Original Article

Effects of dexamethasone to reverse decreased hepatic midazolam metabolism in rats with acute renal failure

Masami Doi, Noriko Kajikawa, and Tetsuya Aiba*

Graduate School of Medicine, Dentistry and Pharmaceutical Sciences,

Okayama University, Okayama 700-8558, Japan

To whom correspondence should be addressed:

Tetsuya Aiba, Ph.D.

Associate Professor

Graduate School of Medicine, Dentistry and Pharmaceutical Sciences,

Okayama University, Shikata, Okayama 700-8558, Japan

Tel./Fax: +81.86.235.7774 / +81.86.251.7926

e-mail: taiba@okayama-u.ac.jp

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Abstract

1. The inductive effects of dexamethasone on hepatic midazolam metabolism were examined in Wistar rats with acute renal failure (ARF) to clarify whether the ARF-related decrease in the hepatic expression of drug-metabolizing enzymes is caused by an impairment in the translation/polypeptide formation process.
2. ARF was induced with intramuscular glycerol injection. Dexamethasone was orally administered. Pooled liver microsomes from 5 rats were prepared with ultracentrifugation for each of 4 groups, namely control and ARF rats, control rats with dexamethasone treatment, and ARF rats with dexamethasone treatment.
3. Hepatic drug-metabolizing activity was examined in an incubation study with the microsomes, where midazolam was employed as a substrate of cytochrome P450 (CYP) 3A enzymes. The hepatic protein and mRNA expressions of CYP3A23/3A1 and 3A2 enzymes were also evaluated.
4. With dexamethasone treatment, the hepatic metabolic rate of midazolam increased 1.4 times in control rats, while it increased 19.6 times in ARF rats, reflecting the greater induction of hepatic protein expressions of CYP3A enzymes in ARF rats

than in control rats.

5. The hepatic protein expression process for CYP3A23/3A1 and 3A2 responds well to dexamethasone treatment in ARF rats, indicating that the translation/polypeptide formation process is not impaired in the presence of ARF.

Key Words:

Acute renal failure; CYP3A2; dexamethasone; hepatic drug metabolism; midazolam.

Running Head:

DEX induces hepatic CYP3A expression in ARF rats

Introduction

Hepatic drug metabolism decreases under the influence of impaired renal function, as demonstrated with the cytochrome P450 (CYP) 3A substrate midazolam (MDZ) in our previous study, in which the hepatic drug-metabolizing activity for MDZ decreased in rats with experimentally induced acute renal failure (ARF) as compared with control rats (Kusaba *et al*. 2012). Propagation mechanism by which an influence of renal failure reaches the liver is not fully clarified to date, but inflammatory factors released into the circulatory system with impaired renal function seem to be partly involved in the decreased metabolism (Abdel-Razzak *et al*., 1995; Assenat *et al*., 2004; Kajikawa *et al*., 2014). Since the decrease in hepatic drug-metabolizing activity in ARF rats is largely due to decreased hepatic protein expression of CYP3A enzymes, it is considered that some processes of gene expression, such as transcription and translation, are impaired in ARF rats. As the mechanism underlying the decreased enzyme expression, a factor influencing the association of nuclear receptors with the promoter region of the target gene, such as an alteration of their phosphorylation and/or a repression of their translocation, may be responsible for the decrease (Elias *et al.*, 2014; Hosseinpour *et al.*, 2007; Krausova *et al.*, 2011; Ourlin *et al.*, 2003). An inhibition of ribosomal polypeptide formation could also be a cause of the decrease (Lang *et al.*, 2000). In addition to these direct suppressions of translation/polypeptide formation, an altered

protein degradation process and a consequent imbalance between protein synthesis and degradation may indirectly cause a decrease in protein expression. It was demonstrated that the phosphorylation and ubiquitination process of CYP3A enzymes results in a reduction of their expression level, largely due to acceleration of their proteasomal degradation (Printsev *et al.*, 2017; Wang *et al.*, 2009). However, little has been elucidated regarding the mechanism behind the decreased hepatic protein expression of drug-metabolizing enzymes in ARF rats. As hepatic drug-metabolizing activity largely influences the pharmacokinetics and therapeutic efficacy of various compounds, it is worth understanding how the hepatic expression and activity of drug-metabolizing enzymes change when homeostasis of the body is impaired by major organ failure.

In this study, we conducted examinations to clarify the underlying mechanisms of decreased hepatic expressions of CYP3A23/3A1 and 3A2 proteins in ARF rats, with a working hypothesis that the translation/polypeptide formation process is inhibited in ARF rats. It is reported that ARF is often accompanied with inflammation (Izuwa *et al*., 2009), and that inflammatory factors, such as interleukin 1β, suppress the hepatic expressions of drug-metabolizing enzymes (Abdel-Razzak *et al*., 1995; Assenat *et al*., 2004). In addition, it was demonstrated that disease model rats showing decreased hepatic expression of drug-metabolizing enzymes poorly respond to protein induction treatment with the potent protein inducer dexamethasone (Blouin *et al*., 1993; De Martin

et al., 2014; Gabbia *et al*., 2018). It is therefore considered that dexamethasone induces hepatic protein expression of enzymes to a lesser extent in ARF rats than in normal rats, since some inhibitory mechanism working behind the translation/polypeptide formation process in ARF rats would counteract dexamethasone's induction process. Dexamethasone has been reported to stimulate the hepatic protein expression of CYP3A enzymes in a transactivation manner, which is associated with the pregnane X receptor to form a heterodimer with the retinoid X receptor, and then the heterodimer binds to the response element of the 5'-flanking region of the gene to stimulate protein expression (Kishida *et al*., 2008; Kliewer *et al*., 1998; Kliewer *et al*., 2002). If some part of these induction processes is impaired in ARF rats, a difference may be observed in dexamethasone's induction effects between normal and ARF rats. With these considerations, we performed this study, in which we subjected normal and ARF rats to dexamethasone treatment, and then we prepared the liver microsomes to evaluate the difference in their hepatic drug-metabolizing activities. We subsequently examined and compared the hepatic protein and mRNA expressions of the CYP3A enzymes between normal and ARF rats to assess whether an impairment of the translation/polypeptide formation process is a cause of the decreased hepatic expression of CYP3A proteins in ARF rats.

