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Hepatic flavin-containing monooxygenase gene regulation in different mouse inflammation models

Jun Zhang*, Madhusudana R. Chaluvadi*, Rob Reddy, Meike S. Motika, Terrilyn A. Richardson, John R. Cashman, and Edward T. Morgan

Human BioMolecular Research Institute (JZ, RR, MSM, JRC), San Diego, CA 92121; and Department of Pharmacology (MRC, TAR, ETM), Emory University School of Medicine, Atlanta, GA 30322

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Running Title:

Hepatic *Fmo* regulation in mouse inflammation models

Address correspondence to:

Dr. Edward Morgan

Emory University School of Medicine

5119 Rollins Research Center

1510 Clifton Road

Atlanta, GA 30322, USA

Tel: +1 404-727-5986

Fax: +1 404-727-0365

E-mail: etmorga@emory.edu

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List of abbreviations used: *C. rodentium, Citrobacter rodentium; E. coli*, *Escherichia coli;* LPS, lipopolysaccharide; DSS, dextran sulfate sodium; TNBS, 2,4,6-trinitrobenzene sulfonic acid; FMO or *Fmo*, flavin-containing monooxygenase; q-PCR, quantitative polymerase chain reaction; NO, nitric oxide; P450s, cytochrome P450s; SEM, standard error of the mean; TLR4, Toll-like

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receptor 4; HeOu, C3H/HeOuJ; HeJ, C3H/HeJ; TNFα, tumor necrosis factor-α; IL, interleukin; RT-PCR, reverse transcription polymerase chain reaction; HNF, hepatocyte nuclear factor.

Abstract

The objective of the study was to investigate the regulation of hepatic flavin-containing monooxygenases (*Fmo*) *1, 3, 4,* and *5* in three different mouse models of inflammation, including treatment with *Citrobacter rodentium* (*C. rodentium*), lipopolysaccharide (LPS), and dextran sulfate sodium (DSS). Quantitative real-time RT-PCR was used to evaluate the steady state mRNA levels for the various *Fmo* isoforms in these mouse models of inflammation during different treatment time courses. *Fmo3* mRNA was most significantly down-regulated in *C. rodentium*-treated female mice. *Fmo 1, 3* and *5* mRNAs were also found to be down-regulated in LPS models of inflammation. The significant down-regulation of hepatic FMO3 protein during *C. rodentium* treatment was confirmed with Western blot analysis of liver microsomes from treated animals. Toll-like receptor (TLR) 4 is known to be responsible for LPS signaling in association with several proteins. To investigate if TLR4 was responsible for regulation of *Fmo* genes in both LPS and *C. rodentium* animal models, *Fmo* mRNA levels in female wild-type (C3H/HeOuJ) and TLR4 mutant (C3H/HeJ) mice were compared in both inflammatory models by real-time RT-PCR. The results showed that *Fmo3* down-regulation during *C. rodentium* infection is independent of TLR4. Whereas TLR4 is likely to play only a partial role in *Fmo1* gene regulation in LPS treated animal, our results show that the down-regulation of *Fmos 3* and *5* in this model is TLR4-dependent. Unlike P450 regulation measured in the same mouse strains, *Fmo3* expression was largely refractory to down-regulation in the DSS model of inflammatory colitis.

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Introduction

Inflammation is a defensive response to microbial invasion or physical, chemical, and/or UV irradiation damages, as well as other disease states. Inflammation responses are mediated by cytokines and chemokines to activate a variety of inflammatory signaling receptors and adaptor molecules, and trigger multiple protein kinase cascades that lead to diverse transcriptional and translational regulation of downstream functional genes. During inflammation, the expression, activity, and functions of many hepatic drug-metabolizing enzymes and drug transporters have been found to be regulated and to have exerted profound effects on the metabolism, distribution, and elimination of many drugs (see reviews by Renton, 2001; Renton, 2004; Morgan et al., 2008). These include Phase I and Phase II enzymes, and drug transporters, most of which are down-regulated during inflammation impacting pharmacokinetics and leading to potentially unpredicted drug toxicity. Thus, in addition to drug-drug interactions, drug-disease interactions can modify individuals' responses to drugs via disease-mediated effects on drug metabolism and pharmacokinetics.

Many different inflammation models have been developed to mimic inflammation-related disease states. Microbial infection is one of the leading causes of inflammation, and treatment with bacterial lipopolysaccharide (LPS) represents a classical inflammatory model for severe systemic infection caused by endotoxemia. LPS stimulates monocytes and macrophages to release early proinflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin -1β (IL-1β), which initiate an inflammatory cascade. The majority of the biological effects of LPS have been attributed to its activation of Toll-like receptor 4 (TLR4) (Poltorak et al., 1998). Live bacterial infection of mice with the murine pathogen *Citrobacter rodentium* (*C. rodentium*) is a model of human enteropathogenic *Escherichia coli* (*E. coli*) infection (Schauer and Falkow, 1993). *C. rodentium* colonizes the colons of infected mice and elicits colonic inflammation and pathological changes resembling inflammatory bowel disease. Treatment with dextran sulfate

sodium (DSS) (Masubuchi and Horie, 2004) or 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Weidenbach et al., 2000) are well-established chemical models of colonic inflammation designed to mimic ulcerative colitis. Examining gene regulation in different models provides opportunities to compare and differentiate the common and specific responses to different inflammatory stimuli.

Regulation of cytochrome P450s (P450s) enzymes has been characterized extensively in the above-noted inflammation models. In both the TNBS and the DSS model, colitis induced in rats was associated with a decrease in P450-dependent metabolism (Weidenbach et al., 2000; Masubuchi and Horie, 2004). Studies in rodents (*in vivo* and *in vitro*) have shown decreases of hepatic P450 mRNA and protein expression, as well as corresponding enzyme activity after treatment with bacterial LPS (Warren et al., 1999; Siewert et al., 2000; Ferrari et al., 2001; Ashino et al., 2004; Richardson and Morgan, 2005). In a *C. rodentium* infection study, several P450 mRNAs were decreased and a few were up-regulated (Richardson and Morgan, 2005; Richardson et al., 2006). Comparing the pattern of hepatic P450 regulation in live infections as well as LPS and chemically-induced inflammatory responses suggests that while a general trend of metabolic enzyme down-regulation is shared among these inflammation models, isoform-specific regulatory factors are certainly involved. The significance of such regulation in clinical drug use in human is not fully understood. One well-recognized case is the dramatic loss in CYP1A2 during an influenza virus infection that led to severe toxicity by theophylline in affected children (Kraemer et al., 1982). It is believed that the impact of such drug-disease interaction will depend on the specific drug metabolism pathways, genetic polymorphisms of the biotransformation enzymes, the specific type of inflammation response involved, and animal species being studied.

Flavin-containing monooxygenases (FMOs) represent another enzyme family that also plays significant roles in the hepatic metabolism of xenobiotics and endogenous substrates in humans and other mammals. While *FMOs* are not known to be regulated by drugs (Cashman, 2003; Motika et al., 2007), down-regulation of hepatic *Fmo1* in LPS treated rats has been reported (Park et al., 1999; Ryu et al., 2004a). However, there is a significant gap in knowledge about *FMO* regulation during inflammation in general. In this study we examined the effect of inflammation on hepatic *Fmo* mRNA levels in LPS, DSS, and *C. rodentium*-treated mice. The most significant regulation of *Fmo1* and *Fmo3* at the mRNA level in *C. rodentium* model was confirmed at protein levels using Western blot analysis. In addition, we compared TLR4-deficient mice with control mice in these inflammation models, to explore the effect of TLR4 on *Fmo* regulation.

