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Ex vivo stimulation of renal tubular *p*-aminohippurate transport by dexamethasone and triiodothyronine in human renal cell carcinoma

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Abstract This paper is the third of a long-term planned series of papers dealing with ex vivo investigations of drug transport in human kidney. The aims of this study are (a) to investigate whether or not human renal cell carcinoma (RCC) can actively accumulate *p*-aminohippurate (PAH) and (b) to test the response of RCC on dexamethasone or triiodothyronine (T3) using tissue slices ex vivo. By this approach, the accumulation capacity of RCC should be stimulated as a prerequisite for an increased uptake of anti-tumour drugs. Tissue slices of RCC samples of 30 patients were incubated for 24 h in Williams medium E containing 0.01–50 μM dexamethasone or T3. Thereafter, slices were placed in PAH-containing Cross–Taggart medium, and PAH uptake into kidney tissue was measured for 2h under standardised conditions as described previously. In intact human renal cortical slices, PAH uptake capacity, expressed as slice to medium ratio ($Q_{S/M}$), was about 2.8 ± 0.16 after 24 h of incubation and increased significantly in dexamethasone-containing medium in a concentration-dependent manner, up to ~150%, whereas T3 did not influence PAH accumulation. On the other hand, in RCC the PAH accumulation capacity was completely abolished ($Q_{S/M}$~1). However, after administration of dexamethasone, the accumulated amount of PAH increased significantly in RCC tissue in a concentration-dependent manner, up to ~190%. T3 was without effect in RCC, too. Surprisingly, the dexamethasone-mediated stimulation could be differentiated into responders and non-responders, with maximal effects at different concentrations for each patient. Nevertheless, the maximal transport rates remained low in RCC, even under hormone influence. In conclusion, a moderate stimulation of tubular transport capacity can be shown ex vivo in human RCC. This phenomenon is only of a relatively low degree compared with intact renal tissue. However, in principle, the response of RCC on dexamethasone could form a basis for further therapeutic strategies to overcome multi-drug resistance in RCC patients. For this purpose, additional experiments analysing the expression of transporters of the ABC cassette-type are in progress.

Key words Renal tubular transport · *p*-Aminohippurate · Stimulation · Renal cell carcinoma · Dexamethasone · Triiodothyronine

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Introduction

The present paper is the third part of a long-term planned series of investigations dealing with ex vivo characterisation of drug transport in human kidney. In previous experiments, it could be shown that *p*-aminohippurate (PAH) accumulation in intact human renal tissue can be stimulated after incubation of renal cortical slices in dexamethasone-containing medium [10]. PAH is transported across the basolateral membrane of the renal proximal tubule, a mechanism indirectly coupled to sodium co-transport [32]. The general possibility of stimulating the renal excretion capacity of PAH in rats after repeated administration of dexamethasone or triiodothyronine (T3) and other suitable substances in vivo has been reviewed previously [7]. The following reasons could be responsible for the stimulation of renal tubular transport capacity: (a) an increase in protein de novo synthesis [23]; (b) an increase in glomerular filtration rate (GFR) [4]; (c) a raise in renal blood flow [8]; (d) systemic hormone effects; and (e) metabolic changes after hormone pre-treatment. The latter two reasons could not be differentiated clearly as yet. On the basis of the results obtained on rats and on intact human kidney tissue, in this study the renal PAH transport capacity

should be characterised in human renal cell carcinoma (RCC). We could show previously that the accumulation capacity of RCC tissue is significantly reduced [10]. The question arose whether or not the accumulation capacity of RCC is under hormonal control and can be enhanced after incubation with dexamethasone or T3.

