Different induction of gulonolactone oxidase in aromatic hydrocarbon-responsive or -unresponsive mouse strains

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Abstract The role of aromatic hydrocarbon receptor (AhR)-mediated signal transduction pathways was investigated in the regulation of ascorbate synthesis by using Ah-responsive and Ah-unresponsive mouse strains. In vivo 3-methylcholanthrene treatment increased hepatic and plasma ascorbate concentrations only in the Ah-responsive strain. The mRNA level of gulonolactone oxidase and the microsomal ascorbate production from *p*-nitrophenyl glucuronide, D-glucuronic acid or gulonolactone in the liver of Ah-responsive and Ah-unresponsive mice were compared. In Ah-responsive mice, these parameters were higher originally, and they further increased upon in vivo addition of 3-methylcholanthrene, while in Ah-unresponsive mice the treatment was not effective. These results suggest that the transcription of gulonolactone oxidase gene is regulated by an Ah receptor-dependent signal transduction pathway.

Key words: Ascorbate; Gulonolactone oxidase; Aromatic hydrocarbon receptor; Glucuronide; 3-Methylcholanthrene

1. Introduction

Ascorbate is a major water-soluble antioxidant of the cell [1]. The maintenance of the adequate level of tissue ascorbate pools is of vital importance as can be seen in scurvy. The last step of ascorbate synthesis is catalysed by L-gulono-γ-lactone oxidase, which produces ascorbate and hydrogen peroxide [1,2]. In ascorbate-synthesising species (i.e. in the majority of animals, except for primates and some other species) the regulation of the enzyme may be responsible for the tuning of ascorbate level. In fact, it has been reported that L-gulono-γ-lactone oxidase (GLO) activity can be altered by end-product inhibition [3]. Other studies demonstrated that ascorbate synthesis is stimulated by the administration of a variety of xenobiotics including 3-methylcholanthrene (3-MC) [4–6]. Carcinogenic polycyclic aromatic hydrocarbons, such as 3-MC, are archetypical inducers of the genes belonging to the aromatic hydrocarbon-responsive gene battery [7]. The upregulation of UDP-glucuronosyltransferase 1A6 (UGT1A6), a member of this gene battery by Ah inducers [8], is accompanied by increased urinary excretion of ascorbate [9]. Therefore, the importance of UGT1A6 induction in 3-MC-stimulated ascorbate synthesis has been suggested [10]. Horio et al. proposed that the first major precursor for microsomal ascorbate synthesis, D-glucuronic acid is produced from UDP-glucuronic acid involving X(phenol)-β-D-glucuronide formation catalysed by UGT1A6 and the subsequent hydrolysis of the glucuronide by β-glucuronidase [10,11].

Besides the above mechanism the direct transcriptional upregulation of microsomal ascorbate synthesising enzymes by 3-MC is also possible. As an AhR agonist stimulates ascorbate production it is a question whether the increased substrate supply itself (by UGT1A6 induction) causes this phenomenon or certain enzymes of ascorbate formation from D-glucuronic acid are induced by an AhR-mediated signal transduction pathway. In vivo experimental systems were chosen for this study: two different mouse strains with different abilities to respond to Ah stimulation. By genetic analysis, the Ah locus appears to be complex consisting of at least two genes with six different alleles, but in crosses between some mouse strains (e.g. C57BL/6 Ah-responsive mice (B6) and DBA/2 Ah-unresponsive mice (D2)) the locus behaves as a single Mendelian trait [12]. B6 mice are high responders and possess high-affinity aryl hydrocarbon receptors, whereas D2 mice are low responders and have small amounts of detectable receptor [12] or express identical amount of AhR but their ligand-binding affinity is lower [13]. The microsomal ascorbate production from D-glucuronic acid and the activity and expression of GLO after in vivo 3-MC treatment were compared in the livers of B6 and D2 mouse strains. Based on our findings an AhR mediated GLO induction is proposed.

2. Materials and methods

2.1. Treatment of mice

Animals received human care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health, USA. Sexually immature (4–6-week-old) male mice colonies from C57BL/6 (B6 as Ah-responsive) and DBA/2 (D2 as Ah-non-responsive) strains were provided by Charles River, Budapest, Hungary and were housed in an animal room of our Department with controlled temperature, humidity, pressure, dark-light cycle and free access to standard food. Single injection of the inducing compound 3-MC dissolved in corn oil (200–250 mg/body weight (kg)) was administered intraperitoneally. Control group of animals received corn oil [12,13]. The dose of corn oil never exceeded 25 ml/kg body weight.

