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DECREASE IN SERUM THYROXINE LEVEL BY PHENOBARBITAL IN RATS IS NOT NECESSARILY DEPENDENT ON INCREASE IN HEPATIC UDP-GLUCURONOSYLTRANSFERASE

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Running title: PB-mediated decrease in serum T_4 level

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ABBREVIATIONS: ID-I, iodothyronine deiodinase; PB, phenobarbital; PCB, polychlorinated biphenyl; T_3, triiodothyronine; T_4, thyroxine; TTR, tranthyretin; TSH, thyroid-stimulating hormone; UDP-GT, UDP-glucuronosyltransferase.

Abstract

We have previously reported that there is a poor correlation between increase in the levels of UGT1A1 and UGT1A6 and decrease in the levels of serum total thyroxine (T_4) and free T_4 in phenobarbital (PB)-treated rats, although the PB-induced decrease in rats is generally thought to occur through induction of the UDP-glucuronosyltransferase (T_4-UDP-GT: UGT1A1 and UGT1A6). In the present study, to clarify a relationship between the decrease in serum T_4 level and the increase in the T_4-UDP-GT activity by PB in rats, we examined the relationship using Gunn rats, a mutant strain of Wistar rats deficient in UGT1A isoforms. Levels of serum total T_4, free T_4 and total triiodothyronine (T_3) were markedly decreased in not only Wistar rats but also Gunn rats 1 day after the final administration of PB (80 mg/kg, i.p., once daily for 4 days), and no significant difference in magnitude of the decrease between Wistar and Gunn rats was observed. On the other hand, the level and activity of T_4-UDP-GT were significantly increased by treatment with PB in Wistar rats but not in Gunn rats. Furthermore, significant decrease in the activity of hepatic type-I iodothyronine deiodinase, which mediates the deiodination of T_4 and T_3, by PB treatment was observed in both Wistar and Gunn rats. In addition, no significant change in the level of serum thyroid-stimulating hormone, the activity of hepatic sulfotransferase and the binding of [^{125}I]T_4 to serum transthyretin and albmin by PB treatment was observed in either Wistar or Gunn rats. In conclusion, the present results demonstrate that the decrease in serum total T_4 level by PB in Gunn rats is not dependent on the increase in hepatic T_4-UDP-GT activity and suggest that even in Wistar rats, the PB-induced decrease in serum T_4 level dose not occur only through increase in hepatic T_4-UDP-GT.

Introduction

Phenobarbital (PB) is well known to decrease the level of serum thyroid hormone and to increase the activities of hepatic drug-metabolizing enzymes in rats and mice (Hood et al., 2003; O'Connor et al., 1999). Furthermore, PB increases levels of serum thyroid-stimulating hormone (TSH) and thyroid gland growth in rats (Hood et al., 1999). As a possible mechanism for PB-induced decrease in level of serum thyroid hormone, enhancement of thyroid hormone metabolism by PB is considered (Capen 1997; McClain 1989). Especially, the decrease in the level of serum thyroxine (T_4) by PB in rats is thought to occur mainly through the induction of T_4-UDP-glucuronosyltransferase (T_4-UDP-GT) responsible for glucuronidation of T_4 (Barter and Klaassen, 1992a; Liu et al., 1995). This hypothesis is supported by the previous reports that a number of T_4-UDP-GT inducers, such as polychlorinated biphenyl (PCB), 3-methylcholanthrene, and pregnenolone-16α-carbonitrile, show ability to decrease serum thyroid hormone (Barter and Klaassen, 1994; De Sandro et al., 1992; Saito et al., 1991). However, the magnitude of decrease in level of serum total T_4 by PB is not necessarily correlated with that of increase in T_4-UDP-GT activity (Saito et al., 1991; Hood et al., 2003). Likewise, our preliminary study (Suzuki et al., 2004) has indicated that there is a poor correlation between increase in the levels of UGT1A1 and UGT1A6 and decrease in the levels of serum total T_4 and free T_4 in PB-treated rats.

In the present study, therefore, we examined a relationship between the decrease in serum total T_4 level and the increase in hepatic T_4-UDP-GT (UGT1A1 and UGT1A6) by PB using UGT1A-deficient Wistar rats (Gunn rats) and demonstrated that

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PB-induced decrease in serum total T_4 level in rats was not necessarily dependent on the increase in hepatic T_4-UDP-GT activity.