PAH uptake into renal cells is quite different from that of anticancer drugs. Nevertheless, the final goal of our experiments is the stimulation of the uptake of substances like cytostatics into human kidney tissue, especially RCC, to overcome the so-called multi-drug resistance (MDR) in RCC, because MDR is a major obstacle to effective chemotherapy of RCC [34]. It is well known that RCC and its metastases are quite insensitive to any kind of cancer chemotherapy [2, 25]. MDR is reported to be caused by the expression of the MDR 1 gene and it is characterised by a broad spectrum cross-resistance to many anticancer drugs [31, 33]. Human kidney proximal tubules express the ATP-dependent export pump for anionic conjugates encoded by the multi-drug resistant protein (MRP 2) [16] or the lipoprotein receptor-related protein (LRP 1) gene [18], too. This MRP is localised to the apical brush-border membrane domain of proximal tubules. MRP 2, the first cloned ATP-dependent export pump for anionic conjugates detected in human kidney, may be involved in renal excretion of various anionic endogenous substances, xenobiotics and cytotoxic drugs. This conjugate-transporting ATPase has a similar substrate specificity to that of MRP 1, and may contribute to the MDR of renal clear-cell carcinomas [27]. The favoured hypothesis to explain the phenomenon of MDR is an increase in transport of anticancer drugs out of RCC cells [15, 17, 22]. Therefore, a stimulation of the uptake of cytostatic drugs could be beneficial in the treatment of RCC and its metastases. However, it has to be kept in mind that RCC metastases have altered MDR 1 expression, potentially due to altered differentiation relative to the primary tumour. Thus, the drug resistance phenotype of primary RCC may not reflect that of its metastases [11]. The investigation of the stimulation of the well-known PAH transport in RCC is a first step in this direction. In further experiments, results obtained with PAH have to be proven with anticancer drugs used in RCC chemotherapy as described for the combination between interferon and dexamethasone [1].

Material and methods

Patients

Between June 1998 and December 1999, in vitro accumulation experiments were performed on human kidney slices. Intact renal cortical tissue, i.e. macroscopically inconspicuous material of tumour-bearing kidney (control), and RCC tissue were obtained from 30 patients undergoing radical nephrectomy of the tumour in the Department of Urology, Friedrich Schiller University, Jena, Germany. This study was performed in accordance with internationally accepted ethical standards for human experimentation and was approved by the Ethical Review Committee of the Medical Faculty of the University of Jena. RCC were classified into groups pT_1–pT_3 without multi-focal localisation. The distance between intact renal tissue and RCC was $\leq$4 cm. The patients were 16 men and 14 women with an average age of 61.1 $\pm$ 11.7 years. Tissue samples (about 1 g) were stored in normal saline on ice (4 °C) immediately after kidney removal. After transportation (30 min), slices were prepared as described below.

Accumulation experiments

Acute experiments (2 h)

Renal cortical slices (intact renal tissue or RCC) with pool sizes of about 100 mg ($\sim$1 mm thick) were prepared free-hand and were incubated in 50-ml Erlenmeyer flasks with PAH (E. Merck, Darmstadt) with bidirectional shaking (about 100 rpm) in 3 ml of Cross–Taggart buffer (pH 7.4; 30 °C; oxygen gassing (2.5 l/h per sample); incubation time 120 min; PAH concentration: 8.5×10^{-5} M). To exclude acute hormone effects, the medium was supplemented with either dexamethasone or T3 as described below. Following incubation, PAH was determined in the supernatant fraction of the homogenate and in the incubation medium. The active uptake of PAH was expressed as the ratio between PAH concentration in the tissue and in the medium after the end of incubation (slice to medium concentration ratio, $Q_{S/M}$) according to Stopp and Bräunlich [30]. The concentrations of PAH were determined using the colorimetric method introduced by Bratton and Marshall [3].

Sub-chronic experiments (24 h)

Experiments were performed with a 24-h incubation of renal cortical slices ($\approx$100 mg) of intact renal tissue or of RCC tissue in 50-ml Erlenmeyer flasks loaded with 10 ml of Williams medium E (WME; Bio Whittaker), which was supplemented with L-glutamine (292 mg/l), insulin (1 μM), gentamicin (50 mg/l), and different concentrations of dexamethasone (Fortecortin Mono; E. Merck, Darmstadt) or T3 (Sigma, St. Louis, Mo., USA) under carbogen gassing (95% O_2/5% CO_2; 2.5 l/h per sample), adjusted to pH 7.4 and 25 °C. Thereafter, slices were placed in Cross–Taggart buffer (pH 7.4) containing only PAH, and the uptake of PAH from the medium into the slices was measured in 2-h accumulation experiments, as described above.