2.2. Preparation and treatment of mouse liver microsomes

Liver microsomal vesicles were prepared from B6 and D2 male mice

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Abbreviations: Ah, aromatic hydrocarbon; AhR, aromatic hydrocarbon receptor; Arnt, AhR nuclear translocator; B6, C57BL/6 Ah-responsive mice; CYP1A1, cytochrome P-450 1A1; D2, DBA/2 Ah-unresponsive mice; EROD, 7-ethoxyresorufin *O*-deethylase; GLO, L-gulono-γ-lactone oxidase; 3-MC, 3-methylcholanthrene; MOPS, 4-morpholinepropanesulfonic acid; UGT1A6, UDP-glucuronosyltransferase 1A6

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as previously described [16]. Microsomes were resuspended in 20 mM 4-morpholinepropanesulfonic acid (MOPS) (pH 7.2) containing 100 mM KCl, 20 mM NaCl, 3 mM $MgCl_2$. The suspensions were frozen and maintained under liquid nitrogen until used. In some series of experiments vesicles were permeabilised by alamethicin using its optimally activating concentration (0.05 mg/mg protein).

2.3. RNA extraction and Northern blotting with GLO probe

Total RNA was extracted from the liver of each mouse by the acid guanidinium thiocyanate method [17]. The isolation of a rat GLO cDNA was described previously [18]. An approximately 1 kb long fragment of the rat GLO cDNA, designated 15L in pUC19 plasmid [19] was kindly provided by Dr Morimitsu Nishikimi (Wakayama Medical College, Department of Biochemistry, Wakayama, Japan). 15L and glyceraldehyde-3-phosphate dehydrogenase cDNA fragments were denatured and labelled with [α-^{32}P]dCTP using the High Prime DNA labelling kit from Boehringer Mannheim. Then these were used to probe the Northern blots. Northern blotting of RNA samples was performed using essentially the method described by Sambrook et al. [20].

2.4. Measurement of metabolites

Microsomes were incubated in 20 mM MOPS (pH 7.2) containing 100 mM KCl, 20 mM NaCl, 3 mM $MgCl_2$. For the determination of *p*-nitrophenol UGT1A6 activities, permeabilised mouse liver microsomes (0.1 mg protein/ml) were incubated in the presence of 5 mM UDP-glucuronic acid and 500 μM *p*-nitrophenol for 30 min at 37°C. Incubations were terminated by the addition of 0.05 volume of 100% trichloroacetic acid. *p*-Nitrophenol UGT1A6 activities were measured spectrophotometrically on the basis of aglycone disappearance from TCA soluble supernatants as previously described [21]. Cytochrome P450 1A1 activity was measured in intact mouse microsomal vesicles by 7-ethoxyresorufin *O*-deethylase assay according to the method of Prough et al. [22]. Plasma and hepatic ascorbate content were measured by reverse phase HPLC following the specific sample preparation as described earlier [23]. For the determination of gulonolactone oxidase activity intact microsomal vesicles (usually 1 mg protein/ml) were incubated in the presence of 5 mM gulonolactone for 30 min at 37°C. To determine ascorbate formation from D-glucuronic acid or *p*-nitrophenyl glucuronide microsomes were permeabilised by alamethicin and were incubated in the presence of 5 mM ascorbate precursor with 5 mM NADPH for 30 min at 37°C. Incubations were terminated by the addition of 100% trichloroacetic acid (5% final concentration). Ascorbate content was measured from trichloroacetic acid soluble supernatants by the method of Omaye et al. [24], based on the reduction of Fe^{3+} with the oxidation of ascorbate and the subsequent determination of the Fe^{2+}-α,α′-dipyridyl complex. β-Glucuronidase activities were detected in the presence of 5 mM *p*-nitrophenyl glucuronide; the formation of *p*-nitrophenol aglycone was measured in the TCA soluble supernatants.

2.5. Miscellaneous

Protein concentration of microsomes was determined by using Bio-Rad protein assay solution with bovine serum albumin as standard according to manufacturers' instructions. For the quantitation of the amount of GLO mRNA, bands were scanned densitometrically. All data were expressed as means ± S.E.M. Statistical analysis was carried out by using Student's *t*-test.