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Materials and Methods

Chemicals. PB was purchased from Nakakita Yakuhin Co., Ltd. (Aichi, Japan). The [^{125}I]-reverse triiodothyronine (T_3) and [^{125}I]T_4, radiolabeled at 5' position of the outer ring, was obtained from PerkinElmer Life Sci., Inc. (Boston, MA). All other chemicals were obtained commercially in appropriate grades of purity.

Animal treatments. Male Wistar rats (160-200 g) and UGT1A-deficient Wistar rats (Gunn rats, 190-260 g) were obtained from Japan SLC., Inc. (Shizuoka, Japan). Male Wistar and Gunn rats were housed in three or four per cage with free access to commercial chow and tap water, maintained on a 12-h dark/light cycle (8:00 a.m.-8:00 p.m. light) in an air-controlled room (temperature: 24.5 ± 1℃, humidity: 55 ± 5%), and handled with human care under the guidelines of the University of Shizuoka (Shizuoka, Japan). Rats were received four consecutive intraperitoneal injection of PB (80 mg/kg) dissolved in 0.9% saline (5 ml/kg). Control animals were treated with a vehicle alone (5 mg/kg). All rats were killed by decapitation on 1 day after the final administration. The liver was removed, and hepatic microsomes and cytosols were prepared according to the method of Kato et al. (1995) and stored at –85℃ until used. Blood was collected from each animal between 10:30 and 11:30 a.m. After clotting at room temperature, serum was separated by centrifugation and stored at –50℃ until used.

Analysis of serum hormones. Levels of serum total T_4, free T_4, total T_3 and TSH

were measured by radioimmunoassay using the Total T4 and Free T4 kit (Diagnostic Products Corporation; Los Angels, CA), T-3· RIABEAD (ABOTT JAPAN Co., Ltd.), and rTSH [^{125}I] Biotrak assay system (Amersham Biosciences UK Limited; Little Chalfont, UK), respectively.

Hepatic T_4-metabolizing enzyme assays. Amounts of proteins of hepatic subcellular fractions, microsomes and cytosols, were determined by the method of Lowry et al. (1951) with bovine serum albmin as a standard. The activity of microsomal UDP-GT toward T_4 was determined by the methods of Barter and Klaassen (1992b). The activity of microsomal type-I iodothyronine deiodinase (ID-I) was determined by the method of Hood and Klaassen (2000). The activity of cytosolic sulfotransferase toward T_4 was determined by the methods of Kaptein et al. (1997).

Western blot analysis. Polyclonal anti-peptide antibodies against the common region of UGT1A isoforms and specific antibodies against UGT1A1, UGT1A6, and UGT2B1 were used (Ikushiro et al. 1995, 1997). Western blot analyses for microsomal UGT isoforms were performed by the method of Luquita et al. (2001). Isolated proteins corresponding to UGT1A1, UGT1A6, and UGT2B1 were detected using chemical luminescence (ECL detection kit, Amersham Pharmacia Biotech), and the level of each protein was determined densitometrically with LAS-1000 (FUJIFILM, Japan).

Analysis of [^{125}I]T_4 binding to serum proteins. At 1 day after consecutive four day-treatment with PB, rats were anesthetized with 50 mg/ml sodium pentobarbital

combined 1:1 with 1 mg/ml potassium iodide at 2 mg/ml. The femoral artery was cannulated (Polyethylene tube SP31, NATUME Inc., Tokyo, Japan) and primed with heparinized saline (33 units/ml). Fifteen minutes later, rats were given 1 ml of [^{125}I]T_4 iv, at 15 μCi/ml in 10 mM NaOH saline + 1 % normal rat serum. After the administration of [^{125}I]T_4, a portion (0.3 ml) of blood was sampled from the artery at the indicated time, and serum was collected and stored at -50℃ for assay. Serum was diluted in 100 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol and 30% glycerol, and subjected to electrophoresis on 4-20% gradient native polyacrylamide gels PAG Mid "DAIICHI" 4/20 (DAIICHI PURE CHEMICALS CO., LTD, Japan), using 0.025 M Tris (pH 8.4) containing 0.192 M glycine as running buffer, for 11 h at 20 mA at 4℃. Human albmin and tranthyretin (TTR), which were incubated with [^{125}I]T_4, were also applied on the gel as templates. After the electrophoresis, a gel was dried and radioautographed for 20 h at room temperature using Imaging Plate 2040 (Fuji Photo Film Co., Ltd, Japan). The levels of [^{125}I]T_4-albmin and [^{125}I]T_4-TTR complexes were determined by counting the gel fractions identified from Bio Imaging Analyzer (BAS-2000II IP Reader, Fuji Photo Film Co., Ltd, Japan).