Determination of glutathione and potassium content of renal cortical slices

Potassium was measured by flame photometry in the renal slice supernatant. For details see [10]. Reduced (GSH) and oxidised (GSSG) glutathione were determined in accordance with Kretzschmar and Klinger [14] and Hissin and Hilf [12] , respectively, with slight modifications.

Statistics

The results are given as arithmetic mean $\pm$ SEM of four to six independent slice preparations. In every patient, three to four slice preparations were used per medium concentration to minimise methodological variances. Statistically significant differences between various experimental groups were analysed using the Mann–Whitney test or paired *t*-test ($P \leq 0.05$).

Results

In the acute accumulation experiments (2-h incubation), the human tissue slices were in good condition: both

potassium concentrations and GSH or GSSG content are in the normal range measured previously in comparable experiments (Table 1). As shown in Fig. 1, in freshly prepared intact human kidney tissue, PAH is accumulated about fivefold compared with medium concentration ($Q_{S/M} = 5.03 \pm 0.41$). These values are in good accordance with previous results and correspond well with the accumulation capacity of, e.g. rats [6]. On the other hand, in RCC, the PAH uptake is completely abolished ($Q_{S/M} \leq 1$). Only in patients 24 and 28 could a distinct PAH accumulation be shown ($Q_{S/M} \sim 1.5$).

After 24 h of incubation in WME, the markers for the quality of the slices (intracellular K^+ concentration, GSH, GSSG) are distinctly deteriorated (e.g. reduction in GSH and GSSG by about 50%, see Table 1); nevertheless, intact human kidney tissue accumulates PAH by a factor of ~ 3 (Fig. 2). This significant reduction in PAH accumulation capacity is known from preliminary experiments on rats and men [10], but it does not disturb the further interpretation of these experiments because the enrichment of PAH is high enough for further experimental approaches. Interestingly, in RCC the PAH uptake seems to be slightly enhanced compared with the acute experiments performed immediately after kidney removal ($Q_{S/M}$: 1.2 vs 0.98; compare Figs. 1 and 2). However, this moderate increase in PAH accumulation is of minor importance, despite the accumulation capacity of 4 from 22 RCC tissue samples is in the same range as that of intact kidney tissue (patients 4, 5, 7, 25).

The main goal of the present paper was to clarify the possibility of stimulating the PAH uptake in RCC by 24-h incubation of the tissue slices in hormone-containing WME. Also under these experimental conditions, the general parameters characterising the slice survival after a 24-h incubation were not distinctly changed (Table 1). Preliminary experiments have shown that the accumulation capacity in both rat's and man's intact kidney tissue can be stimulated 1.5-fold after incubation in dexamethasone containing medium, whereas T3 was without effect. Interestingly, these stimulatory effects were quite uniform in rats and, this is important, in *intact* human tissue. That means the effect occurred with only small variations independently of the respective rat or patient. In the case of RCC, T3 was without effect, too (see Fig. 5). However, concerning the responsiveness of RCC to dexamethasone, the 13 patients investigated can be divided into six "responders" and seven "non-responders" (Fig. 3). The stimulation after dexamethasone reaches about 190 ± 32% in the group of responders. The reasons for these different reactions after dexamethasone incubation could depend on the tumour stage or tumour histology and will be considered in more detail in the discussion.

