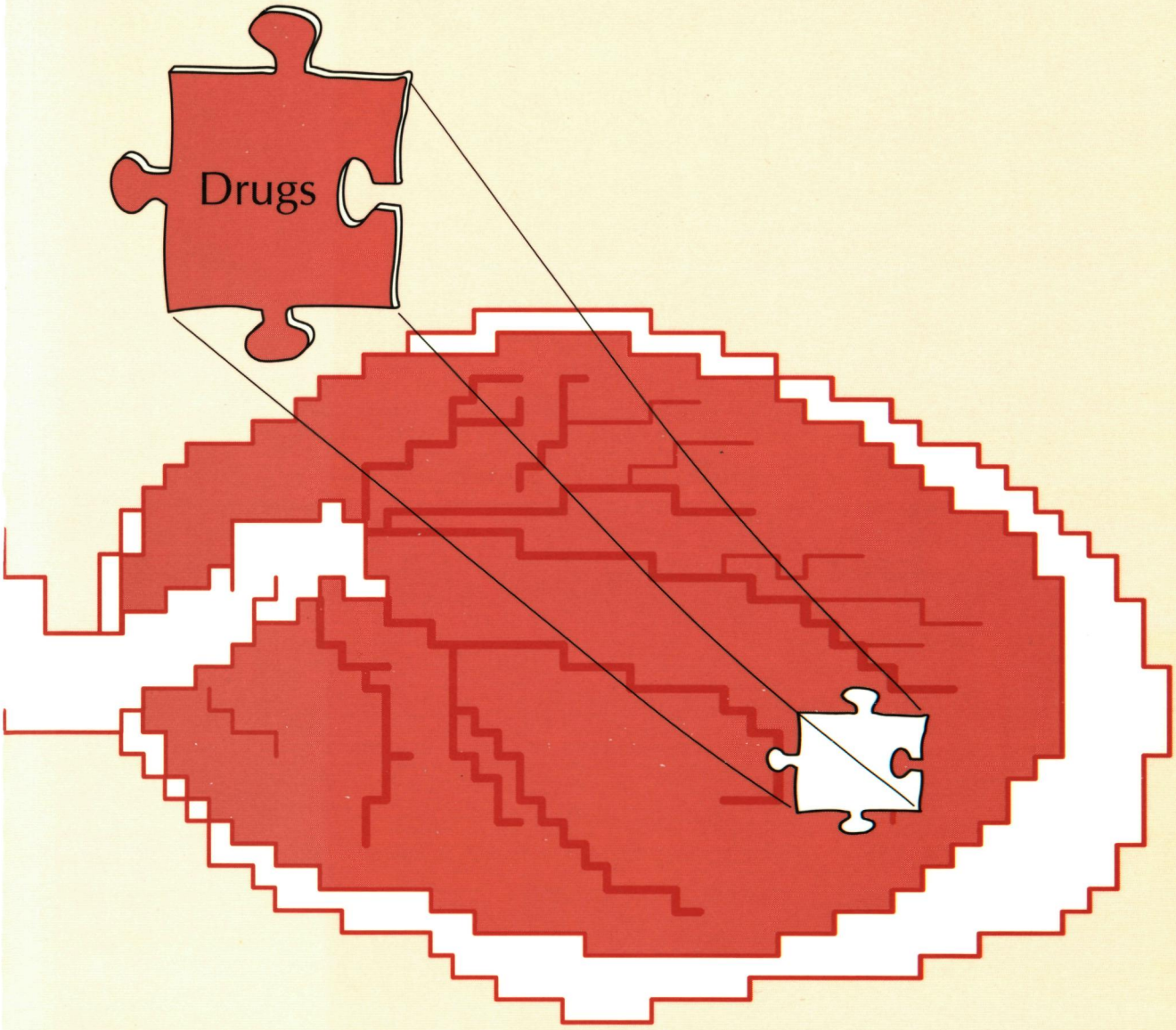
Drug transport and drug-nutrient interactions in the human placenta



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DRUG TRANSPORT AND DRUG-NUTRIENT INTERACTIONS IN THE HUMAN PLACENTA

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Contents

Abbreviations

AIB	α-aminoisobutyric acid
ATP	adenosinetriphosphate
BM	basal membrane
BMV	basal membrane vesicles
DIDS	4,4'-diisothiocyanostilbene-2,2'disulfonic acid
EDTA	ethylenediaminetriacetic acid
FCCP	carbonyl cyanide p-trifluoromethoxyphenylhydrazone
HC-3	hemicholinium-3
HEDTA	N-hydroxyethyl-ethylenediaminetriacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Performance Liquid Chromatography
M_o	starting mince
mepi	mepiperphenidol
MES	2-(N-morpholine)ethanesulfonic acid
MHT	mannitol-Hepes-Tris buffer
NMN	n-methylnicotinamide
NTA	nitrilotriacetic acid
PAH	p-aminohippurate
pH_i	pH inside the vesicle
pH_o	pH outside the vesicle
SMM	syncytial microvillous membrane
SMMV	syncytial microvillous membrane vesicles
TBA	tetrabutylammonium-hydrogensulfate
TEA	tetraethylammonium-bromide
THA	tetrahexylammonium-bromide
THM	Tris-HCl-$MgCl_2$ buffer
TMA	tetramethylammonium-bromide
TPeA	tetrapentylammonium-bromide
TPrA	tetrapropylammonium-bromide
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
val	valinomycin

PART I

INTRODUCTION

Chapter 1

THE HUMAN PLACENTA AND ITS TRANSPORT FUNCTION
- emphasis on mechanisms of drug transfer -

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INTRODUCTION

The placenta is an important organ for optimal growth and development of embryo and fetus during pregnancy. It combines functions performed by separate organs in adult life. Absorption of nutrients and excretion of waste products are major functions of the placenta, which in neonates are performed mainly by intestine and kidney, respectively. For gas transfer, after birth performed by the lungs, the fetus is also dependent on the placenta. Furthermore, hormone synthesis by the placenta, as an endocrine gland and metabolic activity, mainly performed by the liver in later life, are of great importance. The first to maintain the state of pregnancy and the latter to assure energy supply for continuous synthesis and (active) transport processes. (1,2). The complex function of the placenta justifies the concept that transport systems must be present to assure fetal nutrition and excretion.

In this review literature data are summarized concerning human placental transport mechanisms with special reference to drugs. Active trans-placental transport processes, placental metabolism, pH differences between maternal and fetal blood and differential maternal/fetal protein binding might influence fetal drug exposure and hence fetal growth and development (3). For a better understanding of the placenta as a transport organ we start with an overview of placental characteristics, which are important for the trans-placental transfer of compounds in general.

THE PLACENTA AS A TRANSPORT ORGAN

The placenta in history

The importance of the afterbirth was already clearly recognized by the ancient Egyptians. The 'royal placenta' was carried in ceremonial processions right before the pharaoh, indicating the special value given to this organ. The 'placenta', important for good health of the pharaoh and his kingdom, was believed to be the home of the external soul (4,5)

Although the importance of this organ was known far before our era, the term placenta firstly appeared in 1559 A.D. in 'DE RE ANATOMICA' by Realdus Columbus (1516-1559). However, it lasted until the period of William and John Hunter (1718-1793) before the relationship of the unit mother-placenta-fetus was understood. The discovery of the circulation of blood and consequently the explanation of the fetal circulation by William Harvey (1578-1657) and the demonstration by John Mayow (1643-1679) that particles were transferred from maternal to fetal circulation, was of great help to the Hunters for their description of the maternal-placental-fetal axis. In the first half of the 20th century several schemes were proposed to describe and classify the different mammalian placentae of different species, in an attempt to correlate placental function to placental structure (6,7). Only the classification of Grosser (8), based on the number of tissue layers between the maternal and fetal circulation is still useful to explain several transport characteristics across the placenta. His scheme, however, implies that the placenta is purely a passive filter and does not take into account the possible active transport mechanisms, as described for instance for amino acids in the late 1940's (9). Factors like placental shape and vessel geometry are also ignored (10). The general idea that the structure of the placenta is of importance for its function, however, did stand the test of time.

Placental anatomy in relation to its transport function

Anatomy of the human term placenta

The human placenta is a discoid organ with a diameter of approximately 20 cm, a thickness of about 3 cm and a weight of approximately 500 grams at term. The placenta is formed by the chorion frondosum (fetal tissue) and decidua basalis (maternal tissue). Decidua septa formed by the decidua basalis divide the placenta

into several compartments called cotyledons. Such a cotyledon can be considered as a functional unit within the placenta. It contains a villus tree, in which the maternal and fetal circulation are separated by means of the placental barrier. This barrier consists of the endothelium of the fetal capillaries and the trophoblast, containing the villus stroma, the cytotrophoblast and the syncytiotrophoblast. As pregnancy proceeds the thickness of the barrier reduces due to the partial disappearance of the cytotrophoblast layer. The syncytiotrophoblast is bordered at the maternal side by the syncytial microvillous membrane, which contacts directly to the maternal blood in the intervillous spaces. The basal membrane covers the syncytiotrophoblast at the fetal side. Transport across the placenta is greatly regulated by these two membranes. Therefore the trophoblast can be considered as a polar epithelium, as is also present in other organs like kidney, intestine, blood-brain barrier, and through which almost all materials must pass in their transfer from mother to fetus and vice versa. A diagrammatic representation of the human placenta is given in Figure 1.

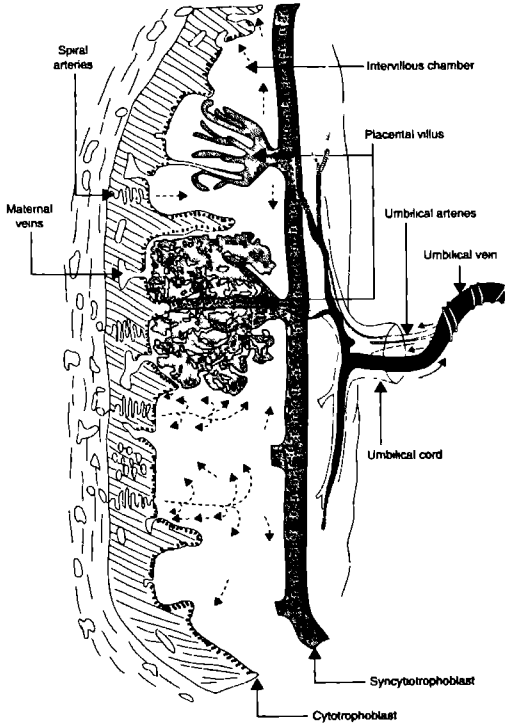

Figure 1. The human placenta with its arterial and venous supply. Adapted from Bourget et al. (11) with permission.

Species differences in placental anatomy

As mentioned before, Grosser's classification is still useful for the explanation of structural-functional relationships of the placenta. Table 1 shows the type of placentae and the number of layers between maternal and fetal blood. Placentae having maternal epi- or endothelia are classified as epithelio- or endotheliochorial. Placentae in which maternal blood directly contacts the trophoblast are called haemochorial, further subdivided according to the number of trophoblastic layers between maternal and fetal blood. Not only factors as mentioned in the classification of Grosser indicate the differences in placental structure between species, the shape of the mature placenta also differs. For humans in the early stage of pregnancy the placenta has a diffuse shape, viz. villi cover the entire surface, whereas the human placenta in late gestation is discoid, characterized by formation of the villi in a circulate plate with defined margins. In ruminants, like sheep and cow for instance, the placenta, has villi restricted to defined regions of the uterus (1).

Assuming that such differences in placental structure must have their influence on placental transfer properties, data from animal studies can not be completely predictive for the situation in the human placenta.

Table 1. Grosser's classification.

placental type		tissue layers								species
		mb	mce	mct	me	is	tr	fct	fce	fb	
epitheliochorial			+	+	+		+	+	+		sheep, pig
endotheliochorial			+	-	-		+	+	+		dog, cat
haemochorial	mono		-	-	-		+	+	+		human, guinea pig
	di		-	-	-		++	+	+		rabbit
	tri		-	-	-		+++	+	+		rat, mouse

Modified from Page (1), the Physiology of the Human Placenta, pp 13. +: number of cell layers; fb: fetal blood; fce: fetal capillary endothelium; fct: fetal connective tissue; is: intervillous space; mb: maternal blood; mce: maternal capillary endothelium; mct: maternal connective tissue; me: maternal epithelium; tr: trophoblast.

Placental anatomy and permeability

The permeability of the (human) placenta is comparable to other lipid membrane containing structures. Lipid soluble substances will freely cross the membrane barrier and lipid insoluble compounds will encounter difficulties in crossing the membranes.

Lipophilic compounds will cross the placenta depending on their molecular size, degree of ionization, protein binding and lipid solubility. Molecules with a molecular weight up to 600 Da, non-ionized and lipid soluble will show unimpeded diffusion. The rate of transfer of such compounds is not dependent on the diffusibility across the membranes, but depends on factors that regulate maternal and fetal blood flows. This kind of transfer is therefore called 'flow-limited'. In this case the rate of passage across the placental membranes is identical to the delivery-rate by the blood stream (1).

In contrast, polar, ionized, hydrophilic compounds will cross the placenta more slowly. The rate of membrane transfer is slower than the rate of delivery by the blood stream. This process is called 'membrane limited' transfer, and the constitution of the membranes determines the rate of transfer.

Especially for hydrophilic molecules, large differences among species have been shown for placental permeability (12). The permeability of human placenta is considerably higher than sheep placenta (13) and comparable to guinea-pig placenta (14). In the sheep there is no significant diffusional flux for hydrophilic compounds with a molecular weight up to 400 Da. However, in the guinea-pig there is no restriction in diffusion for molecules with a weight up to 5000 Da. Permeability data of the human placenta are mainly derived from in vitro experiments. In the perfused human placental cotyledon a close correlation was observed between placental permeability and molecular weight for hydrophilic compounds (15). More recently, permeability of the human placenta determined in vivo, at the time of Caesarian section, showed a reasonable agreement between in vitro and in vivo data (12,16-18). In human placental membrane vesicles it was shown that the permeability of the basal membrane was substantially higher than of the syncytial microvillous membrane for hydrophilic non-electrolytes (19).

The observed species differences in placental permeability are predominantly the reflection of structural differences. The high leakyness of the human and guinea-pig placenta, as compared with the sheep placenta, is due to the fact that the former two have a haemochorial type of placenta, whereas the latter has a epitheliochorial type of placenta, in which more tissue layers separate the maternal and fetal

bloodstreams. There is physiological evidence for paracellular pathways in the human placenta as an explanation for the high leakyness (20). No unequivocal morphological proof for water filled membrane channels has yet been given. By electron microscopy, vesicular and tubular structures have been shown in the guinea-pig placenta, but there is no evidence for such structures in the human placenta (21). Thus, in the haemochorial placenta the trophoblast and the endothelium are responsible for the diffusional resistance to hydrophilic compounds, such that the trophoblastic components determine the overall diffusion barrier and intercellular spaces in the endothelium restrict the diffusion of larger molecules (22). A model has been proposed by Stulc (20) in which the trophoblast contains a limited number of wide openings yielding a low permeability and the second, endothelial, layer possesses a high density of narrow pores, resulting in a high permeability.

Placental ageing and permeability

As pregnancy proceeds placental structure changes. These changes are often regarded as ageing of the placenta, but they can also be viewed (23) as a maturation mechanism. The exponential progress in fetal weight during gestation is not accompanied by a proportional increase in placental weight. After 16 weeks of pregnancy, a reduction in thickness of the barrier between maternal and fetal circulation occurs, due to the partial disappearance of the cytotrophoblast layer. The observable changes in villus structure must be explained as an enlargement of exchange area. This will result in a higher permeability and more efficient exchange of nutrients, necessary for the increased demands of the developing fetus. In early pregnancy the distance between both circulations is about 10 micron and decreases to about 2 micron in late gestation (23,24).

Thus, due to changes in the placental barrier, compound transfer in term placenta is not completely comparable to that in preterm placenta. However, placental structure differences between species are assumed to be of greater influence on transport characteristics than the ageing phenomena and therefore human data are to be preferred.

HUMAN PLACENTAL TRANSPORT MECHANISMS

Drug use during pregnancy

In an overview of epidemiological drug utilization studies it was shown that a considerable amount of drugs was used during pregnancy. Analgesics, antiemetics, antibiotics, tranquilizers, antihistaminics and diuretics were the most widely taken classes of drugs (25). In a Dutch investigation of drug use during pregnancy at least one prescription was received by 86% of the mothers, regarding all three trimesters of pregnancy. As pregnancy proceeds the amount of prescribed drugs generally decreased (except for gastro-intestinals and vitamins), possibly due to the awareness of the mother and physician of the potential risks to the fetus of maternal drug utilization (26). However, in some cases, for instance maternal epilepsy or diabetes, the mother is continuously exposed to drugs during pregnancy. The perinatal use of uterine smooth muscle relaxants (tocolytics like ritodrine and calcium channel blockers) and uterine stimulating drugs (oxytocin and prostaglandines) to inhibit or stimulate the beginning of labour, is another form of obligatory maternal drug use before the end of pregnancy. Furthermore, fetal therapy via maternal drug administration is expected to be employed increasingly. All these factors underline the importance of a good insight into the mechanisms that determine placental drug transfer and fetal plasma concentrations after maternal drug administration.

Placental transport mechanisms

1. As mentioned before, most (pharmacologically active) compounds cross the human placenta by simple diffusion. Transfer in this case is without the use of energy, dependent on the concentration gradient between maternal and fetal blood, the surface area and thickness of the membrane barrier as governed by Fick's law. Placental blood-flow, pH of maternal and fetal blood, physico-chemical characteristics of the compounds and protein binding further determine the capability of crossing the placental membranes.
2. In case of facilitated diffusion placental transfer is carrier mediated, but not dependent on energy. Transfer occurs down the concentration gradient, is inhibitable by structural analogs and is saturable. Especially drugs structurally related to endogenous compounds are assumed to be transported by a facilitated diffusion mechanism.

3. Compound transfer by an active transport mechanism occurs against an electrochemical or concentration gradient, requiring energy. Transport is carrier mediated, saturable and there is competition between related molecules. In case of nutrient transport in fetal direction and probably fetal excretion of xenobiotics into the maternal circulation, placental active transport mechanisms may be involved.
4. A less important route of placental drug transfer can be through pinocytosis. The compound is invaginated into the 'cell membranes' after which it is transferred to the opposite site.

During the past 20 years several transport systems have been identified in the human placenta. Mostly transport systems for endogenous compounds or nutrients are concerned. A summary of the most important transport systems for such substances, studied in isolated placental membrane vesicles, is given in Table 2.

For xenobiotic compounds human placental transport mechanisms are far less well documented. In a few studies the inhibitory effects of certain drugs on transporters for endogenous compounds were investigated: cyclosporine inhibits taurine transport (27), salicylate inhibits sulphate transport (28); loperamide, clonidine and cimetidine inhibit the Na^+/H^+-exchanger (29-31) and amiloride(analogs) (32) and clonidine and imipramine (33) inhibit the guanidine/proton antiporter.

Human placental transfer of drugs is often studied during labour by sampling maternal and umbilical cord blood following maternal bolus drug administration. This technique, however, suffers from several disadvantages such as: dependency of fetal/maternal serum concentration ratios on the time between drug administration and serum sampling, lack of comparison with a proper reference compound or difficulties in evaluating the influence of placental metabolism on drug transfer. For data concerning in vivo fetal-maternal plasma concentration ratio's excellent reviews have been written on placental drug transfer to which we refer to (34-37).

Table 2. Transport systems for endogenous compounds in the human placenta.

Compound	Mechanism	Localisation	Reference
Anorganic anions			
SO_4^{2-}	anion exchange	SMM, BM	38
PO_4^{3-}	sodium-coupled cotransport	SMM	39
Cl^-	anion exchange	SMM	40
	conductive pathway	SMM	41
Organic anions			
lactate	proton-coupled symport	SMM	42
succinate	sodium-coupled cotransport	SMM	43
Anorganic cations			
Na^+	proton exchange	SMM, BM	44
	amiloride sensitive channel	SMM	45
H^+	ATP-dependent	SMM	46
Ca^{2+}	ATP-dependent	BM	47
	proton exchange	SMM	48
Organic cations			
guanidine	proton-coupled antiport	SMM	33
choline	conductive pathway	SMM	49,50
Miscellaneous			
amino acids	sodium-coupled cotransport	SMM, BM	51
	facilitated diffusion	BM	51
glucose	facilitated diffusion	SMM, BM	52,53
dipeptides	facilitated diffusion	SMM	54
bile acids	anion exchange	SMM, BM	55,56

The body of data on mechanisms underlying explicitly human placental drug transport is relatively small. This is in contrast to the amount of information regarding other epithelial organs, like kidney, intestine and liver. The importance of mechanistic studies is indicated by recent results concluding that, although the anatomical structure differs, the placental microvillous membrane has much in common with the basolateral membrane rather than the brush-border membrane of other well characterized transporting epithelia (57), that the human placenta can express pharmacologically distinguishable types of transporters localized at different membranes (58) and that placental transport systems can be pharmacologically distinct from similar transporters in other tissues (59).