Statistics. The data obtained were statistically analyzed according to Dunnett's test after the analysis of variance (ANOVA).

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Results

Serum hormone levels. Constitutive levels of serum total T_4, free T_4, total T_3, and TSH were more than 1.8-fold higher in Gunn rats than in Wistar rats. Effects of PB on levels of serum thyroid hormones were next examined in Wistar and Gunn rats (Fig. 1). In both Wistar and Gunn rats, PB treatment resulted in decreases of serum total T_4, free T_4 and total T_3, and magnitude of the decrease in each serum thyroid hormone was almost the same in the both rats. On the other hand, no significant change in the level of serum TSH by PB treatment was observed in either Wistar or Gunn rats.

Hepatic T_4-metabolizing enzyme activities. It has been reported that T_4 glucuronidation is primarily mediated by UGT1A enzymes, UGT1A1 and UGT1A6, in the rat liver (Visser, 1996). Therefore, we examined effects of PB on hepatic microsomal T_4-UDP-GT activity in Wistar and Gunn rats. Constitutive activity of T_4-UDP-GT was more than 2.1-fold higher in Wistar rats than in Gunn rats. PB treatment resulted in significant increase of T_4-UDP-GT activity in Wistar rats but not in Gunn rats (Fig. 2).

Hepatic microsomal ID-I activity in both Wistar and Gunn rats was significantly decreased by PB (Fig. 3). On the other hand, no significant change in activity of hepatic sulfotransferase by PB treatment was observed in either PB-treated Wistar or Gunn rats (data not shown).

Immunoblot analysis for UGT1As. Levels of immunoreactive proteins responsible for UGT1A isoforms, UGT1A1 and UGT1A6, were increased by PB in Wistar rats but not

in Gunn rats (Figs. 4 and 5). In addition, no constitutive expression of the UGT1A isoforms was confirmed in Gunn rats. On the other hand, level of UGT2B1 was significantly increased by PB in both Wistar and Gunn rats, and magnitude of the increase was higher in Gunn rats than in Wistar rats (Figs. 4 and 5).

Serum protein binding of [^{125}I]T_4. Effect of PB on the binding of [^{125}I]T_4 to serum proteins, TTR and albmin, was examined in Wistar rats. No significant change in the binding level of [^{125}I]T_4 to each serum protein by PB treatment was observed with the exception of decrease in the level of [^{125}I]T_4-TTR complex at 120 min after [^{125}I]T_4 administration (Fig. 6).

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Discussion

In the present study, we found that treatment with PB resulted in a drastic decrease in serum total T_4 and free T_4 levels in both Wistar and Gunn rats, although significant increase in the activity of T_4-UDP-GT occurred only in Wistar rats but not in Gunn rats. The present findings demonstrate that in Gunn rats, PB-induced decrease in level of serum T_4 occurs in a hepatic T_4-UDP-GT-independent fashion. In addition, constitutive levels of serum total T_4 and T_3 were more than 1.8-fold higher in Gunn rats than in Wistar rats, suggesting that the deficit of T_4 glucuronidation results in higher T_4 serum levels. Similar results and suggestions have been obtained by Benathan *et al.* (1983).

In general, T_4-UDP-GT inducers, including PB, clobazam, PCB, 3-methylcholanthrene and pregnenolone-16α-carbonitrile, have been considered to decrease a level of serum T_4 through increase in hepatic T_4-UDP-GT (Barter and Klaassen, 1994; Miyawaki et al., 2003; Van Birgelen et al., 1995). However, it has been reported that difference between rats and mice in magnitude of decrease in level of serum total T_4 by an inducer of T_4-UDP-GT is not well correlated with that in increase in activity of T_4-UDP-GT (Craft et al., 2002, Hood et al., 2003). Furthermore, we have reported previously that PCB-induced decrease in serum T_4 level might occur not only through increase in hepatic T_4-UDP-GT activity but also via formation of hydroxylated PCB metabolites in rats (Kato et al., 2004) and that in PB-treated rats, there was a poor correlation between increase in the levels of UGT1A1 and UGT1A6 and decrease in the levels of serum total T_4 and free T_4 (Suzuki et al., 2004). These previous reports strongly support a possibility that decrease in serum total T_4 level by PB does not occur

only through increase in hepatic T_4-UDP-GT activity.