A further peculiarity of the reaction of RCC tissue slices after dexamethasone incubation consists in its concentration dependency (Fig. 4): The "responders" reacted maximally at different dexamethasone medium concentrations, most pronounced between 0.5 μM and

Table 1 Comparison between the influence of dexamethasone (*Dexa*) and T3 on potassium concentration, reduced glutathione (GSH) and oxidised glutathione (GSSG) in intact renal cortical slices (five controls) and in RCC (seven patients) after 2 or 24 h incubation under oxygen gassing. Arithmetic mean ± SEM; $n = 3$–4 samples per patient

	Two hours incubation						Twenty-four hours incubation					
	Controls			RCC			Controls			RCC		
	NaCl	Dexa	T3	NaCl	Dexa	T3	NaCl	Dexa	T3	NaCl	Dexa	T3
Potassium (μmol/g)	49 ± 4	62 ± 4	53 ± 3	49 ± 4	53 ± 3	48 ± 3	36 ± 4	53 ± 7	42 ± 4	44 ± 5	48 ± 2	43 ± 3
GSH (μmol/g)	1210 ± 150	1520 ± 195	1405 ± 120	948 ± 47	1184 ± 62	932 ± 34	725 ± 30	710 ± 60	860 ± 40	984 ± 51	731 ± 103	846 ± 39
GSSG (μmol/g)	320 ± 35	290 ± 25	169 ± 65	307 ± 94	267 ± 74	283 ± 74	150 ± 10	185 ± 15	182 ± 44	131 ± 36	182 ± 14	131 ± 24

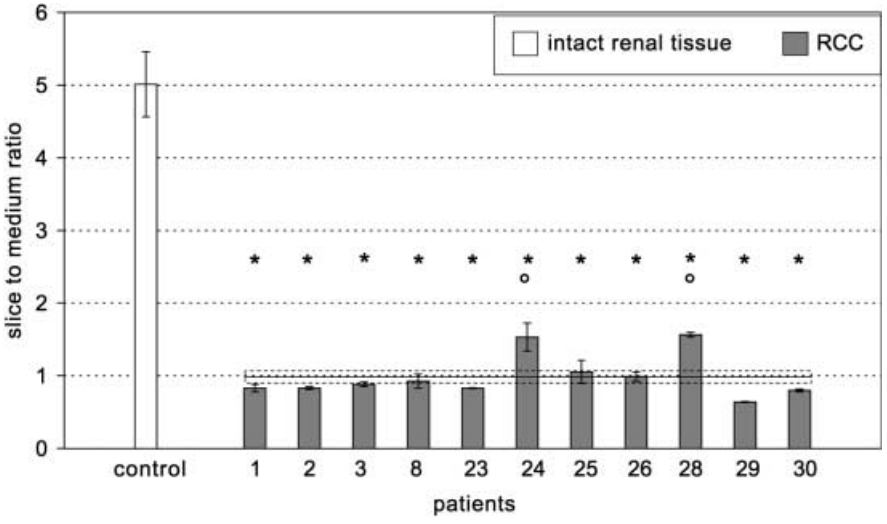

Fig. 1 PAH accumulation capacity ($Q_{S/M}$, 2-h incubation) in renal cortical slices of intact human kidney (mean value = control) and in 11 RCC patients. Arithmetic mean ± SEM; $n = 3$–4 samples per patient; *significant reduction of $Q_{S/M}$ in RCC, °significant differences between single value of RCC and RCC mean value (*line*); ($P \leq 0.05$)

