one-way ANOVA followed by post hoc Scheffe test or Student's t-test where appropriate. Results are expressed as mean ± standard error of mean (SEM). P-values <0.05 were considered statistically significant.

RESULTS

Effects of choline, TMA and TMAO on FMO3 expression in mice livers

In the present study, choline, TMA and TMAO were administrated to animals as a single dose of intraperitoneal injection. Animals treated with choline (n=6) and TMAO (n=7) and one group of controls (distilled water was injected ip (n=7)) were sacrificed 48h after treatment and those TMA treated mice (n=7) and the other control group (n=6) were sacrificed 12h after treatment. The livers mice were removed and immediately were frozen in liquid nitrogen for later use. The relative expression of FMO3 mRNA was shown in Figure1. As it can be seen, FMO3 mRNA level shows a remarkable change after treatment with choline and TMA (Figure 1a). The results indicated a decrease of FMO3 mRNA expression of 45% ($P<0.05$) compared to the control group when mice were treated with choline. TMA administration was caused an increase in mRNA expression of FMO3 to approximately 8-fold ($P<0.0001$). The changes in gene expression at the protein level were presented in Figure 2. Relative levels of FMO3 protein after exposure to TMA rose up to 2.5-fold ($P<0.0001$) than controls (control12h) animals (Figure 2a and 2d), while following choline treatment, FMO3 protein levels only decreased 12% compared to controls that was not significant (Figure 2a and 2c). Analysis of FMO3 mRNA expression by real-time PCR, although was shown a decrease in FMO3 mRNA levels to approximately 20% in mice of TMAO treated relative to the controls, but there was no significant difference statistically between treated mice and control group ($P=0.34$) (Figur1a). Also, no significant difference was detected at protein level between animals TMAO treated and respective control (control48h) as can be seen in Figure 2a and 2c ($P=0.086$).

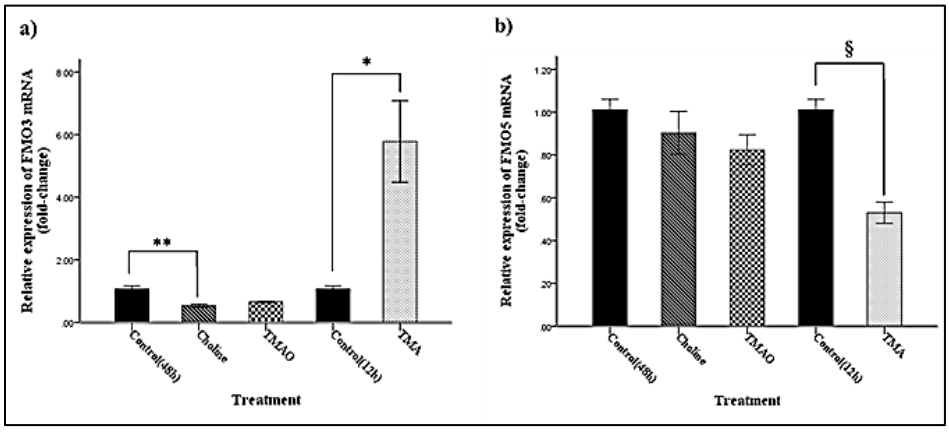

Figure 1: Relative expression of FMO3 and FMO5 mRNAs in liver mice. Animals received choline (139mg/kg), trimethylamine N-oxide (3g/kg) or trimethylamine hydrochloride (95.6mg/kg) as intraperitoneal injection and were killed after 48h or 12h, and their livers were removed. mRNA expressions were measured by real-time PCR. Hypoxanthine phosphoribosyl transferase (HPRT) mRNA levels were measured and were used as a reference gene. a) FMO3 mRNA levels that normalized with HPRT mRNA. b) FMO5 mRNA levels that were normalized with HPRT. Values present means±SEM of 6-7 mice per group *: P<0.0001, **: P<0.05, §: P<0.003 compared to controls

Effects of choline, TMA and TMAO on FMO5 expression in mice livers

Analysis of real-time PCR results and western blots for FMO5 did not show correlation between FMO5 mRNA levels and its protein levels. When mice were treated with TMA, FMO5 mRNA expression decreased to 50 % ($P=0.003$) than to control group that was statistically significant (Figure 1b) but decrease of FMO5 protein levels was only 10% compared to controls ($P=0.35$) (Figure 2b and 2d). In contrast, after treatment with choline, FMO5 mRNA did not change significantly ($P=0.55$) (Figure 1b), However, a significant decrease ($P<0.018$) was observed in FMO5 protein levels in mice treated with choline relative to animal control (Figure 2b and 2c). FMO5 expression at mRNA and protein levels were decreased after administration of TMAO to mice but these changes was not significant statistically Figure 1b, 2b and 2c.