2.6. Materials

Alamethicin, α,α′-dipyridyl, D-glucuronic acid (sodium salt), L-gulono-γ-lactone, 3-MC, MOPS, β-nicotinamide adenine dinucleotide phosphate in reduced form, *p*-nitrophenyl-β-D-glucuronide, D-saccharic acid 1,4-lactone, and UDP-glucuronic acid (sodium salt) were purchased from Sigma, St. Louis, MO, USA. μBondapak C_{18} HPLC column (average particle size 10 μM, 300×4 mm I.D.) was obtained from MZ-Analysentechnik, Mainz, Germany. Protein assay solution and 3MM filter papers were purchased from Bio-Rad Laboratories, Budapest, Hungary. The Hybond-N nylon membrane and the Hyperfilm were provided by Amersham International, Amersham, UK. The α-^{32}P-labelled dCTP was obtained from the Institute of Isotopes, Budapest, Hungary. The High Prime DNA labelling kit was purchased from Boehringer Mannheim, Mannheim, Germany. All other chemicals and reagents were from Sigma and were of analytical grade purity or better.

3. Results

3.1. Comparison of GLO induction by 3-MC in the livers of C57BL/6 and DBA/2 mice

3-MC dissolved in corn oil or the pure solvent was administered to Ah-responsive B6 and Ah-unresponsive D2 mice. The dose of 3-MC applied in the study was reported to be optimally effective in the induction of phase II enzymes of biotransformation [15]. Microsomes and total RNA were prepared from their livers. RNA samples underwent Northern blot analysis using GLO probe. The amount of GLO mRNA was significantly higher in the liver of 3-MC-treated B6 mice compared to either corn-oil-treated B6- and D2-, or 3-MC-treated D2 mice indicating the intensive transcription and/or stabilisation of GLO mRNA upon addition of 3-MC in high responder B6 animals (Fig. 1). According to these data, microsomal GLO activity was hardly stimulated by 3-MC in Ah-unresponsive animals while in high responders more than double elevation occurred (Table 1). The plasma and hepatic ascorbate content exhibited approximately threefold elevation in B6 strain contrary to the moderate increase in ascorbate concentration in the plasma and liver of D2 mice upon addition of 3-MC (Table 1). In control experiments the induction of enzymes known to be the members of the Ah-battery was investigated. 7-ethoxyresorufin *O*-deethylase (EROD) activity attributed to cytochrome P-450 1A1 (CYP1A1) showed more than 200-fold increase in B6 mice

Table 1
The effect of 3-MC treatment on the microsomal GLO, CYP1A1 and UGT1A6 activities in the liver of Ah-responsive or Ah-unresponsive mice

Strain	Treatment	Plasma ascorbate concentration (μM)	Hepatic ascorbate content (μmol/g liver)	Gulonolactone oxidase activity (nmol/min/mg microsomal protein)	EROD activity (nmol/min/mg microsomal protein)	*p*-Nitrophenyl UGT activity (nmol/min/mg microsomal protein)
DBA/2 (D2)	Corn oil	20.5 ± 1.5	0.85 ± 0.02	1.96 ± 0.05	0.04 ± 0.004	20.2 ± 0.7
	3-MC	35.5 ± 1.3[b]	1.37 ± 0.05[b]	2.85 ± 0.04[b]	0.09 ± 0.004[b]	25.3 ± 1.0[b]
C57BL/6 (B6)	Corn oil	46.7 ± 0.8[a]	1.74 ± 0.02[a]	4.50 ± 0.07[a]	0.11 ± 0.008[a]	36.7 ± 1.3[a]
	3-MC	154.2 ± 5.3[a,b]	5.06 ± 0.10[a,b]	9.97 ± 0.55[a,b]	24.73 ± 1.36[a,b]	64.5 ± 3.1[a,b]

DBA/2 and C57BL/6 male mice were treated with corn oil and 3-MC as detailed in Section 2. After the killing of the animals microsomal vesicles were prepared from their livers according to Section 2. At the same time plasma ascorbate concentrations and hepatic ascorbate contents were measured in each mouse. Gulonolactone oxidase, EROD (CYP1A1) and *p*-nitrophenyl UGT (UGT1A6) activities were determined in each microsomal preparation as described in Section 2. For the measurement of UGT1A6 activities microsomal vesicles were permeabilised by alamethicin (0.05 mg/mg protein). Data are expressed as means ± S.E.M. from four individual animals of various experimental groups.
[a]Significant differences between the values of D2 and B6 mice: $P < 0.01$.
[b]Significant differences from the corresponding controls: $P < 0.01$.