Materials and Methods

Chemicals, Animals, and Treatment. The female C57BL/6 mice (Jackson laboratories, Bar Harbor, ME) treated with *C. rodentium* (American Type Culture Collection, Manassas, VA) and their pairfed controls were the same mice described previously (Chaluvadi et al., 2008a). Infection was achieved by allowing the mice to drink a 20% sucrose solution containing *C. rodentium* for 24 h, at a nominal bacterial concentration calculated to result in an average dose of 7 x 10^8 cells/mouse. The actual average doses for each group were determined retrospectively by plating on MacConkey agar and they were 8, 7, 6, and 6 x 10^8 bacteria/mouse for the 7 day, 10 day, 15 day, and 24 day groups, respectively. Control (pairfed) mice received only 20% sucrose in the drinking water. The amount of food consumed by the infected group was calculated and the same amount of food was offered to the pairfed control group the next day. Mice from each group were killed at 7, 10, 15, and 24 days after post infection. Livers were collected, rinsed in cold 1.15% potassium chloride, and stored at -80 °C until RNA or microsome preparation was initiated.

The female C3H/HeOuJ (HeOu) and TLR4-mutant C3H/HeJ (HeJ) mice (Jackson laboratories, Bar Harbor, ME) infected with *C. rodentium*, and their pairfed controls, were the same mice described previously (Richardson et al., 2006). HeOu and HeJ mice derive from the same C3H background strain, and therefore they are genetically very similar except for a naturally occurring spontaneous mutation in the HeJ mice inactivating TLR4 gene. These mice received a dose of 2 x 10^8 *C. rodentium* or saline by oral gavage, and were killed 6 days after infection. Livers were collected as described above.

For induction of colitis with DSS, 10 week old female HeOu or HeJ mice were administered 3% DSS in their drinking water for 5 or 7 days, then killed and their livers removed for analysis. Control animals drank water and were allowed food ad libitum because it was

observed that DSS consumption did not affect food consumption. In the experiment to study the acute effects of LPS injection, ad libitum fed, 10 week old female HeOu or HeJ mice were given a single i.p. injection of either 1 mg/kg *E. coli*, LPS, or saline. The animals were killed 24 h later and livers collected as described above. The treatment groups studied and numbers of animals are summarized in Table 1.

Western Immunoblotting. Liver microsomes from control and treated mice were prepared as previously described (Richardson et al., 2006). FMO1 and FMO3 protein levels in mouse liver microsomes were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting. Polyclonal goat antibodies recognizing mouse FMO1 and 3 were generously provided by Professor Ernest Hodgson (North Carolina State University, Raleigh, NC). Briefly, 30 µg (for FMO1 detection) and 60 µg (for FMO3 detection) of liver microsomal protein from five *C. rodentium* infected and four control mice were fractionated on a 10% SDS-PAGE under denaturing conditions as described by (Laemmli, 1970). Proteins were transferred onto PVDF membranes (Millipore, Bedford, MA), and equal protein loading and transfer were verified by Ponceau S staining of the membrane. Membranes were blocked in 5% milk in PBS-Tween-20 (0.2%) and a 1:1000 dilution of the primary antibodies against FMO1 or FMO3 was applied for 1 hour. After thorough washing, a 1:10,000 dilution of horseradish peroxidase-coupled rabbit anti-goat antibodies (Pierce, Rockford, IL) was applied for 1 hour. Bound antibodies were detected using SuperSignal Western Substrates (Pierce, Rockford, IL). FMO quantification in each microsomal sample was achieved by densitometric analysis employing Kodak Molecular Imaging software (Eastman Kodak Company, Rochester, NY).

Reverse transcription. Liver RNA was prepared from control and treated mice as previously described (Richardson et al., 2006). Each RNA sample was reverse transcribed to cDNA using

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1 µg RNA and Superscript III RT kit (Invitrogen, Carlsbad, CA) including random hexamers as well as oligo dTs in a 20 µl total reaction volume following manufacturer's instruction.

Real-time PCR. Sequences for mouse *Fmo1* (MMU87456), *Fmo2* (AF184981), *Fmo3* (MMU87147), *Fmo4* (AF461145), and *Fmo5* (MMU90535) were aligned to identify gene-specific sequences for primer synthesis. Primers designed for amplification of specific *Fmo* isoforms are listed in Table 2. Comparison of all *Fmo* primers with *Fmo6* and reported pseudogene clusters (Hernandez et al., 2004) revealed that mF3f193 has 95% alignment with mouse *Fmo6*. However, the reverse primer mF3r419 showed no alignment with relative genes, insuring that the amplified PCR products produced by the primer pairs were from a single template. All other primers showed no alignment to these loci. All quantitative PCR (q-PCR) reactions were run in 96-well PCR plates using an iQ5 Thermal Cycler (BioRad, Emeryville, CA). The PCR reactions were prepared in 15 µl volumes in duplicate for each sample using the following components: 1x SYBR super mix (BioRad), gene specific primer pairs (10 pmol), cDNA (10 ng), and H_2O. The q-PCR was run at 95 ^oC for 5 minutes followed by 40 cycles of 95 ^oC for 15 seconds and 58 ^oC for 30 seconds. Duplicate no-template controls were included on the same plate for each gene analyzed. At the end of the PCR cycling steps, product melting curves were inspected and confirmed to have a single amplification peak.

Quantification of relative mRNA level by a standard curve method. A pool of cDNA from control C57BL/6 mice during *C. rodentium* experiment (Control Day 7, n=6, Table 1) were used to generate a standard series (no dilution, 10-fold, 100-fold, 1,000-fold, and 10,000-fold dilution) used for all gene analyses. Standard curves were developed by plotting *Ct* value against log of fold dilutions. To normalize the inter-sample variation in quality inherently associated with RNA preparation, the transcription level of housekeeping gene *Gapdh* was quantified for all samples.

The value obtained from each target gene was then normalized by *Gapdh* value to calculate the relative *Fmo* mRNA levels in comparison with the corresponding control groups.

Statistical analysis. For both FMO protein level analysis and *Fmo* mRNA relative level analysis, treated mouse groups (*C. rodentium,* LPS, and DSS) were compared with the corresponding control mice group via unpaired *t*-test analysis using GraphPad Prism Programs (Version 3.00, La Jolla, CA). The level of statistical significance was set at $P<0.05$.

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Results

Effect of *C. rodentium* infection on *Fmo* mRNA expression. Real time RT-PCR analysis revealed down-regulation of hepatic *Fmo1, 3*, and *4* mRNAs in *C. rodentium*-treated C57BL/6 female mice. *Fmo1* mRNA was slightly suppressed at 7 days of treatment; however, this did not achieve statistical significance. The mRNA level for *Fmo1* decreased modestly at day 10 post treatment to 42%, and started to increase toward control level at day 15 post treatment to 64% of control animals (Figure 1A).

C. rodentium treatment was associated with significant decreases in the mRNA level for *Fmo3* at all time points studied. In the day 7 and day 10 treatment groups, *Fmo3* levels were 5% and 0.6% of control, respectively. At day 15 and day 24 post *C. rodentium* treatment, the *Fmo3* mRNA level increased back to 13% and 27% respectively. However, the mRNA level for *Fmo3* at 24 days post treatment was still significantly lower than the control group (Figure 1B).