Materials and Methods

Materials

MDZ was purchased from Sandoz (Tokyo, Japan) as a commercially available injectable solution in a sterilized isotonic buffer (pH 2.8–3.8). It was used without further purification (Hori *et al*., 2018; Kajikawa *et al*., 2014). Two MDZ metabolites, 4-Hydroxy MDZ (4-OH MDZ) and 1'-hydroxy MDZ (1'-OH MDZ), were obtained from BD Biosciences (San Jose, CA, USA) and Cerilliant (Round Rock, TX, USA), respectively. Dexamethasone was from Tokyo Chemical Industry (Tokyo, Japan). β-NADPH was from Oriental Yeast (Tokyo, Japan). Glycerol and dimethyl sulfoxide were purchased from Wako Pure Chemicals (Osaka, Japan). Purified rabbit polyclonal antibody to rat CYP3A23/3A1 (P/N: AB1253) and that to rat CYP3A2 (P/N: AB1276) were from Millipore (Billerica, MA, USA) (Kusaba *et al.*, 2012). The primer pairs for performing polymerase chain reaction (PCR) and Oligo$(dT)_{20}$ primer for reverse transcription (RT) were obtained via a custom primer synthesis service of Invitrogen/Thermo Fisher Scientific (Waltham, MA, USA). The sequences of the primer pairs are shown in Table 1. RNase inhibitor was obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were of the finest grade available from local distributers.

[Table 1]

Animals and animal specimen preparation

Male Wistar rats at 7 weeks old (190–300 g) were obtained from Japan Charles River (Yokohama, Japan). They were caged in an air-conditioned room with controlled temperature and relative humidity at 20–25°C and 40–50%, respectively. The light/dark cycle was set at 12 hours. All animal experiments were performed in accordance with the guidelines for animal experimentation of Okayama University with approval by the institutional animal ethics committee (OKU-2013022/OKU-2015421).

[Figure 1]

After a 24-hour acclimatization period, rats were randomly assigned to either the control or ARF group. The scheme of animal treatment is shown in Fig. 1. The rats in the ARF group were subjected to 24-hour water deprivation, and then they received intramuscular injections of 50% glycerol (10 mL/kg) in the left and right thighs to induce ARF (Aiba *et al*., 2006; Kusaba *et al.*, 2012). The rats assigned to the control group experienced no water deprivation, and they were injected with saline instead of glycerol. After that, rats in both groups were normally fed with free access to water for 24 hours until the experiments. The establishment of glycerol-induced ARF was checked based on the increased serum creatinine concentration (Kusaba *et al.*, 2012), for which a serum specimen was procured before the experiment. The creatinine concentrations in the control and ARF rats were 0.37 ± 0.02 and 1.26 ± 0.15 mg/dL, respectively, being

significantly different from each other ($p<0.05$).

The dexamethasone treatment to induce hepatic protein expression of the drug-metabolizing enzymes was performed as follows (Cotreau *et al.*, 2000; Kanazu *et al.*, 2012). The dexamethasone solution was prepared by dissolving dexamethasone in dimethyl sulfoxide at a concentration of 40 mg/mL. This dexamethasone solution was orally administered to the control and ARF rats at a dose of 80 mg/kg. The administration was performed once a day at 9:00 am for 3 days.

Rat liver microsomes were prepared from control rats, ARF rats, and rats that received dexamethasone treatment, respectively (Fig. 1). Each of the microsome preparations was carried out with excised livers from 5 rats. They were gently diced and minced in ice-cold saline to be homogenized together, a part of which was spared for the RNA extraction mentioned later, and the microsome preparation was obtained from the liver homogenate with conventional ultracentrifugation (Hori *et al*., 2018; Kusaba *et al.*, 2012). The protein content of the microsome preparation was measured with the Bradford method using the protein assay kit (Bio-Rad, Hercules, CA, USA). The preparation was stored at −80°C until being used in the incubation study to evaluate the hepatic drug-metabolizing activity and in Western blot analysis to assess hepatic protein expressions of CYP3A enzymes.

Evaluation of hepatic drug-metabolizing activity in rats

The hepatic drug-metabolizing activity was evaluated focusing on CYP3A enzymes, in which NADPH-dependent MDZ elimination was examined in incubation experiments involving liver microsome preparations as previously reported with slight modification (Hori *et al*., 2018; Kajikawa *et al*., 2014). In brief, isotonic potassium phosphate buffer (pH 7.4) containing 200 μg of the microsomal protein was used as the incubation mixture. MDZ was dissolved in the mixture at final concentrations of 1, 2.5, 5, 7.5, 10, and 20 μM, respectively. Following a 5-min pre-incubation period, 950 μL of the incubation mixture was spiked with 50 μL of 20 mM β-NADPH solution to start the metabolic reaction. The metabolic reaction was allowed to continue for 5 min at 37°C. A 200-μL aliquot of the incubation mixture was then quickly transferred into 600 μL of ice-cold methanol and vigorously agitated to stop the metabolic reaction. After that, the mixture was centrifuged at 10,000×*g* at 4°C for 3 min to precipitate microsomal protein, and then the supernatant was collected for the determination described later. The incubation experiments were also carried out without β-NADPH, in which potassium phosphate buffer was used instead of the β-NADPH solution.

The elimination rates of MDZ, and the formation rates of its two major metabolites, 4-OH MDZ and 1'-OH MDZ, were characterized with the Michaelis-Menten equation (Eq. 1) (Kajikawa *et al*., 2014):

$$v = \frac{V\mathrm{max} \cdot C}{K\mathrm{m} + C} \quad (1)$$

where v is the β-NADPH-dependent elimination rate of MDZ, or the formation rate of the metabolite. C is the MDZ concentration in the incubation mixture. Km is the apparent Michaelis-Menten constant. Vmax is the maximum MDZ elimination rate, or the maximum formation rate of the metabolite. The parameter estimates were calculated with the non-linear least square method (Kajikawa *et al*., 2014). In this study, we considered that the MDZ elimination process is accounted for by the drug-metabolizing activities of CYP3A23/3A1 and 3A2 proteins, since it was demonstrated that the hepatic MDZ metabolism in rats is almost exclusively mediated by those two proteins, where the 4-OH MDZ formation is mainly mediated by CYP3A2 protein, while the 1'-OH MDZ formation was carried out by both CYP3A23/3A1 and 3A2 proteins (Kobayashi *et al*., 2002; Carr *et al*., 2006).

The hepatic drug-metabolizing activity toward dexamethasone was also examined in the incubation experiments with the liver microsomes in a similar manner as that for MDZ, in which dexamethasone was applied at a final concentration of 20 μM, and 500 μg of microsomal protein was prepared. Following the incubation, the metabolic reaction was subjected to vigorous agitation with ice-cold methanol and centrifugation for protein precipitation. The resultant supernatant was collected for the determination

described later. The NADPH-dependent hepatic metabolism of dexamethasone was evaluated by comparing the amount of dexamethasone in the incubation mixture before and after the incubation period.