Techniques for in vitro perfusion of placental tissue and isolation of placental membrane vesicles were introduced in the seventies. They have been playing an important role in overtaking the relative arrearage in knowledge, as is shown by the expanding literature during the last decade on placental drug transport mechanisms. The dually perfused isolated human placental cotyledon, introduced by Schneider et al. (60), enables the study of mechanisms underlying for instance low umbilical cord/maternal serum concentration ratios. With this method placental drug transfer can be evaluated in relation to flow-, and membrane-limited reference compounds, such as antipyrine and inulin. Furthermore, the influence of placental metabolism, the preferential direction of transport and the presence of overall active transport can be evaluated. Isolated membrane vesicles of human (term) placenta, can contribute further to the elucidation of driving forces, inhibitory susceptibility, specificity and saturability of membrane bound transport systems for exogenous compounds or transport systems primarily present for endogenous compounds, which have also affinity for xenobiotics (61).

In this chapter we summarized literature data on the mechanisms of human placental drug transport studied in the isolated perfused placental cotyledon, placental membrane vesicles or trophoblastic cell cultures. Table 3 summarizes the present knowledge on transport characteristics of groups of drugs, clinically relevant during pregnancy or labour, further subdivided into individual agents. For abbreviations used in Table 3 see page 22.

Table 3. Human placental drug transfer characteristics

Drug		Technique	Transfer	Kinetic parameters	Metabolism	F/M ratio	Tissue/serum ratio	Reference
antibiotics	cephalosporins	ipc	CI=0.04-0.33 T=1-7%					62-66
		smmv	Na indep fac diff	K_m=2.3mM V_{max}=3nmol/mg/60s				67
	penicillins	ipc	CI=0.13-0.22 T=5-7%					68
	sulphonamides	ipc	CI=0.06 T=9%				0.06	63,69
	chloramphenicol	ipc	T=0.17% encap T=1.1% free					70
	pentamidine	ipc	CI=0.04 T=1%				≈1	71
	trimethoprim	ipc	T_f=0.08				0.18	69
	timentin	ipc	CI=0.04-0.06					72
	spiramycin	ipc	T=9%, T_f=0.23					73
analgesics	aspirin	ipc	T=15%			≈1		74
	acetaminophen	ipwp			–	0.8	>1	75
	propoxyphen	ipwp			+	0.6-0.8	>1	75
	phenylbutazone	ipc	T=26%, T_f=0.6					76
	indomethacin	ipc	T=36%, T_f=0.9					76
anti-epileptics	phenytoin	ipc	CI=1.08, 1.6		–	0.94	3.5-3.7	77-79
	phenobarbitone		CI=0.12-0.52		–	≈1		77
	carbamazepine	ipc	CI=1.24		–			80
	valproic acid	ipc	CI=0.95					81

Table 3. continued

Drug		Technique	Transfer	Kinetic parameters	Metabolism	F/M ratio	Tissue/serum ratio	Reference
antivirals	azidothymidine	ipc	CI=0.3-0.6 T_f=0.7 TI=1.5-1.7	A=–,I=–, S=–	+		1	82-85
		smmv		I=–, S=–				83
		tc			+			86
	dideoxyinosine	ipc	CI=0.15-0.3 TI=1.0-1.2		–			82,87,88
		th			–			89
	d4T	ipc	CI=0.4 T_f=0.8	S=–				90
	acyclovir	ipc	CI=0.18 T_f=0.13-0.3	I=–, S=–				91
		smmv		I=+, temp dep				91
	gancyclovir	ipc	CI=0.18 T_f=0.18-0.3	I=–, A=–				92,93
		smmv		I=+, S=+, temp dep, Na^+ indep K_m=0.3mM V_{max}=4nmol/mg/30s				92
	bisheteroypiperazine	ipc	CI=0.72	S=–			≈1	94
ß-antagonists	atenolol celiprolol	ipc	T=3.1-3.4%					95
	timolol labetalol propranolol	ipc	T=17-21%			equal for enantio.	significant tissue binding	95

Table 3. continued

Drug		Technique	Transfer	Kinetic parameters	Metabolism	F/M ratio	Tissue/serum ratio	Reference
ß-agonists	ritodrine	ipc	T=2 4%, T_f=0 6	A=–			17% of dose	96-98
	fenoterol	ipc	T=2 3%					98
	hexoprenaline	ipc	T=1 1%					98
	salbutamol	ipc	T=2 8%, CI=0 4, T_f=0 13	flow dep				98, 99
cardiac glycosides	digoxin	ipc				0 36	3 4	100
drugs of abuse	cocaine and derivates	ipc	CI=0 55-0 99 TI=3 23 T_f=0 89	A=–, S=–	–		1 2-1 8	101-103
corticosteroids	corticosteron	smmv	Na^+ indep temp dep NAD^+ dep	I=+ K_m=7nM V_{max}=2 7nmol/mg/min				104,105
	dexamethasone	ipc	CI=0 37		+			106,109
	prednisolon	ipc	CI=0 38		+			107-109
	betamethasone	ipc	CI=0 41		+			109
	cortisol	ipc	CI=0 48		+			109
diabetics	glyburide	ipc	T=62-89%	S=–			low	110
diuretics	triamterene	ipc	CI=0 74-0 85			0 92		111
respiratory drugs	theophylline	ipc	T=22%, Cl_t=0 9			0 45	0 16	112,113
gastro-intestinals	cimetidine	ipc	CI=0 23-0 4	I=–, A=–, S=–	–	0 46	0 02	114-116
		smmv		S=–				114

Table 3. continued

Drug		Technique	Transfer	Kinetic parameters	Metabolism	F/M ratio	Tissue/serum ratio	Reference
minor tranquilizers	(nor)diazepam	ipc	T_f=0.85				≈2	117,118
	clorazepate		T_f=0.84					117
	fosazepam		T_f=0.2				≈1	118
miscellaneous	metoclopramide-derivates	ipc	CI=0.4				0.01-0.08	119
	bupivacaine	ipc		preferential M→F protein binding dep. fetal pH dep (acidic) I=–, S=–			significant tissue binding	120
	alfentanil	ipc	CI=0.63-0.89	S=–, M→F=F→M			≈1	121
	heparin	ipc	CI=0.02 T=0.3%			0.12		122
	mefloquine	ipc	CI=1.6, T=11%			0.75		123
	chloroquine	ipc	CI=0.44, T=14%, T_f=0.44					124
	carbacyclin	ipc			+	0.02		125,126
	acipimox	ipc	CI=0.25			0.58		127
	cyclosporine	ipc	T<5%				significant tissue binding	128

Clearance index $CI=Cl_{compound}/Cl_{antipyrine}$; Transport index $TI=Cl_{compound}/Cl_{L\text{-}glucose}$; T=% transfer in maternal to fetal direction; Transport fraction T_f= amount of transfer of compound/amount of transfer of antipyrine; ipc=isolated dually perfused cotyledon; tc=trophoblast cells; th=trophoblast homogenates; ipwp= isolated perfused whole placenta; encap=encapsulated into liposomes; A=accumulation against a concentration gradient; I=inhibition by structural analogs; S=saturability; (in)dep=(in)dependent; temp=temperature; enantio=+ and –enantiomers; d4T=2'3'-didehydro-3'-deoxythymidine

Table 3 shows that for almost all drugs the transfer rates did not exceed the transfer rate of the diffusion markers antipyrine (flow-dependent diffusion) and L-glucose (simple diffusion), viz. CI and TI < 1. A CI greater than unity was observed only for the antiepileptic drug carbamazepine due to its higher lipophilicity than antipyrine (80) and for the anti-malaria drug mefloquine (123), which probably resulted from its biexponential kinetic behaviour; a rapid placental uptake followed by slower transfer into the fetal circulation. Transplacental transfer of azidothymidine (84) was faster than the diffusion of L-glucose, due to its greater lipophilicity. However, azidothymidine did not equilibrate as fast as antipyrine, probably because of extensive placental metabolism.

Transfer fractions and percentages of transfer in maternal to fetal direction varied from almost zero to 100 %. In general, this variability was mainly correlated with the physico-chemical characteristics of the compounds studied, such as lipophilicity, degree of ionisation or degree of protein binding. For instance, the more lipid soluble ß-antagonists timolol, labetolol and propranolol showed a 4-times higher steady-state diffusion rate than the more hydrophilic congeners atenolol and celiprolol (95). Heparin showed an extremely low trans-placental transfer, due to its high molecular weight (15000 Da) and its negative charge (121). Other factors of impaired trans-placental transfer are significant tissue binding, as was the case for aspirin (74), theophyline (112,113) and digoxine (100), or significant placental metabolism, which was found for azidothymidine (84,85), dexamethasone (106,109), prednisolon (107-109) and carbacyclin (125,126). These compounds are structural analogs of endogenous compounds and appeared to be substrates for placental metabolic systems. This was also seen for p-aminobenzoic acid, which was acetylated by the human placenta, resulting in a low placental transfer of only 16 % (129). All major drug metabolic pathways present in other tissues are also found in the human placenta, mostly to a lesser extent but they can be quite efficient (130). The implications of these systems for placental drug tansfer, however, need further investigation.

In perfusion studies overall placental drug transfer appeared to occur by simple diffusion. However, in placental membrane vesicles facilitated diffusion has been found for cephalosporins (67), gancyclovir (92) and corticosterone (104,105). It is assumed that these drugs are transported by systems primarily present for endogenous dipeptides, nucleosides and hormones, respectively. For pharmacologically active compounds, such as dopamine (131), noradrenaline (132) and serotonine (133), sodium-dependent active transport systems have been found in human placental SMMV. Although several drugs were able to inhibit transport of

these endogenous drugs (134,135), the implications of the presence of these systems and their transport capacity for placental transfer of for instance antipsychotics, antidepressants and anti-parkinson drugs have not been investigated yet.

For most vitamins active transport systems have been found in the human placenta. Pharmacologically important vitamins, such as retinol and folate, appeared to be exceptions. Retinol was transferred across the human placenta with a transfer index of 0.73 (for comparison, the actively transported vitamins thiamin and riboflavin had transfer indices of 2.1 and 3.4), indicating transport by simple diffusion (136). High affinity binding sites for folate were found (137) in human placental microvilli, however, their role in placental folate transport is not fully elucidated. Preliminary experiments showed that membrane binding accounted for the main part of uptake into microvillous membrane vesicles (138).

Transport systems for sex steroids, another group of endogenous compounds with pharmacological implications, have not been described until now. Possibly, the membrane bound P-glycoprotein (multi-drug resistance protein), which is abundantly present in the human placenta, although its exact membrane localization has yet to be determined, plays a role in placental hormone transport. Additionally, preliminary experiments have suggested that this protein may also act as a Cl^--channel, establishing and maintaining the observed trans-placental (fetal to maternal) chloride gradient (139).

In summary, overall human placental drug transport rarely exceeds the transfer of flow-dependent and membrane-limited marker compounds. Interestingly, relatively often placental drug transfer appeared to be much smaller, indicating impaired trans-placental transport, depending on the physico-chemical characteristics of the drug or placental factors such as tissue binding or metabolism. Although in perfusion studies overall human placental drug transport occurred by simple diffusion, at the membrane level several drug transport systems have been found, mainly for drugs structurally related to endogenous compounds.

SCOPE OF THIS THESIS

The aim of the studies described in this thesis was a systematic approach to the unanswered questions concerning placental drug transport mechanisms. In contrast to other tissues, such as kidney, liver and intestine, the characteristics of classic transport systems for anionic and cationic drugs have not been investigated in the human placenta. In the kidney, the basolateral membrane of the proximal

tubular cell posesses a tertiary active transport system for anionic drugs. This system mediates efficient tubular uptake of these drugs from the blood, resulting in the establishment of a concentration gradient, which acts as a driving force for urinary excretion across the brush-border membrane by facilitated diffusion (140). For cationic drugs, tubular uptake across the basolateral membrane occurs by (facilitated) diffusion, after which active urinary excretion is achieved by an organic cation-proton antiporter in the brush-border membrane (140). In this thesis we tried to elucidate whether comparable transport mechanisms are present in the human placenta, assuming that the placenta may play a role in the excretion of drugs from the fetal circulation. Drugs can reach this compartment by diffusion due to the trans-placental concentration gradient formed after maternal drug administration. It can be also speculated that high and possibly toxic fetal plasma concentrations are achieved when such transport mechanisms accumulate maternally taken drugs into the placenta, forming a driving force for transport into the fetal circulation by (facilitated) diffusion. Furthermore, we tried to characterize quantitatively the interaction of various anionic and cationic drugs with transport systems for nutrients such as amino acids and the cation choline. For all these mechanistic studies, isolated syncytial microvillous membrane vesicles were used. This technique is very well suited for the elucidation of transport driving forces, susceptibility to inhibition by foreign compounds and saturability of transport systems.

In the second part of this thesis we describe the use of a perfusion system of the isolated human placental cotyledon. By mimicking the maternal and fetal blood circulations, contacting this functional unit, factors underlying the observed low trans-placental transfer of the antibiotic spiramycin were investigated.

By studying drug transport and drug-nutrient interactions in the human placenta, we tried to contribute to the insight into factors determining placental and fetal exposure to drugs after maternal administration.

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PART II

DRUG TRANSPORT AND INTERACTIONS IN ISOLATED MEMBRANE VESICLES

Chapter 2

ISOLATION OF SYNCYTIAL MICROVILLOUS MEMBRANE VESICLES FROM HUMAN TERM PLACENTA AND THEIR APLLICATION IN DRUG-NUTRIENT INTERACTION STUDIES

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Summary

The initial step in placental uptake of nutrients occurs across the syncytial microvillous membrane of the trophoblast. This study was designed to isolate syncytial microvillous membrane vesicles (SMMV) of human term placenta, to validate their purity and viability and to investigate the interaction of several commonly used drugs with the transport of two essential nutrients: alanine and choline. SMMV were isolated according to an established procedure, but instead of homogenization the initial preparation step was replaced by mincing of placental tissue followed by gently stirring to loosen the microvilli. These modifications doubled the protein recovery and increased the enrichment in alkaline phosphatase, whereas no substantial contamination with basal membranes nor interfering subcellular organelles was found. The functional viability of the vesicles was evaluated through the transport of alanine. In accordance with literature uptake was sodium-dependent, inhibitable by structural analogues and saturable. A number of cationic drugs were able to inhibit choline uptake, whereas no effect on alanine transport was observed. Anionic drugs, drugs of abuse and catecholamines did not interfere with alanine transport either. In conclusion, our isolated SMMV provide a suitable tool for screening drug-nutrient interactions at the level of membrane transport. In view of the very low susceptibility of the alanine transporter to drug inhibition and the relatively high drug concentrations necessary to inhibit choline transport, it seems unlikely that clinically important drug interactions may occur

with these nutrients.

Introduction

In the human haemochorial placenta the trophoblast forms a true syncytium without intercellular spaces. This polarized epithelium separates maternal and fetal circulation by means of the syncytial microvillous membrane (SMM) facing the maternal circulation and the basal membrane (BM) facing the fetal circulation. Fetal supply of nutrients and excretion of waste products across the placenta is mediated by these membranes forming, together with a core of connective tissue, the functional unit of the human placenta, called chorionic villus or cotyledon (Page, 1993).

During the past 20 years several procedures have been described for the isolation of membranes from human term placenta. Isolation of SMM was introduced by Smith et al. (1974), who separated microvilli from their syncytiotrophoblastic surface by isotonic agitation. Nowadays, widely used modifications of this method involve homogenization or mincing of placental tissue followed by differential centrifugation, with (Booth et al., 1980; Glazier et al., 1988; Illsley et al., 1990) or without (Boyd and Lund, 1981) Mg^{2+}-aggregation of non-microvillous membranes. Isolation procedures for syncytial basal membranes (BM), however, are less often described. The main reason for this is the poor accessibility of these membranes in the syncytial structure of the trophoblast. A method relatively often referred to in literature involves sonication, hypotonic lysis and EDTA incubation to detach separate membranes from the connective tissue, followed by discontinuous Ficoll-gradient centrifugation (Kelley et al., 1983) or differential centrifugation (Hoeltzli et al., 1990). Membrane vesicles are a pre-eminent tool to investigate mechanisms of transport and drug interactions on nutrient transporters, independently of other cell types or placental metabolism.

Fetal growth depends highly on placental transport of nutrients from the maternal to the fetal circulation. The initial step in placental transfer involves active uptake across the SMM into the trophoblast. The neutral amino acid alanine is the most abundant amino acid in maternal circulation (Philipps et al., 1978) and essential for fetal protein synthesis or as energy source for placental or fetal metabolism. The quaternary ammonium compound choline must be transported from maternal circulation into the trophoblast, because the placenta is unable to synthesize choline (Welsch, 1978). Obviously, interaction with the transfer of these

nutrients may impair fetal growth.

Transfer of alanine across the SMM occurs predominantly via the sodium-dependent system A. The system is responsible for the accumulation of alanine from the maternal circulation into the trophoblast. The gradient established enables (facilitated) diffusion of this amino acid into the fetal circulation, resulting in a higher fetal than maternal alanine concentration (Yudilevich and Sweiry, 1985). Since several drugs, like ouabain and digoxin, can interfere with Na^+/K^+ ATP-ase, an enzyme essential for maintaining the transmembrane sodium-gradient, an inhibitory effect on alanine transport can be assumed (Kihlstrom and Kihlstrom, 1981). Drugs may also interact directly with the human placental alanine transporter as was shown by Dicke et al. (1993) for S(-) cocaine in human microvillous membrane vesicles. Barnwell and Rama Sastry (1983) found reduced active uptake of the system A-dependent amino acid AIB in the presence of high concentrations of cocaine, nicotine or morphine in human placental villi, but the type of inhibition could not be attributed to competition with the amino acid at the transport site. In a human cotyledon perfusion system transfer of AIB was not impaired by ethanol (Schenker et al., 1989[a]) or nicotine and a combination of nicotine and ethanol (Schenker et al., 1989[b]). Furthermore, acetaldehyde did not inhibit sodium-dependent alanine uptake into SMMV (Asai et al., 1984). Acetylsalicylic acid has been shown to inhibit alanine transfer into the trophoblast in a guinea pig placenta perfusion system, but this was assumed to be an indirect effect, due to an influence on the uterus muscle by-pass flow (Rybakowski et al., 1993). The metabolic inhibitors dinitrophenol and iodoacetate did reduce alanine transfer in sheep placenta (Sepulveda and Wooding, 1984). The local anaesthetic lidocaine and the anti-cholinergics scopolamine and atropine did interact with AIB uptake in a nonspecific nature (Fant and Harbison, 1981). Inhibitors of choline-acetyltransferase were able to inhibit AIB uptake in placental villi. These results suggest that neutral amino acid transport is linked to the cholinergic system (Rowell and Rama Sastry, 1981).

The mechanisms of placental transport of choline, a precursor in the cholinergic system and phospholipid synthesis were recently investigated in our laboratory. Uptake into SMMV was found to be membrane potential-dependent and saturable with a K_m of 550 μM (van der Aa et al., 1994[b]).

Hardly any information is available on the mechanisms of drug interactions with nutrient transporters. We designed this study to isolate SMMV, validate their fuctional viability, and investigate the potential of various commonly used drugs to interact with membrane transport of alanine and choline.

Materials and Methods

Isolation of membrane vesicles

SMMV were isolated essentially according to method 3 as described by Glazier, Jones and Sibley (1988), based on the method reported by Booth and Kenney (1974) for rabbit kidney brush-border membrane vesicles. However, we introduced some modifications in order to improve the isolation performance.

