Induction of phase I and II drug metabolism in rat small intestine and colon in vitro

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List of non-standard abbreviations:

DME: Drug metabolizing enzyme; TT, testosterone; TOH, hydroxytestosterone; 7HC, 7-hydroxycoumarin; 7HC-GLUC, 7-hydroxycoumarin glucuronide; 7HC-SULF, 7-hydroxycoumarin sulphate; 7EC, 7-ethoxycoumarin; AP, alkaline phosphatase; bNF, β-naphthoflavone; dimethylsulfoxide, DMSO; DEX, dexamethasone; PB, phenobarbital; 3MC, 3-methylcholanthrene; IR, indirubin; tBHQ, tert-butylhydroquinone; Ct value, Cycle threshold value

Abstract

The aim of this study was to evaluate drug metabolism in rat small intestinal and colon precision-cut slices during 24 h of incubation and the applicability of these slices for enzyme induction studies. Various parameters were evaluated: Intracellular levels of ATP (general viability marker), alkaline phosphatase activity (specific epithelial marker), villin expression (specific epithelial marker) and metabolic rates of 7-ethoxycoumarin (CYP1A), testosterone (CYP3A, 2B) and 7-hydroxycoumarin (glucuronide and sulphate conjugation) conversions. ATP and villin remained constant up to respectively 5 and 8 h in small intestine and up to 24 h in colon. The metabolic rate remained constant in small intestinal slices up to 8 h and decreased afterwards to 24-92%, depending on the substrate studied. The inducibility of metabolism in small intestinal and colon slices was tested with several inducers at various concentrations and incubation times. The following inducers were used: 3-methylcholanthrene, β-naphthoflavone, indirubin and tert-butylhydroquinone (AhR ligands), dexamethasone (PXR/GR ligand) and phenobarbital (CAR ligand). After incubation with inducers, metabolic rates were evaluated with 7-ethoxycoumarin and testosterone (phase I), and 7-hydroxycoumarin (phase II) as substrate. All inducers elevated the metabolic rates consistent with the available published *in vivo* induction data. Induction of enzyme activity was already detectable after 5 h (small intestine) and after 8 h (colon) for 3-methylcholanthrene and β-naphthoflavone and was clearly detectable for all tested inducers after 24 h (up to 20-fold compared with non-induced controls). In conclusion, small intestinal and colon precision-cut slices are useful for metabolism and enzyme induction studies.

Introduction

The intestine is highly sensitive for drug-drug and drug-diet interactions, influencing drug metabolism by inhibition and induction of drug metabolizing enzymes (DMEs) (Pelkonen et al., 2001). This can lead to major changes in bioavailability of drugs and may also cause an imbalance between toxification and detoxification (Lin and Lu, 1998).

An unfortunate and serious example of adverse drug-drug interactions is the induction of CYP3A4 and Pgp by St Johns Wort in transplantation patients, causing a serious decrease in cyclosporine plasma concentration, which can lead to organ rejection after transplantation (Barone et al., 2000; Ruschitzka et al., 2000). The importance of the induction of CYP3A4 by St Johns Wort in the human intestine was demonstrated using the jejunal perfusion technique (Tannergren et al., 2004).

Pharmaceutical research would greatly benefit from an *in vitro* system to study drug-drug interactions in the human and animal intestine, to be able to predict potential substrate interactions and undesirable side effects/toxicity of drugs. Preferably, this should be a system that could also make use of human intestinal material, since many drug interactions are highly species specific. Such a system should consist of intact cells that remain viable, expressing genes and proteins and capable of metabolizing drugs for at least 24 h of incubation. Only a few *in vitro* systems have been reported to meet this criteria. Caco-2 cells have been used for induction studies at enzyme activity level (Galijatovic et al., 2000) and LS180 cells for studies at mRNA level (Aiba et al., 2005). However, these cell cultures clearly differ from the complex structure and metabolic function of normal intestinal tissue. The mouse intestinal explant technique is an intact tissue system that has been reported to be useful for studying the induction of fatty acid binding proteins (Mallordy et al., 1995). Important in this respect is the precision-cut slice

system, an intact tissue system in which induction at mRNA level has been described using rat small intestinal tissue (Martignoni et al., 2004). At activity level, a first indication of the inducibility of DMEs in intestinal slices with β-naphthoflavone (bNF) was reported by our laboratory (van de Kerkhof et al., 2005).

In the present study, we further assessed (A) the viability and metabolic capacity of rat small intestinal and colon precision-cut slices after long-term incubation (e.g. up to 24 h) and B) their applicability for DME induction studies. Viability was assessed after incubation by measuring the intracellular ATP levels in both small intestinal and colon slices. ATP levels measured in slices are indicative for the viability of all cell types in the tissue, but for drug metabolism studies the viability of the enterocytes is of particular interest. As viability marker for enterocytes, alkaline phosphatase activity was determined in small intestinal slices. In addition, gene expression of villin (epithelial marker) and GAPDH (housekeeping gene for all cell types) was monitored up to 24 h of incubation. Finally, enzyme activity levels were evaluated using 7-ethoxycoumarin O-deethylation (7EC, CYP1A, phase I), testosterone hydroxylation (TT, CYP2B, CYP3A, phase I) and 7-hydroxycoumarin conjugation (7HC, glucuronidation and sulphation, phase II).

To study DME induction, we focused on 3 major induction pathways: Induction via CAR (regulating CYP2B and CYP3A, (Wang and LeCluyse, 2003)), PXR (mainly regulating CYP3A, but also CYP2B (Wang and LeCluyse, 2003)) and AhR (CYP1A1 (Lin and Lu, 1998)). We chose β-naphthoflavone (bNF) (Lin and Lu, 1998), 3-methylcholanthrene (3MC) (Abdelrahim et al., 2006), indirubin (IR) (Guengerich et al., 2004) and tert-butylhydroquinone (tBHQ) (Schreiber et al., 2006) as AhR ligands. Dexamethasone (DEX) was used as a GR/PXR ligand (Pascussi et al., 2001) and phenobarbital (PB) as

a CAR mediator (Wang and LeCluyse, 2003). Slices were incubated with several

inducers at various concentrations and incubation periods (0, 5, 8 and 24 h), followed by

3 h of incubation with a substrate (7EC, 7HC or TT). For PB studies, induction was also

monitored at mRNA level (CYP2B15, CYP3A9, CYP1A1 and CAR).

Materials and methods

Chemicals

Para-nitrophenylphosphate (pNPP), para-nitrophenol, 6β-, 11β-hydroxytestosterone (TOH), testosterone, androstenedione, 7HC, 7HC-glucuronide (7HC-GLUC), low gelling temperature agarose (type VII-A), DMSO and tertbutylhydroquinone (tBHQ) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Gentamicin, Williams medium E with glutamax-I and amphotericin B (Fungizone)-solution were obtained from Gibco (Paisley, UK). 7EC and bNF were obtained from Fluka Chemie (Buchs, Switserland). 2-amino-2-methyl-1,3-propanediol (ammediol) was purchased from Aldrich (Steinheim, Germany). 16α-TOH was obtained from Steraloids Inc. (Newport RI, USA). Acetic acid, sodium azide, sodium chloride and calciumchloride were obtained from Merck (Darmstadt, Germany). HEPES was obtained from ICN Biomedicals, Inc (Illkirch, France). 7HC-sulphate (7HC-SULF) was a kind gift from Mr. P. Mutch, GlaxoWellcome (Herts, UK). Phenobarbital (PB) was obtained from Bufa B.V. (Uitgeest, Holland). Dexamethasone (DEX) was purchased from Genfarma B.V. (Maarssen, Holland). 3-methylcholanthrene (3MC) was obtained from Supelco (Bellefonte, USA). Indirubin was obtained from Tebu-bio (heerhugowaard, Holland). All reagents and materials were of the highest purity that is commercially available.

