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Regulation of Human β-Glucuronidase by A23187 and Thapsigargin in the Hepatoma Cell Line HepG2

BERNHARD SPERKER, CÉLINE TOMKIEWICZ, OLIVER BURK, ROBERT BAROUKI, and HEYO K. KROEMER

Institut für Pharmakologie, Ernst Moritz Arndt Universität Greifswald, Greifswald, Germany (B.S., H.K.K.); Institut National de la Santé et de la Recherche Médicale U490, Université René Descartes, Paris, France (C.T., R.B.); and Dr. Margarete Fischer-Bosch Institut für Klinische Pharmakolgie, Stuttgart, Germany (O.B.)

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ABSTRACT

A novel approach to reducing organ toxicity of anticancer agents is the application of nontoxic glucuronide prodrugs from which the active drug is released by human β-glucuronidase, an enzyme present at high levels in many tumors. In view of high interindividual variability in β-glucuronidase expression, regulation of this enzyme is an essential factor modulating bioactivation of glucuronide prodrugs. However, data on regulation of human β-glucuronidase expression are not available. Preliminary evidence from animal experiments points to a role of intracellular calcium in regulation of β-glucuronidase activity. Therefore, we investigated regulation of β-glucuronidase by the calcium ionophore A23187 and the calcium ATPase inhibitor thapsigargin in the human hepatoma cell line HepG2. The enzyme was characterized on activity, protein, and mRNA levels by cleavage of 4-methylumbelliferyl-β-D-glucuronide, Western blotting, Northern blotting, and nuclear run-on transcription. Incubation of HepG2 cells with A23187 and thapsigargin, respectively, revealed a time and concentration dependent down-regulation of β-glucuronidase activity to about 50% of the control level. This effect could also be demonstrated in several other cell lines (e.g., HL-60, ECV 304, 32M1, Caco-2/TC7). Effects on protein and mRNA levels paralleled those obtained on enzymatic activity. In line with these data, A23187 and thapsigargin decreased β-glucuronidase transcriptional rate. Our data demonstrate regulation of human β-glucuronidase by xenobiotics. Down-regulation of β-glucuronidase by A23187 and thapsigargin is at least partly mediated by a transcriptional mechanism. Based on our findings, we speculate that β-glucuronidase activity and hence bioactivation of glucuronide prodrugs in humans can be modulated by exogenous factors.

A novel approach to improve drug targeting in cancer chemotherapy is the use of nontoxic glucuronide prodrugs that are selectively bioactivated by the human enzyme β-glucuronidase localized within or on the surface of tumors (for review see Sperker et al., 1997a). To synthesize suitable prodrugs that are bioactivated by β-glucuronidase, a wide variety of different anticancer drugs have been conjugated to glucuronic acid via different kinds of chemical spacers (Houba et al., 1996; Lougerstay-Madec et al., 1998; Papot et al., 1998). These prodrugs are applicable for various therapeutic strategies. For example, antibody-directed enzyme prodrug therapy is based on a prodrug-activating enzyme such as β-glucuronidase that is fused to antibodies specific for tumor antigens like carcinoembryonic antigen or CD20 (Bosslet et al., 1994; Haisma et al., 1998). An alternative approach is a one-step prodrug monotherapy using endogenous tumoral β-glucuronidase activity that selectively cleaves the prodrug (Bosslet et al., 1995, 1998).

In many tumors β-glucuronidase is present at higher levels than in the surrounding normal tissue (Fishman and Anlyan, 1947; Boyer and Tannock, 1993; Mürdter et al., 1997). In contrast to normal tissue, tumoral β-glucuronidase is in part localized extracellularly, probably because of secretion by inflammatory cells and disintegrating tumor cells (Bosslet et al., 1998). Both higher expression levels and extracellular localization seem to be a prerequisite for tumor-selective release of cytostatics from relatively hydrophilic glucuronide prodrugs that poorly penetrate into living cells.

A common issue in prodrug monotherapy and antibody-directed enzyme prodrug therapy is bioactivation of the glucuronide prodrug by β-glucuronidase. Thus, interindividual and intertissue variability of β-glucuronidase activity as well as factors modulating the enzyme's expression and activity are key determinants for these therapeutic strategies.

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ABBREVIATIONS: MEM, minimum essential medium; MU, 4-methylumbelliferone; MUG, 4-methylumbelliferyl-β-D-glucuronide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SSC, standard saline citrate; GRP, glucose regulated protein.