As possible mechanisms for the PB-induced decrease in serum T_4 level, changes in hepatic ID-I activity and serum TSH level might be considered. However, a significant decrease in activity of hepatic ID-I, which mediates the deiodination of T_4 and T_3, by PB treatment was observed in both Wistar and Gunn rats. Similar results have been reported in PB-treated Sprague-Dawley rats (Hood and Klassen, 2000; O'Connor et al., 1999). Accordingly, PB-induced decrease in serum T_4 level is thought to occur through an ID-I-independent pathway. Furthermore, in the present study, levels of serum TSH in both Wistar and Gunn rats were not significantly changed by PB, indicating that TSH is not related to the PB-induced decrease in serum T_4 level, although a significant increase in serum TSH level has been reported to occur in PB-treated rats (Hood et al., 1999; O'Connor et al., 1999). The difference between the previous results and our present results might be attributed to the difference in the dose examined.

Although it has been reported that the binding of PCB and its hydroxylated metabolites to TTR, a major T_4 transporting protein, might be attributed, in part, to decrease in level of serum T_4 in PCB-treated rats (Brouwer et al., 1998; Lans et al., 1993; Kato et al., 2004), displacement of T_4 from serum TTR by PB did not occur in PB-treated rats, with the exception of the slight displacement in Wistar rats at 120 min after PB treatment. Accordingly, decrease in the level of serum T_4 in PB-treated rats occurs in a TTR-independent pathway. In addition, no change in activity of hepatic sulfotransferase, which efficiently catalyzes the sulfation of iodothyronines (Kester et al., 1999), by PB treatment was observed in either Wistar or Gunn rats.

In conclusion, the present findings demonstrate that the decrease in serum total T_4 level by PB in Gunn rats occurs without increases in hepatic T_4-metabolizing enzymes (T_4-UDP-GT, ID-I and sulfotransferase), the binding of PB to serum TTR, and level of serum TSH, although an exact mechanism for the PB-induced decrease remains unclear. In Wistar rats, PB-induced T_4-UDP-GT might also contribute, in part, to the decrease in serum T_4 level. To clarify exact mechanisms for PB-induced decrease in serum thyroid hormones, further studies involving T_4-transporters, non hepatic T_4-UDP-GT and exchangeable thyroid hormone pools in non hepatic tissues would be necessary.

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Footnotes

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Legends for figures

FIG. 1. Effect of PB on levels of serum total thyroxine (T_4), free T_4, total triiodothyronine (T_3), and thyroid-stimulating hormone (TSH). Animals were killed 1 day after the final administration of PB (80 mg/kg, ip, once daily for 4 days), and levels of serum thyroid hormones were measured as described in Materials and Methods. Constitutive levels: total T_4 (μg/dl), 3.32 ± 0.21 (Wistar, N=7) and 7.99 ± 0.30 (Gunn, N=4); free T_4 (ng/dl), 1.54 ± 0.07 (Wistar, N=7) and 2.77 ± 0.07 (Gunn, N=4); total T_3 (ng/ml), 0.34 ± 0.03 (Wistar, N=6) and 0.96 ± 0.05 (Gunn, N=4); TSH (ng/ml), 8.37 ± 1.25 (Wistar, N=6) and 20.85 ± 1.79 (Gunn, N=4). Each column represents the mean ± SE (vertical bars) for four to seven animals. *P<0.01, significantly different from each control.

FIG. 2. Effect of PB on the activity of hepatic microsomal UDP-glucuronyltransferase. Each column represents the mean ± SE (vertical bars) for four animals. Constitutive levels: T_4-UDP-GT (pmol/mg protein/min), 12.60 ± 0.69 (Wistar) and 5.95 ± 1.06 (Gunn). *P<0.01, significantly different from each control.

FIG. 3. Effect of PB on the activity of hepatic microsomal type I iodothyronine deiodinase (ID-I). Each column represents the mean ± SE (vertical bars) for four animals. Constitutive levels: ID-Ⅰ (pmol/mg protein/min), 161.8 ± 3.1 (Wistar) and 126.3 ± 9.5 (Gunn). *P<0.01, significantly different from each control.