Human term placentas from uncomplicated pregnancies were obtained within 15 min after vaginal or ceasarian delivery and brought into 0.9% NaCl (4°C). All subsequent steps were performed at 4°C. After removing the cord, amniochorion and decidua, placental tissue was cut from the maternal side and washed three times in 0.9% NaCl. The tissue was minced in a Waring blender in 300 mM Mannitol, 10 mM Hepes-Tris, pH=7.4 and 1 mM $MgSO_4$ (MHT buffer). The mince was stirred for 30 min to loosen the microvilli and filtered through 4 layers of woven cotton gauze. A sample of this starting mince was taken for enzyme analysis. $MgCl_2$ was added to the filtrate to a final concentration of 10 mM and the filtrate was stirred for 15 min. It was centrifuged at 2200*G for 10 min in an Heraeus sepatech Minifuge RF (Kalkberg, Germany). The pellet was discarded and the supernatant was centrifuged at 23500*G for 40 min in a IEC B-60 ultracentrifuge (Daiment Div., Needham, Massechuttes). G-forces were appointed to the bottom of the tube. The pellet from this run was suspended in MHT buffer with a 19G and 25G syringe needle (5 strokes). This homogenate was again exposed to 10 mM $MgCl_2$. After repeated differential centrifugation, syncytial microvillous membrane vesicles were obtained and suspended with a 25G syringe needle (10 strokes) in a desired intravesicular buffer, frozen in liquid nitrogen and stored at -80°C for four weeks at the maximum.

BMV were isolated from human term placenta according to Hoeltzli et al. (1990) for comparison of the Ca^{2+} uptake characteristics into SMMV and BMV. Briefly, tissue was minced in a warring blender and sonicated for 10 sec in portions of 30g/100ml 50 mM Tris-HCl buffer, pH=7.4. Subsequently, to free basal membranes from connective tissue, the mince was incubated for 30 min in hypotonic 5 mM Tris-HCl buffer, pH=7.4, followed by incubation for 30 min at room temperature (all other steps were performed at 4 °C) in 50 mM Tris-HCl, 250 mM sucrose and 10 mM EDTA buffer, pH=7.4 and resonication for 20 sec. Basal membranes were purified by Mg^{2+}-aggregation and differential centrifugation, frozen in liquid nitrogen and stored at -80 °C.

Analytical methods

The activity of marker enzymes was analyzed according to previously described methods in M_0 and SMMV: alkaline phosphatase for SMM (Mircheff and Wright, 1976), Na^+/K^+ ATP-ase for BM (Schoot et al. 1977), succinate dehydrogenase for mitochondria (Pennington, 1961) and NADPH cytochrome-C-reductase for smooth endoplasmatic reticulum (Omura and Takesue, 1970). Protein was assayed with a coomassie blue kit (Biorad, München, Germany) with bovine serum albumin as standard.

Uptake of [^{3}H]-alanine and [^{3}H]-choline into SMMV was measured in quadruplicate at room temperature using a rapid filtration technique (Russel et al., 1988) by the addition of 10 or 5 µl (approximately 8-4 µg protein) vesicles to 40 or 195 µl extravesicular medium containing a tracer amount of radiolabeled solute, respectively. Uptake of $^{45}CaCl_2$ into SMMV was determined according to van Heeswijk et al. (1984). Uptake was started by the addition of 10 µl vesicles (7-10 µg) to 90 µl extravesicular medium, containing 0.5 mM $CaCl_2$, EGTA, NTA and HEDTA with 5.13 mM $MgCl_2$ and 3 mM ATP to obtain 1 µM free Ca^{2+} and 1.5 mM Mg^{2+} in the presence of ATP and 2.3 mM $MgCl_2$ to obtain 1 µM free Ca^{2+} and 1.5 mM Mg^{2+} in the absence of ATP, all containing a tracer amount of $^{45}Ca^{2+}$ and adjusted to 310 mosmol with 100 mM KCl and mannitol. For the calculations the dissociation constants between EGTA, NTA, HEDTA, Ca^{2+} and Mg^{2+} were used according to Ghijsen et al. (1982) with the computer program proposed by van Heeswijk et al. (1984). At appropriate time intervals the reaction was stopped by adding 2 ml ice cold intravesicular buffer or in case of Ca^{2+} uptake studies, 1 ml ice cold intravesicular buffer to which 0.1 mM $LaCl_3$ was added to remove extravesicular bound Ca^{2+}. The diluted samples of the alanine and choline uptake studies were filtered under vacuum through a glass fibre filter (Whatman GF/F) and in case of the Ca^{2+} uptake studies 900 µl of the diluted samples through a presoaked acetate/nitrate filter (Schleicher & Schull ME25). The filters were washed twice and the radioactivity remaining on the filters was counted in a Beckman LS 6000 LL liquid scintillation counter. Corrections were made for aspecific filter binding. Uptakes are expressed as nmol or pmol/mg protein or as percentage of control uptake (mean ± SD or SE with N representing amount of experiments with different vesicle preparations). Transport conditions and control uptake in pmol/mg protein are given in the legends.

For electron microscopy, the membrane suspension was centrifuged at 1500*G for 10 min. Pellets were fixed in 2% glutardialdehyde in 0.1 M phosphate

buffer, pH=7.4 for 5 hours, washed and suspended in 2% agar (60 °C). The cured (at 4 °C for 30 min) agar was cut into small pieces, fixed in 2% glutardialdehyde, washed and post-fixed in 1.5% osmiumtetroxide in 0.1 M phosphate buffer. The blocks were washed and dehydrated in a series of graded alcohols. The dehydrated blocks were embedded in epon. Ultrathin sections were cut on a Reichert microtome (Vienna, Austria) and mounted on a 150 mesh uncoated copper grid. The sections were contrasted with 3.36% uranylacetate with lead citrate and examined in a Philips 300 transmission electron microscope (Eindhoven, the Netherlands) at 60KV.

Chemicals and materials

[^{3}H]-L-alanine (77 Ci/mmol) was obtained from New England Nuclear (Dreieich, Germany). $^{45}CaCl_2$ (17 mCi/mg) and [^{3}H]-choline (83 Ci/mmol) were obtained from Amersham (Buckinghamshire, UK). S(+)- and S(-)adrenaline were purchased from Sterling-Winthrop Research Institute (New York, NY), S(+/-)cocaine from Genfarma (Maarssen, the Netherlands), and S(-)nicotine from Janssen Chimica (Beerse, Belgium). All other chemicals were purchased from either Sigma (St. Louis, MO), Merck (Darmstadt, Germany) or Boehringer Mannheim (Mannheim, Germany) and were of analytical grade. GF/F filters were obtained from Whatman Int. Ltd. (Maidstone, UK) and ME25 filters from Schleicher & Schüll (Dassel, Germany).

Data analysis

Paired Student's *t*-test was used to determine statistical significance ($P<0.05$). Curve fitting was done by least squares nonlinear regression analysis using the computer program PCNONLIN (Metzler and Weiner, 1989).

Results

Isolation characteristics

Marker enzyme analysis revealed a 21-fold enrichment in alkaline phosphatase for SMMV. Addition of 0.1% saponin, triton X-100 or sodium-deoxycholate to SMMV prior to incubation increased alkaline phosphatase slightly,

showing that approximately 90% of the vesicles were orientated right-side out, as is common for SMMV (Glazier et al., 1988). Na^+/K^+ ATP-ase activity was nearly 5-fold enriched. The SMMV preparation was not or to a very small extent enriched in succinate dehydrogenase and NADPH-cytochrome-C-reductase. Protein yield and specific activities and enrichment factors of the different marker enzymes are given in Table 1. Electron micrographs of the SMMV preparation are shown in Figure 1.

Table 1. Protein recovery and specific activities of marker enzymes.

	Starting mince	SMMV	Enrichment factor	Yield (%)	N
Protein	32 ± 8	0.4 ± 0.1		1.2 ± 0.3	21
Alkaline phosphatase	52 ± 16	1080 ± 390	21 ± 5		21
+saponin (0.1%)		1210 ± 190			3
+triton X-100 (0.1%)		1250 ± 380			3
+deoxycholate (0.1%)		1210 ± 340			3
Na^+/K^+ ATP-ase	0.60 ± 0.02	3.0 ± 1.6	4.9 ± 4.3		5
Succinate dehydrogenase	3.2 ± 0.7	2.9 ± 0.7	0.90 ± 0.08		6
Cytochrome-C-reductase	4.6 ± 1.2	6.6 ± 2.5	1.4 ± 0.8		6

Data are presented as mg protein/g placenta wet weight for protein or μmol/h/mg protein for marker enzymes (means ± SD).

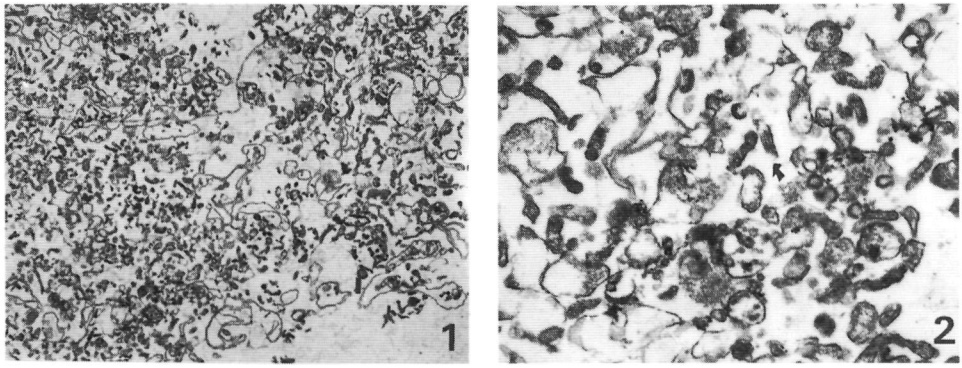

Figure 1. Electron micrographs of SMMV. At low magnification (16000x, bar represents 1 μm) the membrane preparation appeared to be homogenous, vesicular and free of subcellular organelles (1). At high magnification (40000x, bar represents 200 nm) fragments of microvilli are visible and the microfilaments can be clearly distinguished (arrow) (2).

Ca^{2+} and alanine uptake

$^{45}Ca^{2+}$ uptake into SMMV, as shown in Figure 2, was slightly stimulated by ATP and addition of A23187 did not result in Ca^{2+} release, indicating that Ca^{2+} was not accumulated into the vesicles. The inset curve shows ATP-dependent intravesicular accumulation of free Ca^{2+} into BMV, releasable with A23187.

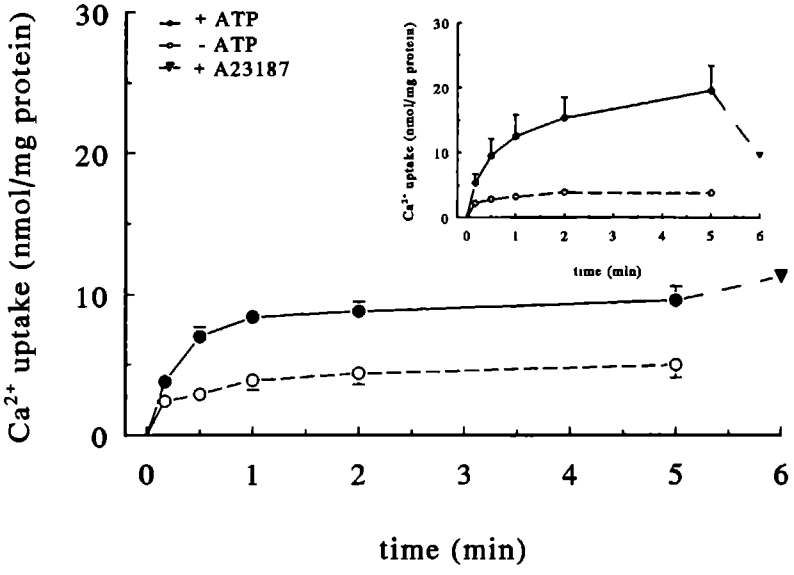

Figure 2. Effect of ATP and A23187 on the uptake of $^{45}Ca^{2+}$ into SMMV. Concentration of free Ca^{2+} was 1 µM, free Mg^{2+} 1.5 mM, ATP 3 mM and A23187 10 µg/ml. Vesicles were suspended in 100 mM mannitol, 100 mM KCl and 10 mM Hepes-Tris, pH=7.4. Composition of the extravesicular media are described in Materials and Methods. Data are presented as nmol/mg protein (mean ± SE; N=3). Inset curve: Effect of ATP and A23187 on the uptake of $^{45}Ca^{2+}$ into BMV. Conditions were the same as described for SMMV.

Time-dependent uptake of 100 µM [^{3}H]-alanine into SMMV is shown in Figure 3. An overshoot above equilibrium (peak vs. equilibrium at 30 sec=1.5) was seen in the presence of an inwardly directed Na^+-gradient, providing evidence for sodium-dependent uptake against a concentration gradient. By permeabilizing SMMV in the presence of 0.1% saponin, uptake at 60 min (equilibrium) was reduced to approximately 20% of uptake without saponin, indicating intravesicular accumulation rather than non specific membrane binding.

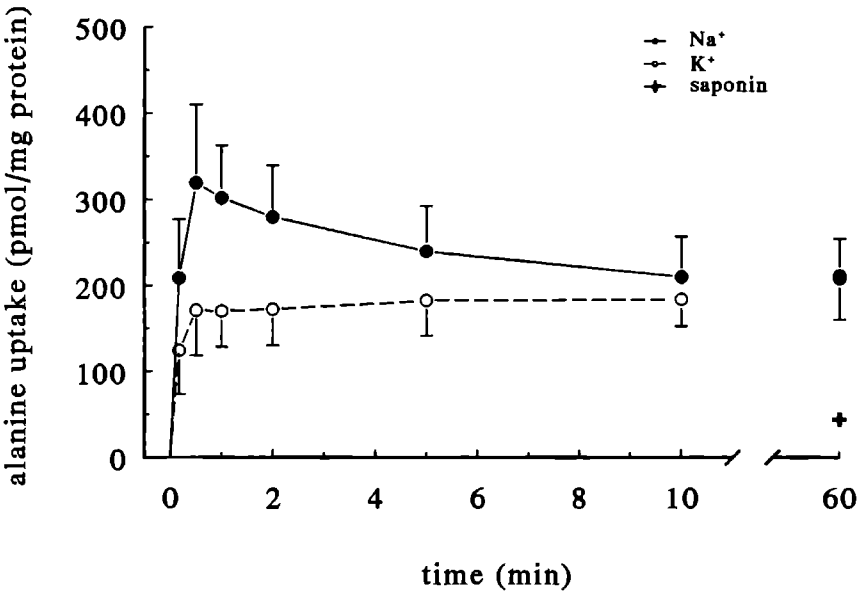

Figure 3. Time-dependent uptake of 100 µM [^{3}H]-alanine into SMMV in the presence and absence of an inwardly directed Na^+-gradient. SMMV were suspended in 300 mM mannitol and 10 mM Hepes-Tris, pH=7 4 Extravesicular media consisted of 50 mM NaCl or 50 mM KCl, 200 mM mannitol and 10 mM Hepes-Tris, pH=7 4 Data are presented as pmol/mg protein (mean ± SD, N=10)

The existence of mediated transport for alanine in SMMV was further evaluated by determining the concentration-dependent alanine uptake in the presence or absence of an inwardly directed Na^+-gradient, which is shown in Figure 4. The curve resulting from subtraction of the sodium-independent component (50 mM K^+-gradient) from the total uptake (50 mM Na^+-gradient) shows saturability and could be described according to Michaelis-Menten kinetics. Nonlinear regression analysis revealed a K_m of 0.23 ± 0.07 mM and a V_{max} of 359 ± 22 pmol/mg/15 sec for sodium-dependent alanine uptake.

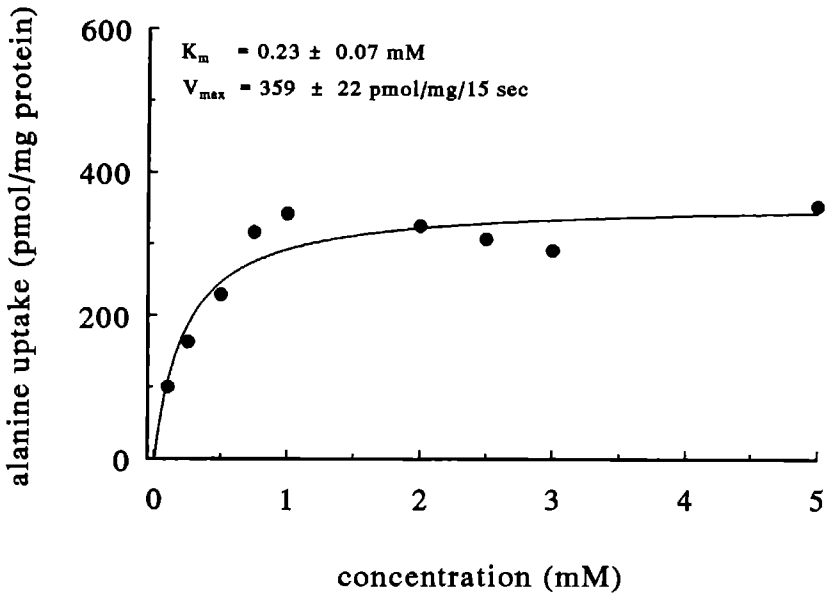

Figure 4. Concentration-dependent uptake of [^{3}H]-alanine at 15 sec into SMMV. The curve represents the difference between uptake in the presence of an inwardly directed Na^+- and K^+-gradient. Vesicles were suspended in 300 mM mannitol and 10 mM Hepes-Tris, pH=7.4. Extravesicular media consisted of 50 mM NaCl or 50 mM KCl and 10 mM Hepes-Tris, pH=7.4 and 0.1-5 mM alanine adjusted to 310 mosmol with mannitol. Data are presented as pmol/mg protein (mean; N=2).

Uptake at 15 sec of 100 μM alanine in the presence of an inwardly directed sodium gradient into SMMV was inhibited by 35 % in the presence of 5 mM glycine and by 52% in the presence of 5 mM phenylalanine as shown in Table 2. Uptakes at 1 min showed no overshoot and equilibrium uptake at 60 min in the presence of these amino acids was not affected. These results further confirmed the presence of mediated alanine transport and showed that interactions with active alanine transport can be demonstrated in our vesicles without an influence on vesicle volume.

Table 2. Effect of amino acids on alanine uptake into SMMV.

Inhibitor	mM	15sec	60sec	60min
control		170 ± 60	220 ± 70	160 ± 40
glycine	5	110 ± 40*	145 ± 16	190 ± 30
phenylalanine	5	80 ± 30*	140 ± 50	180 ± 30

Uptake of 100 μM [^{3}H]-alanine into SMMV in the presence of an inwardly directed sodium gradient. Vesicles were suspended in 300 mM mannitol and 10 mM Hepes-Tris, pH=7.4. Extravesicular media consisted of 50 mM NaCl, 5 mM inhibitor, 10 mM Hepes adjusted to pH=7.4 with Tris and to 310 mosmol with mannitol. Data are presented as pmol/mg protein or % of control uptake (mean ± SD, N=4). * $P<0.05$.

Interaction with alanine and choline transport

The effect of several drugs on alanine uptake is shown in Table 3. None of the drugs were able to inhibit alanine uptake significantly, except for indomethacin. Since uptake at 60 min (equilibrium) was also reduced to 60 % of control uptake it is difficult to ascribe this inhibition to a direct effect on the alanine transporter. In the presence of all other inhibitors peak uptake at 60 sec and equilibrium uptake at 60 min was not altered (data not shown).

Since cocaine competes with re-uptake of catecholamines in adrenergic neurons and a direct interaction of S(-)cocaine with the alanine transporter was suggested by Dicke et al. (1993), the potency of S(+)adrenaline, S(-)adrenaline and S(+/-)noradrenaline to interfere with alanine uptake into our SMMV was also evaluated. Uptake of alanine at 15 sec was not influenced by either of the compounds, viz. 99 ± 17, 97 ± 13 and 93 ± 4 % (mean ± SD, N=4) of representative control uptake, respectively.

Initial uptake of 250 μM choline in the presence of an outwardly directed choline gradient (trans stimulation conditions) was significantly inhibited by several cationic drugs as is shown in Table 4.

Table 3. Effect of several drugs on alanine uptake into SMMV.