In *C. rodentium*-treated mice, the expression of *Fmo4* seemed to follow a similar trend to that of *Fmo1*, but due to a relatively large variability in *Fmo4* expression the difference was not statistically significant even at the lowest point we observed (27%, p=0.06) (Figure 1C). In contrast to the other *Fmos*, the mRNA level of *Fmo5* did not change significantly over the 7-15 days monitoring period post *C. rodentium* treatment (Figure 1D).

Effect of *C. rodentium* infection on FMO protein regulation. Through Western blot analysis, we also evaluated protein levels of FMO1 and FMO3 in hepatic microsomes prepared from the corresponding animals examined above (Figure 2). *C. rodentium* infection significantly down-regulated FMO3 protein expression in mouse liver to 65% of control at day 7 after infection, and FMO3 proteins were further down-regulated to 14% of control on day 10 post infection. Thereafter, the protein expression increased to 22 and 36% of control on days 15 and 24,

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respectively (Figure 2A). The FMO3 protein level regulation followed the same trend as the mRNA level identified from real-time PCR described above. The effect of *C. rodentium* infection on FMO1 protein expression was less dramatic. Nevertheless, significant down-regulation of FMO1 protein occurred at days 10 and 24 to 56% and 78% of control, respectively (Figure 2B). In general, the FMO1 protein regulation pattern also agreed with the mRNA regulation.

Role of TLR4 on *Fmo3* regulation in *C. rodentium* infection. TLR4, in association with several proteins, is responsible for LPS signaling (Hoshino et al., 1999), and mice lacking TLR4 have been used as an essential and convenient tool to examine the role of TLR4 in infection models (Richardson et al., 2006). To investigate the functional involvement of TLR4 in *Fmo* regulation during inflammation response, we used two mouse strains (i.e., HeOu and HeJ) whose genetic backgrounds are similar except that HeJ strain lacks TLR4 due to a naturally occurring mutation (Richardson et al., 2006). The *Fmo3* mRNA level was significantly down-regulated during *C. rodentium* infection in both HeOu and HeJ mice, to 26% and 41% of control respectively (Figure 3).

Regulation of hepatic *Fmos* in the LPS model of bacterial sepsis. A single time point (i.e. 24 hours post treatment) was chosen to examine the effect of acute LPS treatment on *Fmo* regulation because this is an optimal time to monitor LPS-mediated hepatic gene regulation based on our past experience with P450 studies. The effects of a single dose of *E. coli* LPS on hepatic expression of *Fmo* mRNAs are shown in Figure 4. *Fmo1*, *Fmo3*, and *Fmo5* mRNAs were found to be significantly down regulated in the livers of HeOu mice. In the HeJ strain, that lacks TLR4, *Fmo3* and *Fmo5* mRNAs were unaffected by LPS treatment (Figure 4B, 4D), whereas a small but significant reduction in *Fmo1* mRNA was observed (Figure 4A). *Fmo4* mRNA was significantly up-regulated in HeJ, but not in HeOu mice treated with LPS (Figure 4C).

Effect of DSS treatment on *Fmo* mRNA regulation. Distinct from the *C. rodentium* and LPS inflammation responses, no significant differences were found in the mRNA levels for *Fmo1*, *Fmo3*, and *Fmo4* in HeOu or HeJ mice treated for 5 days with DSS, a chemically induced model of ulcerative colitis (Figure 5). This was also true at 7 days of treatment (data not shown). Only *Fmo5* was found to be up-regulated by two-fold in HeJ treated mice. This only occurred after 5 days of treatment for HeJ, and not after 7 days of treatment for HeJ or at either time point in HeOu mice (Figure 5 and data not shown).

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Discussion

While FMO expression in mammalian species is known to be species-, tissue-, age-, and gender-dependent, and the association of defective FMO3 mutations in human with the primary form of trimethylaminuria, a fish odor syndrome associated with poorly metabolized trimethylamine, has been clearly demonstrated at the genetic level, the physiological mechanisms controlling and regulating FMO expression are not well understood (Cashman, 2003; Motika et al., 2007). Evidence exists for the regulation of FMOs by sex hormones (Dannan et al., 1986; Lemoine et al., 1991; Falls et al., 1995; Falls et al., 1997; Coecke et al., 1998) and glucocorticoids in rodents (Dixit and Roche, 1984). Transient trimethylaminuria has been reported to be associated with menstruation in women (Cashman, 2003; Shimizu et al., 2007). FMO3 activity alteration has also been associated with severe hepatic diseases in both human patients and rodent models (Nakajima et al., 1998; Mitchell et al., 1999). In this study, we examined the hepatic mRNA and protein regulation of four FMO isoforms in different inflammation mouse models. A general trend of down-regulation of *Fmos* in both the *C. rodentium* infected as well as LPS treated mice, but not the DSS treated mice, was observed. The regulation of each *Fmo* isoform also showed a different profile.

***Fmo1* regulation.** In the *C. rodentium* model, down regulation of *Fmo1* mRNA correlated well with the time course of bacterial colonization of the colon, which was significant at 7 days post infection, reached its peak at 10 days, and then declined to negligible counts at days 15 and 24 (Chaluvadi et al., 2008a). This temporal profile is similar to that of the regulation of many P450 mRNAs studied in these same mice as well as of the plasma profile of cytokine including IL-6, TNF-α, and interferon-γ (Chaluvadi et al., 2008a). The *Fmo1* protein level followed the same time course as the down-regulation of mRNA, indicating a pre-translational physiologically relevant regulation. Acute LPS treatment also decreased *Fmo1* mRNA. Attenuated *Fmo1* down-regulation in the TLR4-deficient HeJ strain indicated that *Fmo1* mRNA down-regulation in

the LPS model is at least partially TLR4-mediated. However, although the reduction of *Fmo1* mRNA in HeJ strain was attenuated in HeJ strain post LPS treatment, the difference with the control group was still significant, indicating that there is also a TLR4-independent component in LPS induced *Fmo1* regulation.

***Fmo3* regulation.** *Fmo3* mRNA was the most significantly decreased *Fmo* isoform after *C. rodentium* infection. The gradual recovery of *Fmo3* mRNA in the *C. rodentium* treated mouse was noticeably slower than what we observed previously for P450 mRNAs, most of which were back to normal at 15 days (Chaluvadi et al., 2008a). Even at day 24 when bacterial infection was completely cleared and no pathological symptoms were apparent for these animals, the levels of *Fmo3* mRNA (13%) and protein (36%) were still far from normal. Down-regulation of *Fmo3* is likely to be pre-translational based on the parallel protein level regulation. *C. rodentium* infection-mediated *Fmo3* down-regulation is TLR4-independent whereas regulation of *Fmo3* after LPS infection is largely TLR4-mediated. Comparing between the experimental animal strains, HeOu mice are much more sensitive to the pathogenic effects of *C. rodentium* infection than C57BL/6 mice and the P450 down-regulation was associated with significant morbidity in the animals in our previous study (Richardson and Morgan, 2005). It is interesting that the down-regulation of *Fmo3* was much more profound in the C57BL/6 strain, one that showed less pathogenesis, than in the HeOu mice. This was not true of the most profoundly down-regulated P450 studied, the CYP4As, which were more affected in the HeOu mice (Richardson and Morgan, 2005; Chaluvadi et al., 2008a). These results suggest that regulatory factors distinct from that involved in P450 regulation are required for the down-regulation of *Fmo3* during infection and the restoration of *Fmo3* transcription post *C. rodentium* infection of mice.