Human β-glucuronidase (EC 3.2.1.31) is an acid hydrolase that is involved in the lysosomal degradation of glucuronic acid-containing glycosaminoglycans (Paigen, 1989). Deficiency of β-glucuronidase leads to a lysosomal storage disease known as mucopolysaccharidosis type VII (Sly et al., 1973). The tetrameric glycoprotein has a molecular mass of 332 kDa and consists of identical monomers of 651 amino acids (Jain et al., 1996). During transport from the endoplasmic reticulum to lysosomes, the 83- to 80-kDa monomers are proteolytically processed to a 79- to 77-kDa polypeptide, which in turn can be cleaved to 64-kDa and 18-kDa forms (Tanaka et al., 1992; Islam et al., 1993; Gehrmann et al., 1994). The human β-glucuronidase cDNA as well as the gene and the promoter-containing 5′ flanking region have been cloned and characterized (Oshima et al., 1987; Miller et al., 1990; Shipley et al., 1991). The 200-bp region upstream of the translation initiation site has a high G + C content and lacks a TATA box, features commonly associated with housekeeping genes. Interestingly, in contrast to human β-glucuronidase, the murine promoter does contain a putative TATA box (Shipley et al., 1991). Whereas the murine gene is inducible by androgens (Thornton et al., 1998) regulation of human β-glucuronidase expression has not been reported. However, there is considerable interindividual variability of β-glucuronidase expression in different human tissues (Corrales-Hernández et al., 1988; Sperker et al., 1997b), which can be caused by gene dose effects or regulation events. Regulation of expression of human β-glucuronidase by exogenous factors (e.g., drugs or xenobiotics) may be a pivotal issue for metabolic bioactivation of glucuronide prodrugs.

Preliminary evidence from animal experiments suggests involvement of intracellular calcium in modulation of β-glucuronidase activity (Belinsky et al., 1984). We therefore studied regulation of human β-glucuronidase by the calcium ATPase inhibitor thapsigargin and the calcium ionophore A23187 in the human hepatoma cell line HepG2. Using cleavage of the substrate 4-methylumbelliferyl-β-D-glucuronide, Western and Northern blot analyses, as well as nuclear run-on assays, we were able to show regulation of β-glucuronidase expression on functional, protein, and transcriptional levels.

Experimental Procedures

Materials. HepG2, ECV 304, and HL-60 cells were purchased from the American Type Culture Collection (Manassas, VA), Caco-2 subclone TC7 was a gift from U. A. Meyer (Basel, Switzerland) and 32 M1 cells were kindly supplied by G. Jaques (Marburg, Germany). Minimal essential medium (MEM), Dulbecco's modified Eagle's medium, medium 199, RPMI 1640, fetal calf serum, L-glutamine, and MEM nonessential amino acids were from Life Technologies (Karlsruhe, Germany). A23187 and thapsigargin were from Calbiochem (Schwalbach, Germany). 4-Methylumbelliferone (MU), 4-methylumbelliferyl β-D-glucuronide (MUG), Trypan Blue (0.4%), and D-saccharic acid 1,4-lactone were supplied by Sigma (Deisenhofen, Germany) and Pefabloc was from Roth (Karlsruhe, Germany). The monoclonal antibody 2156/42 was a gift from K. Bosslet (Hoechst Marion Roussel, Marburg, Germany). Human β-glucuronidase full-length cDNA was kindly supplied by W.S. Sly (St. Louis, MO); pGem 4Z was from Promega (Mannheim, Germany); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was from the American Type Culture Collection (Rockville, MD); glucose-regulated protein (GRP) 78 cDNA was a gift from Bruce Spiegelman (Boston, MA); and 18S rRNA cDNA has been described previously (Edgar et al., 1998).

Cell Culture. HepG2 cells were grown in MEM containing 10 mM L-glutamine and 1% MEM nonessential amino acids. ECV 304 cells were grown in medium 199, Caco-2/TC7 were grown in Dulbecco's modified Eagle's medium containing 1% MEM nonessential amino acids, HL-60 and 32 M1 were grown in RPMI 1640. All media were supplemented with 10% fetal calf serum. Cell lines were incubated at 37°C in an atmosphere containing 5% CO_2. For treatment with A23187 and thapsigargin cells were seeded in six-well dishes at a density of $6 \times 10^4/cm^2$ (HL-60 at 10^5/ml). Medium was changed after 3 days of culture (except of HL-60 cells) and compounds (dissolved in ethanol) were added at a final ethanol concentration of 0.1%. For determination of enzymatic activity and Western blotting, cells were scraped, resuspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 0.2% Triton X-100, 1 mM Pefabloc) and incubated on ice for 30 min with several intermediate mixing steps. After centrifugation (5 min at 13,000 rpm in a table top centrifuge) protein concentration of the supernatant was determined by the bicinchoninic acid method (Smith et al., 1985). Determination of cell viability after A23187 and thapsigargin treatment, respectively, was performed by Trypan Blue exclusion tests using 0.1% Trypan Blue.