Semi-quantitative evaluation of hepatic protein expression of CYP3A enzymes with Western blot analysis

The hepatic protein expressions of CYP3A23/3A1 and CYP3A2 were evaluated with Western blot analysis in a semi-quantitative manner, as previously reported (Hori *et al*., 2018; Kusaba *et al.*, 2012). The liver microsome preparations were diluted with isotonic potassium phosphate buffer to prepare the specimens for electrophoresis. A 10-μL specimen was applied to an SDS-polyacrylamide gel (10%) at 3 μg of microsomal protein/lane. After the electrophoresis, the proteins were transferred to a nitrocellulose membrane, and the target proteins were detected with the corresponding antibodies to be visualized with the Vectastain® Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) and DAB substrate kit (Vector Laboratories). The target band signals were semi-quantitatively evaluated with densitometry.

Evaluation of hepatic mRNA expressions of CYP3A enzymes with real-time RT-PCR

Total RNA extraction from liver tissue was performed with the RNeasy® Mini kit (QIAGEN, Germantown, MD, USA) according to the manufacturer's instructions. As the tissue specimen for the kit, the ice-cold liver tissues that were diced and minced as mentioned before were finely ground into a paste with a chilled pestle and mortar. A total of 30 mg of the paste was supplied for the kit, producing 100 µL of total RNA solution. The RNA yield in the solution was evaluated spectrometrically at a wavelength of 260 nm. Following total RNA extraction, the RT reaction was performed with the Omniscript® RT kit (QIAGEN). In brief, the reaction mixture was prepared with Oligo$(dT)_{20}$ primer and RNase inhibitor, according to the manufacturer's instructions, and then an adequate volume of the total RNA solution was added to the mixture so that it contained 2 µg of total RNA. The RT reaction was conducted at 37°C for 60 min. After the reverse transcriptase was inactivated by incubating the reaction mixture at 93°C for 5 min, the mixture was maintained at 4°C until use for the subsequent process.

With the reaction mixture obtained in the RT reaction, the hepatic mRNA expressions of CYP3A23/3A1 and CYP3A2 enzymes were evaluated by real-time PCR employing the THUNDERBIRD® SYBR® qPCR Mix (Toyobo, Osaka). Real-time PCR was conducted with the StepOnePlus™ Real-Time PCR System (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA). PCR was carried out according to the thermal cycle program for amplification with a denaturation step at

95°C for 15 seconds, an annealing step at 51°C for 15 seconds, and an extension step at 72°C for 45 seconds in this order. In the extension step of the amplification cycle, the fluorescence was measured with normalization based on fluorescence coming from the internal standard dye ROX. The threshold cycle number was determined as the earliest cycle number at which the fluorescence started to increase in a log-linear manner. The mRNA expressions of *Cyp3a23/3a1* and *Cyp3a2* were evaluated relative to those of *Gapdh*, and they were expressed with their common logarithms.

Analytical methods

MDZ and its metabolites, 4-OH MDZ and 1'-OH MDZ, were simultaneously determined by HPLC equipped with an octadecyl silica column (3 μm, 4.6×150 mm, InertSustain® C18, GL Science, Tokyo, Japan) (Hori *et al*., 2018; Kajikawa *et al*., 2014). Dexamethasone was determined in the same manner as that for MDZ. The aforementioned supernatant specimen obtained in the incubation experiment with the liver microsomes was filtered with a nylon membrane syringe filter (0.2-μm pore size, Advanced Microdevices, Ambala Cantt, India). The filtered specimen was subjected to HPLC for determination. MDZ, its two metabolites, and dexamethasone were eluted with a mobile phase containing 40% methanol and 60% sodium phosphate buffer (10 mM, pH 3.1). They were detected spectrophotometrically at a wavelength of 229 nm.

Data analysis

Data are expressed as the mean ± S.E. The significance of differences between two values was evaluated by Student's *t*-test, and $p<0.01$ was considered significant.

Results

Evaluation of dexamethasone-induced increase in hepatic MDZ metabolism in ARF rats

First, the hepatic drug-metabolizing activity was examined with MDZ in liver microsomes prepared from control rats, ARF rats, and rats that underwent dexamethasone treatment. Regarding MDZ metabolism without dexamethasone treatment, it was shown as expected that the MDZ-metabolizing activity in ARF rats was noticeably lower than that in control rats (Fig. 2A and 2C). As for the dexamethasone-induced increase in MDZ metabolism, besides control rats (Fig. 2A and 2B), the treatment increased hepatic MDZ metabolism in ARF rats (Fig. 2C and 2D), and the extent of increase in ARF rats was larger than that in control rats (Fig. 2B and 2D). With calculation of the Michaelis-Menten parameters for MDZ elimination, the *V*max value increased by 1.4 times with dexamethasone treatment in control rats, while it increased by 19.6 times with treatment in ARF rats (Table 2). A greater increase in the *V*max value was shown in a corresponding manner for metabolite formation. That is, with dexamethasone treatment, the value for 4-OH MDZ formation increased by 1.7 times in control rats, while it increased by 62.8 times in ARF rats (Table 2). In the case of 1'-OH MDZ formation, the values in control and ARF rats increased by 1.9 and 24.7 times, respectively. For MDZ metabolism in ARF rats, a change in *K*m values was also observed with dexamethasone treatment (Table 2), suggesting that the hepatic

expressions of CYP3A23/3A1 and 3A2 proteins were induced in an imbalanced manner.

[Figure 2]

[Table 2]

Evaluation of hepatic drug-metabolizing activity toward dexamethasone in ARF rats

It was considered that the ARF-related decrease in hepatic drug metabolism also affects dexamethasone metabolism, so that dexamethasone used for the treatment could differently accumulate in control and ARF rats. To examine this possibility, the hepatic drug-metabolizing activity toward dexamethasone was evaluated in control and ARF rats. As a result, it was shown that the dexamethasone elimination rate in ARF rats was markedly decreased to 48.5% of that in control rats (Fig. 3), indicating that the ARF rats were exposed to about two times more dexamethasone compared with the control rats.

[Figure 3]

Evaluation of dexamethasone-induced increase in hepatic protein expression of CYP3A enzymes in ARF rats

Since the observed increase in hepatic MDZ-metabolizing activity with dexamethasone treatment was considered to be related to an increase in hepatic

expression of drug-metabolizing enzymes, we subsequently evaluated the hepatic expressions of CYP3A23/3A1 and 3A2 proteins. They are known to largely contribute to MDZ metabolism in rats. As shown in Fig. 4, the hepatic expressions of CYP3A23/3A1 and CYP3A2 proteins that decrease or tend to decrease in ARF rats noticeably increased with dexamethasone treatment. In addition, the induction effects of dexamethasone were greater in ARF rats than control rats. That is, the CYP3A23/3A1 expression increased by 21% in control rats, while it increased by 90% in ARF rats (Fig. 4A). In the case of CYP3A2 expression, although it increased by 4.6 times with dexamethasone treatment in ARF rats, it showed a slightly non-statistically significant decrease in control rats (Fig. 4B).