***Fmo4* and *Fmo5* regulation.** The variability of expression of *Fmo4* in the C57BL/6 mice indicates that no significant effects could be detected in the *C. rodentium* model, although the

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overall pattern was similar to that of *Fmo1*. In the LPS model, a slight increase of *Fmo4* mRNA was observed in the HeJ strain. It is difficult to assess the role of TLR4 in this effect, since a similar but non-significant trend was observed in the HeOu mice. *Fmo5* mRNAs did not change significantly in the *C. rodentium* model. However, LPS treatment caused TLR4-dependent down-regulation of *Fmo5*.

Inflammation model-specific *Fmo* regulation. A clear difference between the *C. rodentium* model of colonic inflammation, which down-regulated *Fmos 1, 3* and *4* in C57BL/6 mice, and the DSS model, where no *Fmo* was affected in HeOu mice, was observed. A comparison of basal *Fmo* mRNA levels among control mice of the three experimental strains revealed that C57BL/6 strain expressed 30-40% less *Fmo1, 3,* and *5*, ($p<0.05$) mRNAs as compared to the HeOu and/or HeJ strains. Basal levels of Fmo4 mRNA in HeOu and HeJ were 2-3 fold higher than that in C57BL/6 respectively ($p<0.01$, data not shown). No significant differences in the basal levels of Fmo mRNAs were observed between HeOu and HeJ strains. As *Fmo3* was down-regulated by *C. rodentium* infection in both strains, we believe the basal level *Fmo* differences among the strains are not the reason for the significant differences observed comparing *C. rodentium* infection and DSS treatment. Livers of DSS treated mice showed dramatic effects on P450 enzymes similar to those in *C. rodentium* treated mice thus confirming acute colitis during DSS treatment (Chaluvadi et al., 2008b). Based on these differences, we hypothesize that general disruption of intestinal microflora and the release of endotoxins following DSS treatment is not sufficient to trigger *Fmo* regulation. The effect of gastrointestinal infection with *C. rodentium* on hepatic *Fmos* is not likely to be solely due to the colonic inflammation it evokes, but that factors unique to the infecting organism are important.

Inflammation-mediated *Fmo1* and *3* regulations observed here are likely controlled at the level of gene transcription or RNA stability. The down-regulation of *Fmo3* was largely

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independent of TLR4, in agreement with our previous work on P450 regulation in this model (Richardson and Morgan, 2005). A number of transcription factors including CCAAT/enhancer-binding protein-α and –β, hepatic nuclear factor HNF-1α, and HNF3 have been shown to play an essential function in sepsis-associated decreases in the transcription of a number of hepatic genes in mice and have been suggested to have similar roles in other inflammatory states (Haaxma et al., 2003). HNF1α and HNF4α, among several transcription factors, have been suggested to be involved in *FMO1* and *FMO3* regulation in humans (Klick and Hines, 2007, Luo and Hines, 2001; Hines et al., 2003). Comparison of human *FMO3* promoter elements with other mammalian species revealed striking differences (Klick et al., 2008). A number of regulatory elements identified in the human FMO3 promoter region, including the CCAAT/enhancer-binding protein-β element, are absent in the specific region examined, suggesting the gene is possibly under very different regulatory mechanisms in human and mice. In rats treated with LPS, FMO enzyme activity as well as FMO1 contents in liver were decreased, and this was at least partially prevented by treatment with inhibitor of inducible nitric oxide (NO) synthase (Park et al., 1999). A later study showed that the decrease in FMO activity is due to a cGMP-independent destabilizing effect of NO on *FMO* mRNA that results in a decreased half-life of the mRNA (Ryu et al., 2004a; Ryu et al., 2004b). Whether the effects of inflammation on *Fmo* mRNA levels in our study are associated with any of the transcriptional and/or post-transcriptional factors identified from other model systems remain to be explored further. .

Many of the effects of inflammation and infection of hepatic P450 expression are thought to be due to proinflammatory cytokines since purified cytokines can regulate P450 expression in rodents *in vivo* as well as in hepatocyte cultures (Aitken et al., 2006). Although little is known about the abilities of cytokines to regulate FMO expression, it is reasonable to speculate that some of the *in vivo* effects that we report in this study are due to proinflammatory cytokines

acting on the hepatocytes. Research is also needed to clarify the role of hormones in the expression of FMO, and to explore whether hormone level fluctuation due to inflammation-induced stress might be involved in alternation of FMO levels.

Conclusions: The results of this study showed isoform-specific down regulation of mouse liver *Fmo* mRNAs during inflammation mediated by *in vivo* infection and LPS treatment with *Fmo3* being most-significantly down-regulated. The suppression of *Fmo3* in infected mice was much slower to recover than was P450 expression shown in previous studies. In contrast to P450s, *Fmo* expression in wild-type mice was not significantly affected in the DSS model of colonic inflammation. Overall, mouse *Fmo* mRNA down-regulation in the LPS model of sepsis was TLR4-dependent, whereas the down-regulation of *Fmo3* during *C. rodentium* infection was TLR4-independent. Nevertheless, other mechanisms must also be involved because even in mice lacking the TLR4 a slight decrease in *Fmo1* mRNA levels was observed after LPS treatment. Further research is in progress to clarify the mechanism of inflammation-mediated down-regulation of *Fmos*. Our work suggests that the pharmacokinetics of drugs cleared by FMO enzymes in mice, especially FMO3, are likely to be altered during inflammation and infection, and provides strong evidence that the isoforms and drugs affected are likely to be disease model dependent.

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References:

Aitken AE, Richardson TA and Morgan ET (2006) Regulation of drug-metabolizing enzymes and transporters in inflammation. *Annu Rev Pharmacol Toxicol* **46:**123-149.

Ashino T, Oguro T, Shioda S, Horai R, Asano M, Sekikawa K, Iwakura Y, Numazawa S and Yoshida T (2004) Involvement of interleukin-6 and tumor necrosis factor alpha in CYP3A11 and 2C29 down-regulation by Bacillus Calmette-Guerin and lipopolysaccharide in mouse liver. *Drug Metab Dispos* **32:**707-714.

Cashman JR (2003) The role of flavin-containing monooxygenases in drug metabolism and development. *Curr Opin Drug Discov Devel* **6:**486-493.

Chaluvadi MR, Kinloch RA, Nyagode BA, Richardson TA, Raynor MJ, Sherman M, Antonovic L, Strobel HW, Dillehay DL and Morgan ET (2008a) Regulation of Hepatic Cytochrome P450 Expression in Mice with Intestinal or Systemic Infections of Citrobacter rodentium. *Drug Metab Dispos* Oct 23 [e-pub ahead of print).

Chaluvadi MR, Nyagode BA, Kinloch RD and Morgan ET (2008b) TLR4-Dependent and -Independent Regulation of Hepatic Cytochrome P450 in Mice with Chemically-Induced Inflammatory Bowel Disease. *Biochem Pharmacol* Nov 6 [e-pub ahead of print).

Coecke S, Debast G, Phillips IR, Vercruysse A, Shephard EA and Rogiers V (1998) Hormonal regulation of microsomal flavin-containing monooxygenase activity by sex steroids and growth hormone in co-cultured adult male rat hepatocytes. *Biochem Pharmacol* **56:**1047-1051.

Dannan GA, Guengerich FP and Waxman DJ (1986) Hormonal regulation of rat liver microsomal enzymes. Role of gonadal steroids in programming, maintenance, and suppression of delta 4-steroid 5 alpha-reductase, flavin-containing monooxygenase, and sex-specific cytochromes P-450. *J Biol Chem* **261:**10728-10735.