Determination of β-Glucuronidase Activity. Activity of β-glucuronidase was essentially measured as described previously (Sperker et al., 1996). Briefly, incubation mixtures contained 2.25 μg of total protein from cell lysates in 50 μl of assay buffer [200 mM sodium acetate, pH 5.0, 10 mM EDTA, 0.01% (w/v) bovine serum albumin, 0.1% (v/v) Triton X-100, 2.5 mM MUG]. Enzymatic reactions were carried out for 2 h at 37°C and stopped by adding 150 μl of 200 mM sodium carbonate. After centrifugation of the mixture for 5 min at 13,000 rpm MU concentration in the supernatant was determined by high-performance liquid chromatography using a C_8 column followed by fluorescence detection (excitation at 355 nm, emission at 460 nm). Calibration samples consisted of heat-inactivated cell lysates and standard solutions of MU and MUG. Calibration curves were linear over a concentration range of 100 nM to 20 μM. Specific enzymatic activities are expressed in nanomoles of released MU per milligram of protein per hour (nmol MU/mg/h).

Western Blotting. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and Western blot analysis of HepG2 cell lysates were carried out following standard protocols (Harlow and Lane, 1988). Briefly, 50 μg of total protein were subjected to 10% SDS gels, transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany) using a semidry blotter (Bio-Rad, Hercules, CA) and incubated with the monoclonal β-glucuronidase antibody 2156/42 (diluted 1:250) for 1 h at room temperature. As second antibodies, alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (DAKO, Hamburg, Germany) were used. Blots were scanned with an HP ScanJet IIc (Hewlett Packard, Greeley, CO, USA) and determination of signal intensity was performed using the ONE-Dscan software (Scanalytics, Billerica, MA).

Isolation of RNA and Northern Hybridization. Total RNA was isolated using the RNeasy Kit from Qiagen (Hilden, Germany). Briefly, cells grown in six-well dishes were washed once with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4), lysed in a guanidinium isothiocyanate-containing buffer, and RNA was purified using silica-gel-based spin columns. Concentration was determined by UV absorbance measurement and 5–10 μg of RNA per lane were electrophoresed in 1% agarose/6.6% formaldehyde agarose gels followed by capillary transfer to Hybond N membranes (Amersham Pharmacia Biotech, Freiburg, Germany) in 20 × standard saline citrate (SSC; 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and UV crosslinking with 1 J/cm^2. Labeling of cDNA and hybridization (at 65°C) was performed using the rediprime labeling kit and rapid-hyb buffer from Amersham Pharmacia according to the manufacturer's instructions. Blots were washed 20 min in 2 × SSC, 0.1% (w/v) SDS at room temperature followed by two washing steps in 0.1 × SSC, 0.1% (w/v) SDS at 65°C. Autoradiography was performed using intensifying screens at −70°C.

Nuclear Run-On Transcription. Analysis of transcription rate was essentially performed as described previously (De Waziers et al., 1995). In brief, after 2 h of incubation with A23187 or thapsigargin HepG2 cells (six dishes per treatment, 8×10^6 cells/dish) were scraped in ice-cold phosphate-buffered saline, pelleted, and resuspended in 2 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40). Nuclei were separated by centrifugation and after a washing step they were finally stored in 50 mM Tris-HCl, pH 8.0, 0.5 mM $MgCl_2$, 0.1 mM EDTA, and 40% glycerol. Nuclei (15×10^6) were incubated for 30 min at 30°C in a transcription buffer containing 100 mM Tris-HCl, pH 7.9, 50 mM NaCl, 0.4 mM EDTA, 1.2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 350 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 10 mM creatine phosphate, 10 μg/ml creatine phosphokinase, 2 mM each CTP, GTP, and ATP, 500 U/ml RNasin, and 1 mCi/ml [α-^{32}P]UTP (3000 Ci/mmol). For isolation of labeled transcripts the preparation was successively treated with 10 μg/ml DNase (RNase-free) for 15 min at 37°C, 40 μg/ml proteinase K for 15 min at 37°C, and 100 μg/ml proteinase K/1% SDS for 30 min at 37°C followed by sequential guanidinium chloride extraction and sodium acetate/ethanol precipitation. Labeled RNA (1.5×10^7 cpm) was incubated for 72 h at 42°C with slot blots containing 5 μg of each of the cDNA probes (pGem4Z, β-glucuronidase, GRP78, 18S rRNA). Subsequently blots were washed, treated with RNase A and signal intensity was determined by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Relative transcription rates were calculated by dividing values obtained for β-glucuronidase and GRP78 by those obtained for 18S rRNA and are expressed as percentage of the corresponding ratio found in control cells (treated with the carrier solvent).