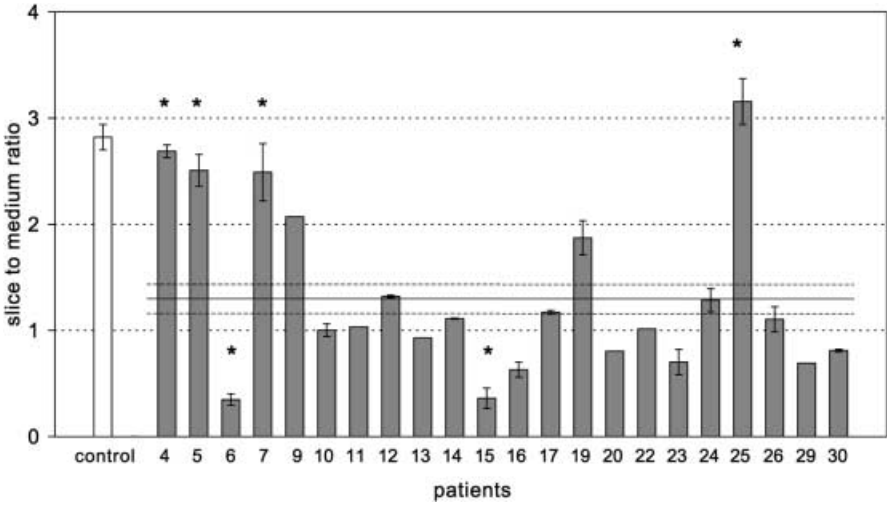

Fig. 2 PAH accumulation capacity ($Q_{S/M}$) after a 24-h incubation of renal cortical slices of intact human kidney (mean value = control) and in 22 RCC patients. Arithmetic mean ± SEM; $n = 3$–4 samples per patient and $n = 9$ patients for controls; *significant difference between single RCC value and RCC mean value (*line*); ($P \leq 0.05$)

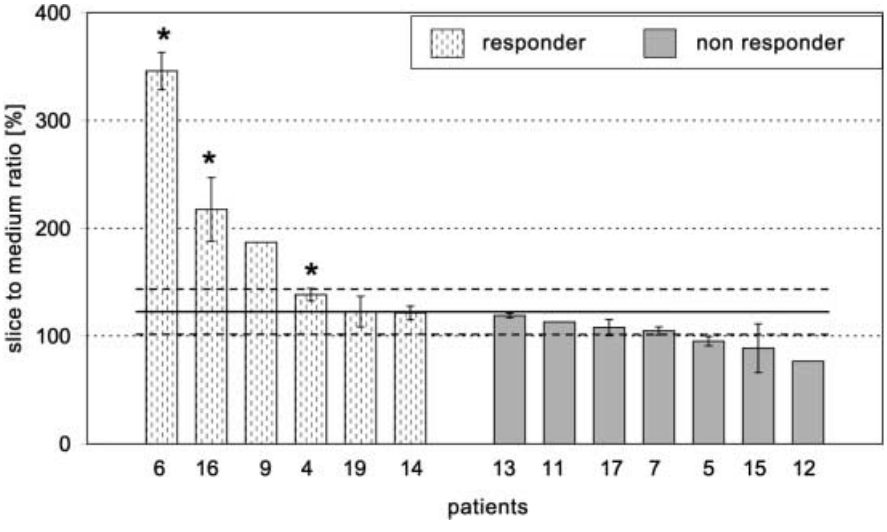

Fig. 3 Influence of dexamethasone on the PAH accumulation capacity of 13 RCC (the number of patients corresponds with Fig. 2) after a 24-h incubation in dexamethasone-containing WME. The patients were classified into ''responders'' and ''non-responders'' to dexamethasone. Arithmetic mean ± SEM; $n = 3$–4 samples per patient; *continuous line* = mean ± SEM, * significant differences between RCC and controls (= 100%); ($P \leq 0.05$)

1 μM. Only in patient 9, is a further increase in dexamethasone medium concentration above 1 μM followed by a decline in PAH accumulation capacity.

For the characterisation of the energy dependence of hormone effects, experiments were performed under oxygen or nitrogen gassing (Fig. 5). In principle, without