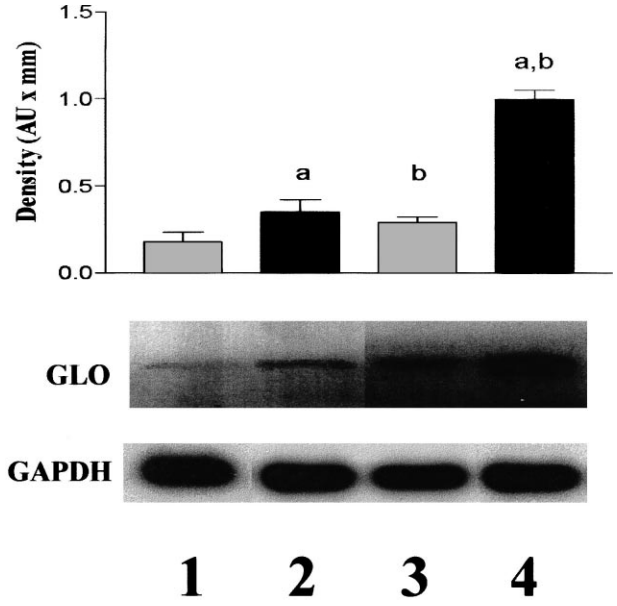

Fig. 1. The effect of 3-MC treatment on GLO mRNA level in the liver of mice belonging to Ah-responsive or unresponsive strains. Total RNA (30 μg/sample) isolated from control and 3-MC-treated mice of both strain (B6, D2) was subjected to electrophoresis in agarose gel containing formaldehyde followed by transfer to Hybond-N nylon membrane as detailed earlier. The membrane was hybridised with [α-^{32}P]dCTP labelled GLO and GAPDH cDNA probes, then autoradiography was used to visualise mRNAs. Northern blot analysis with GLO and GAPDH probe was carried out with each RNA preparation from each animal involved in this experiment and one of the typical results was presented. The results of densitometric scanning of the GLO mRNA bands were also presented. Bars express average densities ± S.E.M. of mRNA bands from four individual animals of various experimental groups. Lane or bar 1, control D2; lane or bar 2, control B6; lane or bar 3, D2 treated with 3-MC; lane or bar 4, B6 treated with 3-MC. The density of B6 mRNA bands significantly differed from the density of D2 mRNA bands ($^{a}P < 0.01$). The significant difference from the corresponding controls within each strain is also indicated ($^{b}P < 0.01$).

while in D2 animals only 2.5-fold stimulation could be observed indicating the expected response to 3-MC (Table 1). Similarly to CYP1A1 the stimulation of *p*-nitrophenyl-UGT activity as a function of UGT1A6 in Ah-responsive mice was in accordance with previous observations (Table 1) [15].

To envisage the role of direct and indirect mechanisms in the induction of GLO, the time-dependence of the induction of EROD, *p*-nitrophenyl-UGT and GLO activities was studied in Ah-responsive mice. The Ah-receptor activation occurred early (after 6 h of 3-MC treatment) as it was reflected in elevated EROD activities, while the increase in *p*-nitrophenyl-UGT and GLO activities appeared later, 12 or 24 h after the treatment with 3-MC, respectively (Fig. 2).

Interestingly, in liver microsomes of corn oil-treated high responder mice the basal GLO activity was more than two times higher compared to the microsomes prepared from the livers of low responder controls. Consequently, higher hepatic ascorbate content and higher plasma ascorbate concentrations were found in Ah-responsive corn oil-treated animals compared to the D2 mice (Table 1). To determine whether the enhanced initial GLO activity in B6 mice occurs at mRNA level, total RNA was isolated from livers of both B6 and D2 controls and was subjected to Northern hybridisation with GLO cDNA probe as described. The autoradiograph showed a higher level of GLO mRNA in high responder controls against corn oil-treated D2 mice (Fig. 1). A similar pattern of difference could be observed between the controls of the two strains in the case of both EROD and *p*-nitrophenyl-UGT activities (Table 1).