Animals

Male Wistar (HsdCpb:WU) rats weighing ca. 350 g were purchased from Harlan (Horst, The Netherlands). Rats were housed in a temperature- and humidity-controlled room on a 12-h light/dark cycle with food (Harlan chow no 2018, Horst, The Netherlands) and tap

water *ad libidum*. The animal ethical committee of the University of Groningen approved the use of animals for these experiments.

Preparation of precision-cut slices

Under isofurane/N_2O/O_2 anaesthesia, the small intestine and colon were excised from the rat and put in ice-cold, oxygenated Krebs-Henseleit buffer (containing 10 mM HEPES and 25 mM D-glucose, pH 7.4). Segments of 3 cm were excised from the colon or small intestine (between 25 and 40 cm from the stomach) and subsequently flushed with ice-cold Krebs-Henseleit buffer. One side of the segment was tightly closed, filled with 3% (w/v) agarose solution in 0.9% NaCl (37°C) and then cooled in ice-cold Krebs-Henseleit buffer, allowing the agarose solution to gel. Subsequently, the filled segment was embedded in 37°C agarose solution using a pre-cooled (0°C) tissue embedding Unit (Alabama R&D, Munford, AL USA). After the agarose solution had gelled, precision-cut slices (thickness about 400 μm and slice wet-weight of about 2 mg) were cut using a Krumdieck tissue slicer as described earlier (de Kanter et al., 2005).

Incubation of precision-cut slices

The slices were incubated individually in a 12-wells culture plate (Greiner bio-one GmbH, Frickenhausen, Austria) in 1.3 ml Williams Medium E (with Glutamax-I), supplemented with D-glucose (final concentration 25 mM), gentamicin (final concentration 50 μg/ml) and amphotericin B (final concentration 2.5 μg/ml). The culture plates were placed in a pre-warmed cabinet (37°C) in plastic boxes, under humidified carbogen (95% O_2 and 5% CO_2) and shaken back and forth 90 times per minute.

Viability testing

Alkaline phosphatase activity: The precision-cut slices were incubated in triplicate for 0, 5, 8 and 24 hours. After incubation, the slices were taken out of the wells, placed in 1 ml 0.05 M ammediol buffer (pH 9.8, at 4°C) and medium and slices were stored separately at 4°C until further analysis. AP activity was determined as described before (van de Kerkhof et al., 2005).

Intracellular ATP levels: Intracellular ATP levels in slices were evaluated up to 24 h of incubation. Directly after tissue excision, 3 pieces of tissue were snap-frozen as 'in vivo' controls. Intracellular ATP levels were determined according to the method described earlier (van de Kerkhof et al., 2006). ATP content was determined in 2-8 experiments in triplicate.

Gene expression levels: 2 μg of total RNA, isolated from 6 snap-frozen slices using the RNeasy Mini Kit (Qiagen, Hilden, Germany), was used to synthesize 50 µl of cDNA using the Promega Reverse Transcripion System (Promega, Madison, WI, USA). 1.25 µl cDNA was used in real-time PCR reactions using SYBRgreen reaction mixture (Applied Biosystems, Warrington, UK) and the appropriate primers listed in table I. Agarose gel electrophoresis and dissociation curves confirmed homogeneity of the PCR products. Only for CAR, a minor second product was formed in colon slices. The cycle treshold value (Ct value) is inversely related to the abundance of mRNA transcripts in the initial sample. Mean Ct value of duplicate measurements was used to calculate the difference of Ct value for the target gene and the reference villin gene (ΔCt), which was compared to the corresponding delta Ct value of the control experiment ($\Delta\Delta$Ct). Data are expressed as fold-induction of the gene of interest according to the formula $2^{-\Delta\Delta Ct}$.

Induction studies

Precision-cut slices were prepared from small intestine (25-40 cm from the stomach) and colon and incubated with the selected inducers for several incubation times at various concentrations: bNF (0, 5, 8 and 24 h pre-incubation at 50 µM), 3MC (0, 5, 8 and 24 h of pre-incubation at 5 µM), IR (0 and 24 h pre-incubation at 10, 100 and 1000 nM), tBHQ (0, 5, 24 h at 50 µM), DEX (0, 5, 24 h at 100 µM) and PB (0, 8, 24 h at 2, 2.5, 4 and 8 mM). Subsequently, slices were transferred to fresh medium and incubated for another 3 h with model substrates: TT (250 µM), 7EC (500 µM) or 7HC (500 µM). The substrates were added as 100 times concentrated stock dissolved in MeOH (final medium concentration: 1% MeOH). As controls, slices were incubated without substrate and medium was incubated with substrate without slice.

The model inducers were added as a 200 times concentrated stock solution in DMSO (100 times for tBHQ) with a final concentration of 0.5 or 1% DMSO, with the exception of PB, which was directly added to the medium (0% DMSO). Control slices were incubated in medium supplemented with the same DMSO concentration (0, 0.5 or 1%).

After 24 h of PB incubation (4 mM), slices were also harvested for RNA isolation. Expressions of several genes as a ratio to villin expression were tested: CYP2B15 (same primers are coding for CYP2B1 according to others (Caron et al., 2005)), CYP3A9, CYP1A1 and CAR. Further, slices were incubated with a combination of PB (4 mM) and DEX (1 µM) during 24 h; slice incubations with only DEX (1 µM) for 24 h were then used as controls.

Metabolite analysis

Testosterone analysis: After TT incubation, slice and medium were collected together and stored at -20°C until further use. Sample extraction and HPLC analysis was performed as described earlier (van de Kerkhof et al., 2006).

7EC and 7HC analysis: As it was previously shown that 7EC and 7HC and their metabolites are not significantly retained in the tissue, analysis was performed on medium samples only (de Kanter et al., 2005). Medium samples were stored at −20°C and analysed for 7HC-GLUC and 7HC-SULF using HPLC analysis as described earlier (van de Kerkhof et al., 2005). Phase I metabolism of 7EC was calculated by adding up the amount of formed 7HC, 7HC-GLUC and 7HC-SULF.

Protein determination:

After incubation with 7EC or 7HC, slices were stored at -20°C until further use. After thawing, 20 µl 5 N NaOH was added to the slice followed by 40 minutes of incubation at 37°C to dissolve the tissue. 980 µl water was added to dilute the NaOH concentration to 0.1 M, after which the mixture was homogenised by 5 seconds of sonication. Samples were diluted and the protein content was determined using Bio-Rad protein assay dye reagent (Bio-Rad, Munich, Germany) using BSA as standard. After testosterone incubation, the protein of the slices was not determined. For these slices, the average protein contents of slices that were incubated with 7EC and 7HC within the same experiment were used.

Statistics:

Statistical significance was determined using Student's t test; $p < 0.05$ is considered significant. For gene expression the $\Delta\Delta$Ct values were used to determine the statistical significance of differences.

Results

Slice characterization during 24 h of incubation

The viability of the slices was monitored by several parameters i.e. intracellular ATP levels, AP activity, gene expression levels and activity during incubation.

Intracellular ATP levels: The intracellular ATP levels were measured in small intestinal and colon slices at various time points during 24 h of incubation, to evaluate the viability (figure 1A). In small intestinal and colon tissue, the '*in vivo'* intracellular ATP was respectively 0.8 and 0.9 nmol/mg protein and increased during the slicing procedure to respectively 3.9 and 2.4 nmol/mg protein (0 h), after which it decreased to respectively 1.0 and 1.7 nmol/mg protein after 24 h of incubation. In small intestinal slices, ATP levels remained constant up to 5 h and decreased significantly afterwards to 75% (8 h) and 25% (24 h), but this value at 24 h was not lower than the *in vivo* value. This is in contrast with colon slices in which no significant decrease was observed for 24 h.

Alkaline phosphatase activity: Alkaline phosphatase (AP) activity was measured in small intestinal slice tissue to assess the AP activity of epithelial cells during incubation, as well as in the incubation medium (figure 1B). The AP activity within the slice remained constant (approximately 100 U/min/slice) during incubation, whereas it increased in the incubation medium.