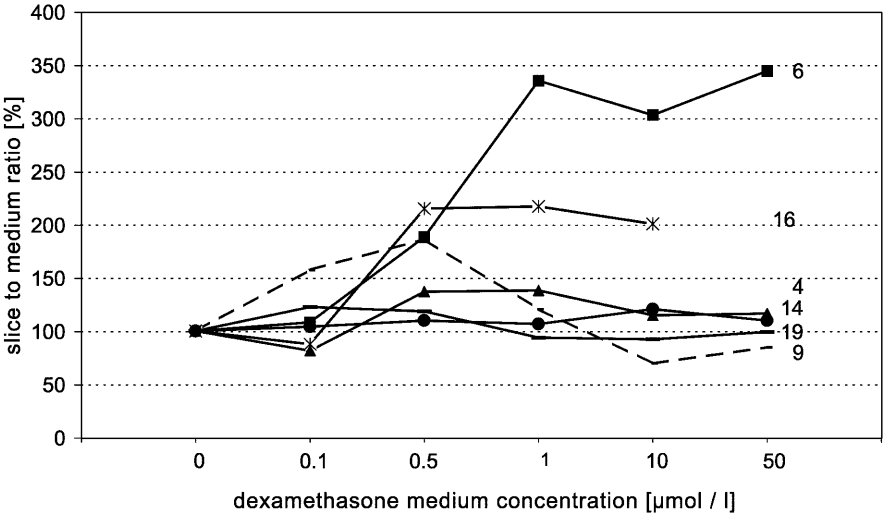

Fig. 4 Dose dependence of the dexamethasone effect on the PAH accumulation capacity after a 24-h incubation in dexamethasone-containing WME of the six "dexamethasone responders" (patients = numbers at curves; see Fig. 3). Extent of stimulation are given in % of slices incubated in WME alone. Arithmetic mean ± SEM; $n = 3$–4 samples per patient; * significant changes in $Q_{S/M}$ ($P \leq 0.05$)