[Figure 4]

Evaluation of dexamethasone-induced increase in hepatic mRNA expression of CYP3A enzymes in ARF rats

The effects of dexamethasone treatment on the hepatic mRNA expression of *Cyp3q23/3a1* and *Cyp3a2* were also examined. Hepatic mRNA expression of *Cyp3a23/3a1* markedly increased with dexamethasone treatment in control and ARF rats, and the extent of the increase was similar between the two groups. (Fig. 5A). As for the expression of *Cyp3a2*, dexamethasone decreased mRNA expression in control and ARF

rats, in contrast to the expression of *Cyp3a23/3a1* (Fig. 5B). In addition, the observed decrease in the mRNA expression of *Cyp3a2* in ARF rats is inconsistent with the fact that dexamethasone increases protein expression of CYP3A2 in ARF rats (Fig. 4B).

[Figure 5]

Discussion

It is well-known that hepatic drug-metabolizing activity is affected by an impaired renal function, as exemplified by the fact that hepatic MDZ metabolism decreases in ARF rats (Kusaba *et al*., 2012), while little is known about the mechanism underlying the decrease. In this study, we examined hepatic drug metabolism in ARF rats with a working hypothesis that the translation/polypeptide formation process of hepatic drug-metabolizing enzymes, such as CYP3A proteins, is impaired with renal failure. It is rational to consider that if the translation/polypeptide formation process is impaired, hepatic protein expression will not be stimulated by chemical inducers, such as dexamethasone. In fact, a decrease in hepatic drug-metabolizing activity in the presence of ineffectiveness of dexamethasone treatment was demonstrated in Zucker fatty rats, an animal model of diabetes (Blouin *et al*., 1993).

Prior to the study with dexamethasone treatment, we examined hepatic MDZ metabolism in ARF rats to confirm that the hepatic drug-metabolizing activity decreased with renal failure. As a result, the hepatic MDZ-metabolizing activity decreased in ARF rats (Fig. 2A and 2C), and the Michaelis-Menten parameters in ARF rats were noticeably different from those in control rats (Table 2). As for the effect of dexamethasone treatment, the hepatic MDZ-metabolizing activity was shown to increase in control rats with dexamethasone treatment (Fig. 2B). On the other hand, it was an unexpected

finding that the hepatic MDZ-metabolizing activity in ARF rats also increased with dexamethasone treatment (Fig. 2D). The observation that dexamethasone induces the hepatic drug-metabolizing activity in ARF rats as if there were little interference with the induction suggests that the translation/polypeptide formation process of the enzymes is not impaired in ARF rats. The results of Western blot analysis support these findings. As shown in Fig. 4, the hepatic protein expressions of CYP3A23/3A1 and CYP3A2 both increased in ARF rats with dexamethasone treatment. It was also shown that their hepatic mRNA expressions measured before the treatment were not different in control and ARF rats (Fig. 5). It is therefore probable that while the decrease in the hepatic MDZ metabolism is caused by a decrease in the hepatic protein expression of CYP3A enzymes in ARF rats, the cause of the decreased protein expression is not related to an impairment of the translation/polypeptide formation process of the enzymes. The process may be intact regarding the responsiveness to dexamethasone treatment.

Although the mechanism underlying the decreased hepatic protein expression of CYP3A enzymes in ARF rats has not been fully elucidated, it can be speculated based on the findings in this study. Besides the fact that the decreased hepatic protein expression in ARF rats is more definitive for CYP3A2 than that for CYP3A23/3A1 (Fig. 4), it is intriguing that while the protein expression of CYP3A2 largely decreases in ARF rats (Fig. 4B), its mRNA expression is not influenced (Fig. 5B). This may be explained

by the fact that the CYP3A proteins are subjected to ubiquitin-dependent proteasomal degradation (Printsev *et al*., 2017; Wang *et al*., 1999; Wang *et al*., 2009). That is, the degradation of CYP3A proteins may be facilitated in ARF rats. It was reported that if the phosphorylation sites in the amino acid sequence of CYP3A protein are phosphorylated to a greater extent, it would be associated with ubiquitin at a faster rate to be degraded (Wang *et al*., 2009). Therefore, it is rational to consider that the phosphorylation process of CYP3A proteins is stimulated, and/or a suppression of ubiquitin-dependent proteasomal degradation is inhibited in ARF rats. Although growth hormone is known to be involved in the gene expression of CYP3A proteins (Dhir et al., 2003; Li *et al*., 2015), it is unlikely that the secretion of growth hormone decreases in ARF rats, as the hepatic mRNA expressions of *Cyp3a23/3a1* and *Cyp3a2* were little affected in ARF rats (Fig. 5).

It is also interesting that the extent of the dexamethasone-induced increase in the MDZ-metabolizing activity of ARF rats was much greater than that in control rats (Fig. 2B and 2D). It may be necessary to consider that the decreased hepatic drug-metabolizing activity in ARF rats influences the pharmacokinetics of dexamethasone. That is, the hepatic drug-metabolizing activity for dexamethasone in ARF rats decreases to about a half of that in control rats (Fig. 3), meaning that ARF rats in the dexamethasone treatment group receive two times more dexamethasone than control rats.

However, even if this is taken into account, it may still be difficult to fully explain the difference in the extents of increase in the MDZ-metabolizing activity between control and ARF rats (Fig. 2). It was shown that the MDZ elimination rate measured at a substrate concentration of 20 μM increases by 13.3 times with dexamethasone treatment in ARF rats (0.79 versus 10.56 nmol/min/mg of microsomal protein) (Fig. 2C and 2D), while the rate increases by 1.5 times in control rats (3.46 versus 5.30 nmol/min/mg of microsomal protein) (Fig. 2A and 2B). Therefore, the inductive effect of dexamethasone on hepatic drug-metabolizing activity may be more potently exerted in ARF rats than in control rats.

The potentiation of the effect of dexamethasone may be reflected by an increased hepatic protein expression of CYP3A enzymes. As shown in Fig. 4, dexamethasone treatment increased the protein expressions of CYP3A enzymes to a greater extent in ARF rats than in control rats. However, regarding the mRNA expression, the dexamethasone-induced increase in ARF rats was similar to that in control rats (Fig. 5). These results suggest that the potentiation of the dexamethasone effect in ARF rats is also related to a change in the protein degradation process. It was reported that a suppression of the degradation of CYP3A protein results in an apparent increase in its protein expression (Printsev *et al*., 2017; Santoh *et al*., 2016), but we could not provide concrete evidence to prove that this reported finding is applicable to our findings in this

study. For future studies, we hypothesized that dexamethasone promotes a suppression process that counteracts the degradation process of CYP3A proteins, probably by modulating phosphorylation and/or dephosphorylation processes of the proteins. It is also likely that dexamethasone alters the substrate affinity regarding protein degradation process, and an altered affinity may contribute to weakening the ARF-related inhibition of the suppression process as well.