Inhibitor	mM	% control uptake	N
None		100	
Anionic drugs			
probenecid	5	102 ± 16	4
acetylsalicylic acid	5	102 ± 8	4
sodium salicylate	5	104 ± 7	4
indomethacin	5	60 ± 12*	4
furosemide	5	87 ± 13	4
valproate	5	99 ± 15	4
Cationic drugs			
cimetidine	5	111 ± 11	4
famotidine	5	98 ± 14	4
Drugs of abuse			
cocaine	1	94 ± 5	3
nicotine	1	103 ± 11	3
cotinine	1	107 ± 4	3
ethanol	87 (0.4%)	99 ± 10	3

Uptake of 100 μM [^{3}H]-alanine at 15 sec into SMMV in the presence of an inwardly directed sodium gradient Vesicles were suspended in 300 mM mannitol and 10 mM Hepes-Tris, pH=7.4 Extravesicular media consisted of 50 mM NaCl, inhibitor at the specified concentration, 10 mM Hepes adjusted to pH=7 4 with Tris and to 310 mosmol with mannitol. Data are presented as % of representative control uptake (mean ± SD) Mean control uptake was 208 ± 71 pmol/mg protein (mean ± SD, N=9) * P<0 05

Table 4. Effect of cationic drugs on choline uptake into SMMV.

Inhibitor	mM	% control uptake
None		100
Hemicholinium-3	5	15 ± 5*
Atropine	5	22 ± 8*
Cimetidine	5	46 ± 4*
Ranitidine	5	48 ± 7*
Propranolol	5	66 ± 14*
Procainamide	5	72 ± 9*

Uptake of 250 μM-[^{3}H]-choline at 10 sec into SMMV in the presence of an outwardly directed choline gradient. Vesicles, suspended in 100 mM mannitol, 100 mM KCl and 10 mM Hepes-Tris, pH=7.4, were pre-equilibrated with 5 mM unlabeled choline and 20 μM valinomycin at 37 °C. Extravesicular media consisted of 100 mM mannitol, 100 mM KCl, 20 μM valinomycin, 10 mM Hepes-Tris, pH=7.4, 5 mM inhibitor and unlabeled choline to achieve an extravesicular concentration of 250 μM. Data are presented as % of representative control uptake (mean ± SD, n=3 or 4). Mean control uptake was 5003 ± 498 pmol/mg protein (mean ± SD, N=4). * $P<0.05$

Discussion

In this report we describe the marker enzyme and transport characteristics of SMMV from human term placenta isolated according to an established, but slightly modified method. The SMMV were used to investigate drug-nutrient interactions of several commonly used drugs with the alanine and choline transporter.

SMMV were isolated according to a procedure originally described for the isolation of rabbit renal brush-border membrane vesicles (Booth et al., 1974), but proven to be useful also for the isolation of human placental SMMV (Glazier et al., 1988). However, to improve the isolation results we decided to replace the homogenization step of placental tissue by mincing the tissue in a Waring blender to pieces of approximately 5 mm^3. Then the mince was stirred for 30 min to loosen the microvilli and filtered through gauze to remove large tissue parts, after which the procedure according to Glazier et al. (1988) was continued. These modifications doubled the protein recovery (0.6% to 1.2%) and increased alkaline phosphatase

enrichment slightly (18-fold to 22-fold). Apparent contamination of basal membranes in our SMMV was in the same range (5-fold enrichment in Na^+/K^+ ATP-ase in our vesicles as compared to 6-fold enrichment in ouabaine binding in theirs). Although it is questioned in literature whether ATP-ases actually are located at the basal side of the human placenta exclusively (Eaton and Oakey, 1994), our modifications did not result in a higher contamination of ATP-ase containing structures. Contamination of subcellular organelles possibly interfering in transport studies, as mitochondria and endoplasmatic reticulum, was very small. Functional contamination of basal membranes is assumed to be negligible since no significant intravesicular accumulation of free Ca^{2+} in the presence of ATP could be determined. This effect was shown in BMV (Fisher et al., 1987 and Lafond et al., 1991) and not in SMMV (Brunette et al., 1991 and Page et al., 1993), providing evidence for the location of an ATP-dependent Ca^{2+}-transporter at the basal side in human placenta. However, Treinen and Kulkarni (1987) found ATP-stimulated uptake into SMMV. They concluded that this uptake might be into inside-out vesicles. Lafond et al. (1991) proposed that this effect might be a result of Ca^{2+}-stimulated alkaline phosphatase activity in SMMV. These factors might also be of influence in the small ATP-stimulated intravesicular Ca^{2+} uptake observed in our SMMV. We conclude that our SMMV are suitable for drug-nutrient interaction studies since alanine uptake was comparable to literature: sodium-coupled, inhibitable by structural analogues and saturable with kinetic parameters comparable to those reported by Johnson and Smith (1988) and Asai et al. (1982), K_m = 0.39 and 0.38 mM, respectively. The degree of inhibition of alanine uptake in the presence of a sodium gradient was the same as observed by Johnson and Smith (1988) and Enders et al. (1976). Obviously, the contribution of system L exceeded the contribution of system A in total alanine transport. As was proposed by Asai et al. (1982), alanine transfer is mediated by system A (inhibitable with glycine) with high affinity and by system L (inhibitable with phenylalanine) with low affinity. System ASC, another alanine transporting system, is assumed to be located in cell membranes other than SMM, e.g. BM (Johnson and Smith, 1988).

Three groups of commonly used drugs during pregnancy were investigated for their potency to interact with alanine transport. Organic anions, as analgesics and the diuretic furosemide, did not inhibit alanine uptake, whereas they did inhibit p-aminohippurate uptake in SMMV (van der Aa et al., 1994[a]). It seems that the amino acid transport systems A and L are insensitive to anionic drugs. The cationic H_2-receptor antagonists had no influence on alanine transfer either. However various cationic drugs were able to inhibit active uptake of the essential cationic

nutrient choline. Inhibitor concentrations used were very high, consequently no conclusions can be drawn yet on the possible clinical implications of the observed inhibition of choline transfer. The drugs of abuse ethanol and nicotine, did not impair AIB transport in human placental perfused cotyledon and microvillous vesicles (Schenker et al., 1989[a], 1989[b]). No inhibition of alanine transport was observed in our vesicles in the presence of high concentrations of nicotine, its metabolite cotinine, and ethanol. Thus, also in our study no evidence was found that fetal growth retardation after extensive ethanol consumption or nicotine exposition is related to a direct effect on amino acid membrane transport. Uptake of several amino acids, however, was decreased in human placental villi in the presence of high concentrations of morphine, nicotine and cocaine, but it was not clear whether the interaction was due to competition for the amino acid carriers (Barnwell and Rama Sastry, 1983). A more complex relationship was assumed between these drugs, Ca^{2+}-fluxes, cholinergic system and amino acid transport (Sastry et al., 1977, 1983). In this respect the report by Dicke et al. (1993), who observed stereoselective inhibition of sodium-dependent alanine uptake into human placental SMMV in the presence of low doses S(-)cocaine, is of special interest. Their results suggest a direct interaction of S(-)cocaine with the alanine transporter and are in contrast with our observations, where no inhibition was found in the presence of cocaine, S(+)- and S(-)adrenaline and S(+/-)noradrenaline. However, the inhibition they observed was low and it is questionable whether uptake at 1 min in their experiments can be considered as a true measure of initial transport rate, since uptake at 30 sec or later clearly deviated from linearity in our experiments (Figure 3). Another difference is that our control uptake at 15 sec was about 3000 times higher than their uptake at 1 min. Probably the sensitivity of the alanine transporter to exogenous compounds is higher at low alanine concentrations. Because maternal plasma contains a relatively high concentration of alanine (0.25 µmol/ml; Philipps et al., 1978), low alanine concentrations are not likely to occur in vivo. Even when a cocaine-addicted mother is underfed, fetal growth retardation would be merely a result of malnutrition itself than an interaction of cocaine with amino acid transport.

In conclusion, our SMMV showed marker enzyme, microscopic and functional characteristics in accordance with literature. Uptake of alanine, the most abundant amino acid in maternal plasma, was sodium-dependent, saturable and inhibitable by structural analogues. Commonly used drugs, like analgesics, H_2-receptor antagonists and drugs of abuse did not interact with alanine transport, whereas several cationic drugs inhibited choline uptake. The membrane vesicles isolated and used in this study provide a suitable tool for screening the inhibitory

potency of drugs on nutrient transporters.

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Chapter 3

p-AMINOHIPPURATE UPTAKE BY SYNCYTIAL MICROVILLOUS MEMBRANE VESICLES OF HUMAN TERM PLACENTA

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Summary

The mechanism of uptake of p-aminohippurate (PAH) by syncytial microvillous membrane vesicles of human term placenta was investigated. Initial PAH uptake and efflux were increased in the presence of a pH-gradient and a Cl^--gradient, respectively. Forced negative and positive membrane potentials did not influence the uptake, which indicated that the transport is not electrogenic. The pH-dependent increase is probably the result of a higher rate of diffusion due to a lower degree of dissociation of PAH. Because several organic anions failed to trans stimulate PAH uptake and FCCP did not decrease the uptake in the presence of an inwardly directed H^+-gradient, ruling out an PAH/OH^- antiport, an anion exchange system does not appear to be present in these membranes. Since electrogenicity and anion exchange seem not to be involved in the Cl^--dependent increase, an allosteric effect of Cl^- on the transporter might be possible.

Various organic anions were able to inhibit pH-stimulated PAH uptake significantly. Kinetic analysis of the probenecid sensitive part of uptake provided further evidence for mediated transport of PAH (K_m= 7.4 ± 2.6 mM and V_{max}= 2.0 ± 0.4 nmol/mg/15sec). Non-inhibitable diffusion accounted for the main part of total transport. Concentration dependent inhibition of PAH transport by probenecid showed a K_i of 2.5 ± 0.9 mM.

It is concluded that human placental syncytial microvillous membrane vesicles possess a low affinity transport mechanism for PAH with low specificity.

The importance of this system, for placental excretion of anionic drugs, will depend on the intrasyncytial concentration of these drugs, caused by the transport across the basal membrane.

Introduction

The human placenta, a polarised epithelium, like intestine, kidney and blood-brain barrier, is the crucial organ for absorption of nutrients and excretion of waste products necessary for fetal growth and development. Transport systems of the syncytiotrophoblast mediating absorption and excretion will be present in the syncytial microvillous membrane (SMM) and the basal membrane (BM) (Whitsett, 1980).

The majority of drugs are sufficiently lipophilic to cross the placenta by passive diffusion and to reach the fetal circulation (Chamberlain, 1986). Very little is known on the role of the placenta in eliminating drugs. Ganapathy et al. postulated that the human placental organic cation-proton antiporter may participate in eliminating cationic drugs from the fetal circulation (Ganapathy et al., 1988). In only one study, on the transfer of the anionic drug p-aminohippurate (PAH), in sheep placenta in vivo, no evidence was found for active transport (Mc Nay et al., 1969). Because of the fact that analgesics are the second most widely taken group of drugs during pregnancy (Bonati et al., 1990) it is relevant to get more information on the transport of anionic drugs across the human placenta. Membrane vesicles isolated from human term placenta have proven their usefulness in studying transport characteristics of compounds across separate membranes of the syncytiotrophoblast, without the limitations of species differences.

The present study was designed to investigate the characteristics of the transport of PAH, a model substrate for anionic drugs in kidney studies, in isolated syncytial microvillous membrane vesicles (SMMV) of human term placenta.

Materials and methods

Vesicle preparation

Human placental SMMV were prepared according to previously described procedures (Glazier et al., 1988; Glazier et al., 1990), which are essentially based

on a method for the isolation of brush-border membranes from rabbit kidney (Booth and Kenny, 1974). Placentas were obtained within 15 min after vaginal delivery. Fetal membranes and decidua were removed and the villous tissue was dissected and homogenized in a Warring Blender and Potter Elvjehem. At this point a modification was introduced: the homogenate was stirred for 30 min to loosen the microvilli. After $MgCl_2$ aggregation of contaminating membranes and differential centrifugation, both steps repeated once, the SMMV were obtained and suspended in an appropriate buffer to reach a final protein concentration of 10-15 mg/ml. The vesicles were frozen in $N_2(l)$ and stored at - 80°C until use. All subsequent steps were performed at 4°C.

Enzyme analysis

Purification was determined by assaying the specific activity in SMMV relative to the specific activity in the homogenate of the following marker enzymes according to previously described methods: alkaline phosphatase for SMM (Mircheff and Wright, 1976), Na^+/K^+ ATP-ase for BM (Schoot et al., 1977), succinate dehydrogenase for mitochondria (Pennington, 1961), NADPH-dependent cytochrome-C-reductase for smooth endoplasmatic reticulum (Omura and Takesue, 1970) and cathepsin-D for lysosomes (Anson, 1938). The orientation of the vesicles was determined by assaying the alkaline phosphatase activity with and without previous treatment with 0.1 % saponin. Protein was assayed with a coomassie blue kit (Biorad, München, Germany) with bovine serum albumin as standard.

Transport studies

The uptake of [^{3}H]-L-alanine and [^{3}H]-PAH into SMMV was measured in quadruplicate at 20°C and 37°C, respectively, using a rapid filtration technique (Schwab et al., 1984). Generally uptake was started by the addition of 10 µl (10-15 µg protein) membrane suspension to 40 µl extravesicular medium. In case of efflux studies 90 µl and in case of trans stimulation studies 190 µl extravesicular medium was used. In general the extravesicular media consisted of 10 mM Hepes-Tris, pH=7.4 or 10 mM Mes-Tris, pH=6.0 with the appropriate gradients adjusted to 310 mosmol with mannitol to which radiolabeled substrates were added. In case of trans stimulation studies, vesicles, suspended in 300 mM mannitol and 10 mM Hepes-Tris, pH=7.4, were preequilibrated with 500 µM unlabelled PAH, 1 mM furosemide, 1 mM probenecid or 1 mM urate for 60 min at 37°C. Efflux studies

were carried out with SMMV preequilibrated with 100 μM [^{3}H]-PAH for 60 min at 37°C. Exact composition of the transport conditions are given in the legends.

At appropriate time intervals the reaction was stopped by adding 2 ml of ice-cold stop buffer with the same composition as the intravesicular medium, except in case of membrane binding studies in which the stop buffers were equal to the extravesicular medium. The sample was filtered under vacuum through a Whatman GF/F-filter and washed twice. The radioactivity remaining on the filter was counted in a Beckman LS 6000 LL liquid scintillation counter. Corrections were made for aspecific filter binding. Uptake is expressed as pmol or nmol/mg protein and efflux as % of equilibrium uptake (mean ± SD). All transport experiments were conducted at least 3 times with 2 placentas.

Paired Student's t-test was used to determine statistical significance ($P<0.05$). Curve fitting was done by least-squares nonlinear regression analysis using the computer programme PCNONLIN (Metzler and Weiner, 1989) for the determination of K_m and V_{max} and the computer programme GraphPad INPLOT (4.0) (GraphPad Software, 1992) for the determination of K_i.

Chemicals and materials

[^{3}H]-L-alanine was obtained from New England Nuclear (Dreieich, Germany) and [^{3}H]-p-aminohippurate from Amersham (Buckinghamshire, U.K.). All other chemicals were purchased from either Sigma (St. Louis, U.S.A.), Merck (Darmstadt, Germany) or Boehringer (Mannheim, Germany) and were of analytical grade. GF/F-filters were obtained from Whatman Int. Ltd. (Maidstone, U.K.)

Results

Vesicle preparation

Enzyme analysis revealed a 14-fold enrichment of alkaline phosphatase for SMMV over homogenate. Specific activities: 550 ± 220 and 40 ± 13 μmol/hr/mg protein (N=11), respectively. This result is in accordance with previous reports (Glazier et al., 1988). The SMMV preparation was not enriched in mitochondria, smooth endoplasmatic reticulum or lysosomes (enrichment factors: 0.7, 1.3 and 0.1, respectively). The vesicle preparation showed a 5-fold enrichment in Na^+/K^+ ATP-ase over homogenate. Specific activities: 16 ± 7 and 3 ± 2 μmol/hr/mg protein

(N=11), respectively. The specific activity of Na^+/K^+ ATP-ase in human placenta is very low and therefore difficult to detect in the starting homogenate. A small contamination would result in an apparently high enrichment (Brunette and Allard, 1985; Karl et al., 1989).

Adding 0.1% saponin previous to enzyme analysis slightly increased the specific activity of alkaline phosphatase in SMMV (285 ± 80 versus 315 ± 115 µmol/hr/mg protein, N=3), which indicated that the vast majority of the vesicles were orientated right-side-out (>90%).

The procedure yielded approximately 50 mg protein/150 g placenta tissue.

Functional validation

The isolated membrane vesicles were validated functionally by measuring the Na^+-gradient dependent L-alanine uptake, which is present in SMM (Johnson and Smith, 1988). Figure 1 shows the time course of 100 µM L-alanine uptake into SMMV in the presence of an inwardly directed 50 mM Na^+- or 50 mM K^+-gradient. In the presence of a Na^+-gradient an overshoot above equilibrium value (peak vs. equilibrium=1.5) of the uptake of L-alanine was observed. Uptake at 2 min in the presence of a Na^+-gradient was 2.3-fold enhanced over that observed in the presence of a K^+-gradient. Freezing N_2(l) and storage (-80°C) for 1 month did not influence the alanine transport (data not shown).

PAH transport

1. Effect of different transport conditions

Uptake of 100 µM PAH at 15 sec into SMMV, as shown in Table 1, was increased significantly in the presence of an inwardly directed 100 mM Na^+- and 100 mM K^+-gradient (pH_i=pH_o=7.4) and an inwardly directed H^+-gradient (pH_i=7.4/pH_o=6.0) compared to pH_i=pH_o=7.4, ($P<0.05$). The pH-dependent PAH uptake was not influenced by the addition of 10 µg/mg protein of the proton ionophore FCCP. As shown in Table 2 a valinomycin induced inside negative or positive membrane potential did not influence PAH uptake at 15 sec compared to short-circuited and control membrane vesicles. Uptakes in the presence of equal intra- and extravesicular concentrations of Na^+ or K^+ were in the same range as uptakes in the presence of inwardly directed gradients of Na^+ or K^+ (Table 1 and 2). Loading the vesicles with 500 µM unlabelled PAH, 1 mM furosemide, 1 mM

probenecid or 1 mM urate did not trans stimulate the PAH uptake (32 ± 6, 37 ± 13, 34 ± 11 and 31 ± 7 (pmol/mg protein), respectively). In the presence of a H^+-gradient uptake of 100 µM PAH could be inhibited significantly by the addition of 5 mM probenecid, ($P<0.05$).

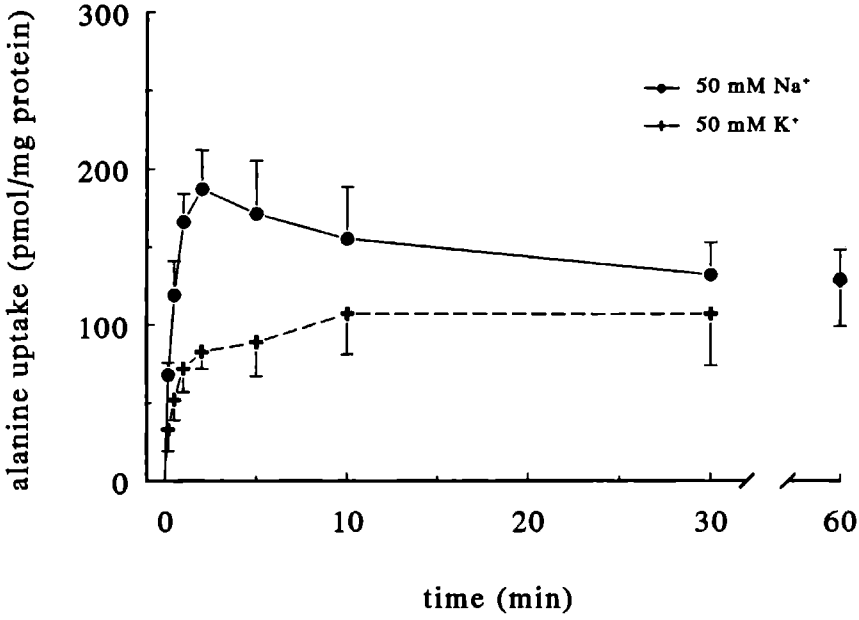

Figure 1. Time dependent uptake of 100 µM [^{3}H]-L-alanine into SMMV in the presence of an inwardly directed 50 mM Na^+- or 50 mM K^+-gradient. The vesicles were suspended in 300 mM mannitol and 10 mM Hepes-Tris, pH=7.4. The extravesicular medium consisted of 200 mM mannitol, 50 mM NaCl or 50 mM KCl and 10 mM Hepes-Tris, pH=7.4. Values are expressed as pmol/mg protein (mean ± SD).