GAPDH and Villin expression: GAPDH and villin expressions were measured at various time points in both small intestine and colon slices. GAPDH (generally applied as housekeeping gene) remained constant up to 24 h of incubation in slices of both organs (figure 1C). In colon slices, villin expression (figure 1D) also remained constant up to 24 h of incubation. In contrast, the villin expression in small intestinal slices was constant up

to 8 h of incubation and decreased afterwards, possibly indicating that small intestinal slices lost epithelial cells after 8 h of incubation.

Metabolic rates during incubation: Slices were pre-incubated for 0, 5, 8 and 24 h after which the metabolic conversion of several model compounds was monitored. In general, in both small intestinal and colon slices (table II and III), the metabolic activity (per mg protein) was constant up to 8-11 h and decreased during further incubation, but the rate of decline of activity varied between substrates. For colon slices, the protein content per slice was constant up to 27 h. For small intestinal slices, however, the protein content per slice decreased during incubation to 71% (5 h), 64% (8 h) and 49% (24 h) compared with 0-3 h incubated slices (data not shown).

In small intestinal slices, 7EC metabolism and androstenedione, 7HC-GLUC and 7HC-SULF formation, remained constant up to 11 h of incubation (Table II). In contrast, the 6β-TOH formation rate had already decreased, non-significantly, after 5 h of pre-incubation. Then, after 24 h of pre-incubation, 7HC and 6β-TOH formation significantly decreased to 38% and 24% respectively compared with 0-3 h of incubation. Androstenedione, 7HC-GLUC and 7HC-SULF formation tended to decrease, although not significantly, to respectively 43 and 77% of the 0-3 h rates.

In colon slices, considerable activity (32-196%) was retained up to 27 h of incubation (table III). The metabolic rates for all tested reactions remained high (> 81%) after 8 h of pre-incubation. 7HC-GLUC, 7HC-SULF and androstenedione formation remained constant up to 27 h of incubation, but 7EC metabolism and 7HC sulphation decreased non-significantly to 41 and 54% respectively. Formation of 6β-TOH increased non-significantly to 196% after 24-27 h of incubation compared with 0-3 h.

Induction studies

bNF and 3MC: Although the rate of 7EC conversion declined to 38 and 32% during incubation up to 27 h in respectively small intestine and colon, a very strong induction of

phase I metabolism of 7EC was found when slices were pre-incubated with either bNF or 3MC (Figure 2). In small intestinal slices (figure 2A) pre-incubated with bNF (50 μM) or 3MC (5 μM) for 5, 8 or 24 h, the metabolic rate increased strongly and was already prominently induced after only 5 h of pre-incubation (> 16-fold compared with DMSO control). The fold induction was the highest after 8 hours of pre-incubation (20-fold for bNF and 19-fold for 3MC) and induction remained prominent after 24 h of pre-incubation with a metabolic rate of 113 pmol/min/mg protein (12-fold for both bNF and 3MC; $p < 0.05$ compared with DMSO control for each time point).

In colon slices (figure 2B), metabolic conversion of 7EC was also induced by bNF and 3MC. When pre-incubated for 8 or 24 hours with bNF or 3MC, the metabolic rate increased from 7.2 to 146 and 62 pmol/min/mg protein respectively ($p<0.05$), but was not significantly elevated after 5 h. Further, the induction (compared with DMSO controls) was the highest after 24 h of pre-incubation (21-fold with bNF and 10-fold with 3MC). After 8 h of pre-incubation, induction was 5-fold for bNF and 4-fold with 3MC.

Slices of small intestine and colon were also incubated with 7HC after 0, 5, 8 and 24 h of pre-incubation with bNF and 3MC. Neither bNF nor 3MC influenced the conjugation rates of 7HC (data not shown).

Indirubin (IR): IR is reported to be an endogenous ligand for the AhR (Guengerich et al., 2004). Therefore, small intestinal slices were pre-incubated with indirubin at various concentrations (10, 100, 1000 nM) for 24 h after which the metabolic rate of 7EC towards 7HC was determined (figure 3). A significant induction (6.1-fold compared with DMSO control) was observed after 24 h of pre-incubation with 1000 nM IR ($p < 0.05$). .

Tert-butyl hydroquinone (tBHQ): Some phase II enzymes are reported to be induced by tBHQ via the AhR pathway (Schreiber et al., 2006). To test the induction potential of

tBHQ in rat intestine, we incubated slices (small intestine and colon) with tBHQ (50 μM) for 0, 5, 24 h after which slices were incubated for 3 h with 7HC. In small intestinal slices (figure 4A), a 1.7-fold induction was significant ($p < 0.05$) after 24 h of incubation, (24 h DMSO control: 220 ± 35 and 24 h induced levels: 365 ± 62 pmol/min/mg protein). No effect could be detected by tBHQ on 7HC glucuronidation after 5 h of incubation. In colon slices (figure 4B), no effect could be observed after pre-incubation with tBHQ. In addition, in both small intestinal and colon slices no effect was observed on 7HC sulphation (data not shown).

Dexamethasone: Small intestinal and colon slices were incubated with dexamethasone (100 μM) to investigate the ability of this compound to induce metabolic enzymes in precision-cut slices (figure 5).

When control slices were incubated with TT, two TT metabolites, namely 6β-TOH and androstenedione, were detected. When small intestinal slices were pre-incubated with DEX, the rate of 6β-TOH formation (figure 5A) increased 1.7-fold, from 11.6 (control) to 18 pmol/min/mg protein ($p < 0.08$ compared with DMSO control) after 5 h and 2.3-fold, from 4.5 (control) to 10.1 pmol/min/mg protein ($p < 0.05$ compared with DMSO control) after 24 h. For androstenedione formation (figure 5B), only a slight, non-significant increase was observed after 24 h of pre-incubation (from 53 to 78 pmol/min/mg protein). In colon slices (figure 5C/D), 6β-TOH formation was not significantly increased by DEX. However, in 5 out of 6 experiments a slight, but non-significant increase of 6β-TOH formation was observed after 5 h of incubation. After 24 h of pre-incubating colon slices with DEX, a small non-significant elevation of androstenedione formation from 68 to 95 pmol/min/mg protein was observed.

Phenobarbital (PB): Small intestinal and colon slices were incubated with PB (0, 2, 2.5, 4 and 8 mM) to study the influence of this compound on the metabolic conversion of TT, 7EC and 7HC (figure 6).

In small intestinal and colon slices, an effect of PB at 4 mM, but not at 2 mM, on 7HC glucuronidation was observed (fig 6A, $p < 0.02$ compared with control (0 mM after 24 h) and fig 6F, $p < 0.04$)). Sulphation, however, was not affected (fig 6B and fig 6G).

Doubling the concentration of PB to 8 mM drastically decreased the glucuronidation rate in small intestine and colon to 0.2 and 21% respectively and the sulphation rate to 60 and 7% respectively. To investigate whether PB affected the viability of small intestinal slices, control incubations with 2, 4 and 8 mM PB were performed and the intracellular ATP levels measured afterwards. Concentrations of 0, 2 and 4 mM PB had no effect on the ATP content (0.9 nmol ATP/mg protein), but ATP levels were drastically decreased when slices were incubated with 8 mM (0.2 nmol ATP/mg protein).

In small intestine and colon, PB (2.5 mM) induced the metabolic rates of 7HC formation (figure 6C and figure 6H). Induction was detectable after 8 h (1.8-fold in small intestine and 2.9-fold in colon compared with control) and 24 h of pre-incubation (3.1-fold in small intestine and 4.1-fold in colon compared with control). Induction was only significant in colon ($p < 0.05$) after 8 h of pre-incubation. However, the induction in small intestine was clear, since prominent increases of metabolic rates were found in 3 out of 4 experiments after 8 h and 3 out of 3 experiments after 24 h.

PB (2.5 mM) did not influence the formation of 6β-TOH (fig 6D and 6I) or androstenedione (fig 6E and 6J).