Dixit A and Roche TE (1984) Spectrophotometric assay of the flavin-containing monooxygenase and changes in its activity in female mouse liver with nutritional and diurnal conditions. *Arch Biochem Biophys* **233:**50-63.

Falls JG, Blake BL, Cao Y, Levi PE and Hodgson E (1995) Gender differences in hepatic expression of flavin-containing monooxygenase isoforms (FMO1, FMO3, and FMO5) in mice. *J Biochem Toxicol* **10:**171-177.

Falls JG, Ryu DY, Cao Y, Levi PE and Hodgson E (1997) Regulation of mouse liver flavin-containing monooxygenases 1 and 3 by sex steroids. *Arch Biochem Biophys* **342:**212-223.

Ferrari L, Peng N, Halpert JR and Morgan ET (2001) Role of nitric oxide in down-regulation of CYP2B1 protein, but not RNA, in primary cultures of rat hepatocytes. *Mol Pharmacol* **60:**209-216.

Haaxma CA, Kim PK, Andrejko KM, Raj NR and Deutschman CS (2003) Transcription factors C/EBP-alpha and HNF-1alpha are associated with decreased expression of liver-specific genes in sepsis. *Shock* **19:**45-49.

Hernandez D, Janmohamed A, Chandan P, Phillips IR and Shephard EA (2004) Organization and evolution of the flavin-containing monooxygenase genes of human and mouse: identification of novel gene and pseudogene clusters. *Pharmacogenetics* **14:**117-130.

Hines RN, Luo Z, Hopp KA, Cabacungan ET, Koukouritaki SB and McCarver DG (2003) Genetic variability at the human FMO1 locus: significance of a basal promoter yin yang 1 element polymorphism (FMO1*6). *J Pharmacol Exp Ther* **306:**1210-1218.

Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K and Akira S (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* **162:**3749-3752.

Klick DE and Hines RN (2007) Mechanisms regulating human FMO3 transcription. *Drug Metab Rev* **39:**419-442.

Klick DE, Shadley JD and Hines RN (2008) Differential regulation of human hepatic flavin containing monooxygenase 3 (FMO3) by CCAAT/enhancer-binding protein [beta] (C/EBP[beta]) liver inhibitory and liver activating proteins. *Biochemical Pharmacology* **76:**268-278.

Kraemer MJ, Furukawa CT, Koup JR, Shapiro GG, Pierson WE and Bierman CW (1982) Altered theophylline clearance during an influenza B outbreak. *Pediatrics* **69:**476-480.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680-685.

Lemoine A, Williams DE, Cresteil T and Leroux JP (1991) Hormonal regulation of microsomal flavin-containing monooxygenase: tissue-dependent expression and substrate specificity. *Mol Pharmacol* **40:**211-217.

Luo Z and Hines RN (2001) Regulation of flavin-containing monooxygenase 1 expression by ying yang 1 and hepatic nuclear factors 1 and 4. *Mol Pharmacol* **60:**1421-1430.

Masubuchi Y and Horie T (2004) Endotoxin-mediated disturbance of hepatic cytochrome P450 function and development of endotoxin tolerance in the rat model of dextran sulfate sodium-induced experimental colitis. *Drug Metab Dispos* **32:**437-441.

Mitchell S, Ayesh R, Barrett T and Smith R (1999) Trimethylamine and foetor hepaticus. *Scand J Gastroenterol* **34:**524-528.

Morgan ET, Goralski KB, Piquette-Miller M, Renton KW, Robertson GR, Chaluvadi MR, Charles KA, Clarke SJ, Kacevska M, Liddle C, Richardson TA, Sharma R and Sinal CJ (2008) Regulation of drug-metabolizing enzymes and transporters in infection, inflammation, and cancer. *Drug Metab Dispos* **36:**205-216.

Motika MS, Zhang J and Cashman JR (2007) Flavin-containing monooxygenase 3 and human disease. *Expert Opin Drug Metab Toxicol* **3:**831-845.

Nakajima M, Iwata K, Yamamoto T, Funae Y, Yoshida T and Kuroiwa Y (1998) Nicotine metabolism in liver microsomes from rats with acute hepatitis or cirrhosis. *Drug Metab Dispos* **26:**36-41.

Park CS, Baek HM, Chung WG, Lee KH, Ryu SD and Cha YN (1999) Suppression of flavin-containing monooxygenase by overproduced nitric oxide in rat liver. *Mol Pharmacol* **56:**507-514.

Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B and Beutler B (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282:**2085-2088.

Renton KW (2001) Alteration of drug biotransformation and elimination during infection and inflammation. *Pharmacol Ther* **92:**147-163.

Renton KW (2004) Cytochrome P450 regulation and drug biotransformation during inflammation and infection. *Curr Drug Metab* **5:**235-243.

Richardson TA and Morgan ET (2005) Hepatic cytochrome P450 gene regulation during endotoxin-induced inflammation in nuclear receptor knockout mice. *J Pharmacol Exp Ther* **314:**703-709.

Richardson TA, Sherman M, Antonovic L, Kardar SS, Strobel HW, Kalman D and Morgan ET (2006) Hepatic and renal cytochrome p450 gene regulation during citrobacter rodentium infection in wild-type and toll-like receptor 4 mutant mice. *Drug Metab Dispos* **34:**354-360.

Ryu SD, Kang JH, Yi HG, Nahm CH and Park CS (2004a) Hepatic flavin-containing monooxygenase activity attenuated by cGMP-independent nitric oxide-mediated mRNA destabilization. *Biochem Biophys Res Commun* **324:**409-416.

Ryu SD, Yi HG, Cha YN, Kang JH, Kang JS, Jeon YC, Park HK, Yu TM, Lee JN and Park CS (2004b) Flavin-containing monooxygenase activity can be inhibited by nitric oxide-mediated S-nitrosylation. *Life Sci* **75:**2559-2572.

Schauer DB and Falkow S (1993) Attaching and effacing locus of a Citrobacter freundii biotype that causes transmissible murine colonic hyperplasia. *Infect Immun* **61:**2486-2492.

Shimizu M, Cashman JR and Yamazaki H (2007) Transient trimethylaminuria related to menstruation. *BMC Med Genet* **8:**2.

Siewert E, Bort R, Kluge R, Heinrich PC, Castell J and Jover R (2000) Hepatic cytochrome P450 down-regulation during aseptic inflammation in the mouse is interleukin 6 dependent. *Hepatology* **32:**49-55.

Vallance BA, Deng W, Jacobson K and Finlay BB (2003) Host susceptibility to the attaching and effacing bacterial pathogen Citrobacter rodentium. *Infect Immun* **71:**3443-3453.

Warren GW, Poloyac SM, Gary DS, Mattson MP and Blouin RA (1999) Hepatic cytochrome P-450 expression in tumor necrosis factor-alpha receptor (p55/p75) knockout mice after endotoxin administration. *J Pharmacol Exp Ther* **288:**945-950.

Weidenbach H, Leiz S, Nussler AK, Dikopoulos N, Bachem M, Buttenschoen K, Reinshagen M, Beger HG, Adler G and Schmid RM (2000) Disturbed bile secretion and cytochrome P450 function during the acute state of experimental colitis in rats. *J Hepatol* **32:**708-717.