Table 1. Effect of different transport conditions

Extravesicular medium	Uptake
pH=7.4	30 ± 8
pH=6.0	65 ± 18
100 mM Na^+	51 ± 7
100 mM K^+	53 ± 9
pH=6.0 + 5 mM probenecid	41 ± 10
pH=6.0 - FCCP	52 ± 15
pH=6.0 + FCCP	50 ± 12

Uptake of 100 µM [^{3}H]-PAH at 15 sec in different extravesicular media in a 10 mM Hepes-Tris, pH=7.4 or 10 mM Mes-Tris, pH=6.0 buffer with the specified gradient adjusted to 310 mosmol with mannitol. Vesicles were suspended in 300 mM mannitol and 10 mM Hepes-Tris, pH=7.4. In case of FCCP studies vesicles were preequilibrated with FCCP, dissolved in ethanol, for 60 min at 37°C. The same amount of ethanol was added to the controls. Values are expressed as pmol/mg protein (mean ± SD).

Table 2. Effect of membrane potential

Transport condition	Uptake
K^+_i=K^+_o=0 mM/Na^+_i=Na^+_o=100 mM	56 ± 8
K^+_i=K^+_o=100 mM/Na^+_i=Na^+_o=0 mM	52 ± 6
K^+_i=100 mM,K^+_o=0 mM/Na^+_i=0mM, Na^+_o=100 mM	53 ± 7
K^+_i=0 mM,K^+_o=100 mM/Na^+_i=100 mM,Na^+_o=0 mM	58 ± 2

Uptake of 100 µM *[^{3}H]*-PAH at 15 sec in different intra- and extravesicular media in the presence of 10 µg/mg protein valinomycin. Vesicles were suspended in 100 mM mannitol, 100 mM K-gluconate or 100 mM Na-gluconate and 10 mM Hepes-Tris, pH=7.4. Extravesicular media consisted of 100 mM mannitol, 100 mM K-gluconate or 100 mM Na-gluconate and 10 mM Hepes-Tris, pH=7.4. Vesicles were preequilibrated at 37°C for 60 min with 10 µg/mg protein valinomycin in ethanol, which was evaporated under N_2. Values are expressed as pmol/mg protein (mean ± SD).

Figure 2 shows the time course of the uptake of 100 µM PAH into SMMV in the presence of an inwardly directed H^+-gradient with and without 5 mM probenecid. PAH was rapidly taken up during the first 60 sec and reached equilibrium after 60 min. Equilibrium uptake was not affected by probenecid (data not shown). As shown in Figure 3, efflux of 100 µM PAH out of SMMV was significantly enhanced in the presence of an inwardly directed Cl -gradient, whereas SO_4^{2-} did not significantly enhance the efflux.

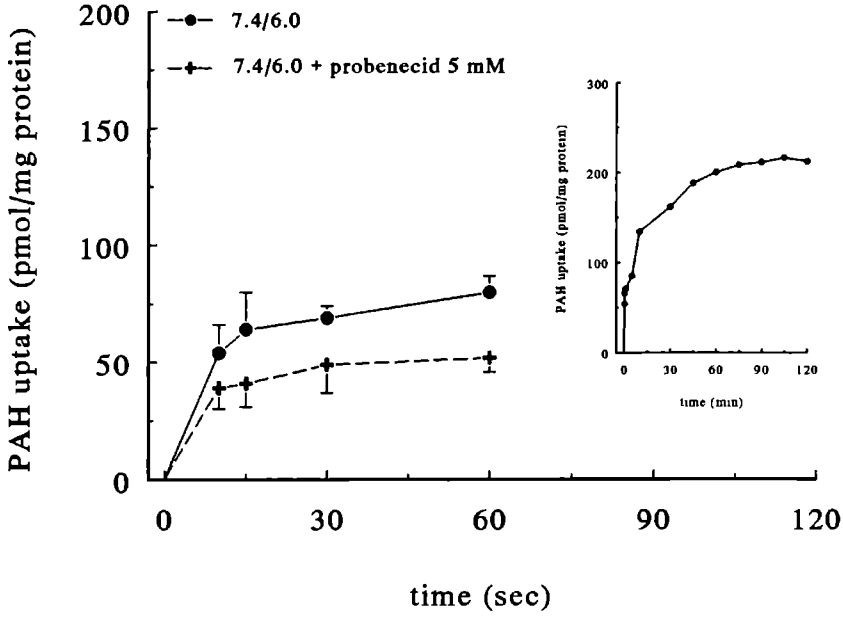

Figure 2. Time dependent uptake of 100 µM [^{3}H]-PAH into SMMV in the presence of an inwardly directed H^+-gradient with or without 5 mM probenecid. Vesicles were suspended in 300 mM mannitol and 10 mM Hepes-Tris, pH=7.4 Extravesicular medium consisted of 300 mM mannitol and 10 mM Mes-Tris, pH=6 0. Values are expressed as pmol/mg protein (mean ± SD). Inset: Time dependent PAH uptake until equilibrium with the same transport conditions.

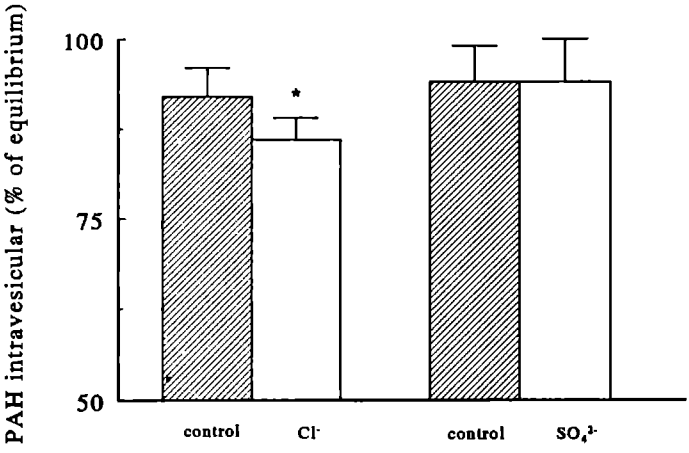

Figure 3. Efflux of 100 μM [^{3}H]-PAH at 15 sec out of SMMV in the presence or absence of an inwardly directed 50 mM Cl^-- or SO_4^{2-}-gradient. Vesicles were suspended in 200 mM mannitol, 50 mM K-gluconate, 10 mM Hepes-Tris, pH=7.4 in case of Cl^-- and 100 mM mannitol, 100 mM K-gluconate, 10 mM Hepes-Tris, pH=7.4 in case of SO_4^{2-}-efflux studies. Extravesicular medium consisted of 200 mM mannitol, 50 mM KCl, 10 mM Hepes-Tris, pH=7.4 in case of Cl^-- and 150 mM mannitol, 50 mM K_2SO_4, 10 mM Hepes-Tris, pH=7.4 in case of SO_4^{2-}-efflux studies. Values are expressed as % of equilibrium uptake (mean ± SD). Equilibrium uptake after 60 min was 370 ± 35 pmol/mg protein for Cl^-- and 277 ± 33 pmol/mg protein for SO_4^{2-}-efflux studies (mean ± SD). * $P<0.05$.

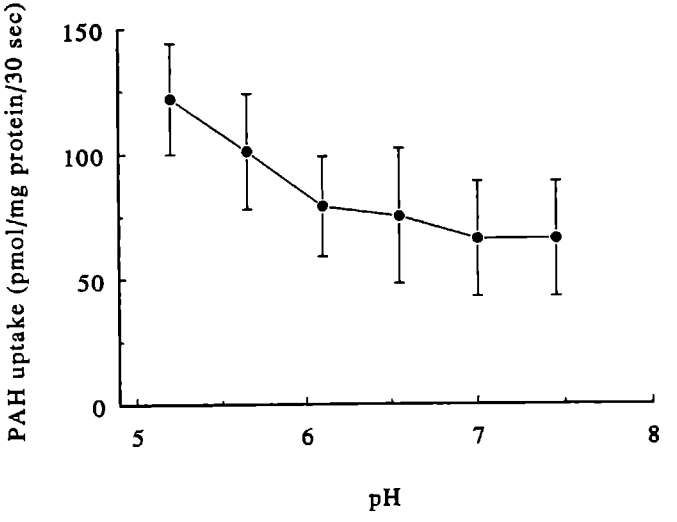

Figure 4. Effect of extravesicular pH on the 30 sec uptake of 100 μM [^{3}H]-PAH into SMMV. Vesicles were suspended in 300 mM mannitol and 10 mM Hepes-Tris, pH=7.4. Extravesicular medium consisted of 300 mM mannitol of which the pH was varied by changing the concentrations of Hepes, Mes and Tris, but total amount of Hepes or Mes + Tris was maintained at 10 mM. Data are expressed as pmol/mg protein (mean ± SD).

2. Effect of extravesicular pH

Since PAH is an organic anion with a pK_a of 3.8 it exists predominantly in the dissociated form at physiologic pH. We determined the effect of extravesicular pH on the uptake of 100 µM PAH. As shown in Figure 4 decreasing the extravesicular pH increased the PAH uptake.

3. Determination of membrane binding

To discriminate between uptake into SMMV and membrane binding we determined the dependence of PAH uptake on medium osmolarity. Figure 5 shows an inverse relation between the uptake of 100 µM PAH and osmotic pressure measured after 90 min ($Y=71.15*X+11.81$, $R^2=0.9998$). Extrapolation to infinite osmolarity indicated a small degree of membrane binding (±5%)

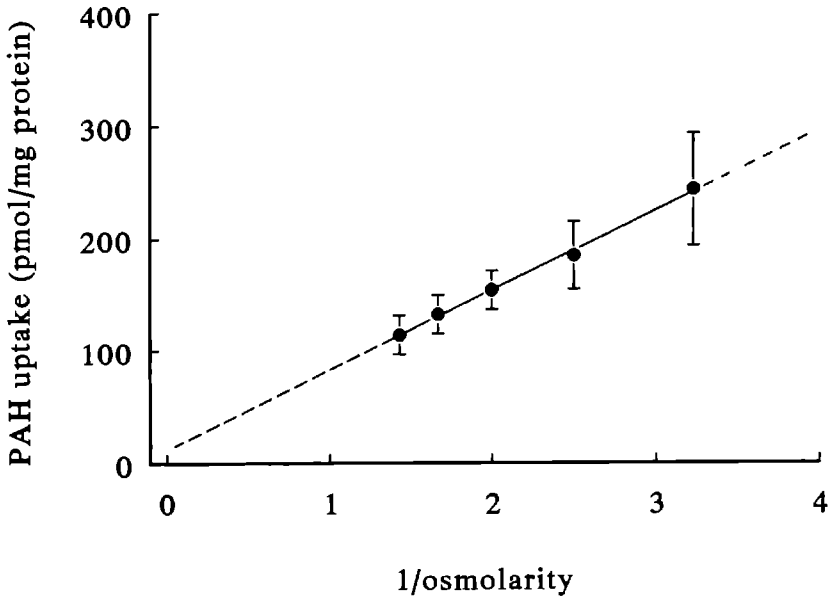

Figure 5. Effect of increasing osmotic pressure on equilibrium uptake (90 min) of 100 µM [^{3}H]-PAH into SMMV Vesicles were suspended in 300 mM mannitol and 10 mM Hepes-Tris, pH=7 4. Extravesicular media consisted of 10 mM Mes-Tris, pH=6 0 and increasing concentrations of mannitol. Stop buffers had the same composition as the extravesicular media Values are expressed as pmol/mg protein against reciprocal osmolarity (mean ± SD).

4. Effect of organic anions

As was shown in Figure 2 and Table 1 probenecid was able to inhibit PAH uptake. This is indicative of the existence of mediated transport for PAH in SMMV. To examine the specificity of the carrier we determined the effect of various organic anions on uptake of 100 μM PAH in the presence of an inwardly directed H^+-gradient. Table 3 shows that anionic drugs, except salicylate, inhibited PAH uptake, whereas urate did not inhibit uptake.

Table 3. Effect of organic anions on PAH uptake

Organic ion	mM	% Uptake
control		100
probenecid	5	62 ± 17 *
DIDS	1	65 ± 15 *
furosemide	5	56 ± 8 *
indomethacin	2.5	62 ± 5 *
salicylate	5	96 ± 23
urate	1	90 ± 27

Effect of various organic anions on the 15 sec uptake of 100 μM [^{3}H]-PAH in the presence of an inwardly directed H^+-gradient. Experimental conditions were similar to those given in the legend of Figure 2. Results are expressed relative to representive control uptakes (mean ± SD). * $P<0.05$.

5. Determination of kinetic parameters

Further evidence for mediated transport was obtained from experiments in which the uptake of increasing concentrations of PAH was determined in the presence of an inwardly directed H^+-gradient with and without 5 mM probenecid (Figure 6). Subtracting simple diffusion (that part of total uptake that could not be inhibited with probenecid) from the total uptake, resulted in a curve which could be described according to Michaelis-Menten kinetics. Least-squares nonlinear

regression analysis revealed a K_m of 7.4 ± 2.6 mM and a V_{max} of 2.0 ± 0.4 nmol/mg protein/15 sec. The inset curve shows the concentration dependent inhibition of the uptake of 100 µM PAH by probenecid in the presence of an inwardly directed H^+-gradient. Nonlinear regression analysis showed a K_i of 2.5 ± 0.9 mM.

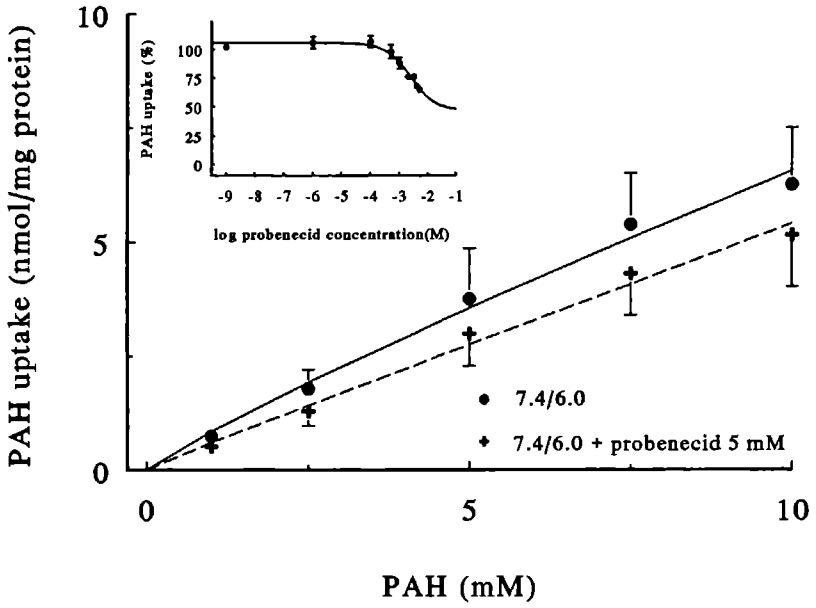

Figure 6. Concentration dependent uptake of [^{3}H]-PAH into SMMV in the presence of an inwardly directed H^+-gradient with or without 5 mM probenecid. Experimental conditions were the same as described in the legend of Figure 2. Uptakes were measured at 15 sec in the presence of increasing concentrations PAH (0-10 mM). Values are expressed as nmol/mg protein (mean ± SD). Inset: Effect of probenecid on the uptake of 100 µM [^{3}H]-PAH in the presence of an inwardly directed H^+-gradient. Experimental conditions were the same as described in the legend of Figure 2. Uptakes were measured at 15 sec in the presence of increasing concentrations probenecid (10^{-6}-5 mM). Values are expressed as % uptake of control (without probenecid). Control uptake was 88 ± 1 pmol/mg protein.

Discussion

Our SMMV were enriched 14-fold in alkaline phosphatase, which was in accordance with the results reported by Glazier et al. (Glazier, Jones and Sibley, 1988). They found a 6-fold enrichment in ouabain binding, which was in the same range as our 5-fold enrichment in Na^+/K^+ ATP-ase as marker for the contamination by basal membrane. Because Na^+/K^+ ATP-ase may also be found on plasma membranes of other placenta cells, the actual contamination of basal membrane will be lower (Illsley et al., 1990).

To be sure that our preparation exhibited transport characteristic of SMM, we validated our vesicles functionally. Alanine uptake into SMMV showed an overshoot within 2 min in the presence of an inwardly directed Na^+-gradient, which indicated a Na^+-dependent uphill transport system for alanine in our membrane vesicles. This transport system has been described in SMMV (Johnson and Smith, 1988) and not in BMV (Hoeltzli and Smith, 1989).

Initial uptakes were increased in the presence of a pH-gradient, a Na^+- and K^+-gradient and equal intra- and extravesicular concentrations of Na^+ or K^+ as compared to uptake in a buffer with only mannitol. Under the experimental conditions an overshoot was not observed, but this does not exclude secondary active transport. The enhanced initial efflux in the presence of a Cl^--gradient, as compared to a SO_4^{2-}-gradient, could be the result of an exchange mechanism as has been described in brush-border membranes of dog kidney (Steffens, Holohan and Ross, 1989). However, since PAH, furosemide, probenecid and urate failed to trans stimulate PAH uptake an exchange mechanism in SMMV seems unlikely. Also the proton ionophore FCCP did not decrease the PAH uptake in the presence of an H^+-gradient, which means that proton transport is not directly involved in PAH uptake and a PAH/OH^- exchange mechanism can be excluded. Forced inside positive and negative membrane potentials did not influence PAH uptake. The small increase in efflux in the presence of a Cl^--gradient and in uptake in the presence of a Na^+- and K^+-gradient is therefore unlikely to be the result of an influence of the membrane potential on PAH transport. Since uptake in the presence of inwardly directed Na^+- and K^+-gradients equals uptake in simply the presence of these ions, inreased uptake must be due to some influence of the ionic strength of the solutions rather than an effect of an altered membrane potential. Since electrogenicity and anion exchange seem not to be involved in the Cl^--dependent increase, an allosteric effect of Cl^- on the transporter, as was described by Inui et al. for rat renal basolateral membrane vesicles (Inui et al., 1986), might be possible. The increased initial uptake in the

presence of an H^+-gradient is probably the result of a higher diffusion due to a lower degree of dissociation of PAH. It can be calculated that at pH=6.0, 0.6% and at pH=7.4, 0.03% of the total amount of PAH is in undissociated form. Membrane binding accounted for approximately 5% of the total uptake at pH_o=6.0, and cannot be an explanation for the increased uptake compared to pH_o=7.4. A pH-dependent increase in vesicle volume cannot be completely ruled out, but this should have minor influence on initial uptake values. The absence of an uphill co-transport system as a driving force for PAH transport, in our vesicles of human placenta, is in accordance with the lack of active transfer for PAH in sheep placenta (Mc Nay et al., 1969).

Various organic anions were able to inhibit the initial pH-stimulated PAH uptake. These results suggest the presence of a mediated transport mechanism for PAH, with a relatively low specificity. The presence of a mediated process was confirmed by the observed saturability of PAH uptake. The kinetic parameters found provided evidence for a low affinity and high capacity transport system comparable with those demonstrated in brush-border membrane vesicles of dog kidney (Russel et al., 1988) and rat kidney (Bresler et al., 1989). Non-inhibital diffusion accounted for the main part of total transport.

The in vivo role of this carrier remains to be elucidated. Fetal kidney starts to excrete waste products into the amniotic fluid approximately 5 months after conception (Smith, 1951). An excretory function of the placenta for organic anions, especially during the first 5 months, would therefore be meaningful. The carrier system may in vivo mediate the transport of anionic drugs across SMM into the maternal circulation by facilitated diffusion. Whether this system plays a role of importance in the elimination of anionic drugs from the fetal circulation in preterm placenta will strongly depend on the intrasyncytial concentration of these drugs, resulting from uptake at the basal membrane.