Gene expression during incubation: PB was expected to induce CYP2B. However, the formation of 16α- and 16β-TOH was below the detection limit in intestinal slices and remained undetectable after 24 h of pre-incubation with PB. Therefore, the presence of CAR and CYP2B15 mRNA was investigated during incubation. Slices were harvested

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after several incubation periods: 0, 5, 8, 24 h in control medium. Slice preparation (small intestine and colon) did not influence the expression of CAR and CYP2B15 (data not shown). Incubation of small intestinal and colon slices for 24 h did not change CAR expression either. CYP2B15 mRNA expression, however, had already decreased in both tissues significantly after 5 h of incubation (small intestine: $12 \pm 0.05\%$ of control value with $p < 0.05$; colon: $28 \pm 14\%$ of control value with $p < 0.05$) and remained low up to 24 h of incubation (small intestine $5 \pm 0.1\%$ of control value; colon: $41 \pm 21\%$ of control value).

In small intestine, PB did not induce the expression of CYP2B15 mRNA (figure 7A). In contrast, CYP3A9 (1.7-fold, $p < 0.05$) was significantly increased. CYP1A1 (8.6-fold), CAR (3.5-fold) mRNA expressions were also induced in all individual experiments (figure 7A/B). Addition of DEX to the incubation medium did not affect the inducing ability of PB for the P450s tested. Furthermore, several pilot experiments were performed incubating proximal jejunum slices for 5 h with either PB (4 mM) alone or with PB (4 mM), DEX (10 μM), insulin (1 μM) and 5% FCS (as described by others (Martignoni et al., 2004)) using proper controls without PB. All above-mentioned incubation conditions did not induce CYP2B15 mRNA levels (data not shown).

In colon slices (figure 7C/D), however, CYP2B15 mRNA expression tended to increase after PB incubation in 3 out of 6 experiments. The induction of CYP1A1 (7-fold), CYP3A9 (2-fold, $p < 0.05$) and CAR (4-fold, $p < 0.05$) expression was found in all experiments performed (n=5-6). Medium supplementation with DEX (1 μM) significantly increased the induction of CYP2B15 from 4 up to 72-fold in colon. Furthermore, CYP3A9 and CYP1A1 induction remained significant after incubation with both PB (4 mM) and DEX (1 μM) compared with only DEX (1 μM) supplementation as a control.

Discussion

Recently, we presented rat intestinal precision-cut slices as a tool to study drug metabolism up to 3 h of incubation (de Kanter et al., 2005; van de Kerkhof et al., 2005). In the present study, we further investigated the applicability of intestinal slices up to 24 h of incubation for both metabolism and induction studies. Therefore, we evaluated the viability up to 24 h of incubation and treated slices up to 24 h with 5 prototypical inducers after which drug metabolism was evaluated.

Intracellular ATP levels are considered to be a proper measure for the overall viability of the tissue. The intracellular ATP levels of small intestinal and colon tissue directly after excision ('in vivo' levels) were 0.9 nmol/mg protein, but increased during slicing, which is similar to what was reported for rat liver and lung slices (De Kanter et al., 2002). It suggests that ATP is also synthesized during the slicing procedure at 4ºC in the presence of oxygen, which is in agreement with findings by others (Minor and Isselhard, 1996). The ATP levels in small intestine remained constant up to 8 h, after which it declined to 25% of control values after 24 h, but it was never lower than the tissue value. Furthermore, the viability of the enterocytes (metabolising cells) was studied in more detail. The AP activity in slices remained constant up to 24 h. In addition, the increase of enzyme activity in medium indicates that the cells are capable of *de novo* synthesis of AP during incubation. In addition, villin expression was evaluated during incubation, as it is commonly considered the housekeeping gene for enterocytes (Engman et al., 2001) in which it is exclusively expressed (West et al., 1988). Assuming that A) the protein content is a measure for the amount of tissue in the slice, B) the expression of villin is directly correlated to the amount of enterocytes in the slice, and taking into account that C) GAPDH remained constant in both small intestinal and colon slices up to 24 h, our

results indicate that in colon slices no cells are lost up to 24 h, since all parameters that were tested, remained constant (villin and GAPDH expression, the amount of protein per slice, ATP and activity levels). In small intestinal slices, based on the decreased protein content, it seemed that some cells are lost during the first 8 h. The constant levels of villin, GAPDH and metabolic rates expressed per protein indicate that this occurs to the same extent for all cell types present in the slice. After 8 h of incubation, however, small intestinal slices have lost more enterocytes in relation to other tissue cells, as judged by the decreased villin expression per total RNA. The protein content of slices decreased to about 50% after 24 h. The activity per mg protein decreased after 8 h of incubation, but remained clearly detectable after 24 h. The decline was different for the various metabolic reactions tested and this might be explained by either lack of endogenous stimuli present in the incubation medium ('de-induction') and/or differences in half-lives of various iso-enzymes, being in agreement with earlier findings by others in rat liver slices (Renwick et al., 2000) and cultured rat hepatocytes (McMillan et al., 1991). The decrease in villin can at least partly be explained by the normal apoptosis of enterocytes, since the in 'vivo' lifespan of these cells is approximately 2 days (Kaminsky and Zhang, 2003). Apparently, in the slices proliferation cannot compensate for the loss of cells by apoptosis. However, further research is needed to support this hypothesis.

Summarizing this data, colon slices remain viable up to 24 h of incubation; in small intestinal slices the ATP content and the amount of enterocytes per slice decrease after 8 h. In addition, some metabolic activity is lost after 8 h, but remains clearly detectable after 24 h of incubation. Therefore, small intestinal slices can be used for quantitative metabolism studies up to 8–11 h and for qualitative studies up to 24-27 h. The observed decrease in metabolic rate is in accordance with those found in hepatocytes, but, like in

hepatocyte studies, this does not necessarily impede detection of induction by inducing drugs (Wortelboer et al., 1991).

Slices were incubated with model inducers to test the applicability of slices for induction studies. In a recent study, we showed the induction of 7EC O-deethylation after 24 h of BNF exposure in intestinal slices (van de Kerkhof et al., 2005). In the current study, induction of 7EC O-deethylation (CYP1A) was observed with bNF, 3MC (small intestine and colon) and IR (small intestine), well-known AhR ligands (Lin and Lu, 1998; Guengerich et al., 2004; Abdelrahim et al., 2006). This is in line with published *in vivo* studies, in which orally administered bNF or 3MC induced 7EC O-deethylation 2-100 fold in small intestine (McDanell and McLean, 1984; Rosenberg, 1991; Spatzenegger et al., 2000).

In our *in vitro* studies, induction (at activity level) was already clearly detectable after 5 h in small intestinal slices and is in line with *in vivo studies* showing CYP1A1 induction in rat small intestine readily 3 h (Zhang et al., 1997) and 12 h after BNF administration (Zhang et al., 1996). Unfortunately, no such data is available for colon tissue, which impedes comparison with results of the present study.

The AhR pathway is known to be involved in the induction of both phase I (Abdelrahim et al., 2006) and II metabolism (Auyeung et al., 2003). tBHQ (AhR ligand (Schreiber et al., 2006)) has been reported to induce UGT activity in caco-2 cells (Munzel et al., 1999) and indeed it induced glucuronidation in small intestine, but not in colon slices. However, two other AhR ligands, 3MC and bNF, did not induce 7HC conjugation (via UGT1A6 (Ikushiro et al., 2004), UGT1A7/8 (Webb et al., 2005)), which is in line with reported findings that UGT1A6-8 mRNA expression was not induced in rat duodenum after *in vivo* administration of bNF (Shelby and Klaassen, 2006).

Dexamethasone, a known rodent PXR agonist at the concentration used, induces CYP3A in rat (Savas et al., 1999). In the present study, induction of 6β-TOH formation

by DEX was observed readily after 5 h (although not significant) and 24 h which is in agreement with *in vivo* studies showing the induction of the CYP3A1/2 protein (Zhang et al., 1996) and mRNA (Martignoni et al., 2004). In colon tissue, 6β-TOH formation was slightly but non-significantly induced after 5 and 24 h in 5 out of 6 experiments. Possibly, it may take longer than 24 h to induce CYP3A activity in colon as was reported in Caco-2 cells (Cummins et al., 2001).