Statistical Analysis and Calculations. Statistical calculations and curve fitting was performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Data are expressed as mean ± standard deviation (S.D.). Comparisons of treated groups to control groups were made using Student's *t* test. Concentration-effect curves were fitted and EC_{50} values were calculated using the Boltzmann equation.

Results

Concentration and Time-Dependent Effects of Thapsigargin and A23187 on β-Glucuronidase Activity. Lysates from the human hepatoma cell line HepG2 show specific β-glucuronidase activities of about 40 to 130 nmol MU/mg/h, depending on the passage level of the cells. Because addition of the specific inhibitor D-saccharic acid 1,4-lactone (1 mM) completely blocked cleavage of the enzyme substrate (data not shown), nonspecific hydrolysis could be excluded.

Incubation of HepG2 cells with different concentrations of the calcium ionophore A23187 and the calcium ATPase inhibitor thapsigargin for 48 h decreased specific activity of β-glucuronidase in cell lysates to a minimum of about 40 to 60% of the control level. IC_{50} values were 154 nM for A23187 and 3.2 nM for thapsigargin, respectively (Fig. 1A). Time course experiments with 1 μM A23187 and 100 nM thapsigargin showed a steady decrease of specific enzyme activity in treated versus control cells up to 48 h (Fig. 1B). Marked differences between control and treated cells were not seen before 24 h of incubation. Removing thapsigargin after 8 and 24 h of incubation, respectively, followed by further incubation with control medium up to 48 h, resulted in 90 and 114% β-glucuronidase activity, respectively, compared with control.

To determine cytotoxicity of A23187 and thapsigargin, respectively, cells were incubated with either 1 μM A23187 or 100 nM thapsigargin. Treatment with A23187 resulted in 92 and 76% viable cells after 24 and 48 h, respectively, compared with controls. After exposure to thapsigargin, 106 and 86% viable cells were observed after 24 and 48 h, respectively.