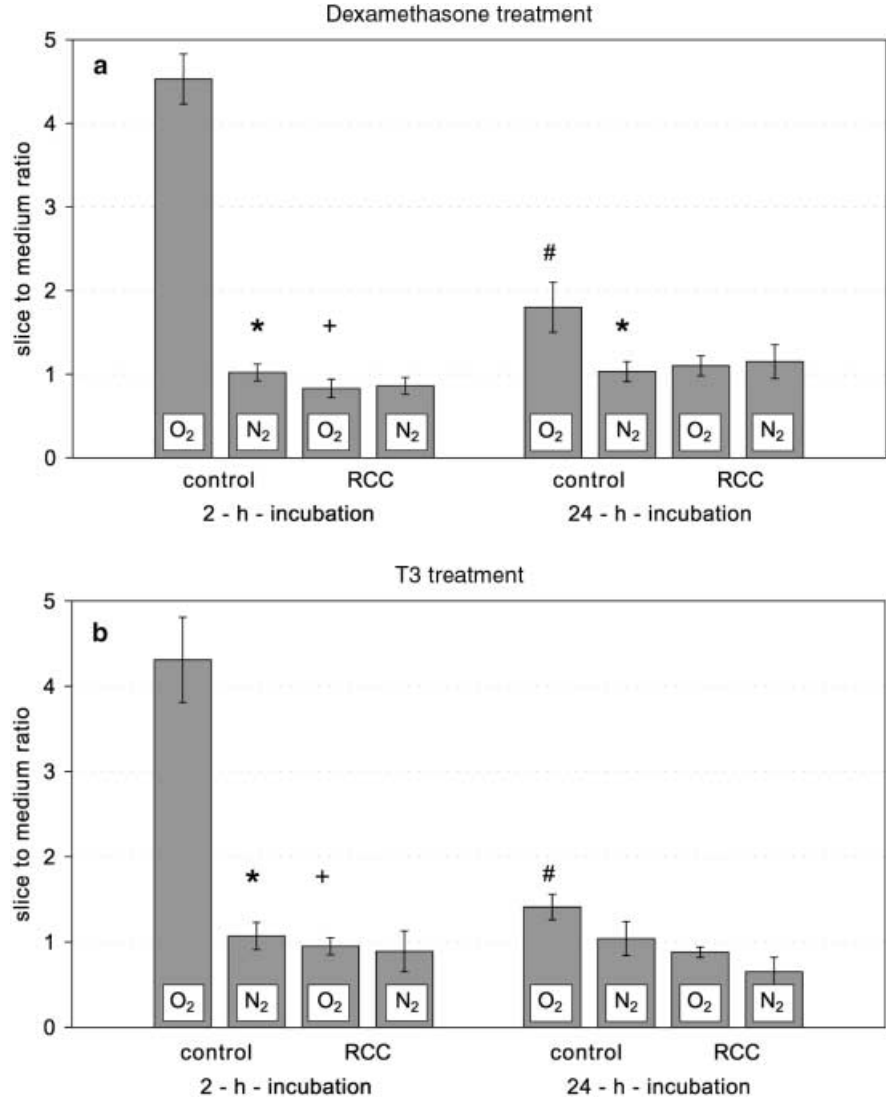

Fig. 5 Comparison between the influence of dexamethasone or T3 on the PAH accumulation capacity ($Q_{S/M}$) after 2 or 24 h incubation of intact renal cortical slices (controls) or of RCC under oxygen or nitrogen gassing. Arithmetic mean ± SEM; $n = 3$–4 samples per patient; 12 controls or RCC were investigated (8 × dexamethasone, 4 × T3); *significant influence of nitrogen, + significant differences between controls and RCC, # significant influence of 24 h hormone pre-treatment ($P \leq 0.05$)

an oxygen supply, no active PAH accumulation occurs in any case. Thus, the stimulatory effect of incubation of intact human kidney tissue and RCC tissue in dexamethasone-containing medium is clearly an energy-consuming active process, whereas in the absence of oxygen, an equilibrium between tissue slices and medium occurs and $Q_{S/M}$ values reach only about 1.

Discussion

Five important findings have emerged from the present study:

1. Ex vivo experiments are qualified to characterise the renal PAH accumulation capacity in human kidney slices.
2. Compared with intact human renal cortical slices, the accumulation capacity of RCC is tremendously diminished. There is nearly no active PAH uptake in RCC.
3. Both in intact kidney tissue and in RCC, the PAH accumulation capacity can be stimulated after 24–h of incubation in dexamethasone-containing WME, whereas T3 is without effect in intact and in RCC slices.
4. Evidently the reactivity of RCC on dexamethasone is not uniform: ''responders'' and ''non-responders'' can be differentiated. A correlation between accumulation capacity and tumour stage cannot be calculated because of the small number of patients.
5. The extent of the dexamethasone effect seems to be more pronounced in RCC than in intact human kidney tissue.

As reported previously [10], the possibility of stimulating the renal tubular transport capacity ex vivo in intact human kidney tissue by dexamethasone could be proven, whereas T3 was without effect. But, as shown by Fleck et al. [10], the PAH transport capacity of RCC disappeared nearly completely. Only patients 24 and 28, whose RCC tissue slices accumulated PAH at least in part, suffered from a relatively early stage of RCC. Therefore it was uncertain whether or not progressive stages of RCC are under hormonal control. The same explanation could be responsible for the differentiation into ''dexamethasone responders'' and ''non-responders'': in principle, the TNM classification of responders was not as serious than that of the non-responders. In other words, a response to dexamethasone pretreatment can be expected only in early stages of RCC. This assumption is supported by the findings of Tobe et al. [31]. Their comparisons of MDR 1 levels between histological types revealed significantly more MDR 1 in clear-cell tumours than in oncocytomas. However, the mean MDR 1 level of the more undifferentiated clear cell tumours was significantly lower than that of adjacent normal kidney. MDR 1 levels in early-stage clear-cell tumours were lower than in tumours that had spread into perinephric tissue or had metastasised. In conclusion, MDR 1 expression decreases in the more undifferentiated tumours, but still remains at levels high enough to be drug resistant. Higher MDR 1 expression in the invasive tumours than in non-invasive tumours suggests that MDR 1 expression and invasiveness may be linked. MDR 1 expression seems to correlate with the differentiation of the RCC; thus, its value as an objective measure of the degree of differentiation should be further explored [13].