PB induces DMEs via CAR (Wang and LeCluyse, 2003). In the present study, 7HC glucuronidation was induced by PB in both organs. In contrast to our findings, *in vivo* administered PB was reported not to induce UGT1A6-8 in rat duodenum (Shelby and Klaassen, 2006). 7EC O-deethylation (CYP1A) was induced by PB (2.5 mM) in both small intestine and colon which is in line with findings in hepatocytes, showing CYP1A1 induction by PB (Sadar et al., 1996).

Testosterone appeared not to be a good substrate for CYP2B metabolism in intestinal slices and therefore mRNA expression was examined. However, no induction of CYP2B15 expression was observed, despite the continuous expression of CAR during 24 h of incubation. In contrast, induction of CYP1A1 (confirming our activity data), CYP3A9 and CAR mRNA was observed in both organs.

In rat hepatocytes, it was shown that addition of DEX (100 nM) to the medium increased the inducibility of CYP2B (Ringel et al., 2002). In the present study in colon, but not in small intestinal slices, DEX appeared to enormously induce CYP2B15 mRNA. This suggests that PB mediated induction is differentially regulated in rat small intestine and colon, but this should be confirmed in further research.

In this study, induction was detected after 5, 8 and/or 24 h of exposure dependent on the inducer. Induction after 3MC and BNF was readily detectable after 5 h and the highest

after 8 h, but induction after tBHQ and DEX was only detectable after 24 h of incubation. This data suggest that for proper detection of inducing capacity of drugs, intestinal precision-cut slices should be incubated with these drugs for several incubation times.

To conclude, colon precision-cut slices are a useful tool for quantitative drug metabolism studies up to 24-27 h whereas slices of small intestine can be used to measure drug metabolism quantitatively up to 8–11 h and qualitatively up to 24-27 h. The current study demonstrates that this model is also very suitable to study drug-induced induction of metabolism *in vitro* in the intestine, since the AhR, PXR and CAR pathway are functional in both small intestinal and colon slices. This provides an opportunity to further investigate mechanisms of induction in various regions of the intestine. Moreover, when applied also to human tissue, it may predict and increase our understanding of drug-induced changes in intestinal metabolism and bioavailability in patients.

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Footnotes

a) unnumbered footnote providing the source of financial support

b) name + full address of person to receive reprint requests

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Figures

Figure 1:

Viability characterization of small intestinal and colon slices up to 24 h of incubation. Several parameters were monitored: intracellular ATP (A), alkaline phosphatase (only small intestine) (B), GAPDH mRNA expression (C) and villin mRNA expression (D). Results are mean ± SEM of slices of 2-8 rats, in each experiment at least 3 slices were incubated per time point.

Figure 2:

Induction of 7EC activity in precision-cut slices prepared from small intestine (25-40 cm from stomach) (A) and colon (B). Slices were pre-incubated for 0, 5, 8 and 24 hours with control medium, 50 μM β-naphthoflavone (bNF), 5 μM 3-methylcholanthrene (3MC), 0.5% DMSO (solvent of bNF and 3MC stock solutions). Subsequently, slices were transferred to fresh medium containing 7EC (500 μM) for 3 h of incubation.

Results are mean ± SEM of slices of 3-7 rats. In each experiment 3 slices were incubated per treatment. Significant differences toward the activities of slices incubated with 0.5% DMSO per incubation time are indicated with   * $p<0.05$, ** *p<0.01*, *** *p<0.001*.

Figure 3:

Induction of 7EC activity in precision-cut slices prepared from small intestine (25-40 cm from stomach). Slices were incubated for 0 and 24 hours with control medium, 0.5% DMSO (solvent of IR stock solutions) and IR at different concentrations: 10 nM, 100 nM or 1000 nM. Subsequently, slices were transferred to fresh medium for 3 h of substrate incubation with 500 μM 7EC. Results are mean ± SEM of slices of 4 rats. In each

27

experiment 3 slices were incubated per treatment. Significant differences toward the activities of slices incubated with 0.5% DMSO are indicated with * p < 0.05.

Figure 4:

Induction of 7HC glucuronidation in precision-cut slices prepared from A) small intestine (25-40 cm from stomach) and B) colon. Slices were pre-incubated for 0, 5 and 24 h with control medium, 1% DMSO or 50 μM tBHQ (1% DMSO) and subsequently transferred to fresh medium and incubated with 500 μM 7HC for 3 h. Results are mean ± SEM of slices of 3-4 rats. In each experiment 3 slices were incubated per treatment. Significant differences are indicated with * *p < 0.05*.

Figure 5:

Induction of TT conversion to 6β-TOH (A,C) and androstenedione (B,D) in precision-cut slices prepared from small intestine (25-40 cm from stomach, A,B) and colon (C,D). Slices were incubated for 0, 5 and 24 hours with control medium, 0.5% DMSO or 100 μM dexamethasone (DEX) and subsequently transferred to fresh medium and incubated with 250 μM TT for 3 hours. Results are mean ± SEM of slices of 3-6 rats. In each experiment 3 slices were incubated per treatment. Significant differences toward the activities of slices are indicated with * *p<0.05*.

Figure 6:

Influence of PB on 7HC-glucuronidation, 7EC and TT metabolism in precision-cut slices prepared from small intestine (25-40 cm from stomach, A-E)) and colon (F-J). Slices were pre-incubated for 0 and 24 h with control medium, 2 mM or 4 mM PB and transferred to fresh medium with 7HC (500 μM, fig 6A/B, F/G) for 3 h of substrate incubation. Slices were pre-incubated for 0, 5, 8 and 24 h with 2.5 mM PB and then

transferred to fresh medium containing either 7EC (500 µM, fig 6C, 6H) or TT (250 µM, fig 6D/E and 6I/J) for 3 h of additional incubation. Results are mean ± SEM of slices of 3-6 rats. In each experiment 3 slices were incubated per treatment. Significant differences towards 24 h control are indicated with * *p < 0.05, ** p < 0.01*.

Figure 7:

Gene expression of small intestinal (25-40 cm from stomach) (A,B) and colon (C,D) slices after 24 h of incubation with control medium, PB (4 mM), PB (4 mM) + DEX (1µM) or DEX (1µM). Several genes were studied: CYP2B15, CYP3A9, CYP1A1 (A, C) and CAR (B,D). Slice incubation Expression is corrected for villin and the control values were set at 1. Horizontal line indicates control levels (fold-induction = 1). Results are mean ± SEM of 4-6 experiments. In each experiment 6 slices were incubated per treatment and harvested together. Significant differences towards 24 h control medium are indicated with   * *p<0.05,* ** *p<0.01*. Significant differences between PB + DEX and DEX incubations are indicated with # *p < 0.05*.

Table 1 – Primer information of the rat genes under study

Gene	GenBank number	Forward Primer (5'-3')	Reverse Primer (5'-3')
Villin	**XM_001057825**	GCTCTTTGAGTGCTCCAACC	GGGGTGGGTCTTGAGGTATT
GAPDH	**XR_008524**	CGCTGGTGCTGAGTATGTCG	CTGTGGTCATGAGCCCTTCC
CYP1A1	**NM_012540**	GGCACTCTGGACAAACACCT	CAGCGGGCATGTTTTAAAGT
CYP3A9	**U60085**	GGACGATTCTTGCTTACAGG	ATGCTGGTGGGCTTGCCTTC
CYP2B15	**XM_001070774***	GCTCAAGTACCCCCATGTCG	ATCAGTGTATGGCATTTTACTGCGG
CAR	**AB105071**	ACCAGATCTCCCTTCTCAAG	CTCGTACTGGAACCCTA

* coding for CYP2B1 according to caron et al (Caron et al., 2005)

Table II – Metabolic rates (pmol/min/mg protein) during 27 h of incubation and percentages of retained metabolic rates compared with 0-3 h of incubation in small intestinal slices. Results (mean ± SEM) are extracted from figures 2 and 6 (n = 3-8 rats), 7HC data are extracted from 'not shown' data.