Acknowledgements

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Chapter 4

UPTAKE OF CIMETIDINE INTO SYNCYTIAL MICROVILLOUS MEMBRANE VESICLES OF HUMAN TERM PLACENTA

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Summary

Uptake of the H_2-receptor antagonist, cimetidine, into syncytial microvillous membrane vesicles of human term placenta was investigated to clarify whether an active transport mechanism can be responsible for the observed barrier of the human placenta for cimetidine. Imposition of an outwardly directed H^+-gradient stimulated cimetidine uptake, resulting in a small transient overshoot. The H^+-gradient-dependent peak uptake was decreased under voltage clamped conditions by carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP), suggesting the presence of an organic cation-proton exchange mechanism. Uptake was partially, but significantly, inhibited by organic cation transport inhibitors, H_2-receptor antagonists and several other cationic drugs, providing further evidence for mediated uptake. H^+-gradient-dependent cimetidine uptake was saturable and characterized by a low affinity (K_m) of 6.3 mM and V_{max} of 17.5 nmol/mg protein/10 sec. We conclude that the system cannot play an important role in the barrier function of the human placenta in the transport of cimetidine. Rather than active transport, other factors, as for instance the degree of ionization of cimetidine at physiological pH, seem to be a more likely explanation for the low clearance of cimetidine across the human placenta.

Introduction

H_2-receptor antagonists, like cimetidine and ranitidine, are frequently used therapeutic agents in the treatment of peptic ulcer in humans. Additionally, cimetidine is sometimes used in the prevention of gastric acid aspiration for women at labour under general anaesthesia (McGowan, 1979).

The guanidine-analogues ranitidine (Mihaly et al., 1982) and cimetidine (Mihaly et al., 1983) cross the placenta in pregnant ewes at near term. Fetal plasma concentrations, however, were much lower than maternal plasma concentrations after maternal dosage. This trans placental gradient was reported to be due to the placenta itself (e.g. active transport from fetus to mother, irreversible placental metabolic elimination), since fetal renal elimination did not play an important role (Mihaly et al., 1983). In a follow-up study the trans placental gradient could not be explained by a low placental permeability or differential plasma protein binding of cimetidine between mother and fetus (Ching et al., 1985).

Cimetidine, administered to women at labour, showed a slow placental transfer finally achieving equal concentrations in maternal and fetal plasma. Protein binding or placental metabolism was a minor influence. Because the concentration of cimetidine in the amniotic fluid was raised and there was a decreasing concentration in the cord blood, a role for fetal renal elimination seemed likely (Howe et al., 1981). However, since the fetal kidney begins to excrete waste products approximately five months after conception, the placenta could play a role in the elimination of cationic drugs before five months.

In the perfused human placental cotyledon, the clearance of cimetidine was low, providing evidence for a barrier function of the placenta in cimetidine transfer (Ching et al., 1987). Since fetal and maternal plasma concentrations did equilibrate, active transport appeared to play an insignificant role in maintaining a barrier across the human placenta. The absence of mediated transport was confirmed by Schenker et al. (1987), using the same experimental technique. The maternal to fetal transfer of cimetidine showed no signs of accumulation, saturability or susceptibility to inhibition by structural analogues. In this study, no evidence was found also for the saturation of cimetidine uptake in microvillous membrane vesicles of human term placenta. However, concentrations of up to only 400 μM were used and no driving forces were applied.

More precise information concerning the driving forces, specificity and saturability of transport could provide better insight into the underlying mechanism of the low clearance for cimetidine across the human placenta. The present study

was designed to investigate in more detail the molecular mechanisms of cimetidine transport across the human term placenta by using isolated syncytial microvillous membrane vesicles (SMMV).

Materials and Methods

Vesicle preparation

Human placental SMMV were prepared according to a modification of the procedure described by Glazier et al. (1988) as method number three. Briefly, tissue obtained within 15 min after uncomplicated delivery was minced in a Warring blender and stirred for 30 min to loosen the microvilli. After $MgCl_2$ aggregation and differential centrifugation, both steps repeated once, SMMV were harvested and suspended in an appropriate buffer. The protein concentration of SMMV used in this study was 10.4 ± 2.2 mg/ml (N=23). All subsequent steps were performed at 4°C. Vesicles were frozen in N_2(l) and stored at -80°C for 4 weeks at the maximum. Protein was assayed with a coomassie blue kit (Biorad, München, Germany). Alkaline phosphatase enrichment of SMMV, measured according to Mircheff and Wright (1976), was 22-fold as compared to starting mince (M_0: 60 ± 20, SMMV: 1350 ± 570 µmol/h/mg, N=14).

Uptake studies

Uptake of [^{3}H]-cimetidine into SMMV was measured in quadruplicate at 37°C, using a rapid filtration technique (Russel et al., 1988). Uptake was started by addition of 10 µl membrane suspension to 40 µl extravesicular medium containing radiolabeled compound. The transport conditions are given in the legends. At appropriate time intervals, the reaction was terminated by adding 2 ml of ice-cold stop solution with the same composition as the intravesicular medium, except for membrane binding studies in which the stop solutions were equal to the corresponding extravesicular media. The samples were filtered under vacuum through a Whatman GF/F glass fibre filter and washed threefold. Radioactivity remaining at the filter was counted in a Beckman LS 6000 LL liquid scintillation counter. Corrections were made for nonspecific filter binding.

Data analysis

Data are presented as means ± SD, with N representing the number of experiments with different vesicle preparations. Statistical significance of differences in cimetidine uptake was determined with the use of two-tailed, paired Student's *t*-test ($P<.05$). Curve fitting was done by least squares nonlinear regression analysis, using the computer program PCNONLIN (Metzler and Weiner, 1989).

Chemicals

[^{3}H]-Cimetidine (21 Ci/mmol) was obtained from Amersham (Aylesbury, U.K.). Cimetidine was generously donated by Smith, Kline & French Laboratories (Herts, U.K.), nizatidine by Eli Lilly & Company (Indianapolis, IN) and trimethoprim by Bergel (the Netherlands). Mepiperphenidol and famotidine were generously donated by Merck, Sharpe & Dohme (Rahway, NY). n-Methylnicotinamide (NMN) and tetraethyl-ammoniumbromide (TEA) were purchased from Janssen Chimica (Beerse, Belgium). All other chemicals were purchased from either Sigma (St Louis, MO), Merck (Darmstadt, Germany) or Boehringer (Mannheim, Germany). GF/F filters were obtained from Whatman Int. Ltd (Maidstone, U.K.).

Results

H^+-gradient-dependent uptake of cimetidine

The outwardly directed proton gradient (pH_i=6.0/pH_o=7.4) stimulated cimetidine (20 μM) uptake into SMMV, resulting in a small, but significant, transient overshoot (peak vs. equilibrium=1.3) as compared to uptake in the absence of a H^+-gradient (pH_i=pH_o=7.4) (Figure 1). The difference in equilibrium uptake at 60 min is probably due to a pH-dependent increase in vesicle volume, but this should have minor effects on initial uptake values.

Effect of ionophores on H^+-gradient uptake

To determine whether the stimulated uptake of cimetidine in the presence of a proton-gradient was due to the activity of an organic cation-proton antiport system

or a H^+-diffusion potential the effect of the proton ionophore FCCP was evaluated. FCCP causes an enhanced H^+-flux down its concentration gradient, which lowers the availability of H^+ for a possible cation-proton exchanger and consequently decreases the uptake of the cation. The FCCP induced H^+-flux also renders the inside of the vesicle more negative resulting in an increase of uptake of the cation if uptake is dependent on a H^+-diffusion potential. As shown in Table 1, FCCP did not increase the H^+-gradient-dependent initial and peak uptake rates. These results show that an inside negative H^+-diffusion potential is unlikely to be a driving force for cimetidine uptake. However, in voltage clamped vesicles (equal amounts of potassium at both sides of the membrane in presence of the K^+-ionophore valinomycin), in which peak and equilibrium uptakes were not significantly altered, FCCP was able to decrease cimetidine uptake, resulting in a diminished overshoot. The decreased uptake suggests an organic cation-proton exchanger responsible for the small transient overshoot.

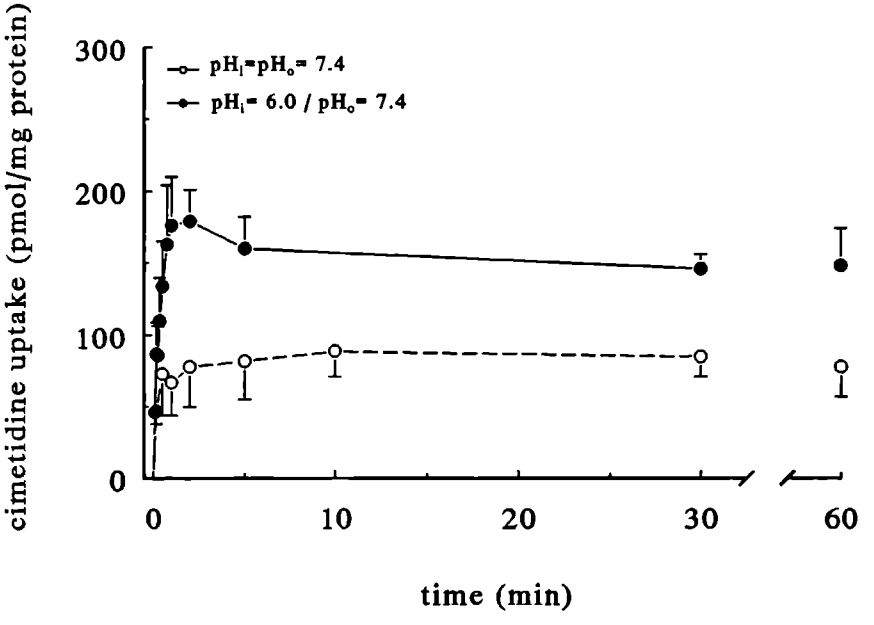

Figure 1. Time-dependent uptake of 20 μM [^{3}H]-cimetidine into SMMV in the presence or absence of an outwardly directed H^+-gradient. Vesicles were suspended in 300 mM mannitol and 10 mM Mes-Tris, pH=6.0 or 10 mM Hepes-Tris, pH=7.4. Extravesicular medium consisted of 300 mM mannitol and 10 mM Hepes-Tris, pH=7.4. Values are expressed as pmol/mg protein (N=11).

Table 1. Effect of H^+-gradients and ionophores on cimetidine uptake

	Condition	Uptake		
		10 sec	60 sec	60 min
A	pH_i=7.4/pH_o=7.4	47 ± 9	67 ± 23	78 ± 21
	pH_i=6.0/pH_o=7.4	87 ± 9	176 ± 34	148 ± 26
	pH_i=6.0/pH_o=7.4+FCCP	84 ± 16	172 ± 36	154 ± 18
B	pH_i=6.0/pH_o=7.4+val+FCCP	57 ± 12	108 ± 34	130 ± 35

Uptake of 20 μM [^{3}H]-cimetidine into vesicles suspended in 300 mM mannitol and 10 mM Hepes-Tris, pH=7.4 or 10 mM Mes-Tris, pH=6.0 (A) or 100 mM mannitol, 100 mM KCl and 10 mM Mes-Tris, pH=6.0 (B). Extravesicular media consisted of 300 mM mannitol and 10 mM Hepes-Tris, pH=7.4 (A) or 100 mM mannitol, 100 mM KCl and 10 mM Hepes-Tris, pH=7.4 (B). Vesicles were preequilibrated for 60 min at 37°C with 20 μM valinomycin (val) and/or 40 μM FCCP. FCCP (40 μM) was also added to the extravesicular medium. Data are presented as pmol/mg protein (N=3).

Determination of membrane binding

Figure 2 shows that the uptake of 20 μM cimetidine was inversely related to medium osmolarity ($R^2 > 0.81$ of individual regression lines), indicating transport into an osmotically responsive intravesicular space. Because of the large variation in uptake data between different placentas, only a rough estimate could be made of the actual intravesicular uptake, which accounted for 77 ± 27 % of total uptake

Effect of organic cations

The inhibitory effect of various organic cations on cimetidine uptake was investigated. Table 2 shows the initial H^+-gradient-dependent uptake of 20 μM cimetidine as percentage of representative control uptake in the presence of 1 or 5 mM inhibitor. The prototypic organic cation transport inhibitors mepiperphenidol and amiloride and several other cationic compounds, like trimethoprim, TEA, and the H_2-receptor antagonists ranitidine, nizatidine and famotidine were able to inhibit cimetidine uptake partially, but significantly. The endogenous organic cation, n-methylnicotinamide (NMN), decreased cimetidine uptake also, whereas choline and guanidine did not.

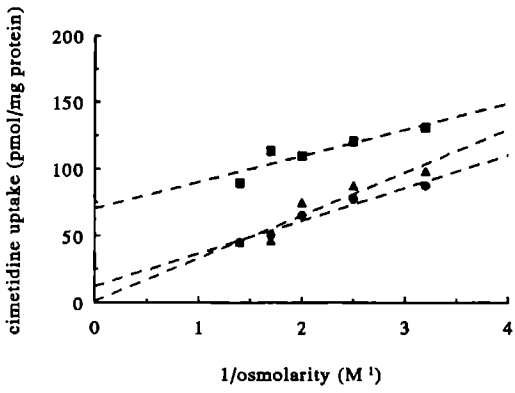

Figure 2. Effect of increasing osmotic pressure on uptake at 30 min of 20 μM [^{3}H]-cimetidine into SMMV. Vesicles were suspended in 300 mM mannitol and 10 mM Hepes-Tris, pH=7.4. Extravesicular media consisted of 10 mM Hepes-Tris, pH=7.4 and increasing concentrations of mannitol. Values of three placentas are expressed individually as pmol/mg protein versus reciprocal osmolarity of the final solution (N=3).

Table 2. Effect of various organic cations on cimetidine uptake

Organic cation	mM	% uptake
control		100
mepiperphenidol	1	55 ± 19 *
amiloride	1	85 ± 2
	5	76 ± 15 *
trimethoprim	1	54 ± 20 *
ranitidine	1	74 ± 13 *
famotidine	1	69 ± 12 *
nizatidine	1	67 ± 14 *
tetraethylammonium	1	71 ± 10 *
N-methylnicotinamide	1	74 ± 16 *
guanidine	1	100 ± 2
choline	1	102 ± 5

Effect of various organic cations (1 or 5 mM) on the 30 sec uptake of 20 μM [^{3}H]-cimetidine in the presence of an outwardly directed H^+-gradient. Experimental conditions were similar to those given in the legend of Figure 1. Results are expressed relative to representative control uptakes (N=4). Mean control uptake was 112 ± 30 pmol/mg protein (N=10). * $P<0.05$.

Kinetics of cimetidine uptake

The presence of mediated uptake was further investigated by evaluating the initial uptake of increasing concentrations of cimetidine in the presence of an outwardly directed H^+-gradient. The relation between cimetidine concentration and uptake rate was nonlinear. Total uptake corrected for estimated nonsaturable uptake showed saturability as can be seen in Figure. 3. Kinetic parameters were determined by fitting an euation combining Michaelis-Menten and linear kinetics:
$v=V_{max} \cdot S / (K_m + S) + k \cdot S$ to the data, where v is the initial cimetidine uptake rate and S is the cimetidine concentration. Nonlinear regression analysis revealed a K_m of 6.3 ± 4.4 mM, a V_{max} of 17.5 ± 10.2 nmol/mg/10 sec and a k of 1.6 ± 0.3 nmol/mg/10sec/mM.

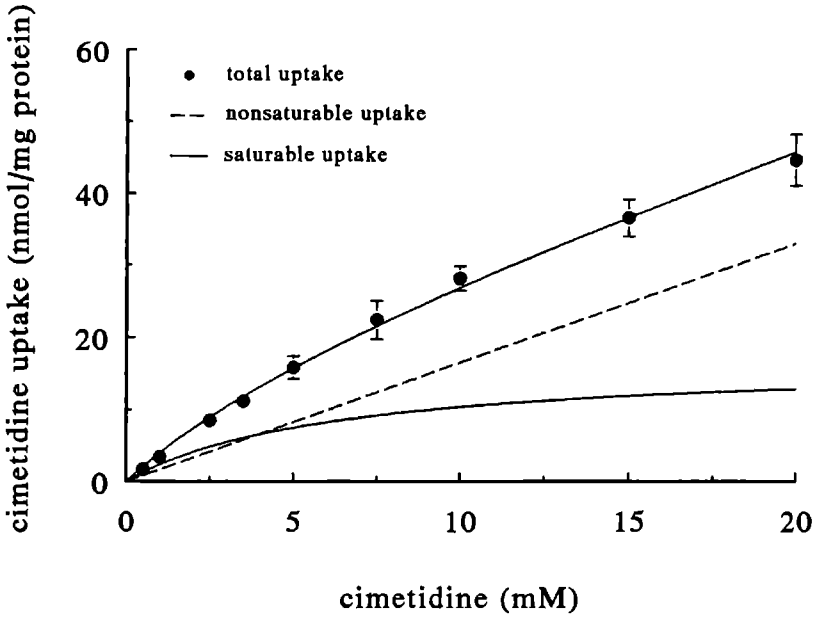

Figure 3. Concentration-dependent uptake of [^{3}H]-cimetidine at 10 sec into SMMV in the presence of an outwardly directed H^+-gradient. Experimental conditions were the same as described in the legend of Figure 1. Cimetidine concentrations ranged from 10 µM to 20 mM. Values are expressed as nmol/mg protein (N=3).

Discussion

In human in vivo studies and in the artificially perfused human placental cotyledon, no evidence was found for mediated transport of cimetidine (Howe et al., 1981, Ching et al., 1987). It was concluded that transfer of cimetidine across the human placental trophoblast occurs by passive diffusion. However, if diffusional transfer quantitatively exceeds uptake via a mediated pathway, the latter will be masked and not detected in these type of studies. Therefore, isolated membrane vesicles are a more appropriate tool to investigate whether mediated transport occurs across the plasma membrane.

Our results in human placental SMMV show that cimetidine transfer across the syncytial microvillus membrane of the human term placenta will be mainly due to passive diffusion, confirming the results obtained by others (Howe et al., 1981, Ching et al., 1987, Schenker et al., 1987). However, a small, but significant part of total cimetidine uptake into SMMV could be attributed to mediated uptake, since uptake was pH-dependent, inhibitable by several organic cations and saturable. Because the proton ionophore, FCCP, decreased H^+-gradient-dependent cimetidine peak uptake under voltage clamped conditions, we suggest the presence of an organic cation-proton antiport system for cimetidine in human placental SMMV, responsible for the small part of mediated uptake. Cimetidine itself has been reported to interfere with the human placental Na^+/H^+-exchanger present in the syncytial microvillus membrane, in a reversible manner and competitive with respect to Na^+ (Ganapathy et al., 1986). It seems possible that cimetidine, in the presence of a H^+-gradient, is transported by this Na^+/H^+-exchanger. The inhibition of cimetidine uptake we observed in presence of the Na^+/H^+-exchange inhibitor amiloride would support such a view. However, this cationic drug is not a selective inhibitor, because it also inhibits organic cation transport in human placental and rabbit renal cortical brush-border membrane vesicles (Ganapathy et al., 1988, Miyamoto et al., 1989). The inhibition of guanidine uptake into renal cortical brush-border membrane vesicles by cimetidine (Miyamoto et al., 1989) has not been found in human placental brush-border membrane vesicles (Ganapathy et al., 1988). Since guanidine did not inhibit cimetidine uptake significantly in our experiments, the guanidine analogue cimetidine is not likely to be transported by the guanidine-proton antiport system present in human placental SMMV (Ganapathy et al., 1988). Cimetidine did inhibit membrane potential-dependent choline uptake into SMMV (van der Aa et al., 1994). In contrast, choline was not able to inhibit cimetidine uptake significantly in the present study. Therefore it is also unlikely that cimetidine

is transported by the choline carrier.

The affinity of the system is low, as can be concluded from a K_m of 6.3 mM characterizing the concentration-dependent uptake of cimetidine. The clinical relevance of this low affinity system with respect to the use of H_2-receptor antagonists during pregnancy seems therefore limited in terms of a possible accumulation into the trophoblast or extensive elimination from fetal to maternal circulation. Such a low affinity transport system in microvillus membranes would only make sense in combination with a high affinity system in basal membranes. From in vivo and perfused cotyledon experiments no evidence is available for the presence of such a system (Howe et al., 1981, Schenker et al., 1987).