If it were possible to increase the uptake of drugs into RCC under in vivo conditions, it might be feasible to enhance the uptake of anticancer drugs into RCC and its metastases and, therefore, to improve the efficacy of the chemotherapy of RCC. Without doubt this is only one side of the coin: the enhanced uptake of anticancer drugs could be followed by an increase in their export out of the tumour cell. Therefore, the findings of this study may reflect the sum of two different influences: dexamethasone could both increase PAH uptake into RCC and accelerate the efflux out of the RCC. These two phenomena cannot be differentiated by our experimental approach. Furthermore, MDR can be either intrinsic or acquired, and can be caused by several mechanisms. The so-called classical MDR has been held mainly responsible for MDR phenotype on urological malignancies. However, several other MDR pathways have been identified. MDR can be caused by the membrane-bound MDR-associated protein, the detoxifying glutathione metabolism, the antiapoptotic protein BCL2, and changes in levels or activity of the topoisomerase enzymes [33].

A second unsolved problem consists in the chemical differences between PAH (weak organic acid) and most anticancer drugs (bases, alkaloids, nucleotides). Therefore, future experiments will investigate the effect of dexamethasone, T3 and other factors influencing renal transport functions, such as epidermal growth factor (EGF), on the uptake of methotrexate, *cis*-platinum, tomudex or topotecan into RCC. It has to be taken into consideration that, in cases of anticancer drugs, hormones other than dexamethasone, such as T3 or EGF, could enhance the enrichment of these compounds in RCC because they could both increase the uptake and reduce the efflux of anticancer drugs out of RCC. By this approach it could be possible to overcome the MDR of RCC against cytostatics [5, 29].

The following limitations should be considered when interpreting the results of the present study:

1. The accumulation capacity of both intact human kidney tissue and RCC can be stimulated ex vivo. However, it remains open whether the stimulation phenomenon, measured after incubation with dexamethasone, is the consequence of increased de novo synthesis of carrier proteins as assumed previously [4]. Our own preliminary polymerase chain reaction (PCR) data show that evidently PAH carrier synthesis could be enhanced by dexamethasone influence (unpublished results). On the other hand, the stimulation of PAH transport could result from a better nutritional state of the renal cortical slices indicated by the general parameters intracellular K^+ and glutathione (see Table 1).

2. Dexamethasone is a substrate to the MDR 1 gene product [28]. Therefore, it cannot be excluded that ex vivo dexamethasone interferes with the transport proteins evacuating PAH from the tubular cell [32]. Nevertheless, the dexamethasone-mediated stimulation of PAH accumulation indicates the increased PAH uptake compared with its efflux.

3. Anticancer chemotherapy is focussed first of all on RCC metastases. It has been reported previously that cellular expression of P-glycoprotein, which mediates MDR, is associated with an enhanced tumour dissemination, i.e. metastases are more susceptible to anticancer drug therapy [19]. Beside this, the administration of high-dose dexamethasone could disturb the general metabolic status in the organism. Therefore, also in intact tissues, the effect of anticancer drugs could be enhanced and side effects of high-dose dexamethasone therapy should be kept in mind.

Altogether, RCC is a disease known to demonstrate a high degree of intrinsic chemotherapy drug resistance, and this has been shown to be related to intrinsic overexpression of P-glycoprotein [26]. We conclude that advanced RCC is a highly chemo-resistant tumour, and that the addition of high doses of dexamethasone or T3 was not able to modulate this resistance in the patient's renal tissue slices. Nevertheless, combination therapy seems to be the method of choice: the combination of various drugs – cytostatics plus, for example, dexamethasone or, as reported by Naito et al. [21] and Punt et al. [24], different anticancer drugs, significantly increased the life span of mice inoculated with drug-resistant tumour cells without any significant side effects; whereas chemotherapy with the anticancer agent alone did not increase the life span at all.

In the future it is planned to further characterise the expression of molecular transporters of the ABC cassette type [35] such as MDR 1 or MDR 3 [20], MRP 1, 2 and 6 [16] or lipoprotein receptor-related protein (LRP 1) [18]. These experiments are in progress and the results will be reported as soon as an adequate amount of RCC tissue is available from a sufficient number of patients.

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