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* Equal contribution from JZ and MRC

Reprint shipping address:

Dr. Edward Morgan

Emory University School of Medicine

5119 Rollins Research Center

1510 Clifton Road

Atlanta, GA 30322, USA

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Legends for Figures:

Figure 1. Effect of *C. rodentium* treatment on hepatic *Fmo* mRNA regulation in C57BL/6 mice. Hepatic *Fmo1* (A), *Fmo3* (B), *Fmo4* (C), and *Fmo5* (D) mRNA levels in *C. rodentium*-infected female mice after 7, 10, and 15 days in comparison with the corresponding control mice groups. Day 24 post treatment also available for *Fmo3* (B). Number of mice for each group is as listed in Table 1, and mean levels are graphed. Error bars represent standard error of the mean (SEM). Statistically significant differences between the control and *C. rodentium* infected mice are identified by * for $P<0.05$, ** for $P<0.01$, and *** for $P<0.001$.

Figure 2. Effect of *C. rodentium* treatment on hepatic FMO protein levels in C57BL/6 mice. Microsomal FMO3 (A) and FMO1 (B) protein level during *C. rodentium* infection were quantified based on western blot analysis of FMO3 (C) and FMO1 (D) respectively. Protein expression of control mice (n=4 for each group) and of mice at 7, 10, 15, and 24 days after *C. rodentium* infection (n=5 for each group) are compared. Mean levels are graphed and error bars represent SEM. Statistically significant differences between the control and *C. rodentium* infected mice are identified by * for $P<0.05$ and *** for $P<0.001$.

Figure 3. Effect of *C. rodentium* treatment on hepatic *Fmo3* mRNA expression in HeJ and HeOu mice. Hepatic *Fmo3* mRNA level were measured in *C. rodentium*-treated female HeJ and HeOu mice after 6 days of infection, in comparison to the corresponding control mice groups. Number of mice for each group is as listed in Table 1. Mean levels are graphed and error bars represent SEM. Statistically significant differences between the control and *C. rodentium* infected mice are identified by * for $P<0.05$, ** for $P<0.01$, and *** for $P<0.001$.

Figure 4. Effect of LPS treatment on hepatic *Fmo* mRNA regulation. Hepatic *Fmo1* (A), *Fmo3* (B), *Fmo4* (C), and *Fmo5* (D) mRNA levels were measured in female HeJ and HeOu mice, 24 hours after injection with 1 mg/kg LPS or saline (control groups). Number of mice for each group is as listed in Table 1. Mean levels are graphed and error bars represent SEM. Statistically significant differences between the control and LPS treated (LPS) mice are identified by * for $P<0.05$, ** for $P<0.01$, and *** for $P<0.001$.

Figure 5. Effect of DSS treatment on hepatic *Fmo* mRNA regulation. Hepatic *Fmo1, 3, 4,* and *5* mRNA levels in DSS treated female HeJ and HeOu mice after 5 days are shown in comparison to the corresponding control mouse groups. Number of mice for each group is as listed in Table 1. Mean levels are graphed and error bars represent SEM. Statistically significant differences between the control and DSS treated (DSS) mice are identified by ** for $P<0.01$.

Supplemental Figure 1. Relative basal level of *Fmo 1, 2, 3, 4* mRNA in control animals of different strains C57BL/6 (n=16), HeOu (n=11), and HeJ (n=11) mRNA levels are shown in comparison. Mean levels are graphed and error bars represent SEM. Statistically significant differences between the strains are identified by * for $P<0.05$, ** for $P<0.01$, and *** for $P<0.001$.

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Table 1. Experimental group summary. Number of samples from control and treated mice, and *Fmo* isoforms analyzed in each respective groups in following Figures are summarized. Infection models are abbreviated as *C. rodentium* for *C. rodentium*-treated mice, LPS for bacterial lipopolysaccharide infected mice, and DSS for dextran sulfate sodium-treated mice.

Infection Model	Mouse Strain	Treatment Period	Control (n=)	Treated (n=)	*Fmo* Isoform Analyzed
C. rodentium	C57BL/6	7 days	6	5	*Fmo1, 3, 4, 5*
		10 days	5	6	*Fmo1, 3, 4, 5*
		15 days	6	6	*Fmo1, 3, 4, 5*
		24 days	6	6	*Fmo3*
	HeJ	6 day	5	4	*Fmo3*
	HeOu	6 day	4	5	*Fmo3*
LPS	HeJ	1 day	6	5	*Fmo1, 3, 4, 5*
	HeOu	1 day	5	4	*Fmo1, 3, 4, 5*
DSS	HeJ	5 days	6	6	*Fmo1, 3, 4, 5*
	HeOu	5 days	6	5	*Fmo1, 3, 4, 5*
	HeJ	7 days	6	6	*Fmo1, 3, 4, 5*
	HeOu	7 days	6	6	*Fmo1, 3, 4, 5*

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Table 2. *Fmo* gene-specific primers used for real-time PCR analysis.

Gene	Forward primer		Reverse primer	
Fmo1	mF1f393	tcgagaggagcagtgacctg	mF1r676	ccactgtccagagacagcaa
Fmo3	mF3f193	cagcatttaccaatcggtcttc	mF3r419	tgacttcccatttgccagtag
Fmo4	mF4f276	agccatgagaaattctgggacta	mF4r552	cttggctgtgcaggatctgt
Fmo5	mF5f1026	gactaccccatcccagatcat	mF5r1225	acctgctgtttcccttcaca
Gapdh	mGAPDHf47	cgtcccgtagacaaaatggt	mGAPDHr292	ctcctggaagatggtgatgg