We additionally observed a dissimilarity between CYP3A23/3A1 and CYP3A2 regarding the gene expression and responsiveness to dexamethasone treatment. Regarding their difference in responsiveness to dexamethasone treatment, the gene expression of *Cyp3a2* is known to be less induced by dexamethasone as compared with that of *Cyp3a23/3a1*, partly due to a slight difference in their nucleotide sequences in the proximal 5'-flanking region (Huss and Kasper, 1998). It was also reported that dexamethasone induces the gene expression of the nuclear receptor RXRα in rats (Li *et al*., 2015). RXRα suppresses the gene expression of CYP3A proteins (Steineger et al., 1998). Based on these things, dexamethasone may act on mRNA expression of *Cyp3a2* not in an increasing, but in a decreasing manner (Fig. 5). A dissimilarity regarding the gene and protein expression between CYP3A23/3A1 and CYP3A2 enzymes was also reported in cholestatic rats, in which dexamethasone was reported to exert its inductive effect *via* an activation of the nuclear receptor CAR (Gabbia *et al*., 2018). However, the

potentiation of the dexamethasone effect was not observed in the animal model, suggesting that the mechanism underlying the dissimilarity in cholestatic rats is different from that in ARF rats we observed. As for the relationship between mRNA and protein expressions, an increase in the protein expression of CYP3A23/3A1 may follow an increase in mRNA expression (Figs. 4A and 5A), but this is not the case for CYP3A2 expression (Figs. 4B and 5B). This may reflect the difference in their processes for regulating gene expression. It has been demonstrated that the gene expression of *Cyp3a23/3a1* is regulated by microRNA, miR-23b (Sun *et al*., 2016), while it is still unknown whether this is also the case for *Cyp3a2* expression. It is probable that the expression level of CYP3A23/3A1 protein is determined by regulating its mRNA expression, and that of CYP3A2 protein is managed by the protein degradation process.

In this study, we demonstrated with dexamethasone treatment that although the hepatic MDZ-metabolizing activity decreases in ARF rats due to a decrease in hepatic protein expression of CYP3A proteins, the translation/polypeptide formation process of CYP3A proteins is not impaired in ARF rats. Besides the protein expression process, an alteration of the protein degradation process may be involved in the decreased hepatic protein expression in ARF rats.

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Conflict of Interest

The authors declare that there are no conflicts of interest. The authors take full responsibility for any unforeseen conflict of interest that may arise.

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Legends to Figures

Fig. 1. Animal treatment scheme in this study. Twenty male Wistar rats were randomly and evenly assigned to either the control or ARF group. Ten rats in the ARF group were subjected to the glycerol injection to induce ARF, and 10 rats in the control group received saline injection. Then, 5 rats in the control group and 5 rats in the ARF groups were orally treated with dexamethasone at a dose of 80 mg/kg for 3 days. The other rats in both groups were normally fed without receiving any substitutive treatment. After that, rats were sacrificed and livers were excised.

Fig. 2. Evaluation of the dexamethasone-induced increase in hepatic MDZ metabolism in control and ARF rats. The MDZ metabolism was examined with liver microsomes, and the elimination rate of MDZ was evaluated along with the formation rates of its two metabolites, 4-OH and 1'-OH MDZ. In panel A, the elimination and formation profiles of MDZ metabolism in control rats without dexamethasone treatment are shown. Those profiles in control rats with dexamethasone treatment are presented in panel B. The profiles in ARF rats without dexamethasone treatment are shown in panel C, and those in ARF rats with the treatment are indicated in panel D. In all panels, the elimination

profiles of MDZ are indicated with open circles, while the formation profiles of 4-OH and 1'-OH MDZ are shown with open and closed squares, respectively. Data are expressed as the mean ± S.E. from 4–5 independent experiments. Some error bars are behind the symbols. Solid lines are the best-fit lines for the corresponding values obtained with the Michaelis-Menten equation, being applied to characterize the profiles.

Fig. 3. Hepatic metabolism of dexamethasone in control and ARF rats. The NADPH-dependent elimination rates of dexamethasone were examined with liver microsomes from control and ARF rats, in which dexamethasone was used at a concentration of 20 μM. Data are expressed as the mean ± S.E. from 6 independent experiments. *p<0.01: significantly different between the values.

Fig. 4. Induction effects of dexamethasone on the hepatic protein expressions of CYP3A23/3A1 (panel A) and 3A2 (panel B) in control and ARF rats. In both panels, two representative results given in Western blotting performed with anti-CYP3A23/3A1 and 3A2 antibodies are presented above the corresponding bar graph. Data are expressed as the mean ± S.E. from 4 independent experiments. *p<0.01: significantly different between the values with and

without dexamethasone treatment in the corresponding group. $^{\#}p<0.01$: significantly different from the value in the control rats without dexamethasone treatment. Keys: w/o DEX, without dexamethasone treatment; w/ DEX, with dexamethasone treatment; DEX, dexamethasone.

Fig. 5. Effects of dexamethasone treatment on hepatic mRNA expression of *Cyp3a23/3a1* (panel A) and *Cyp3a2* (panel B) in control and ARF rats. In both panels, the mRNA expression level of the CYP3A enzyme was evaluated relative to that of *Gapdh*. The obtained value is expressed using a common logarithmic scale. Data are expressed as the mean ± S.E. from 5–6 independent experiments. $*p<0.01$: significantly different between the values with and without dexamethasone treatment in the corresponding group. Keys: w/o DEX, without dexamethasone treatment; w/ DEX, with dexamethasone treatment; DEX, dexamethasone.

Tables

Table 1. Primer pairs used for PCR amplification to evaluate hepatic mRNA expression of drug-metabolizing enzymes in rats.

mRNA	Forward primer	Reverse primer	Location	Amplicon size	Reference
Cyp3a23/3a1	5’-gga gat cac agc cca gtc aat c-3’	5’-tgg cca gtg ctg tgg atc ac-3’	955–1303	349 bp	Matsubara *et al.*, 2004
Cyp3a2	5’-ttg atc cgt tgt tct tgt ca-3’	5’-ggc cag gaa ata caa gac aa-3’	713–1035	323 bp	Wonganan *et al*., 2009
Gapdh	5’-gtt acc agg gct gcc ttc tc-3’	5’-ggg ttt ccc gtt gat gac c-3’	121–288	168 bp	Naruhashi *et al.*, 2002

Table 2. Michaelis-Menten parameters for MDZ metabolism determined in liver microsomes prepared from control and ARF rats with (w/) and without (w/o) dexamethasone treatment. [a]