To investigate whether the decrease of β-glucuronidase

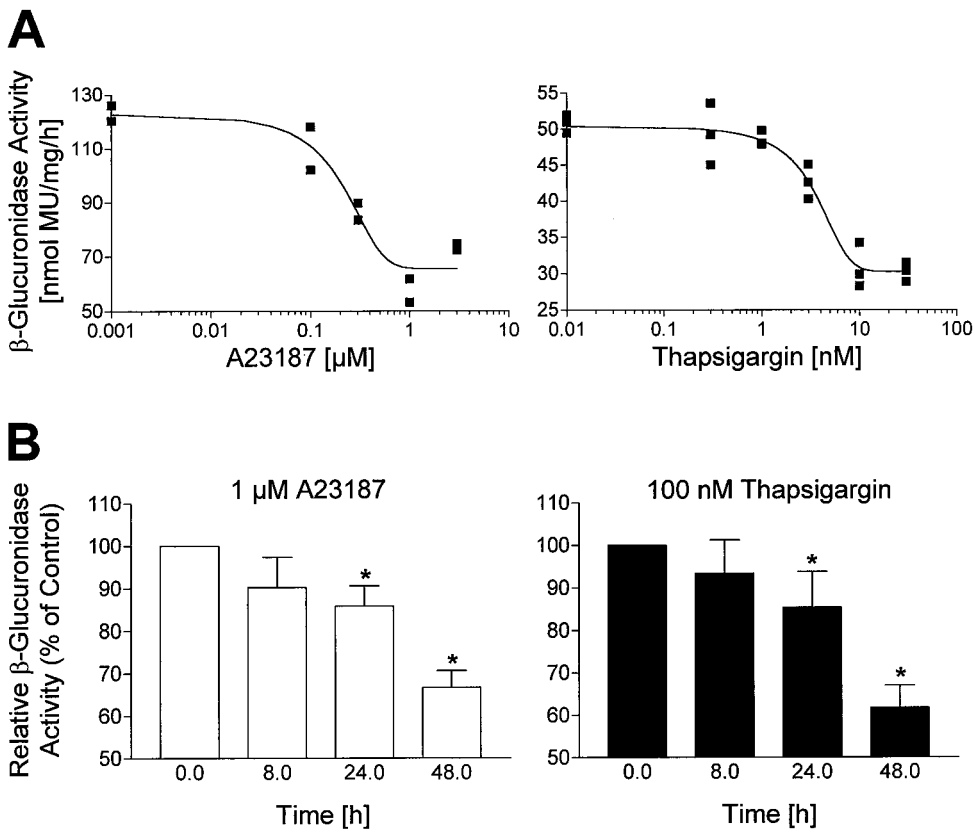

Fig. 1. Effects of A23187 and thapsigargin on β-glucuronidase activity in HepG2 cells. A, concentration dependence of enzymatic activity in cell lysates after 48 h incubation of HepG2 cells with A23187 ($n = 2$) or thapsigargin ($n = 3$). Ethanol contents are represented by 0.001 μM A23187 and 0.01 nM thapsigargin, respectively. B, time course of the effects of 1 μM A23187 and 100 nM thapsigargin in HepG2 cells. Activities of treated cells were compared with respective control cells at each time point (mean ± S.D.; $n = 3$; *, significantly different from the respective control, $p < 0.05$).

activity is cell type-specific, different human cell lines (HL-60, ECV 304, 32 M1, Caco-2/TC7) were incubated with 1 μM A23187 for 24 or 48 h. Although the cell lines exerted different levels of specific β-glucuronidase activity, down-regulation by A23187 to about 32 to 52% was found in all cell lines (Fig. 2).

Down-Regulation of β-Glucuronidase Protein and mRNA Expression by A23187 and Thapsigargin. To investigate the effects on β-glucuronidase protein levels, cell lysates were subjected to SDS polyacrylamide gel electrophoresis followed by Western blotting using a monoclonal antibody directed against human β-glucuronidase. In contrast to human liver homogenate displaying 83-, 79-, and 64-kDa bands, in HepG2 cells, the 64-kDa band is missing and no additional bands were seen under the conditions used. As depicted in Fig. 3A, the amount of immune reactive protein was decreased after incubation with 1 μM A23187 and 10 nM thapsigargin for 48 h, respectively. In addition, in treated cells the ratio of the 83-kDa to 79-kDa protein bands was 0.17 ($\pm$ 0.09, $n = 4$) whereas it was about 0.67 ($\pm$ 0.17, $n = 4$) in control cells (treated with 0.1% ethanol) as determined by densitometry. In contrast, the level of GAPDH immune reactive protein remained unchanged in HepG2 cells after treatment with A23187 and thapsigargin, respectively, as well as in Caco-2/TC7 cells incubated with A23187.

Determination of mRNA levels was performed by Northern blot analysis. Hybridization with either a human β-glucuronidase or GAPDH cDNA probe revealed mRNA bands of 2.2 and 1.2 kb, respectively. Whereas β-glucuronidase mRNA abundance decreased after 24 h of incubation of HepG2 cells with 3 μM A23187 and 100 nM thapsigargin, respectively, no change in signal intensity was seen for GAPDH mRNA (Fig. 3B). Moreover, the amount of low-density lipoprotein receptor mRNA increased after treatment with 3 μM A23187.

Effects of A23187 and Thapsigargin on the Transcription Rate. Nuclear run-on experiments were performed to determine the mechanism of down-regulation of β-glucuronidase. [α-^{32}P]UTP labeled primary transcripts were hybridized to slot blots containing pGem4Z as a negative control DNA, β-Glucuronidase full-length cDNA, GRP78 cDNA as a positive control for up-regulation by A23187 and 18S rRNA cDNA as a reference to correct for loading variability. Treatment of HepG2 cells with 3 μM A23187 or 1 μM thapsigargin for 2 h decreased β-glucuronidase transcription rate to 46 and 67% of the control level, respectively (Fig. 4). In contrast, GRP78 transcription rate was increased by a factor 2 to 2.5.