3.2. *Ascorbate synthesis from p-nitrophenyl glucuronide or D-glucuronic acid in liver microsomes from C57BL/6 and DBA/2 mice treated with 3-MC*

The effect of 3-MC treatment on other microsomal enzymes participating in the ascorbate synthetic pathway was also investigated. To bypass the AhR-regulated UGT1A6 activity, *p*-nitrophenyl glucuronide or D-glucuronic acid were added to microsomes as precursors for ascorbate synthesis. The vesicles were permeabilised with the pore-forming agent alamethicin [25] to promote the entry of the substrates into the intraluminal space. Ascorbate formation from D-glucuronic acid or from *p*-nitrophenyl glucuronide was higher in microsomes of Ah-responsive mice compared to the other strain and it further increased after 3-MC treatment (Table 2). According to previous suggestions, β-glucuronidase can participate in ascorbate synthesis since the inhibition of β-glucuronidase by sac-

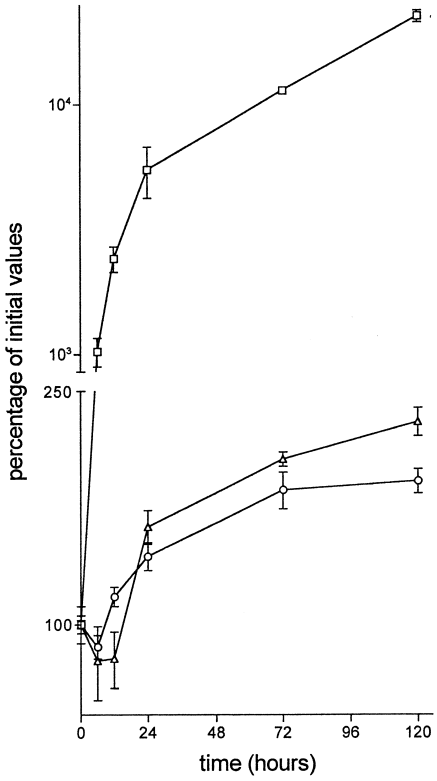

Fig. 2. The time course of the induction of CYP1A1, UGT1A6 and GLO activities after 3-MC treatment in C57/BL6 mice. C57BL/6 male mice were treated with 3-MC as detailed in Section 2. Animals were killed after various periods of the treatment and microsomal vesicles were prepared from their livers according to the experimental section. Gulonolactone oxidase (△), EROD (CYP1A1) (□) and *p*-nitrophenyl UGT (UGT1A6) (○) activities were determined in each microsomal preparation as described in Section 2. For the measurement of UGT1A6 activities microsomal vesicles were permeabilised by alamethicin (0.05 mg/mg protein). Data are expressed as the percentage of initial activities. (For absolute values of initial activities see the corn oil treated C57/BL6 (control) data of Table 1). Means ± S.E.M. of results from four animals

Table 2
Microsomal ascorbate formation from glucuronic acid or p-nitrophenyl glucuronide in the presence or absence of saccharolactone in the liver of 3-MC treated Ah-responsive and Ah-unresponsive mice

Strain	Treatment	Ascorbate formation from 5 mM D-glucuronic acid (nmol/min/mg protein)	Ascorbate formation from 5 mM *p*-nitrophenyl glucuronide (nmol/min/mg protein)		β-Glucuronidase activity (5 mM *p*-nitrophenyl glucuronide) (nmol/min/mg protein)	
			−Saccharolactone	+Saccharolactone	−Saccharolactone	+Saccharolactone
DBA/2 (D2)	corn oil	0.08 ± 0.01	0.08 ± 0.01	0.040 ± 0.003[c]	1.66 ± 0.06	0.69 ± 0.03[c]
	3-MC	0.10 ± 0.01[b]	0.08 ± 0.01	0.047 ± 0.003[c]	1.71 ± 0.06	0.75 ± 0.01[c]
C57BL/6 (B6)	corn oil	0.14 ± 0.01[a]	0.12 ± 0.01[a]	0.043 ± 0.004[c]	1.71 ± 0.04	0.70 ± 0.02[c]
	3-MC	0.26 ± 0.01[a,b]	0.27 ± 0.01[a,b]	0.047 ± 0.008[c]	1.66 ± 0.03	0.73 ± 0.01[c]