Metabolite formed	0-3 h (100%)	5-8 h	(%)	8-11 h	(%)	24-27 h	(%)
7HC (CYP1A, 1B)	18 ± 3.2	14.9 ± 5.9	**(83)**	16.3 ± 4.2	**(91)**	6.8 ± 4.5*	**(38)**
6β-TOH (CYP3A)	16.1 ± 3.4	7.8 ± 1.5	**(48)**	11.3 ± 1.2	**(70)**	3.9 ± 1.1**	**(24)**
Androstenedione (CYPs, 17β-HSD)	147 ± 22	143 ± 32	**(97)**	201 ± 23	**(137)**	93 ± 29	**(63)**
7HC-GLUC	377 ± 93	259 ± 38	**(69)**	341 ± 53	**(91)**	162 ± 31	**(43)**
7HC-SULF	39 ± 9.6	41 ± 8.1	**(105)**	45 ± 8.2	**(115)**	30 ± 8.4	**(77)**

Significantly different from 0-3 h with $p < 0.05$

Significantly different from 0-3 h with $p < 0.01$

Table III: Metabolic rates (pmol/min/mg protein) during 27 h of incubation and percentages of retained metabolic rates compared with 0-3 h of incubation in colon slices. Results (mean ± SEM) are extracted from figures 2 and 6 (n = 3-6 rats), 7HC data are extracted from 'not shown' data.