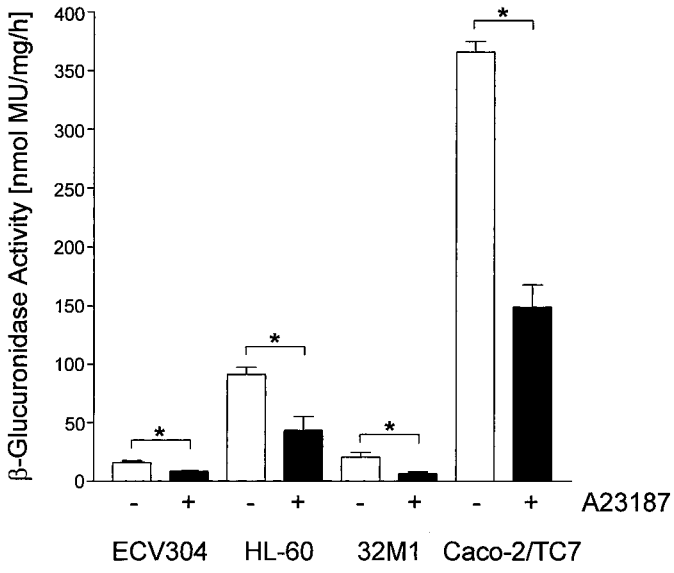

Fig. 2. Effect of A23187 on β-glucuronidase activity in different cell lines. Cells were incubated with 1 μM A23187 (+) or 0.1% ethanol (−) for 24 h (Caco2-TC7, 48 h). Release of MU from MUG was determined in whole-cell lysates (mean $\pm$ S.D.; $n = 4$; Caco2-TC7, $n = 3$; $^*p < .05$).

Discussion

Recently, we were able to prove the concept of one-step prodrug monotherapy by using the glucuronide prodrug *N*-[4-β-glucuronyl-3-nitrobenzyloxycarbonyl] doxorubicin (HMR1826). Treatment of cynomolgus monkeys with high-dose HMR1826 (250 mg/kg) did not reveal any signs of cardiotoxicity, which is a severe problem in chemotherapy with doxorubicin itself (Bosslet et al., 1998). Studies with an isolated, perfused human lung tumor model revealed a 7-fold higher accumulation of doxorubicin in tumor tissue after perfusion with HMR1826 compared with perfusion with doxorubicin, which resulted from high metabolic activity of β-glucuronidase at the tumor site (Mürdter et al., 1997). In line with these experiments, wide interindividual variability of human β-glucuronidase expression and activity has been demonstrated in various tissues (Corrales-Hernández et al., 1988; Mürdter et al., 1997; Sperker et al., 1997b) which could be caused by regulation by exogenous and/or endogenous factors.

Although induction of murine kidney β-glucuronidase by androgens is a well-known phenomenon that is mediated by transcriptional and translational mechanisms (Fishman,

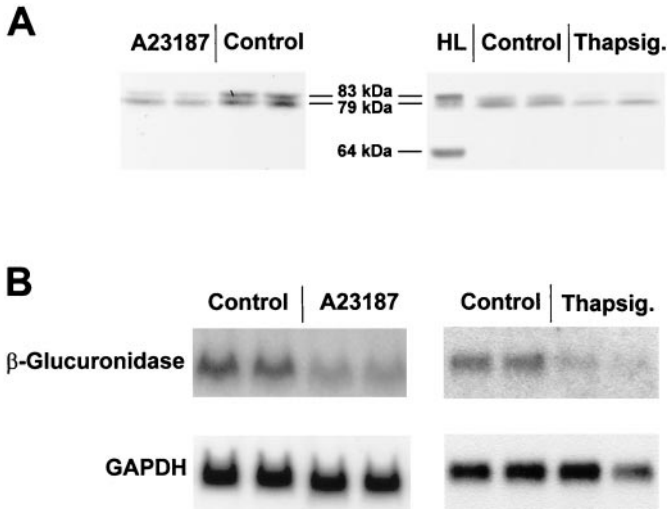

Fig. 3. Determination of the effects of A23187 and thapsigargin on protein and mRNA levels. A, Western blotting of lysates from HepG2 cells treated with 1 μM A23187, 10 nM thapsigargin or 0.1% ethanol (control) for 48 h. Lysate protein (50 μg) was loaded on a 10% SDS polyacrylamide gel, transferred to nitrocellulose, and finally incubated with the monoclonal antibody 2156/42. Human liver homogenate (HL, 20 μg) was used as a positive control for antibody specificity. B, Northern blots of total cellular RNA from HepG2 cells treated with 3 μM A23187 or 100 nM thapsigargin for 24 h, respectively. RNA (10 μg from A23187-treated cells; 5 μg from thapsigargin-treated cells) was electrophoresed in 1% agarose/6.6% formaldehyde gel, transferred to a nylon membrane and successively hybridized with ^{32}P-labeled cDNA probes for β-glucuronidase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively.