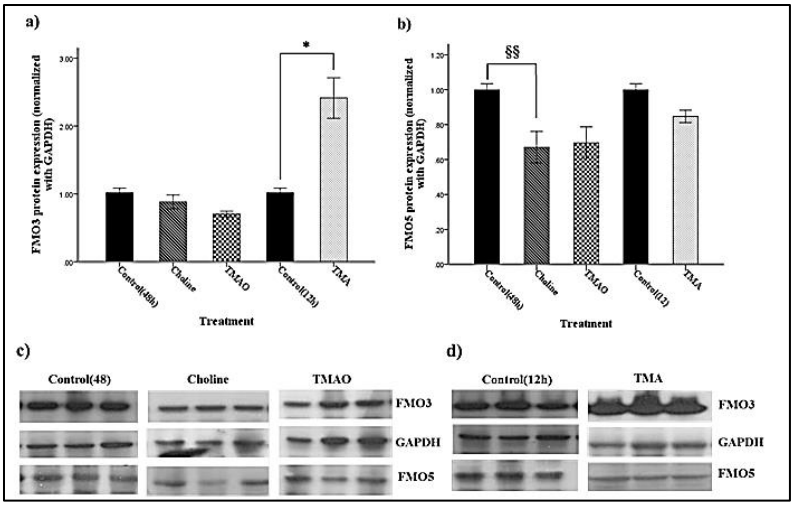

Figure 2: Relative amounts of FMO3 and FMO5 protein. Analysis of western blot bands for FMO3 and FMO5 proteins after densitometry analysis by image J software and normalization with GAPDH protein bands. Relative expression was calculated by a) dividing (FMO3/GAPDH) protein ratio from each sample (treated) to (FMO3/GAPDH) protein ratio from control sample and b) dividing (FMO5/GAPDH) protein ratio from each sample (treated) to (FMO5/GAPDH) protein ratio of the control sample. The values present means±SEM of 6-7 mice per group c) Western blot bands of FMO3, FMO5 and GAPDH proteins in liver samples from mice treated with choline, trimethylamine N-oxide (TMAO) and control 48h (water received as IP injection). d) Western blot bands of FMO3, FMO5 and GAPDH proteins in liver samples from mice treated with trimethylamine (TMA) and control 12h (water received as ip injection). *: P<0.0001 and §§: P<0.018 compared to respective controls