	Control rats				ARF rats			
	w/o dexamethasone		w/ dexamethasone		w/o dexamethasone		w/ dexamethasone	
	Vmax [b]	Km [c]	Vmax [b]	Km [c]	Vmax [b]	Km [c]	Vmax [b]	Km [c]
Elimination rate								
MDZ	4.50 ± 0.28	5.28 ± 0.75	6.42 ± 0.35 [d]	4.50 ± 0.67	0.90 ± 0.10 [e]	2.08 ± 0.38 [e]	17.60 ± 1.08 [d]	12.08 ± 1.38 [d]
Formation rates of metabolites								
4-OH MDZ	2.47 ± 0.20	3.08 ± 0.81	4.38 ± 0.45 [d]	5.18 ± 1.04	0.37 ± 0.02 [e]	1.80 ± 0.60 [e]	23.23 ± 4.90 [d]	50.10 ± 13.24 [d]
1'-OH MDZ	0.58 ± 0.04	3.21 ± 0.61	1.12 ± 0.12 [d]	1.68 ± 0.58	0.15 ± 0.02 [e]	6.13 ± 1.20	3.70 ± 0.50 [d]	10.66 ± 2.77

a Data are shown as the mean ± S.E. from 4–5 independent experiments.

b Data are expressed in units of nmol/min/mg of microsome protein.

c Data are expressed in units of μM.

d $p<0.01$, significantly different between the values with and without dexamethasone treatment in the corresponding animal group.

e $p<0.01$, significantly different from the corresponding value in the control rats.

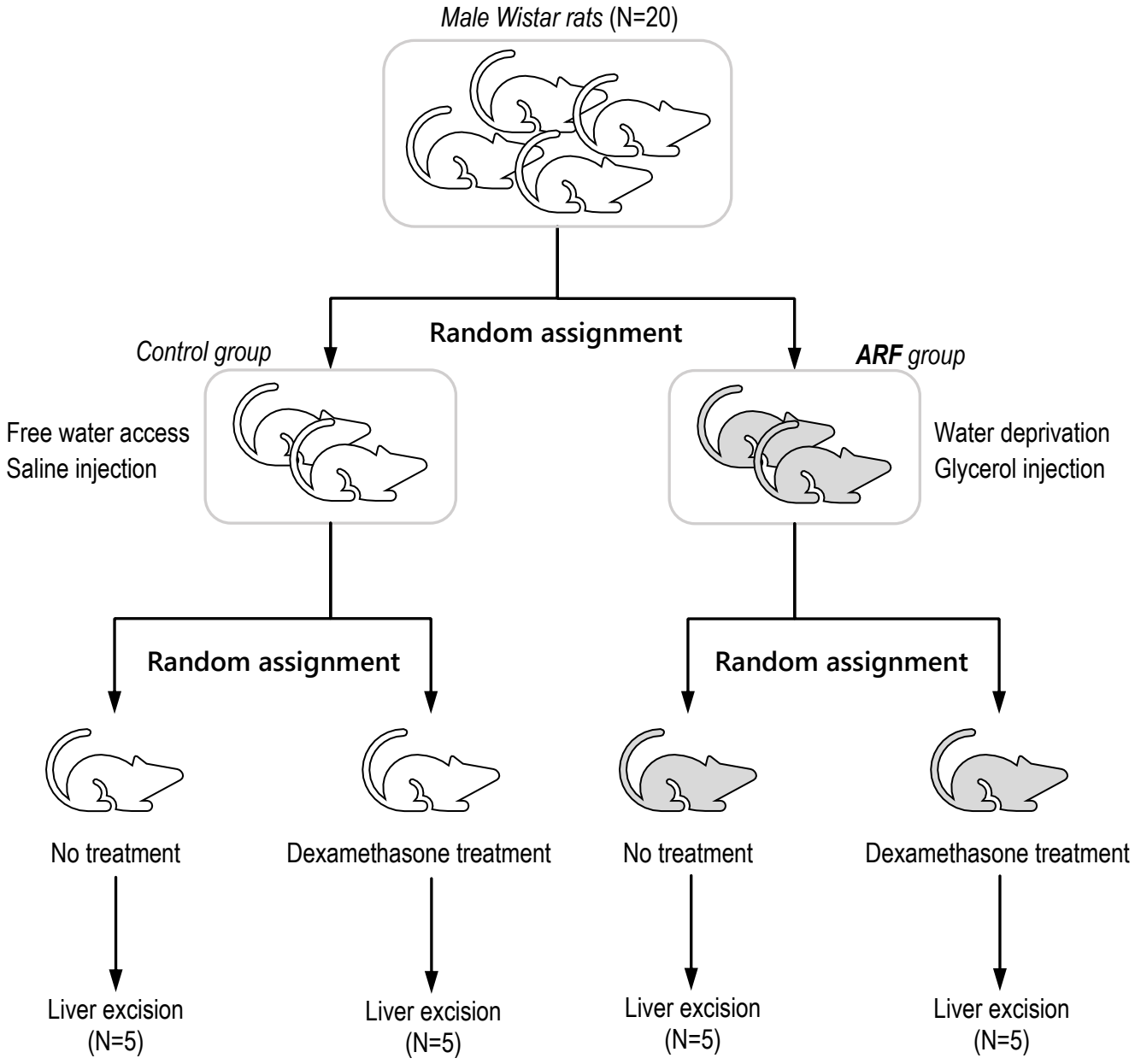
Male Wistar rats (N=20)
Random assignment
Control group
***ARF** group*
Free water access
Saline injection
Water deprivation
Glycerol injection
Random assignment
Random assignment
No treatment
Dexamethasone treatment
No treatment
Dexamethasone treatment
Liver excision
(N=5)
Liver excision
(N=5)
Liver excision
(N=5)
Liver excision
(N=5)