1951; Bracey and Paigen, 1987; Thornton et al. 1998), regulation of expression of the human enzyme has not been reported up to now. Because the promoter structure of the human β-glucuronidase gene shares several features with promoters of "housekeeping genes," the human enzyme was assumed to be a nonregulated "housekeeping protein" (Shipley et al., 1991).

The present article describes, for the first time, regulation of human β-glucuronidase expression by xenobiotics. Because compounds such as the calcium ionophore A23187 that modulate cytosolic calcium concentration seem to play a role in modulation of β-glucuronidase activity in the rat (Belinsky et al., 1984), we investigated regulation of human β-glucuronidase by A23187 and the calcium ATPase inhibitor thapsigargin. Incubation of HepG2 cells with these compounds leads to a considerable down-regulation of β-glucuronidase expression. The parallel decrease of activity, protein, and mRNA amounts suggests that lower mRNA levels result in decreased protein expression followed by reduced enzymatic activity of β-glucuronidase. As proven by nuclear run-on transcription, reduced mRNA levels are caused at least in part by a decreased transcription rate. In contrast, expression of GAPDH and 18S rRNA are unaffected as assessed in Northern blot analyses and nuclear run-on assays, respectively. However, transcriptional induction of GRP 78 by A23187 and thapsigargin was observed that is in accordance with previous reports (Price et al. 1992). Chao et al. (1990) found a coordinated induction of GAPDH and GRP 78 expression in such human cell lines as HL-60, EB-1, and HeLa by treatment with high concentrations of A23187 with a maximum after 4 to 6 h followed by a decrease up to 24 h. We and others (Gong et al., 1995) could not observe any change in GAPDH expression that might be caused by use of different cell lines, lower concentrations of A23187, and different incubation times. Therefore, GAPDH can be used as an unregulated loading control for Northern blot hybridization under the conditions used in our experiments. The lack of effects on expression of GAPDH and 18S rRNA, respectively, as well as induction of GRP78 and low-density lipoprotein receptor, suggest an effect that is not explained by cytotoxicity of the compounds. In line with these data, thapsigargin-mediated down-regulation was reversible. Moreover, viability of treated cells remained unchanged up to 24 h, although expression of β-glucuronidase was reduced significantly.