FIG. 4. Representive Western blot patterns for hepatic microsomal UGT isoforms.

FIG. 5. Effect of PB on levels of hepatic microsomal UGT isoforms. After Western blot as shown in Figure 4, the isolated bands responsible for UGT isoforms were densitometrically quantified as described in Materials and Methods. The data are represented as the mean ± SE (vertical bars) for four animals. **P*<0.05, significantly different from each control. ND, Not detectable.

FIG. 6. Effect of PB on the binding of [^{125}I]T_4 to the serum proteins in Wistar rats. The binding of [^{125}I]T_4 to the serum proteins was assessed by nondenaturing polyacrylamide gel electrophoresis, as described in Materials and Methods. Each colume represents the mean ± SE (vertical bars) for three animals. **P*<0.05, significantly different from each control.

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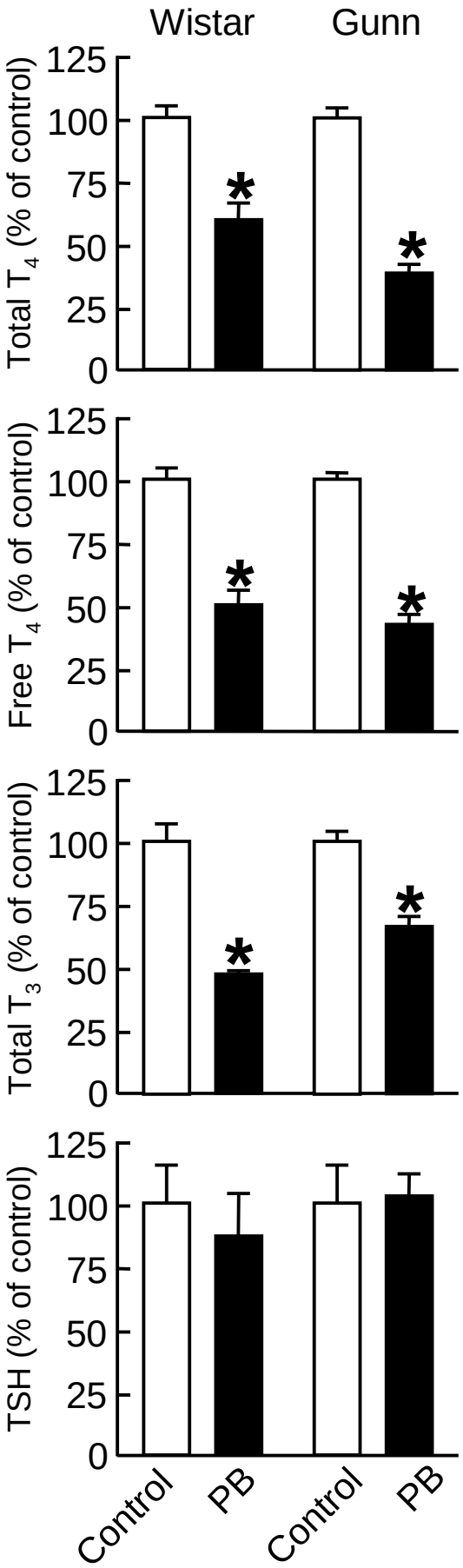

Fig. 1.

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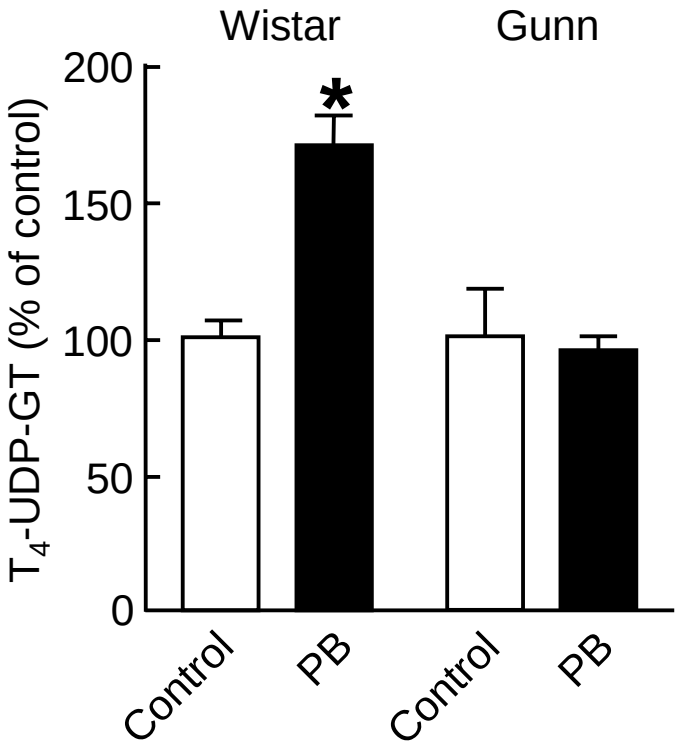

Fig. 2.