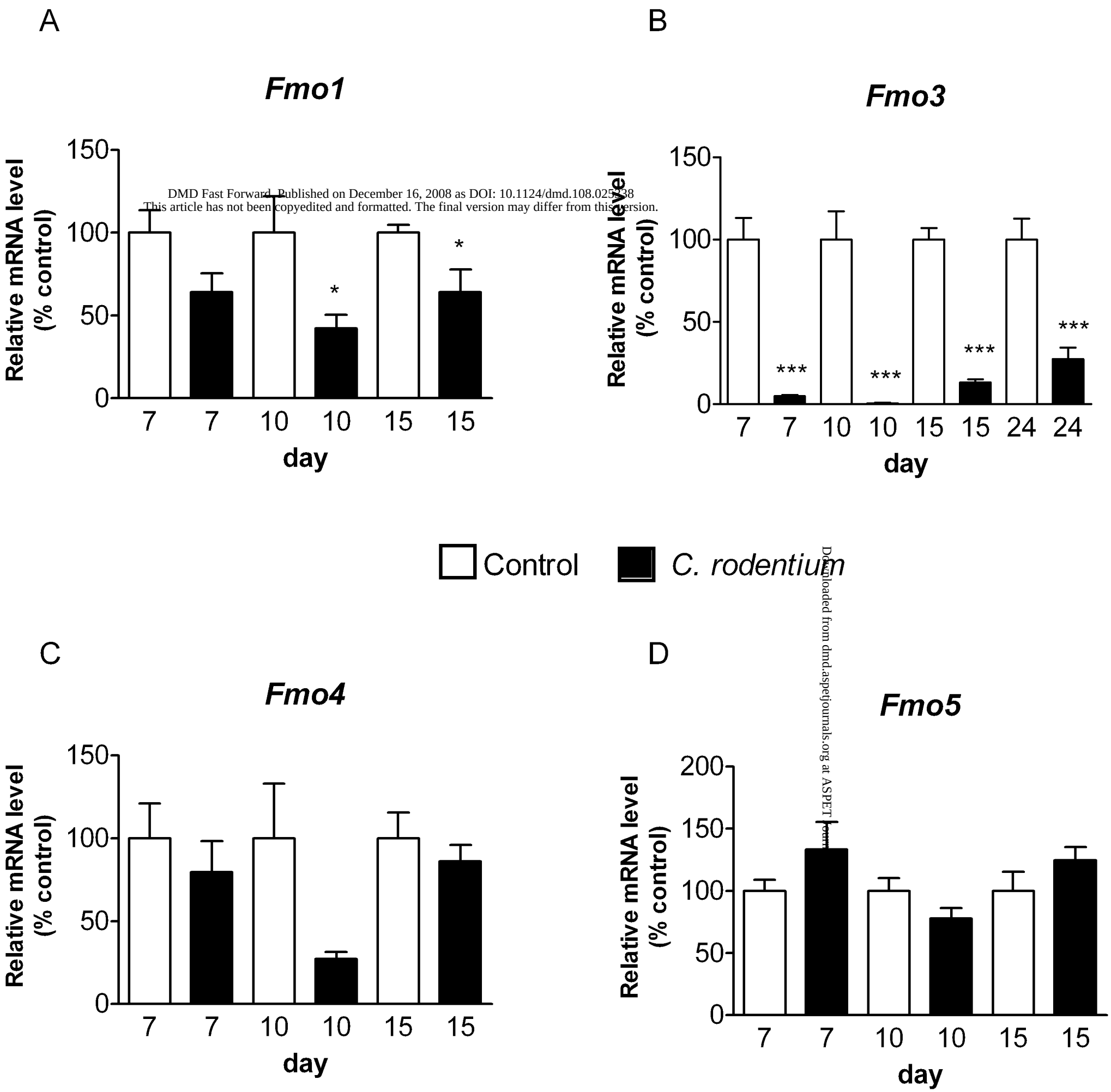

Figure 1

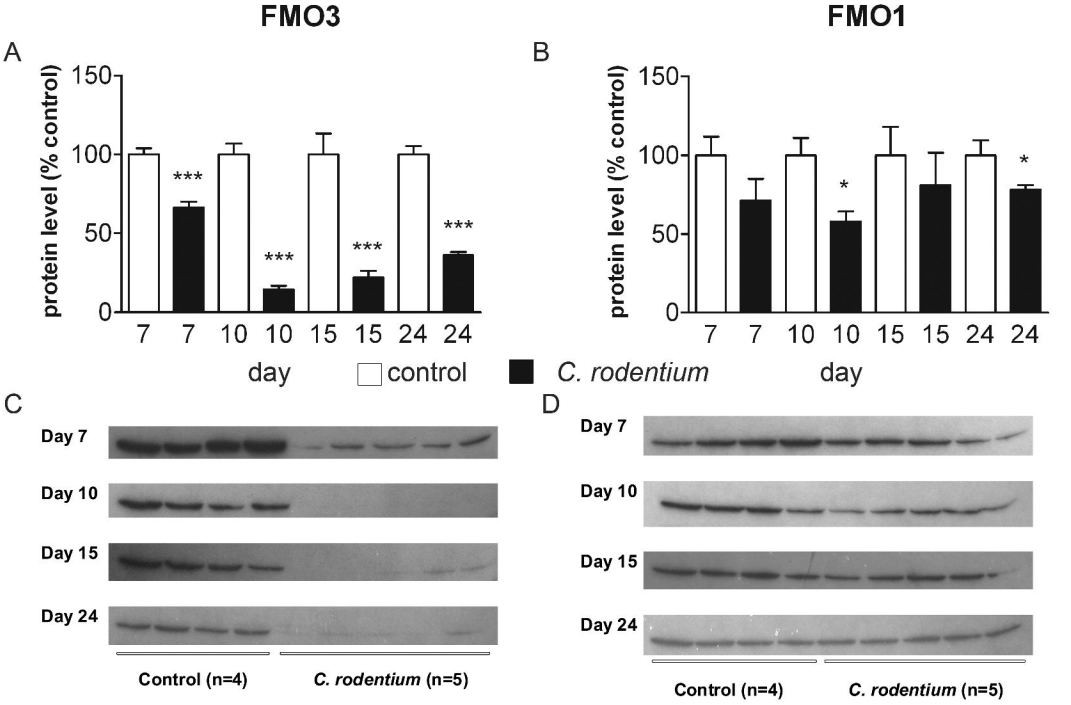
FMO3
FMO1
A
B
protein level (% control)
150
100
50
0
***
*
7 7 10 10 15 15 24 24
day
control
C. rodentium
C
D
Day 7
Day 10
Day 15
Day 24
Control (n=4)
C. rodentium (n=5)