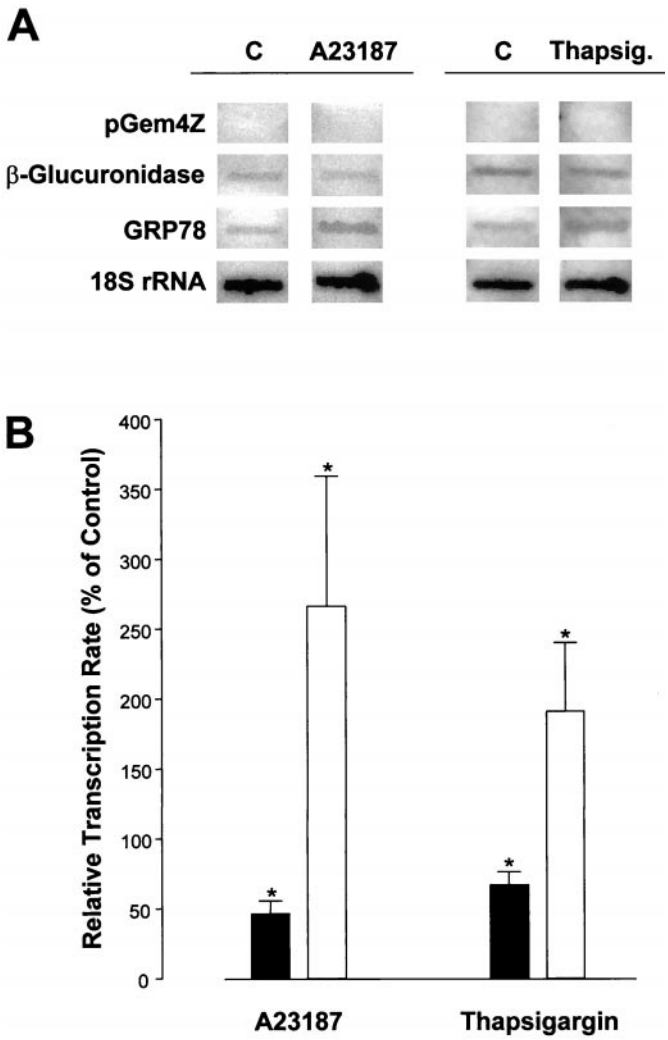

Fig. 4. Nuclear run-on transcription of β-glucuronidase (■) and GRP78 (□) genes. HepG2 cells were treated for 2 h with 3 μM A23187, 1 μM thapsigargin, or 0.1% ethanol as a control (C), respectively. Nuclei were isolated and incubated in the presence of [α-^{32}P]UTP. A, labeled RNA was extracted and hybridized with pGem4Z, β-glucuronidase, GRP78, and 18S rRNA cDNAs that were immobilized on nitrocellulose sheets. Signal intensity was determined by using a Phosphor Imager. B, transcription rate was determined by dividing signals obtained for β-glucuronidase and GRP78 by those obtained for 18S rRNA and are expressed as percentage of the corresponding ratio found in control cells (treated with the carrier solvent 0.1% ethanol). Mean values ± S.D. of three or four independent experiments are given (*, significantly different from control, $p < .05$).

Because the human hepatoma cell line HepG2 is a widely used system for investigation of lysosomal enzymes (Zhu and Conner, 1994; Isidoro et al., 1997) we used this model to determine the effects of xenobiotics on expression of β-glucuronidase. The enzymatic activity found in HepG2 cells is about 10% of that measured in human liver tissue and the processing of the β-glucuronidase protein seems to be somewhat different compared with human liver (Sperker et al., 1997b). As HepG2 cells lack the 64-kDa fragment, a mutation may exist at the proteolytic cleavage site or the cells may be unable to express the corresponding protease. However, the effects of A23187 and thapsigargin seem to be not cell line-dependent, because a variety of other cell lines from different sources, such as leukocytes, lung, endothelium, and intestine, revealed a comparable down-regulation of β-glucuronidase activity.

In addition to reduced expression of β-glucuronidase, A23187 and thapsigargin affect processing of the protein as suggested by reduced ratio of the 82-kDa/79-kDa proteins. Although A23187-mediated release of lysosomal proteins from polymorphonuclear leukocytes has been described by others (Hatzelmann et al., 1994), measurement of β-glucuronidase activity in culture medium of A23187-treated HepG2 cells did not reveal any accumulation of enzymatic activity (data not shown). In addition, it is very likely that the 79-kDa band represents the mature lysosomal protein, whereas the 82-kDa band is related to the microsomal precursor (Paigen, 1989; Islam et al., 1993) suggesting an augmented proteolytic processing of the microsomal form of the enzyme.

The signal transduction pathways by which A23187 and thapsigargin repress β-glucuronidase expression remain to be elucidated. Parallel effects of both the calcium ionophore A23187 and the calcium ATPase inhibitor thapsigargin point

to an influence of increased cytoplasmic Ca^{2+} concentrations as described for a wide variety of different proteins, such as plasminogen activator inhibitor-type 1 or c-*myb* (Peiretti et al., 1996; Schaefer et al., 1996). The 5′ flanking region of the human β-glucuronidase gene contains one potential binding site for the transcription factor AP-2 at position −198 (Shipley et al., 1991). Because AP-2 mRNA has been shown to be repressed by calcium ionophore (Lüscher et al., 1989), a comparable mechanism could also be involved in down-regulation of human β-glucuronidase by A23187.

The reason for increased expression of β-glucuronidase in tumors compared with the surrounding healthy tissue remains unclear. Recently, we demonstrated expression of β-glucuronidase in tumor-associated inflammatory cells, which seem to be involved in activation of the prodrug HMR 1826 (Bosslet et al., 1998). Therefore, in addition to upregulation of the endogenous tumoral enzyme, infiltration of β-glucuronidase-expressing immune cells and induction of the enzyme in these cells could contribute to increased β-glucuronidase levels in tumors.

In summary, our study demonstrates regulation of human β-glucuronidase by xenobiotics. Thus, bioactivation of glucuronide prodrugs can be modulated by exogenous factors that could lead to variable efficacy of prodrug therapy. Particularly, intracellular Ca^{2+} levels are modulated by numerous drugs that may be coadministered in the setting of cancer chemotherapy. Further studies are conducted to understand modulation of expression of human β-glucuronidase on molecular level.

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Send reprint requests to: Dr. Bernhard Sperker, Institut für Pharmakologie, Ernst Moritz Arndt Universität Greifswald, Friedrich Loeffler Str. 23d, D-17487 Greifswald, Germany. E-mail: sperker@uni-greifswald.de