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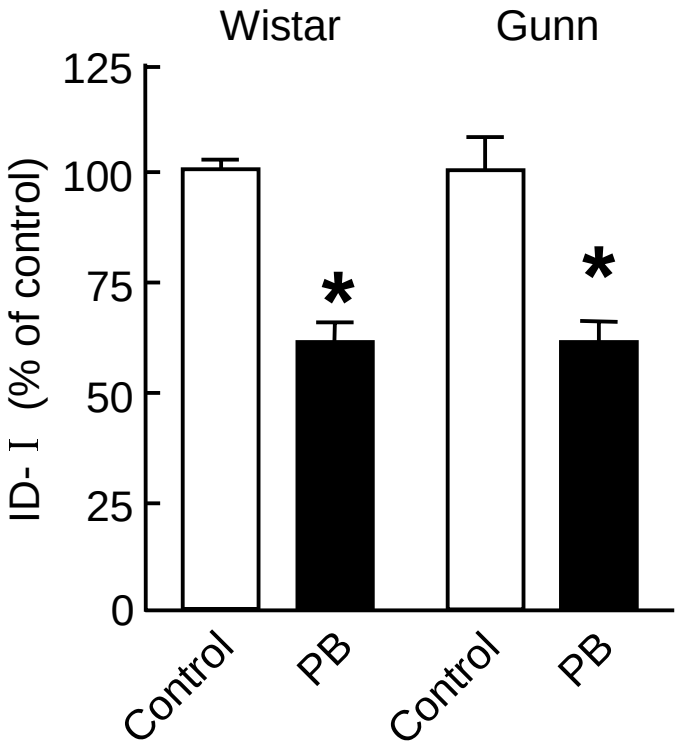

Fig. 3.

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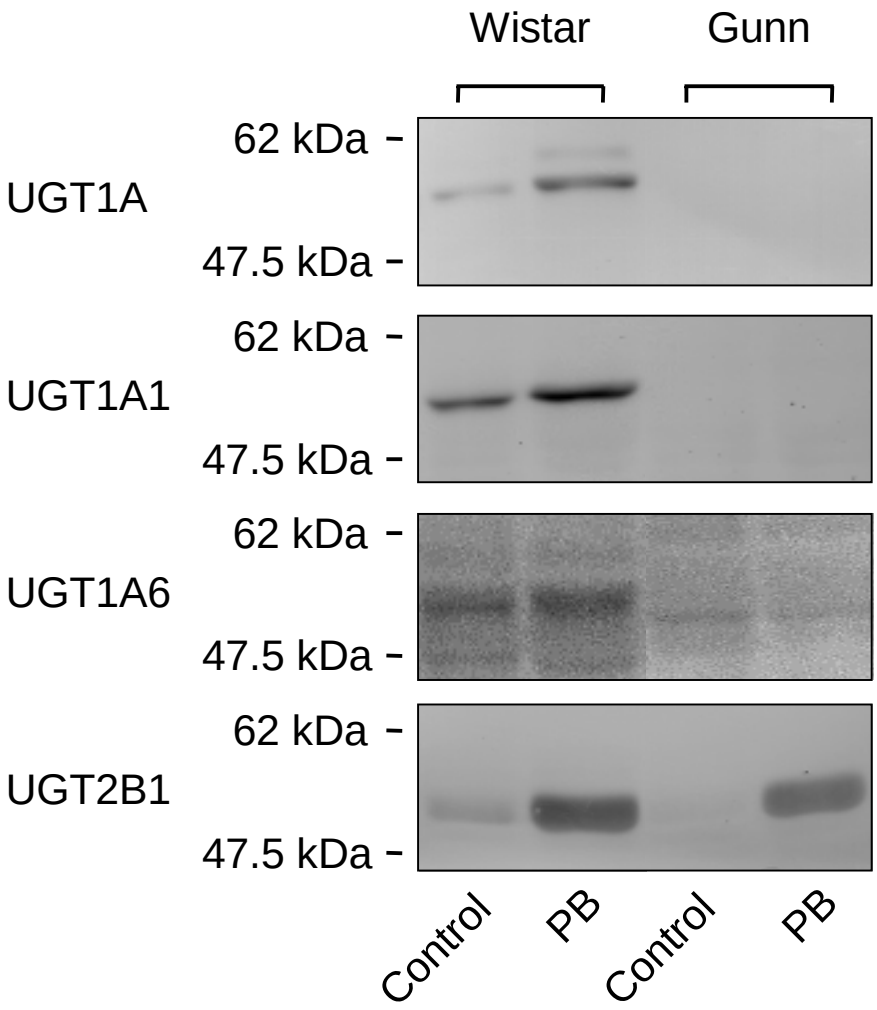