Figure 1

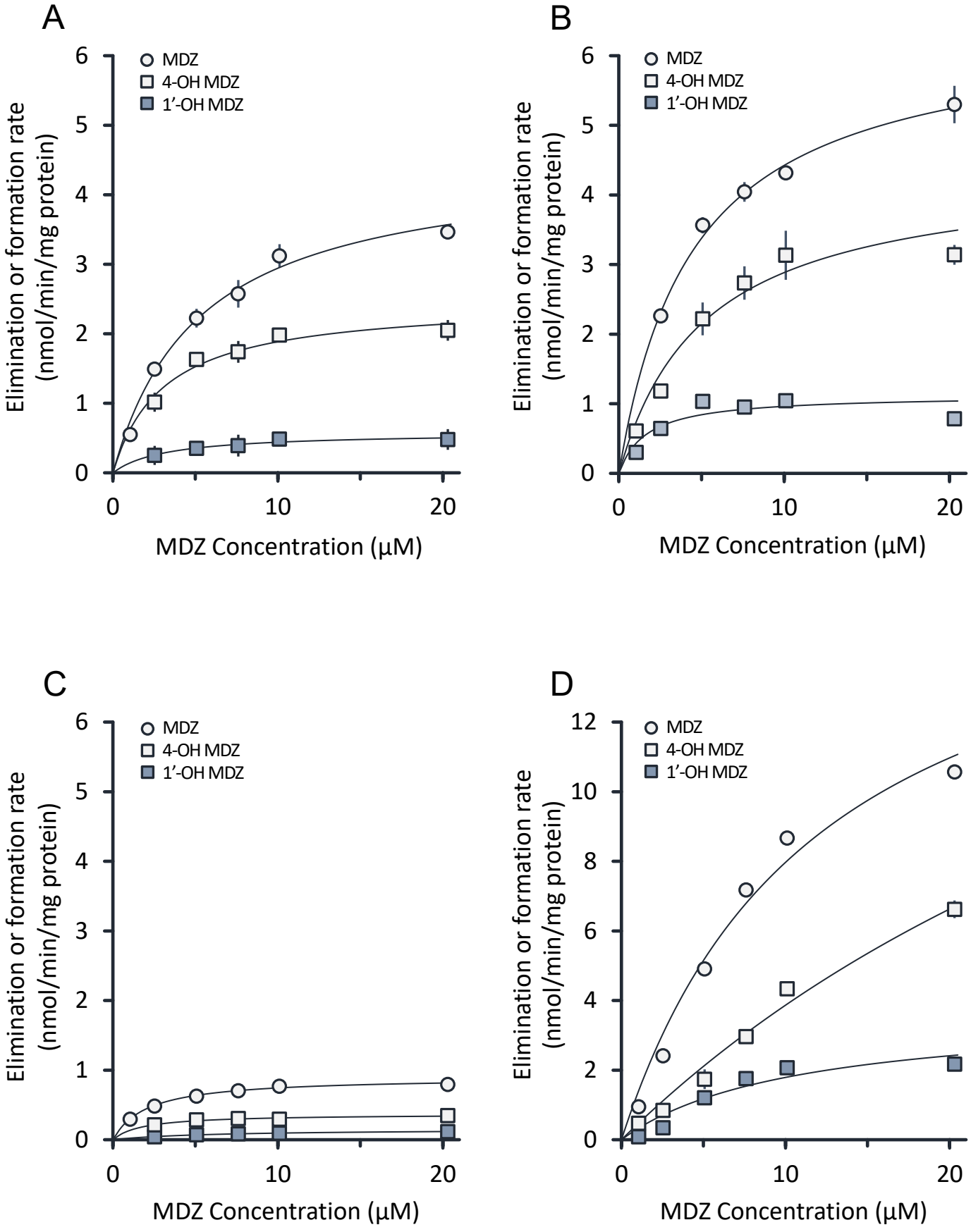
A
B
C
D
MDZ
4-OH MDZ
1'-OH MDZ
Elimination or formation rate
(nmol/min/mg protein)
MDZ Concentration (μM)
0
10
20

Figure 2

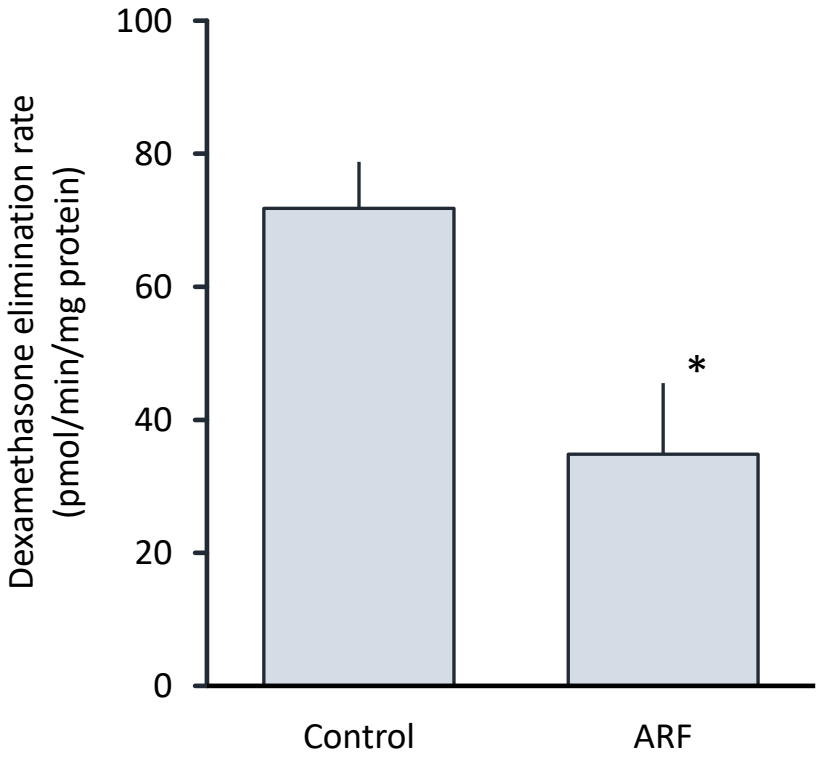
Dexamethasone elimination rate
(pmol/min/mg protein)
100
80
60
40
20
0
*
Control
ARF