DISCUSSION

Recent studies have demonstrated that some members of flavin-containing monooxygenase (FMO) family not only metabolize drugs, but also play important physiological roles in intermediary metabolism [22,26,27]. FMO3 is a member of FMO family that oxidizes trimethylamine (TMA) to trimethylamine-N-oxide (TMAO) [16]. Trimethylamine N-oxide is a compound that to be highly linked with cardiovascular disease [14,17,19]. The increase in TMAO, as a FMO3 enzymatic product, is observed following an increase in TMA production from its precursors (such as choline, L-carnitine) [14,16]. The study of M Warrier et al (2015) showed the role of FMO3 for regulating cholesterol balance via effects on biliary and nonbiliary reverse cholesterol transport (RCT), and it introduced TMA as a candidate involved in the ability of FMO3 to regulate cholesterol balance [15]. It has also been found that despite of direct biochemical relation of FMO3 and TMAO, FMO3 enzyme and TMAO product impact lipid metabolism through distinct mechanisms. In fact, transcription control of FMO3 has been raised as an important regulatory key for cholesterol metabolism so that subtle changes in FMO3 expression, can perturb lipid metabolism significantly [15,22]. In this regard, the present study attempted to investigate impacts choline, TMA and TMAO on FMO3 expression in mouse liver after intraperitoneal administration these compounds. Our experiments showed increase significantly of FMO3 expression after exposure to TMA and a significant decrease after choline administration. Therefore, it can be considered that TMA and choline are metabolites with the ability to change FMO3 expression. A number of studies have described the impact of various factors on FMO3 expression. Hepatic FMO activity is greatly affected by the foods [9,16]. For example, the intake of foods containing indols (e.g. Vegetables) inhibit FMO3 activity [16]. Also, in mice fed with cholic acid-rice diet were observed the increase in FMO3 mRNA and protein expression [16]. In male mice, FMO3 expression is down-regulated by testosterone [10]. The increased FMO3 expression as a result of TMA might influences on lipid and glucose metabolism, since the recent study conducted by Diana M. Shih [22] using Hep3B cells which had overexpression of FMO3 resulting from adevirus mediated gene transduction, showed increased glucose production (gluconeogenesis) and lipogenesis. It is possible that TMA affects lipid metabolism through both increasing TMAO levels (as a proatherogenic metabolite) and increase in FMO3 expression. The results of the current study showed a significant decrease in FMO3 mRNA levels after choline administration to mice. However, changes in protein levels were not significant. The liver and kidneys are responsible for rapid clearance of choline from circulation [28]. In liver choline´s net uptake is result from two processes, one is concentration dependent and transfers choline by a non-saturable mechanism and the other depicts a saturable mechanism. At high concentrations, approximately 60% of choline is metabolized to betaine by choline oxidase system (composed of choline dehydrogenase and betaine-aldehyde dehydrogenase), 30% is metabolized to phosphorylcholine and lecithin and 10 % remains as free choline. At lower concentrations,

phophorylcholine and lecithin constitute approximately 70% of the choline metabolites [29]. Decreased FMO3 mRNA levels on the second day after administration of a single dose of choline (as seen in current study) may be result of the influence of choline metabolites, phosphorylcholine and lecithin [30]. A study conducted by Cashman et al [31] revealed that in rats administered Total Parenteral Nutrition (TPN) for 5 days, adding choline to TPN (TPN+choline) increased hepatic FMO activity. They observed a 1.6 fold increase in FMO3 protein, which was not significantly different, compared to controls. Our results, however, showed a decrease in FMO3 protein, although this difference was also not significant. This difference might be due to the production of different metabolites in periods longer than 2 days that we used in our study. We can therefore conclude that choline does not affect FMO3 protein levels significantly. Analysis of hepatic FMO5 expression in mice treated with TMA showed a significant decrease in FMO5 mRNA levels. This is interesting because TMA is not a substrate for FMO5. It has been found that only two members of the FMO family, FMO1 and FMO3, metabolize TMA, with FMO3 being the more active isoform [16]. In human and mouse liver, mRNA levels of FMO5 are higher than other FMOs [8,32] but, this enzyme has no activity than to typical substrates of FMOs such as trimethylamine and benzydamine [33,34]. Despite this, FMO5 was affected by a number of compounds and chemicals. For example, in primary human hepatocytes, rifampin induces FMO5 expression [35], also FMO5 was induced by lipoic acid in female mouse hepatocytes [36]. Possible implications of changes in FMO5 expression should be investigated. It appears to be important, because the repression of FMO5 gene expression has been observed in liver samples of patients with type-2 diabetes [37].

CONCLUSION

Results from our study did not show any correlation between the expression FMO5 at mRNA and protein levels. This is in agreement with an Overby's study [38] that showed variability in protein and mRNA expression for hepatic FMOs (FMO3 and FMO5). Gene expression is regulated at many levels from DNA to protein. Several processes influence protein levels in the cell: mRNA export from nucleus to cytoplasm, degradation of some mRNA molecules, and selecting mRNAs for translation by ribosomes [39]. FMO5 expression (similar to each protein) may be regulated at each of these steps. In summary, the experiments this study showed the increase of FMO3 expression (at mRNA and protein levels) by TMA and the decrease of FMO3 mRNA levels by choline. FMO5 mRNA amounts were also decreased by TMA, although TMA is not a substrate for FMO5. Understanding of the mechanisms involved in the changes of FMO3 and FMO5 expression requires further experiments.

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