Fig. 4.

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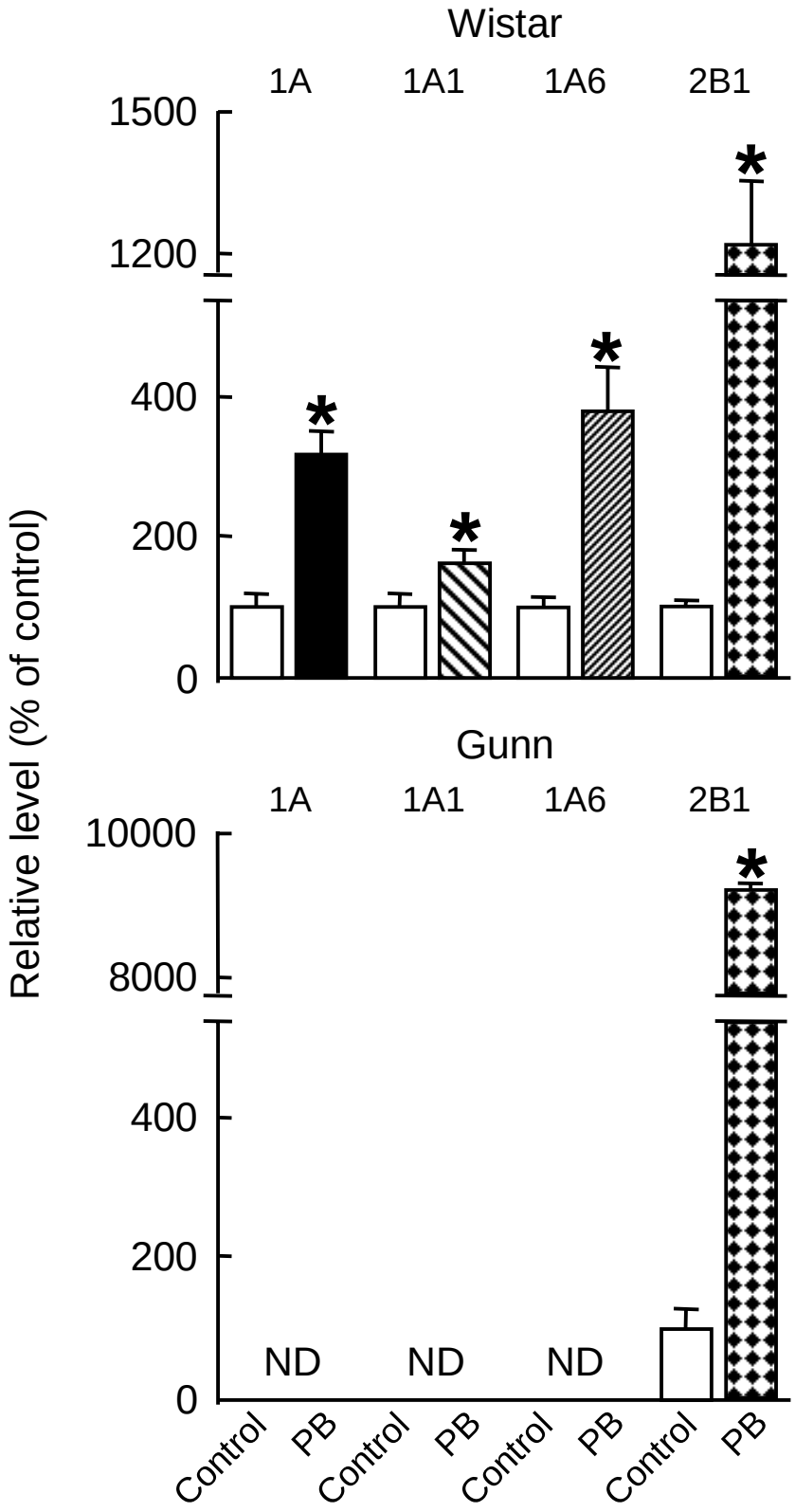

Fig. 5.