Figure 3

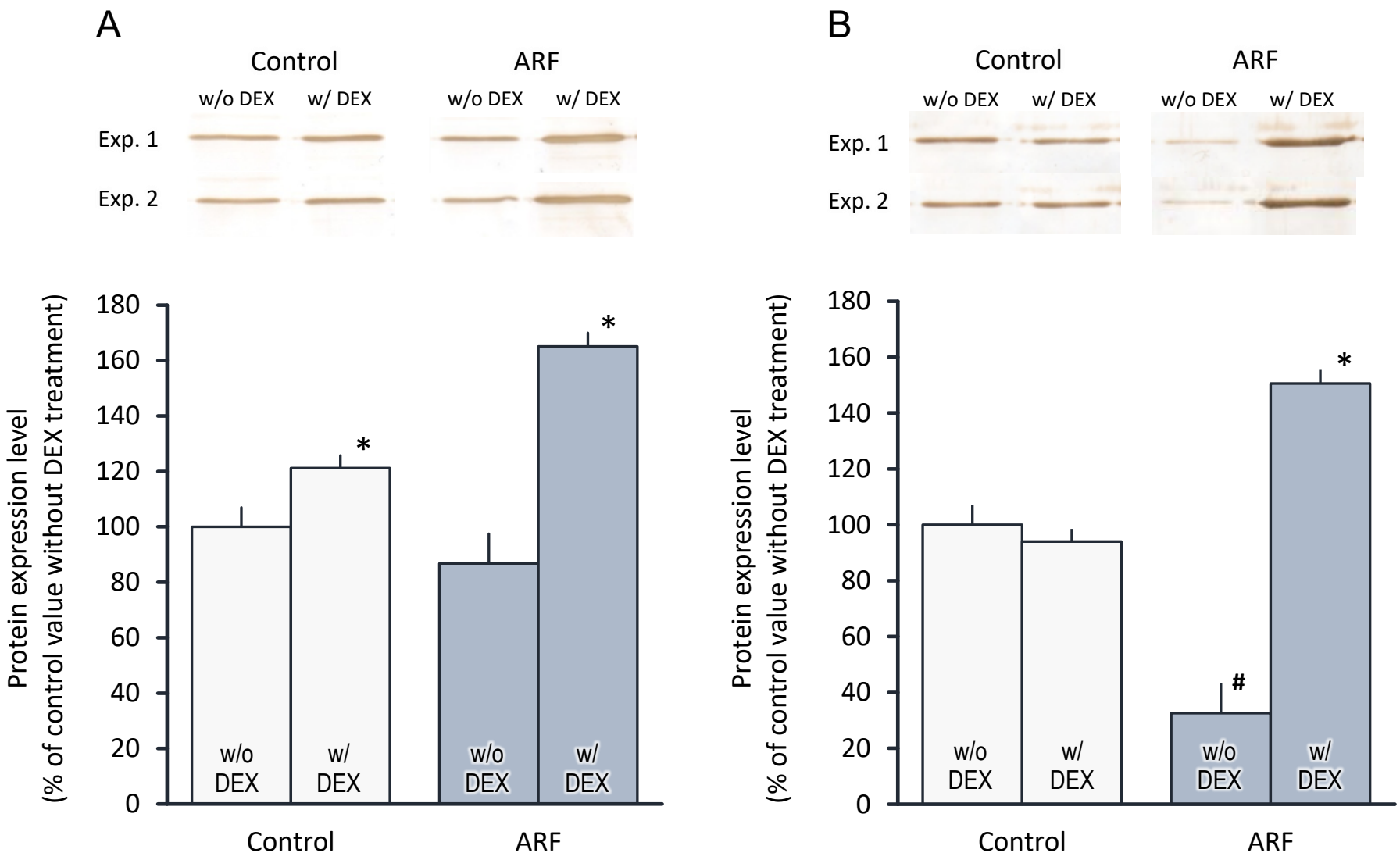
A
Control
ARF
w/o DEX
w/ DEX
w/o DEX
w/ DEX
Exp. 1
Exp. 2
Protein expression level
(% of control value without DEX treatment)
180
160
140
120
100
80
60
40
20
0
*
*
w/o DEX
w/ DEX
w/o DEX
w/ DEX
Control
ARF
B
Control
ARF
w/o DEX
w/ DEX
w/o DEX
w/ DEX
Exp. 1
Exp. 2
Protein expression level
(% of control value without DEX treatment)
180
160
140
120
100
80
60
40
20
0
*
#
w/o DEX
w/ DEX
w/o DEX
w/ DEX
Control
ARF

Figure 4

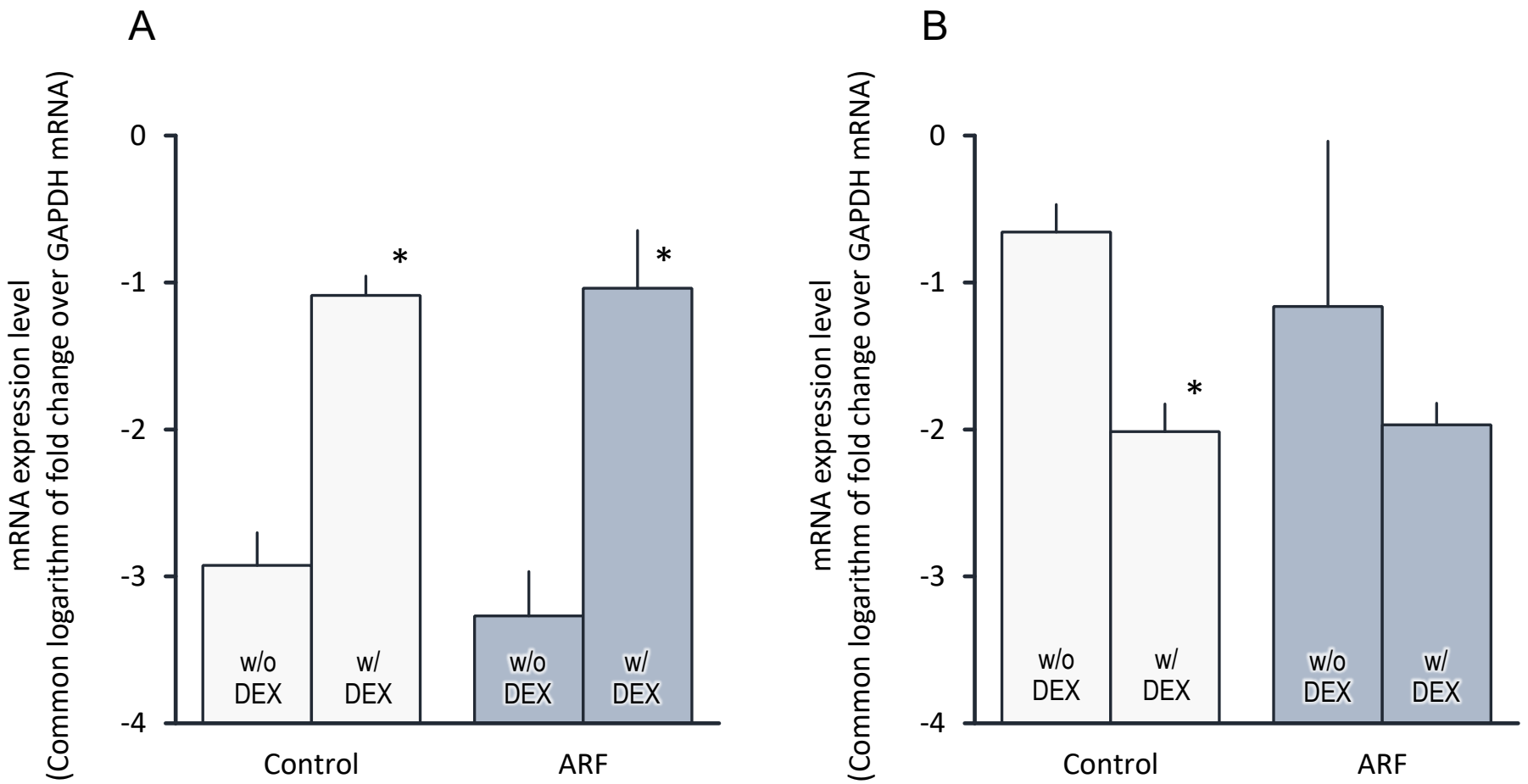
A
B
mRNA expression level
(Common logarithm of fold change over GAPDH mRNA)
0
-1
-2
-3
-4
*
*
*
w/o DEX
w/ DEX
w/o DEX
w/ DEX
Control
ARF

Figure 5