Metabolite formed	0-3h (100%)	5-8h	(%)	8-11h	(%)	24-27h	(%)
7EC (CYP1A, 1B)	22.1 ± 9.5	31.5 ± 16	(143)	17.6 ± 5.9	(80)	7.2 ± 4.6	(32)
6β-TOH (CYP3A)	4.7 ± 1.7	3.0 ± 2	(64)	4.1 ± 1.3	(87)	9.2 ± 1.8	(196)
Androstenedione (CYPs, 17β-HSD)	95 ± 26	62 ± 8	(65)	77 ± 24	(81)	85 ± 24	(90)
7HC-GLUC	653 ± 57	509 ± 45**	(78)	635 ± 194	(97)	579 ± 212	(89)
7HC-SULF	64 ± 6.4	58 ± 6*	(91)	58 ± 16	(90)	50 ± 7.5	(78)

Significantly different from 0-3 h with p < 0.05

**Significantly different from 0-3 h with p < 0.01*

Figure 1

A: ATP

B: AP activity

C: GAPDH expression

D: Villin expression
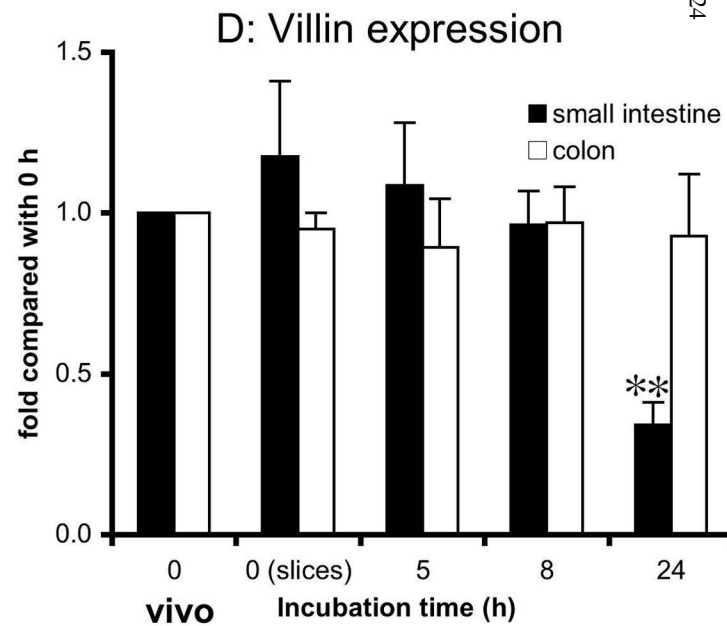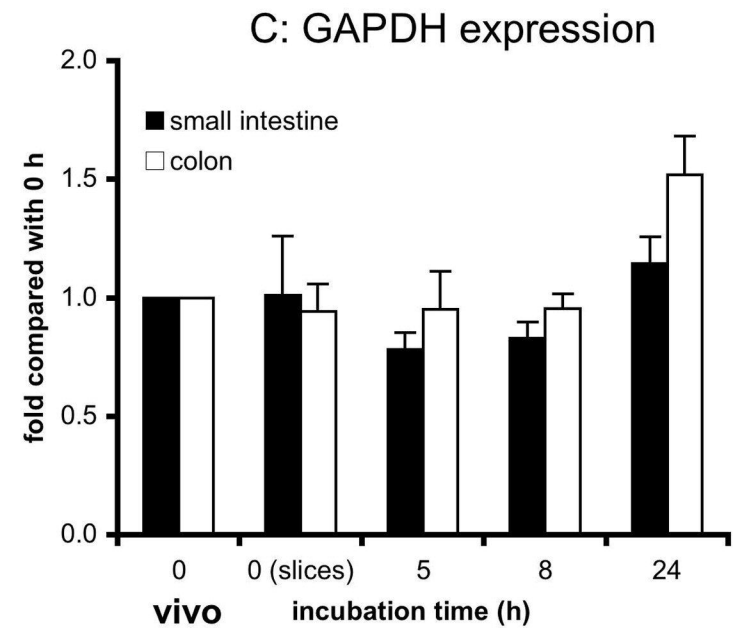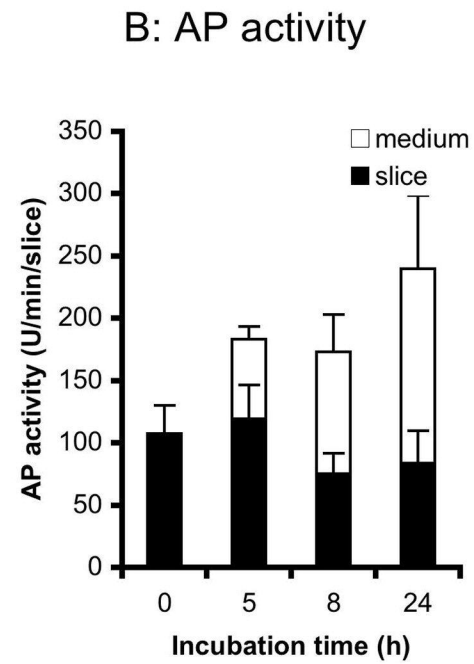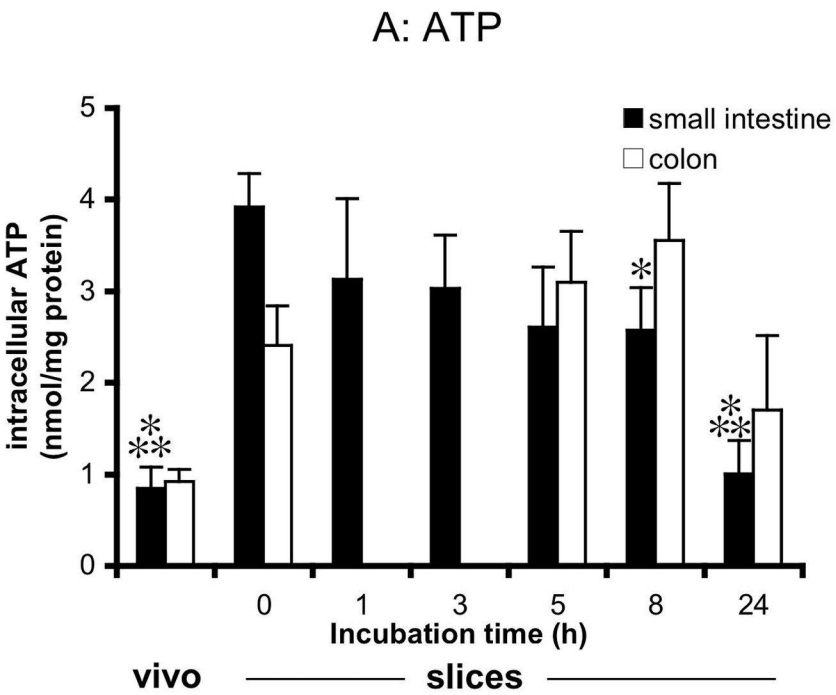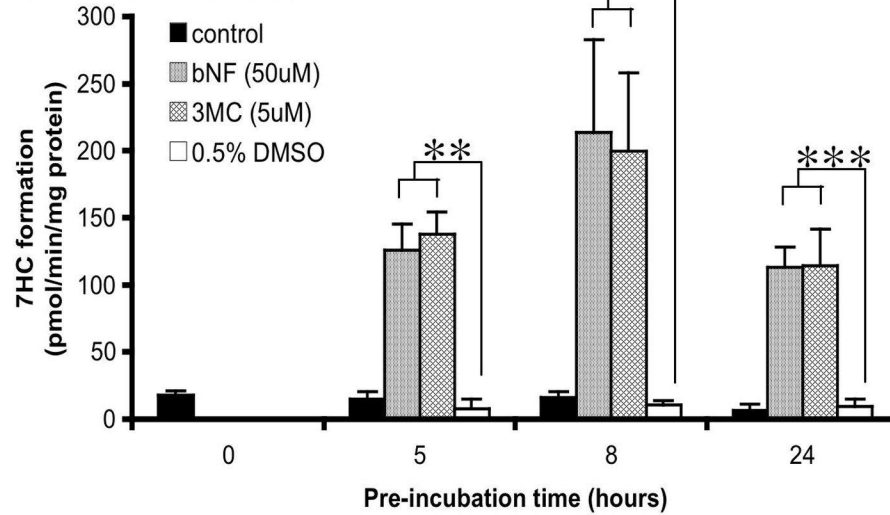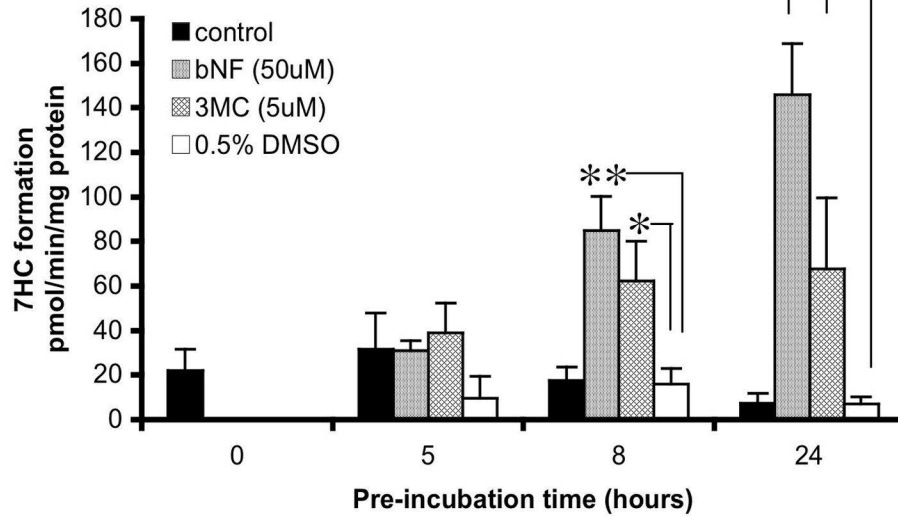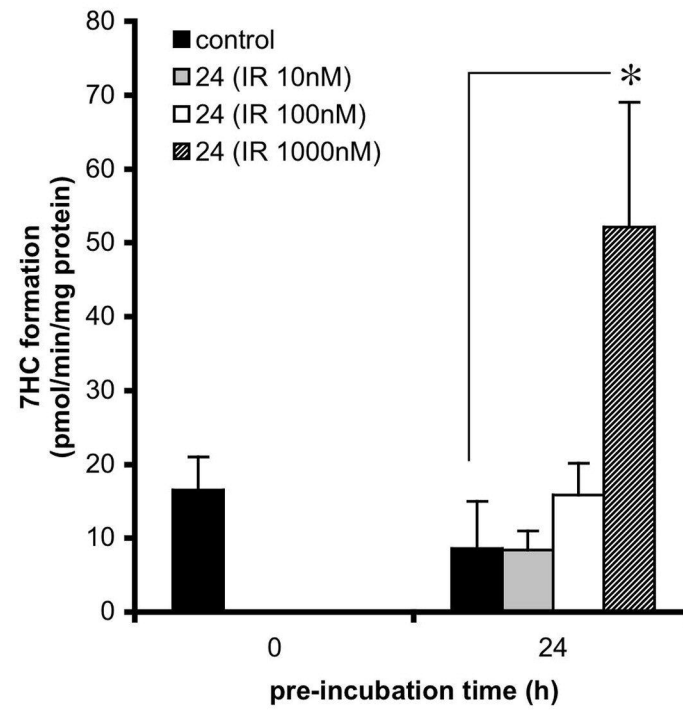
Figure 2