We therefore conclude that the system cannot be an important factor in the barrier function of the human placenta in the transport of cimetidine as observed by Ching et al. (1987). Thus, active transport can indeed be ruled out in maintaining such a barrier. Other factors, as for instance the degree of ionization of cimetidine (pK_a=6.8) at physiological pH, are probably of more importance to the low clearance of cimetidine across the human placental trophoblast.

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Chapter 5

UPTAKE OF CHOLINE INTO SYNCYTIAL MICROVILLOUS MEMBRANE VESICLES OF HUMAN TERM PLACENTA

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Summary

The uptake of the quaternary ammonium compound choline was studied in syncytial microvillous membrane vesicles of human term placenta. Uptake was stimulated by an inside negative membrane potential and by loading the vesicles with unlabelled choline. Imposition of an inwardly directed Na^+- or outwardly directed H^+-gradient did not result in a stimulation of choline uptake. Several organic cations were able to inhibit choline transport: hemicholinium-3 ≥ choline ≥ mepiperphenidol > cimetidine ≥ famotidine. The kinetics of uptake involved a saturable process for choline with high affinity (K_m= 550 μM).

Our results confirm the presence of a carrier mediated transport system in human placental syncytial microvillous membranes. The system appears to be electrogenic, and able to transport choline efficiently from the maternal circulation into the placenta driven by the negative cell interior.

Introduction

The cationic quaternary ammonium compound choline is an essential substrate for the synthesis of phospholipids and acetylcholine. Since the placenta and fetus do not synthesize choline, it must be transported from the maternal plasma by the trophoblast (Welsch, 1978). The human placental concentration of choline is

approximately 1 mM, whereas maternal plasma contains about 20 μM choline. Accumulated choline against this concentration gradient is rapidly converted into acetylcholine (Welsch, 1976). Although acetylcholine is present in large concentrations in the human placenta its main function, in an organ which lacks innervation, remains still unclear. It has been proposed that the placental cholinergic system plays a role in the regulation of amino acid transport (Rowell and Sastry, 1981).

The mechanisms of choline transport have been characterized in various tissues. In neural tissue a high affinity sodium cotransport system has been described (Kuhar and Murin, 1978). In erythrocytes facilitated diffusion is involved, which is not sodium dependent (Deves and Krupka, 1979). In rat intestinal brush-border membranes a choline transporter independent of sodium, H^+ or membrane potential was found with a K_m of 159 μM (Saitoh et al., 1992). Two saturable transport systems were found in rabbit renal brush-border membranes (K_m= 97 μM and K_m≈ 10 mM). Uptake was stimulated by an inside negative membrane potential and by a trans concentration gradient of choline Wright et al., 1992).

The mechanism of choline transport across the placenta has still not been fully elucidated. In placenta fragments choline was concentrated against a concentration gradient. Nonsaturable passive diffusion in combination with carrier mediated transport was found, with K_m= 350 μM and V_{max}= 75 nmol/ml intracellular water/min (Welsch, 1978). Saturable sodium independent choline uptake into the trophoblast was demonstrated in the dually perfused guinea pig placenta (Sweiry et al., 1985) and human placenta (Sweiry et al., 1986). Nonsaturable components, reflecting passive diffusion, were also observed. Driving forces and specificity, however, of choline transport in the human placenta have still not been clarified.

The present study was designed to provide more insight into the mechanisms of choline transport across the human placenta, using isolated syncytial microvillous membrane vesicles (SMMV) of human term placenta.

Materials and methods

Preparation of SMMV

SMMV were prepared from fresh human term placentae, essentially according to a procedure by Glazier et al. (1988) described as method number three. Tissue was minced in a Warring blender. The mince was stirred for 30 min to

loosen the microvilli. After $MgCl_2$ aggregation and differential centrifugation SMMV were harvested and suspended in an appropriate intravesicular buffer to a final protein concentration of 10-15 mg/ml. Vesicles were frozen in liquid nitrogen and stored at -80°C for four weeks at the maximum. The alkaline phosphatase enrichment, measured according to Mircheff and Wright (1976) of SMMV compared to starting mince was 24-fold (M_0= 60 ± 7 and SMMV= 1400 ± 175 µmol/hr/mg, N=6). Protein was assayed with a coomassie blue kit (Biorad, München, Germany).

Uptake studies

Uptake of [^{3}H]-choline into SMMV was measured in quadruplicate at 37 °C using a rapid filtration technique (Russel et al., 1988). The samples were filtered through Whatman GF/F filters and the radioactivity remaining on the filters was counted in a Beckman LS 6000 LL liquid scintillation counter. Corrections were made for nonspecific filter binding. Exact compositions of the transport conditions are given in the legends. Uptake is expressed as pmol or nmol/mg protein or % of control uptake (mean ± SD) of at least three experiments with three placentas. Paired Student's t-test was used to determine statistical significance ($P<0.05$). Curve fitting was done by least squares nonlinear regression analysis using the computer programme PCNONLIN (Metzler and Weiner, 1989).

Chemicals

[^{3}H]-Choline was obtained from Amersham (Buckinghamshire, UK), cimetidine from Smith, Kline & French (Welwyn Garden City, Herts, UK). and mepiperphenidol from Merck, Sharp & Dohme (Rahway, N.Y., USA). All other chemicals were purchased from either Sigma (St. Louis, M.O., USA) or Merck (Darmstadt, Germany) and were of analytical grade. GF/F filters were obtained from Whatman Int. Ltd. (Maidstone, England).

Results

Time dependent uptake of choline

The effect of an imposed gradient of H^+ (pH_i=6.0/pH_o=7.4) and Na^+ (100 mM) on the uptake of 25 µM choline into SMMV is illustrated in Figure 1. An outwardly directed proton gradient did not stimulate the choline uptake (A). Neither did an inwardly directed sodium gradient (B). The effect of an inside negative membrane potential is shown in Figure 2. A stimulation of the uptake above equilibrium was observed (peak versus equilibrium=1.8).

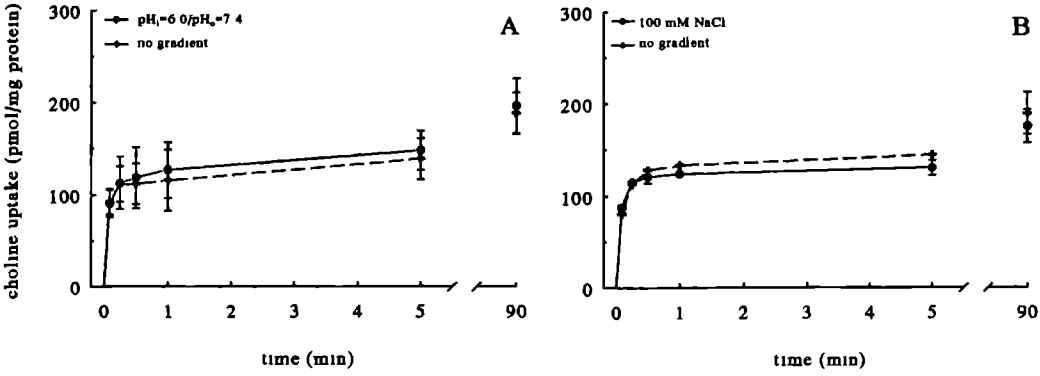

Figure 1. Effect of an outwardly directed proton gradient (A) and inwardly directed sodium gradient (B) on the uptake of 25 µM [^{3}H]-choline into SMMV A Vesicles were suspended in 100 mM mannitol, 100 mM KCl and 10 mM Hepes-Tris, pH=7.4 or 10 mM Mes-Tris, pH=6.0 Extravesicular medium consisted of 100 mM mannitol, 100 mM KCl and 10 mM Hepes-Tris, pH=7.4 B Vesicles were suspended in 100 mM mannitol, 100 mM KCl and 10 mM Hepes-Tris, pH=7.4 Extravesicular media consisted of 100 mM mannitol, 100 mM KCl or 100 mM NaCl and 10 mM Hepes-Tris, pH=7.4 10 µl vesicles were added to 40 µl extravesicular medium Values are expressed as pmol/mg protein (mean ± SD)

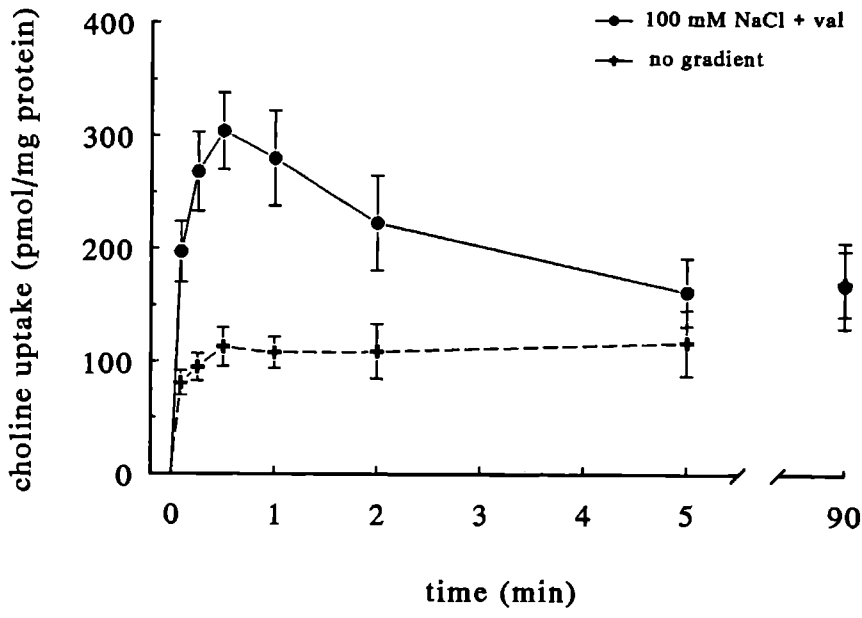

Figure 2. Effect of an inside negative membrane potential on the uptake of 25 µM [^{3}H]-choline into SMMV. Vesicles, suspended in 100 mM mannitol, 100 mM KCl and 10 mM Hepes-Tris, pH=7.4, were preequilibrated with 20 µM valinomycin at 37 °C. Extravesicular media consisted of 100 mM mannitol, 100 mM KCl or 100 mM NaCl and 10 mM Hepes-Tris, pH=7.4. 10 µl vesicles were added to 40 µl extravesicular medium. Values are expressed as pmol/mg protein (mean ± SD).

Counter transport of choline

The effect of loading the vesicles with 5 mM unlabelled choline on the uptake of 250 µM choline into SMMV is shown in Figure 3. Voltage clamp conditions were maintained by adding valinomycin and equal concentrations of intra- and extravesicular K^+. The outwardly directed choline gradient stimulated the choline uptake above equilibrium (peak versus equilibrium=2.1).

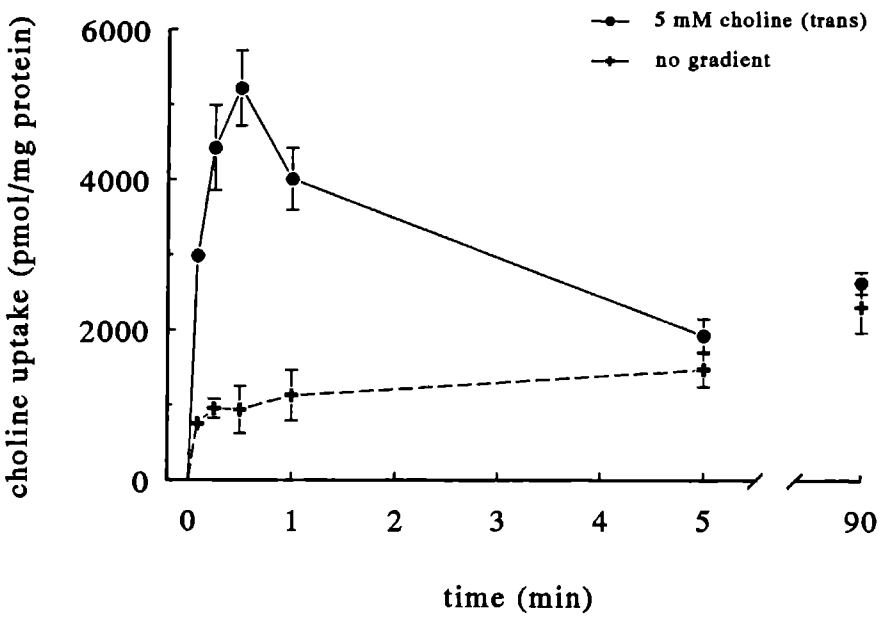

Figure 3. Effect of an outwardly directed gradient of 5 mM choline on the uptake of 250 µM [^{3}H]-choline into SMMV. Vesicles, suspended in 100 mM mannitol, 100 mM KCl and 10 mM Hepes-Tris, pH=7.4, were, in case of the outwardly directed choline gradient, preequilibrated with 5 mM unlabelled choline at 37 °C. Control and with choline loaded vesicles were also preequilibrated with 20 µM valinomycin at 37°C. Extravesicular media consisted of 100 mM mannitol, 100 mM KCl, 20 µM valinomycin, 10 mM Hepes-Tris, pH=7.4 and unlabelled choline to achieve an extravesicular concentration of choline of 250 µM. 5 µl vesicles were added to 195 µl extravesicular medium. Values are expressed as pmol/mg protein (mean ± SD).

Effect of different drugs on choline uptake

Figure 4 shows the effect 5 mM of various drugs on the uptake of 250 µM choline into SMMV, loaded with 5 mM unlabelled choline. Voltage clamp conditions were maintained. The structure analogue hemicholinium (HC-3), choline itself and the organic cation transport inhibitor mepiperphenidol (mepi) reduced choline uptake by 85 %. Cationic drugs like cimetidine (cim) and famotidine (fam)

were able to inhibit uptake by 50 %. Low-molecular weight organic cations like tetraethylammonium (TEA) and n-methylnicotinamide (NMN) showed no inhibitory potency, neither did the organic anion transport inhibitor probenecid.

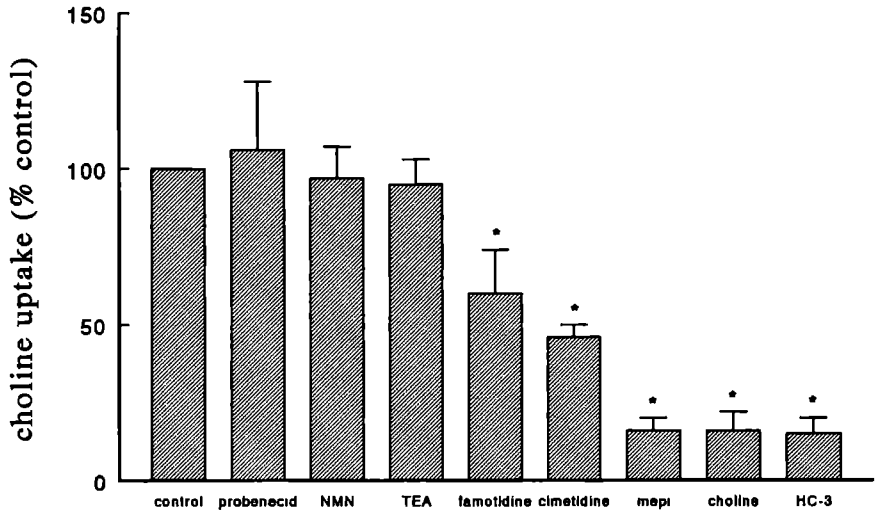

Figure 4. Effect of 5 mM cis concentrations of organic cations and anions on the 10 sec uptake of 250 μM [^{3}H]-choline into SMMV in the presence of an outwardly directed gradient of 5 mM choline. Experimental conditions were the same as described in the legend of Figure 3, except that 5 mM of specified organic drugs were added to the extravesicular media. Values are expressed as % of control uptake (mean ± SD). Control uptake (without inhibitor) was 3130 ± 515 pmol/mg protein. * Statistical significance ($P<0.05$; df=2).

Kinetics of choline uptake

Over a range of 125 μM to 5mM, saturability in the uptake of choline was seen in the presence of an outwardly directed 5 mM choline gradient (Figure 5). Uptake values were measured under voltage clamp conditions. The curve resulting from subtracting simple diffusion (that part of total uptake that could not be inhibited by extravesicular choline) from total uptake was analyzed according to

Michaelis-Menten kinetics. Least-squares nonlinear regression analysis revealed a K_m of 550 ± 80 μM and a V_{max} of 10.0 ± 0.49 nmol/mg protein/10 sec (R^2 = 0.995).

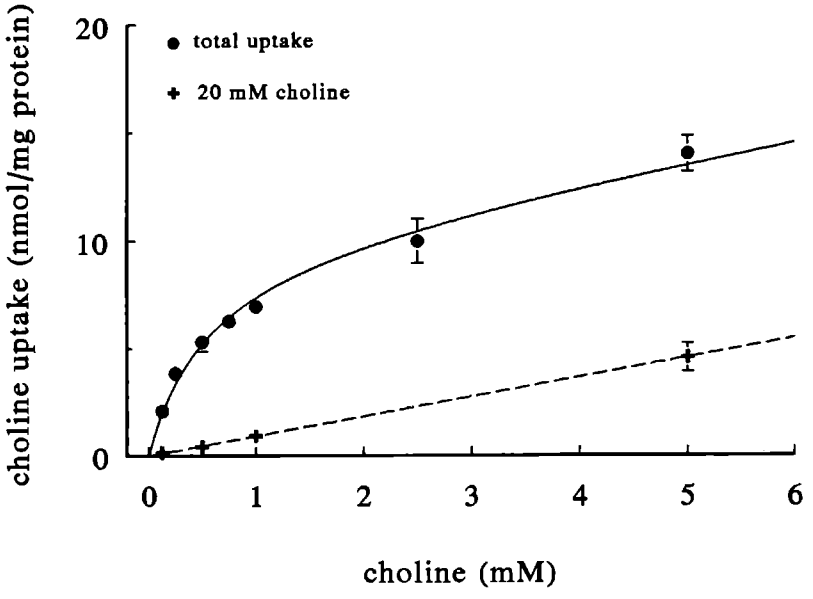

Figure 5. Concentration dependent uptake of choline at 10 sec into SMMV in the presence of an outwardly directed gradient of 5 mM choline with and without 20 mM choline in the extravesicular medium to saturate the carrier in order to determine the simple diffusion component of total uptake. Experimental conditions were the same as described in legend of Figure3. Uptakes were measured in the presence of increasing concentrations choline (0.125-5 mM). Values are expressed as nmol/mg protein (mean ± SD).

Discussion

Our results in SMMV, confirm the existence of an uphill mediated transport mechanism for choline in the human placenta. A transient accumulation of choline was seen in the presence of an outwardly directed choline gradient, which is considered to be indicative for carrier mediated transport. Uptake into SMMV was also stimulated above equilibrium uptake by an inside negative membrane potential. We conclude that the physiologically negative cell interior can act as a driving force

for placental choline uptake from the maternal circulation. The uptake into SMMV was sodium independent. This result is in good agreement with the sodium independent uptake into the trophoblast, as was demonstrated in the dually perfused guinea pig placenta (Sweiry et al., 1985). Choline transport in rabbit renal brush border membranes (Wright et al., 1992), human erythrocytes (Deves and Krupka, 1979) and rat intestinal brush border membranes (Saitoh et al., 1992) is also sodium independent in contrast to the uptake in neural tissue, which is sodium coupled (Kuhar and Murin, 1978). It seems that a sodium gradient, maintained by Na^+/K^+ ATPase, is not directly involved in choline transport in most tissues including the human placenta.

Several cations were able to inhibit choline uptake at relatively high concentrations. The analogue HC-3, choline itself and the organic cation transport inhibitor mepiperphenidol inhibited the choline uptake nearly completely, whereas cationic drugs like cimetidine and famotidine only partly reduced choline uptake. Therapeutic plasma concentrations of these H_2-receptor antagonists are low, which means that the clinical relevance of this inhibition, at concentrations as high as 5 mM, will be limited. NMN and the quaternary ammonium compound TEA did not inhibit the uptake. In rabbit renal brush-border membranes TEA also had no inhibitory potency (Wright et al, 1992), whereas in rat intestinal brush-border membranes TEA and NMN caused both cis-inhibition and trans-stimulation (Saitoh et al., 1992). Because of the strong inhibition of choline and HC-3 as compared to the other cations we suggest a relatively high specificity of the choline transporter in placental SMMV. Whereas our results indicate an affinity of cationic drugs for the choline carrier the reverse, an affinity of choline for the organic cation/proton antiporter for guanidine could not be determined in placental brush-border membranes (Ganapathy et al., 1988). In the same study cimetidine did not inhibit this guanidine carrier. It seems that exogenous organic cations interfere with the choline transporter rather than with the guanidine transporter.