Liver microsomes were prepared from the corn oil-, and 3-MC treated D2 or B6 male mice according to Section 2. 5 mM NADPH was added to alamethicin permeabilised vesicles (0.05 mg/mg protein) and ascorbate formation from 5 mM D-glucuronic acid or 5 mM *p*-nitrophenyl glucuronide were measured. Ascorbate formation from *p*-nitrophenyl glucuronide was determined both in the presence and absence of 10 mM saccharolactone simultaneously with β-glucuronidase activities as described earlier. Data are expressed as means ± S.E.M. from four individual animals of various experimental groups. Significant differences between the values of D2 and B6 mice: [a]$P < 0.01$; significant differences from the corresponding controls: [b]$P < 0.01$.
[a]Significant differences between the values of D2 and B6 mice: $P < 0.01$.
[b]Significant differences from the corresponding controls: $P < 0.01$.
[c]Statistically significant differences of values obtained from saccharolactone treated microsomes versus normal incubations: $P < 0.01$.

charolactone [26] decreased ascorbate production from *p*-nitrophenyl glucuronide (Table 2). β-Glucuronidase activities were similar in microsomes from both strains and remained unchanged after 3-MC treatment, indicating that β-glucuronidase is not responsible for the increased ascorbate synthesis.

4. Discussion

In the present study enhanced GLO activity was observed after 3-MC administration in C57BL/6 mice known to be high responders to AhR-dependent stimuli (Table 1), while in DBA/2 mouse strain possessing low amounts of detectable AhR and/or low-affinity ligand-binding sites [12] this enzyme activity could not be efficiently stimulated (Table 1). According to the Northern analysis the elevation in GLO activity in 3-MC-treated B6 mice can be explained by the increase in GLO mRNA (Fig. 1).

In contrast to the present study it has been reported previously that GLO mRNA level remains unaffected by 3-MC in rats [11]. The non-responsiveness of rat *GLO* gene in 3-MC treatment can be explained by the mutations of AhR-AhR nuclear translocator (Arnt) dimer-specific core sequences within the E-boxes, which occurred in the promoter of rat *GLO*, or by the TATA box-free promoter of this gene [11,27]. The necessity of TATA box in enhancer/promoter communication during the AhR-mediated induction of CYP1A1 is well described [7].

The role of AhR in the regulation of *GLO* expression was emphasised by the comparison of basal values detected in corn oil-treated controls of both strains. The original hepatic and plasma ascorbate levels, as well as the original rate of GLO activities and mRNA levels were significantly lower in Ah-insensitive control mice than in high responders (Table 1 and Fig. 1) similarly to the behaviour of well-known Ah-battery elements: CYP1A1 and UGT1A6 (Table 1). It may be supposed that in Ah-responsive animals the ligands of the AhR can induce the members of the Ah-battery, including GLO.

The enzymes of the Ah-battery may stay under dual transcriptional control: (1) the direct AhR-mediated pathway and/or (2) oxidative stress-initiated route due to the phase I metabolism of AhR ligands and stimulation of electrophile response element of phase II genes [8]. In several cases the two groups of regulatory elements may overlap. On the basis of the time-dependence of UGT1A6 and GLO induction the involvement of secondary mechanisms is also supposed; their induction is lagging compaired to the induction of the EROD activity. However, the time course of the induction of both UGT1A6 and GLO shows a similar pattern; and the AhR-dependent induction of UGT1A6 has been verified [8].

It has been assumed that the Ah-stimulated ascorbate production was caused by AhR-inducible UGT1A6 and β-glucuronidase [10]. The microsomal ascorbate formation from both *p*-nitrophenyl glucuronide and D-glucuronic acid was higher in control Ah-responder mice and it could be stimulated further by 3-MC treatment only in these animals indicating the existence of an inducible step located after β-glucuronidase (Table 2). The close correlation between UGT1A6 and GLO activities calculated from the data of Table 1 refers to the same inductive states of these two enzymes which can be explained by their common regulatory mechanism: the AhR-dependent way. The prevention of ascorbate synthesis from *p*-nitrophenyl glucuronide by the inhibition of β-glucuronidase (Table 2) supports the importance of AhR-regulated UGT1A6 in the substrate supply of Ah-stimulated ascorbate synthesis as proposed by Horio et al. [10,11]. It has been also reported that phenobarbital treatment of rats enhanced hepatic ascorbate level without any effect on GLO activity. It could be explained by the induction of several UGTs increasing the level of glucuronides and consequently the glucuronic acid supply for ascorbate synthesis [11].

In summary, we concluded that gulonolactone oxidase may be a target for the AhR-mediated signal transduction pathway. Further work is needed to evaluate the contribution of direct or indirect mechanisms of the AhR-dependent transcriptional activation to enhanced ascorbate synthesis.

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