A: Small intestine

B: Colon

Figure 3

7HC to 7HC-GLUC formation

A: Small intestine
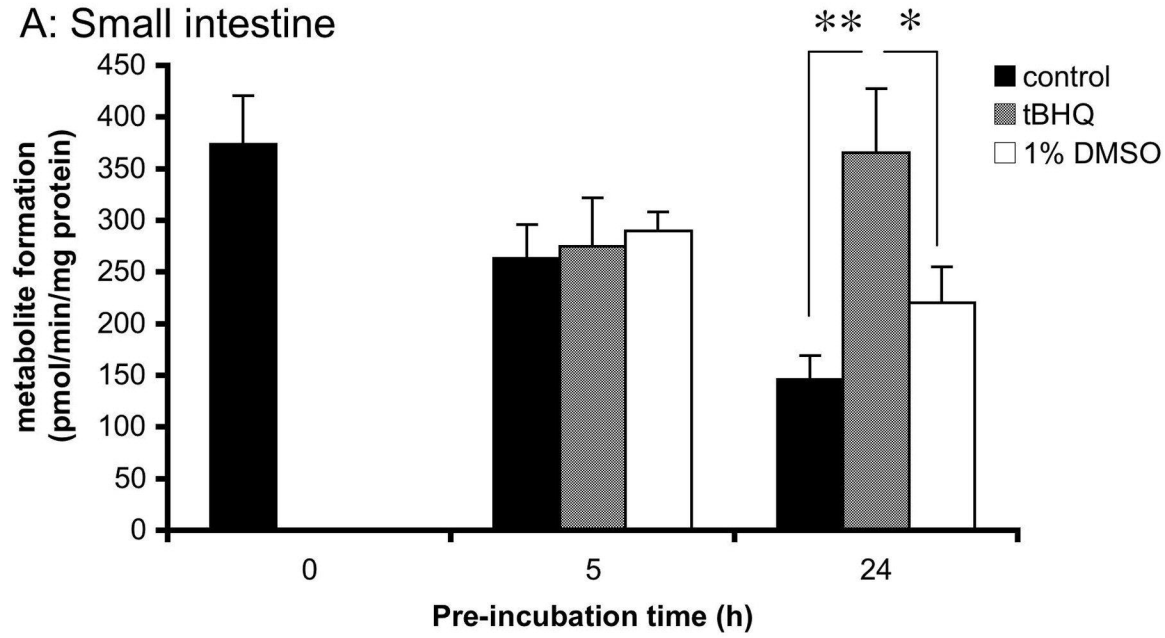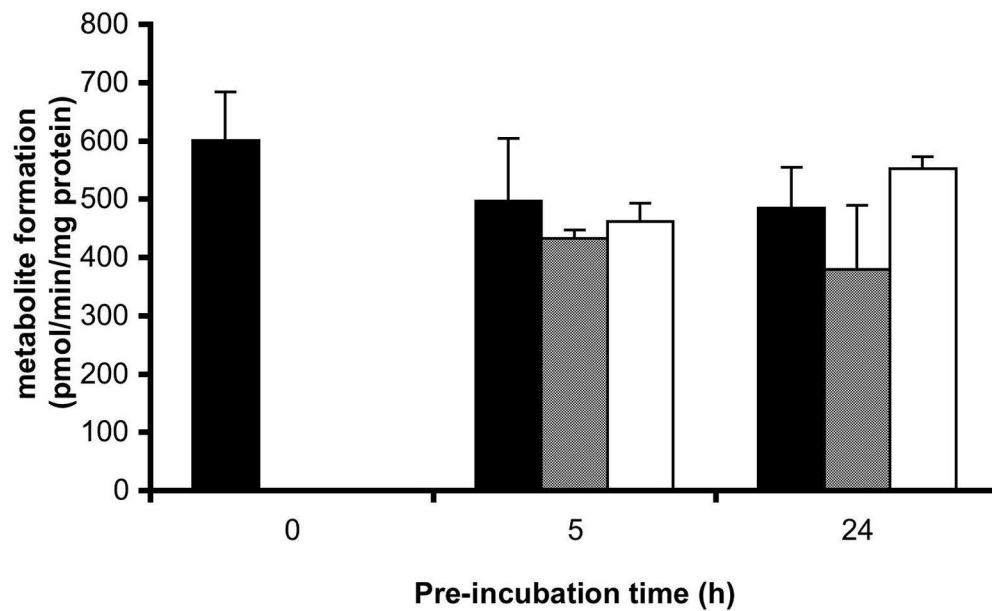
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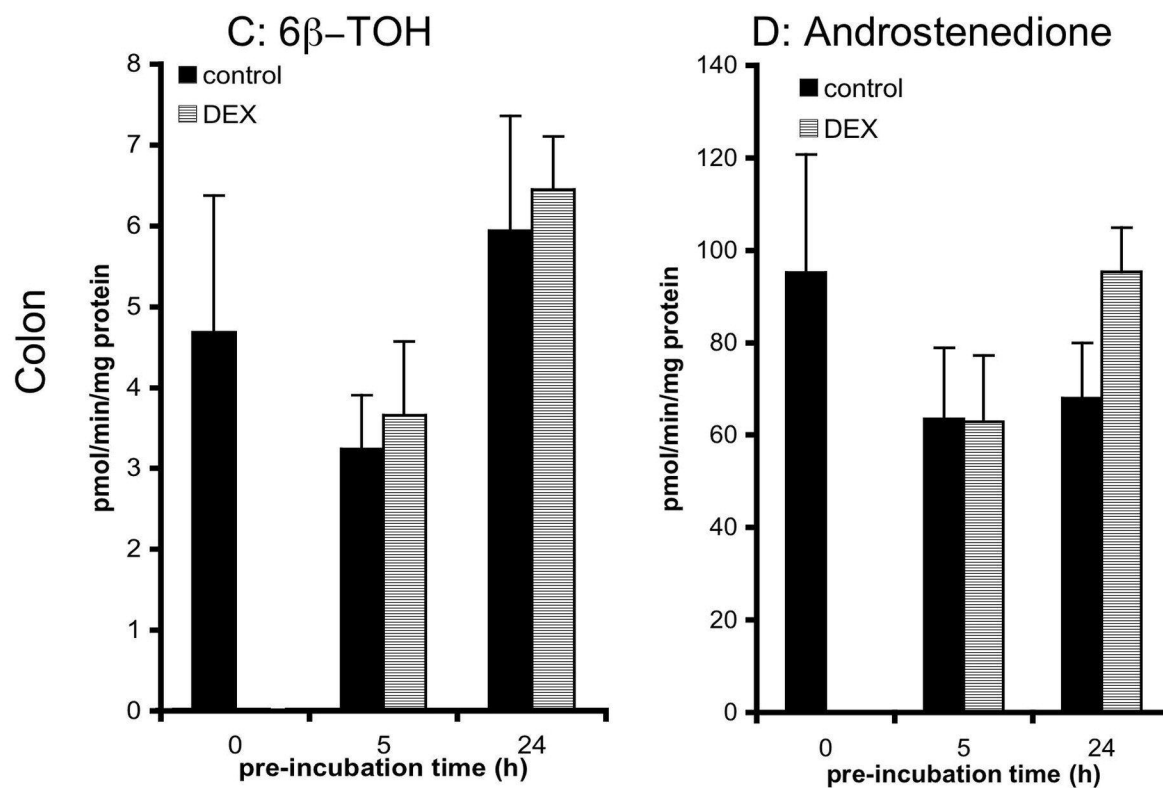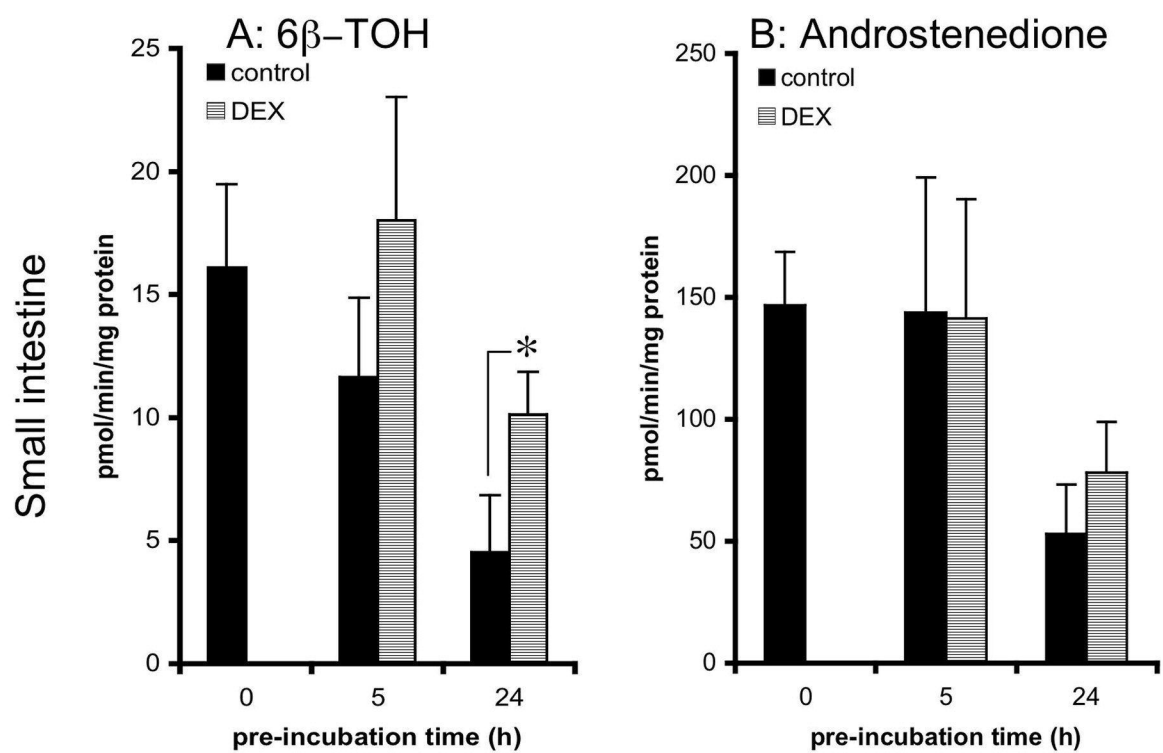
Figure 4

Figure 5

Figure 6

Small intestine

A: 7HC-GLUC   B: 7HC-SULF   C: 7HC   D: 6β–TOH   E: Androstenedione

Colon

F: 7HC-GLUC   G: 7HC-SULF   H: 7HC   I: 6β–TOH   J: Androstenedione

Figure 7