Figure 2

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Fmo3

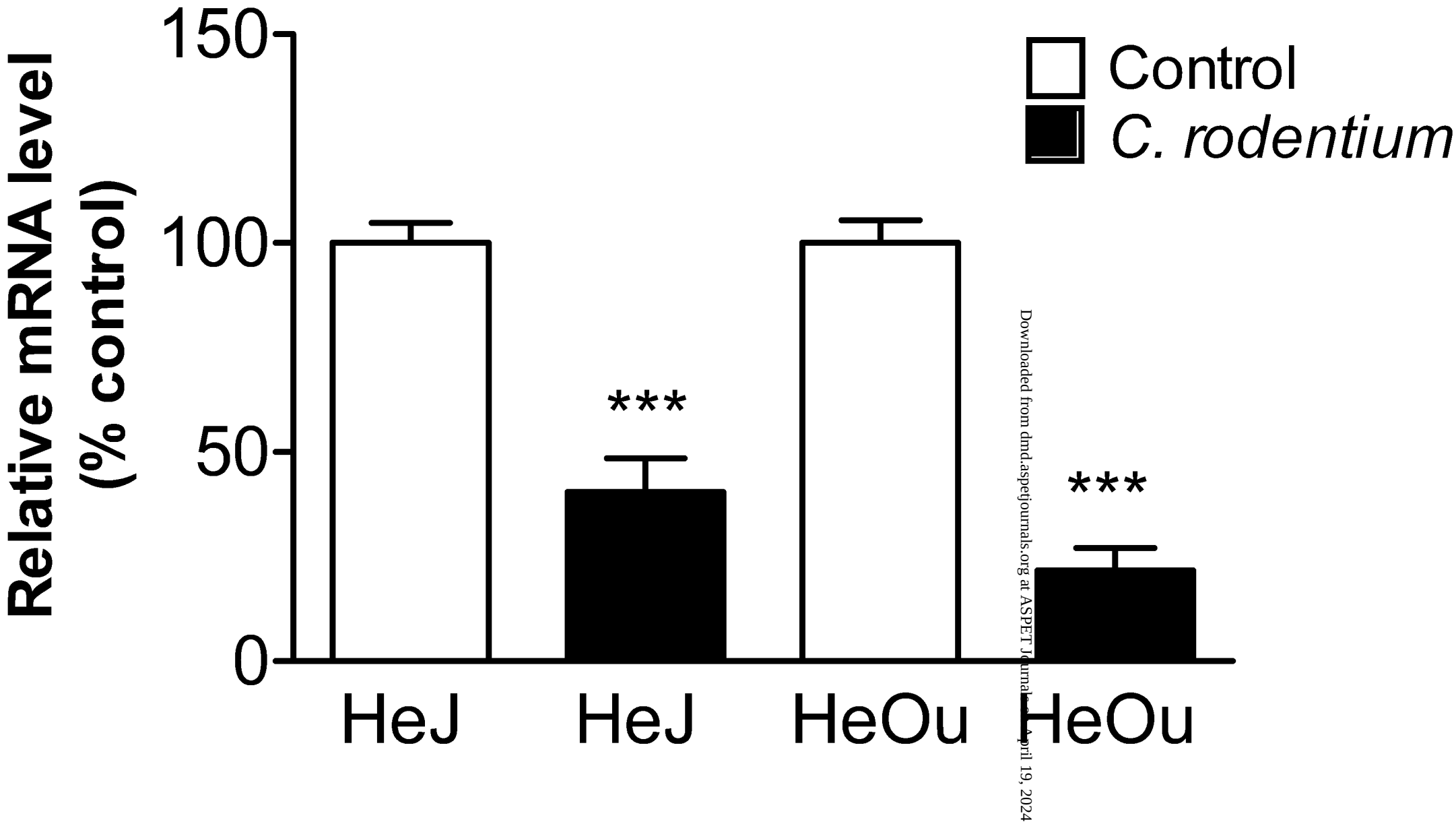

Figure 3

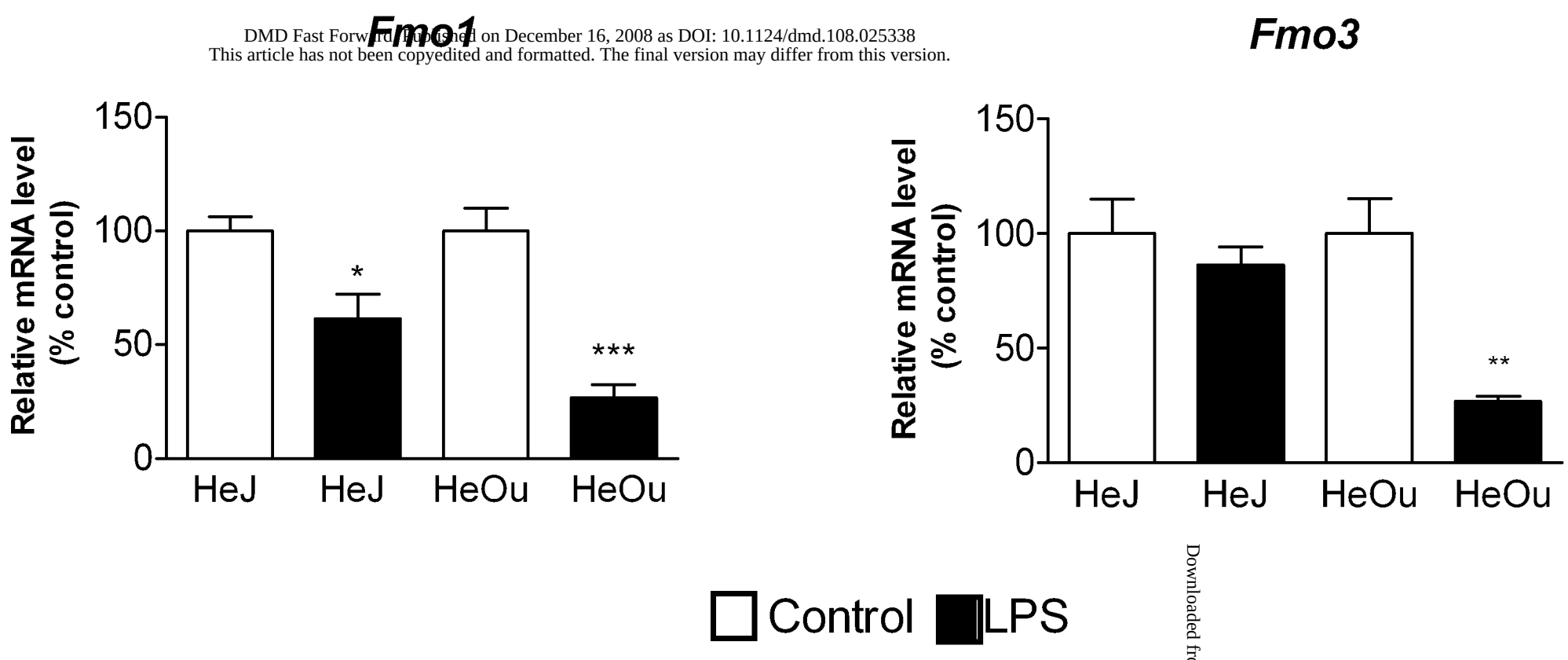

Figure 4

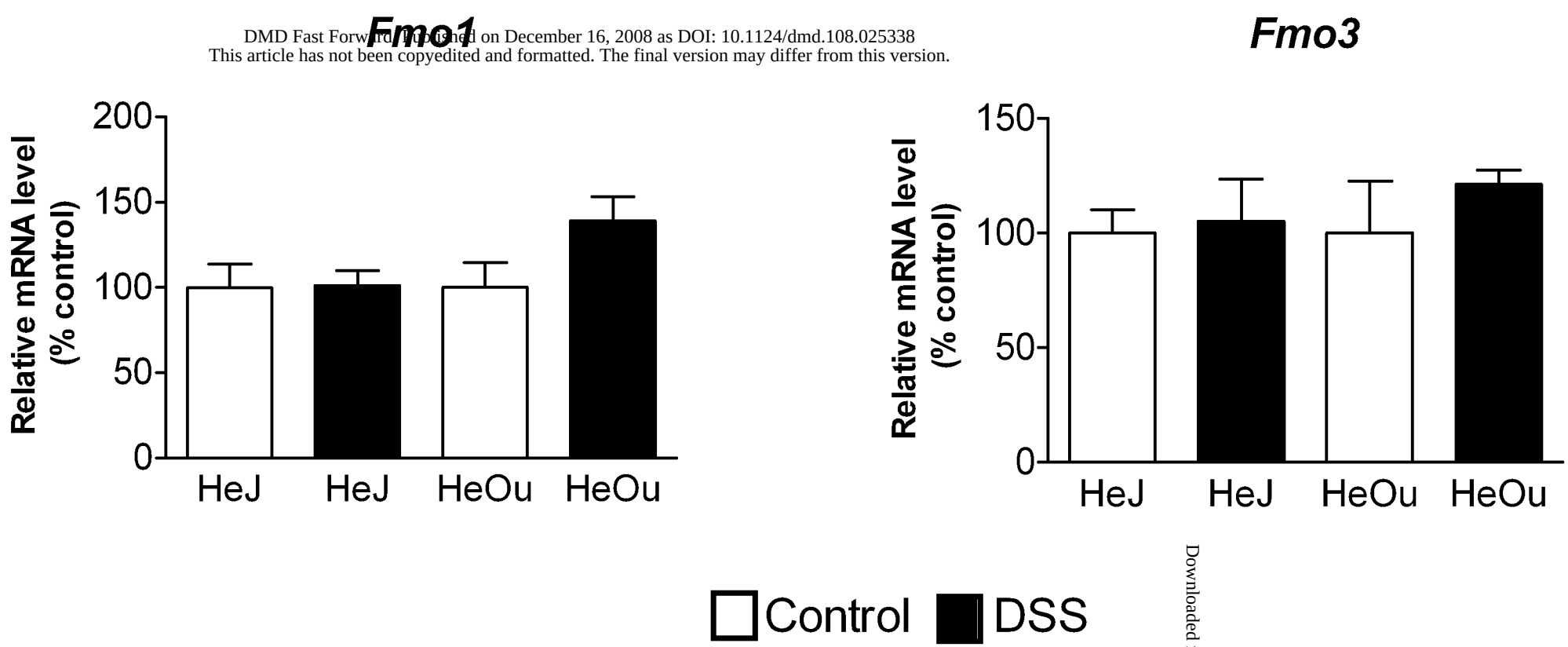

Figure 5