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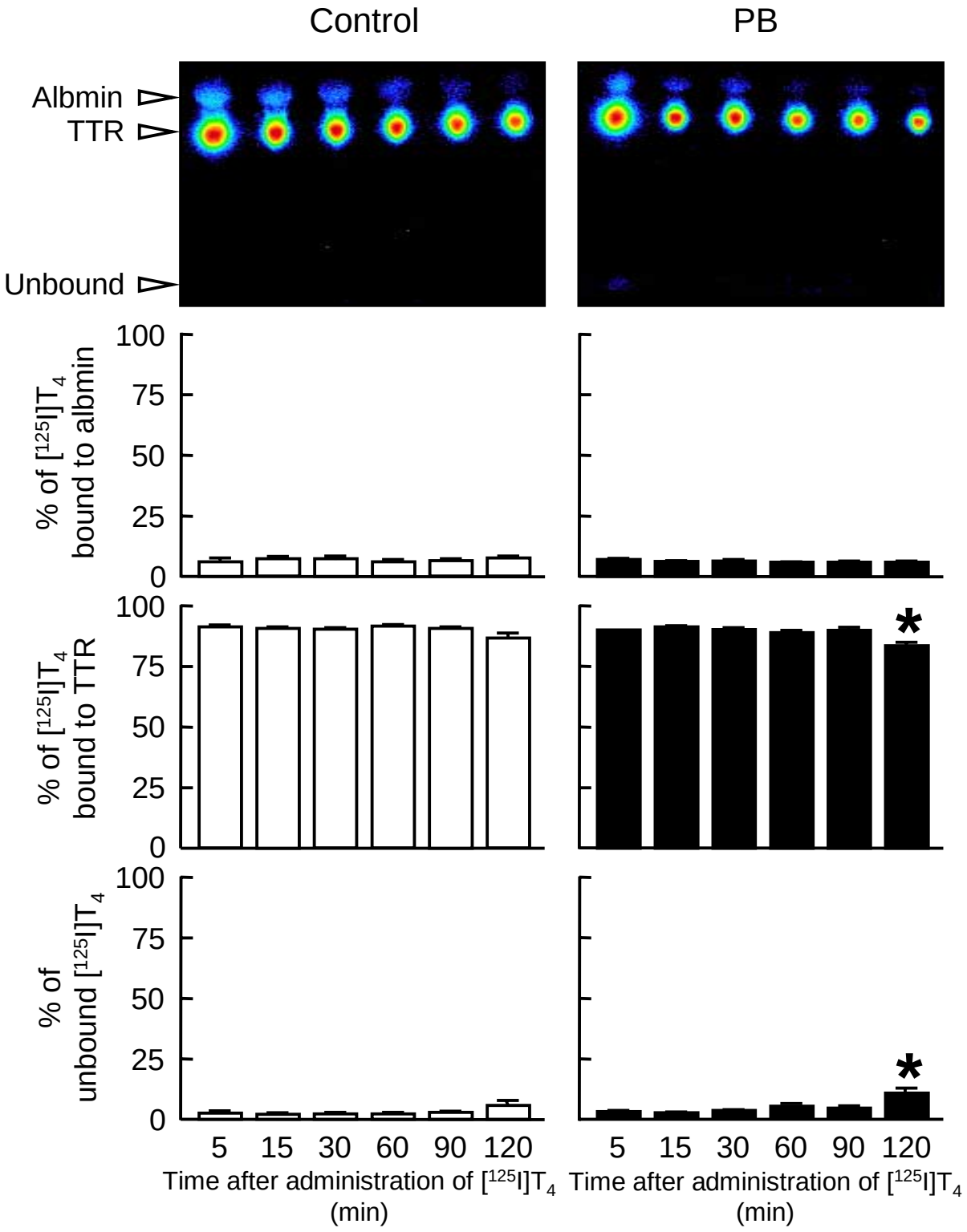

Fig. 6.