The kinetics determined in SMMV involved a saturable process with a K_m of 550 μM, corresponding with a value of 350 μM found in placental fragments (Welsch, 1976) and greater than 97 μM and 159 μM found in other epithelia like rabbit renal brush-border membranes (Wright et al., 1992) and rat intestinal brush-border membranes (Saitoh et al., 1992), respectively. A K_m of 550 μM, which is far above the maternal plasma concentration of 20 μM, assures an efficient uptake of choline into the trophoblast.

In conclusion, the transport system for choline in syncytial micovillous membrane vesicles of human term placenta demonstrated here, can efficiently provide for the placental uptake of choline from the maternal circulation driven by the negative cell interior. It is believed that this syncytial accumulation is essential for the permeability of the trophoblast for amino acids (Rowell and Sastry, 1981) or for the fetal need of choline. The apparent discrepancy between the low placental transfer as shown by Sweiry et al. (Sweiry et al., 1986) and the significant placental uptake of choline suggests that the placenta will function as a buffer for the fetal need of choline. Fetal choline uptake will therefore be regulated mainly by the transfer across the basal membrane of the trophoblast.

Acknowledgements

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Chapter 6

INHIBITION OF CHOLINE UPTAKE IN SYNCYTIAL MICROVILLOUS MEMBRANE VESICLES OF HUMAN TERM PLACENTA: SPECIFICITY AND NATURE OF INTERACTION

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In press: Biochemical Pharmacology

Summary

The potency and nature of the inhibitory effect of various cationic drugs on the transport of choline across the placental syncytial microvillous membrane was investigated. TEA, a model substrate for organic cation transport, was a poor inhibitor. Enlarging the degree of alkylation of the quaternary ammonium increased the inhibitory effect, in proportion with increasing lipophilicity. Log concentration versus % control uptake curves showed marked differences in inhibitory potency for the different cationic drugs. HC-3 inhibited mediated choline uptake in the micromolar range, whereas atropine and mepiperphenidol were less potent. H_2-receptor antagonists cimetidine, ranitidine and famotidine inhibited choline uptake in the millimolar ranges. Dixon analysis revealed a competitive nature of inhibition for HC-3 and atropine (K_i = 40 μM and 1,2 mM, respectively). Cimetidine interacted noncompetitively (K_i = 3.4 mM). Since relatively high concentrations are needed to reach half maximal inhibition, impairment of fetal choline supply due to maternal drug use during pregnancy is not to be expected.

Introduction

Choline, a cationic quaternary ammonium compound, is an essential substrate for adequate growth and development of the fetus. Choline serves as a substrate for

the synthesis of phospholipids and acetylcholine. Since the human placenta and fetus do not synthesize choline, fetal supply is highly dependent on the proper transfer of this nutrient from maternal to fetal circulation (Welsch, 1976). The initial step in placental transfer involves uptake across the syncytial microvillous membrane of the trophoblast. In the perfused human placental cotyledon, *trans*-placental transfer was slow in contrast to the fast and high placental uptake (Sweiry et al., 1986). Since there was a small excess of *trans*-placental choline transfer in comparison with the extracellular marker mannitol, preferentially towards the fetal circulation, the human placenta possesses a unidirectional pathway for choline in the maternal to fetal direction. Placental choline uptake on the maternal side was inhibited by choline itself and on the fetal side by HC-3, a well-known competitive inhibitor of the choline transporter in various tissues, suggesting the existence of a specific transport systems at both sides of the trophoblast. Evidence for mediated trophoblastic choline uptake was also found in human placental fragments, which accumulated choline against a concentration gradient inhibitable by HC-3 with a K_i of 0.45 mM. However, because uptake in fragments is the net result of transport across both the syncytial microvillous and basal membranes, it is impossible to differentiate between inhibition of the choline transporter at both sides of the trophoblast. Isolated vesicles of these membranes are a more appropriate tool for investigating the location and nature of such interactions. Recently, we described the mechanisms of choline uptake into isolated syncytial microvillous membrane vesicles of human term placenta (van der Aa et al., 1994). Uptake was not sodium-dependent or coupled to proton transport. An inside negative membrane potential enhanced choline uptake, showing that a negatively charged inner membrane surface acts as a driving force for trophoblastic choline uptake. Mediated transport was confirmed by the trans-stimulatory effect of unlabeled choline and cis-inhibitory effect of HC-3, the organic cation transport inhibitor mepiperphenidol and H_2-receptor antagonists. Model substrates for organic cation transport TEA and NMN did not inhibit choline transport. Furthermore, we found that uptake under trans-stimulation conditions was saturable with a K_m of 550 μM. Our results were confirmed in a study by Grassl, who used exactly the same approach (Grassl, 1994). In addition he showed on the basis of evaluation of the inhibitory potency of a great amount of organic cations that at least two sites of interaction with the choline transporter can be postulated: a negative site that binds with the positively-charged nitrogen and a site of hydrogen bonding that interacts with the primary alcohol. Furthermore, the degree of nitrogen group alkylation appeared to be of importance.

Since trophoblastic uptake is the rate-limiting step in fetal choline supply,

interaction of maternally-administered drugs with the choline transporter at the microvillous membrane could have clinical implications for fetal growth and development. This study was designed to further investigate the specificity of the choline transporter at the syncytial microvillous membrane of the human placental trophoblast, by characterizing the inhibitory potency and nature of the interaction of various cationic drugs.

Materials and methods

Chemicals

[^{3}H]-Choline was obtained from Amersham (Buckinghamshire, U.K.). Cimetidine was kindly donated by Smith, Kline & French (Welwyn Garden City, Herts, U.K.) and mepiperphenidol and famotidine by Merck, Sharp & Dohme (Rahway, NJ, U.S.A.). All other chemicals were purchased from either Sigma (St. Louis, MO, U.S.A.), Merck (Darmstadt, Germany) or Boehringer Mannheim (Mannheim, Germany) and were of analytical grade. GF/F filters were obtained from Whatman Int. Ltd. (Maidstone, U.K.).

Preparation of SMMV

SMMV were prepared from fresh human term placentae according to an established (Glazier et al., 1988), which was further improved upon our laboratory (van der Aa et al., 1994; van der Aa et al., in press). Briefly, tissue was minced in a Warring blender and stirred for 30 min to loosen the microvilli. After $MgCl_2$ aggregation and differential centrifugation SMMV were harvested and suspended in the appropriate intravesicular buffer for uptake studies, to a final protein concentration of 10-15 mg/mL. Vesicles were frozen in liquid nitrogen and stored at -80°C for four weeks at the maximum. This freezing and storage procedure did not influence choline uptake. The alkaline phosphatase enrichment of SMMV, measured according to Mircheff and Wright (1976), was 24-fold (M_0 = 70 ± 15 and SMMV = 1690 ± 310 μmol/hr/mg, N=14) compared to starting mince. Protein was assayed with a Coomassie blue kit (Biorad, Munich, Germany).

Uptake studies

Uptake of [^{3}H]-choline into SMMV was measured in quadruplicate at 37 °C using a rapid filtration technique (Russel et al., 1988). The samples were filtered through Whatman GF/F filters (average pore size 0.7 μm) and the radioactivity remaining on the filters counted in a Beckman LS 6000 LL liquid scintillation counter. Corrections were made for nonspecific filter binding. The exact conditions of the transport experiments are given in the legends. Uptake is expressed as pmol or nmol/mg protein or % of control uptake (mean ± SD), N representing the number of experiments with different placentae.

Data analysis

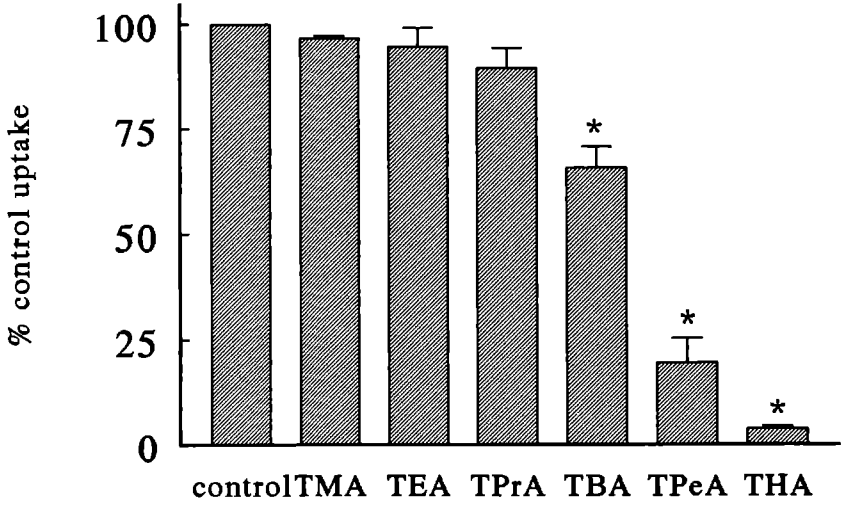
From concentration versus % uptake curves of 3 concentrations of choline below K_m (50, 125 and 250 μM), the concentration required to reach half maximal inhibition (IC_{50}) of several cationic drugs was estimated by least squares nonlinear regression analysis, using the computer program GraphPad Inplot 4.0 (1992). The weighted residual sums of squares of one- and two-site models were compared using the F-test. Transformation of the data according to Dixon (1953) revealed the nature of inhibition. The inhibitory constant (K_i) for a competitive inhibitor was estimated according to the equation of Cheng-Prusoff: $K_i = IC_{50} / 1+(S/K_m)$, where S = choline concentration (1973). For K_m and V_{max} of choline previously determined values were used viz. 550 μM and 10 nmol/mg/10 sec, respectively (van der Aa et al., 1994). In case of a noncompetitive inhibitor, IC_{50} is independent of S, and consequently K_i equals IC_{50}. Paired Student's t-test was used to determine statistical significance ($P < 0.05$).

Results

Inhibitory potency tetraalkylammonium compounds

In our previous study it was found that 5 mM TEA did not inhibit choline (250 μM) uptake into SMMV (van der Aa et al., 1994). We now investigated whether variation in the degree of alkylation of TEA influences inhibitory potency. Enlarging the alkyl chains at the quaternary ammonium resulted in a higher percentage of inhibition (Figure 1). The increase in inhibitory potency of the

compounds corresponded well with the increase in lipophilicity as given by their calculated log P values (Wright et al., 1995). Because of the surface tension lowering properties of the tetraalkylammonium compounds, we verified whether the vesicles stayed intact in the presence of the inhibitors. Only in the presence of THA was the equilibrium uptake of 250 μM choline at 60 min significantly reduced as compared with control uptake. The other compounds did not interfere with the membrane integrity.



Figue 1. Effect of 1 mM *cis* concentrations of a series of homologous quaternary ammonium compounds on uptake of 250 μM [^{3}H]-choline at 10 sec. Vesicles, suspended in 100 mM mannitol, 100 mM KCl and 10 mM Hepes-Tris, pH=7.4, were pre-equilibrated with 5 mM unlabeled choline and 20 μM valinomycin at 37°C. 5 μL vesicle suspension was added to 195 μL extravesicular medium. Extravesicular media consisted of 100 mM mannitol, 100 mM KCl, 20 μM valinomycin, 10 mM Hepes-Tris, pH=7.4, inhibitor at a specified concentration and unlabeled choline to achieve an extravesicular concentration of 250 μM in the final solution. Values are expressed as % of representative control (without inhibitor) uptakes (mean ± SD, N=3). Control uptake was 5115 ± 625 pmol/mg protein. *P<0.05.

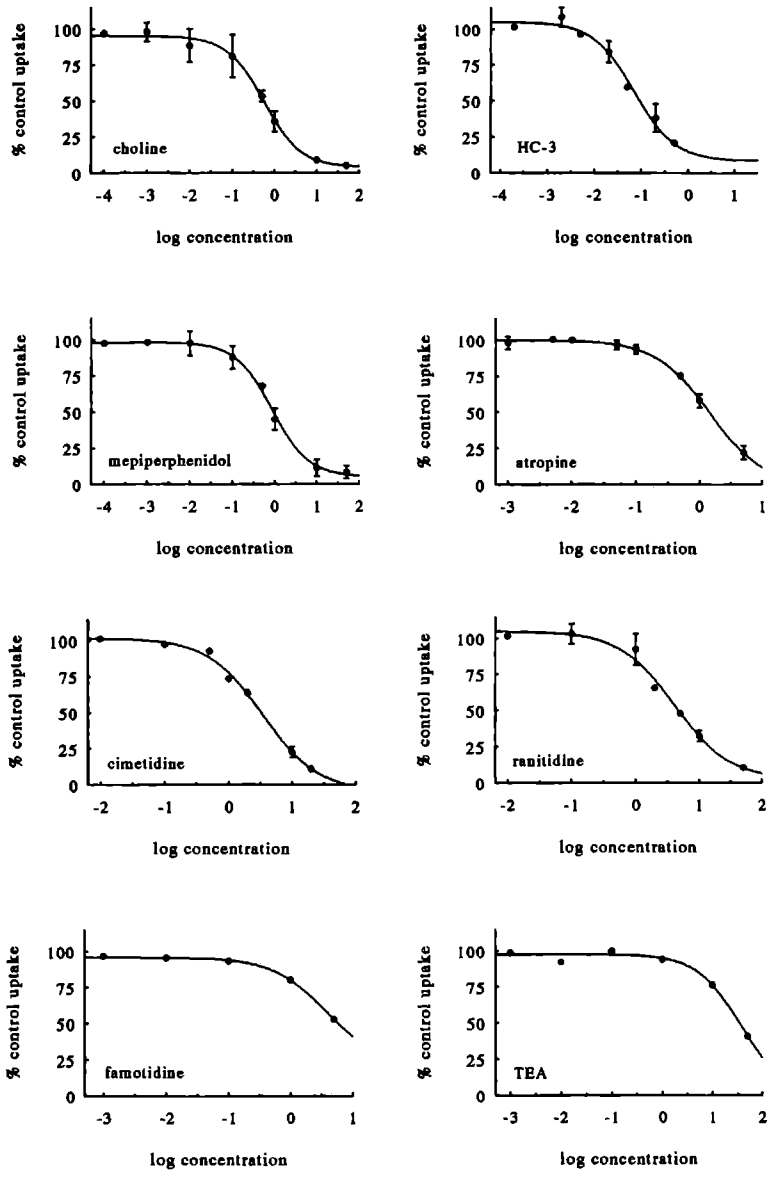


Figure 2. Inhibiton of 250 μM [^{3}H]-choline uptake into SMMV at 10 sec under trans-stimulation conditions by various organic cations. Experimental conditions were the same as described in legend of Figure 1. Values are expressed as % of representative control uptakes versus log concentration inhibitor (mM). Each point represents the mean ± SD of three experiments with three placentae, except for famotidine and TEA (N=1).

Inhibition of choline uptake by several organic cations

Plots of log concentration inhibitor versus % of control uptake of 250 µM choline are shown in Figure 2. The results of the nonlinear regression analysis of the typically sigmoid shaped curves are summarized in Table 1. Marked differences in inhibitory potency can be seen between the organic cations tested. HC-3 inhibited choline uptake for 50 % at a relatively low concentration, whereas the organic cation transport inhibitor mepiperphenidol and the anticholinergic drug atropine were less potent inhibitors. The H_2-receptor antagonists cimetidine, ranitidine and famotidine showed IC_{50} values only in the mM ranges. For TEA only a rough estimate of the IC_{50} value could be made, because of the very high concentrations (> 10 mM) necessary to achieve half maximal inhibition. In all cases analysis according to a two-site model did not fit the data better than a one-site model (P>0.2).

Table 1. Apparent inhibitory constants of cationic drugs on choline uptake.

Compound	IC_{50} (mM)	K_i (mM)
choline	0.55 ± 0.11	-
HC-3	0.069 ± 0.004	0.039 ± 0.009 [a]
atropine	1.45 ± 0.30	1.24 ± 0.10 [a]
mepiperphenid	0.85 ± 0.55	-
cimetidine	3.39 ± 0.47	3.39 ± 0.47 [b]
ranitidine	4.04 ± 0.62	-
famotidine	3.80 (N=1)	-
TEA	>50 (N=1)	-

IC_{50} values determined from inhibition curves at a choline concentration of 250 µM and inhibitory constants K_i (a: competitive; b: noncompetitive) determined from inhibition curves at 50, 125 and 250 µM choline. Values are expressed as means ± SD, N=3, except for famotidine and TEA.

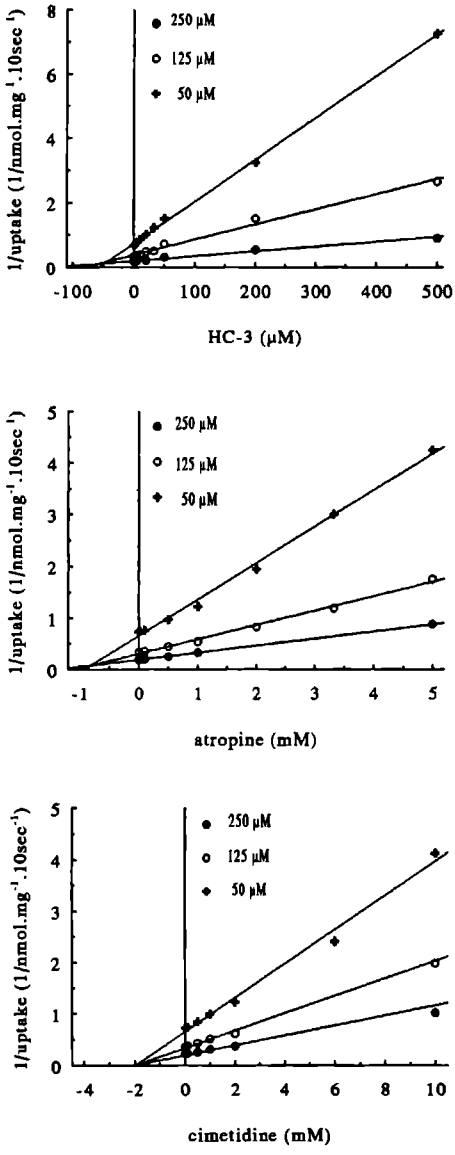


Figure 3. Dixon plots of interaction of HC-3, atropine and cimetidine with choline. Concentrations of extravesicular [^{3}H]-choline were 50, 125, 250 µM. Experimental conditions were the same as described in legend of Figure 1, except that in the case of 50 µM choline 5 µL vesicle suspension was added to 495 µL extravesicular medium.

Nature of interaction with the choline transporter

Although IC_{50} values provide a good measure of inhibitory potency they cannot explain the nature of the interaction. We used the method of Dixon analysis to evaluate the type of choline transport inhibition by HC-3, atropine and cimetidine. The concentration-dependent inhibition of these compounds was measured at three choline concentrations: 50, 125 and 250 μM. Increasing the substrate concentration decreased the inhibitory effectiveness of HC-3 and atropine, resulting in a lower IC_{50} value at a lower choline concentration. Transformation of the data according to Dixon showed that the lines intersected above the X-axis and to the left of the Y-axis, indicating a competitive mode of interaction of HC-3 and atropine with the choline transporter (Figure 3). K_i values for competitive inhibition, calculated from the Cheng-Prusoff equation for the three choline concentrations, were 40 μM for HC-3 and 1.2 mM for atropine (Table 1). In contrast, the IC_{50} value for cimetidine was independent of the choline concentration. Dixon analysis resulted in an intersection of the lines on the X-axis, consistent with a noncompetitive type of interaction (Figure 3). Consequently the K_i for cimetidine equalled the IC_{50} value of 3.4 mM (Table 1).

Discussion

The present study demonstrates that several cationic drugs inhibited human placental choline transport across the syncytial microvillous membrane with different inhibitory potencies. HC-3 and atropine appeared to be competitive inhibitors, whereas cimetidine interacted noncompetitively. TEA, a model substrate for organic cation transport in various tissues, only inhibited choline transport at very high concentrations. Enlarging the degree of alkylation of this quaternary ammonium compound increased the inhibitory effect in proportion with increasing lipophilicity.

Wright et al. (1992) postulated a set of structural elements important for interaction with the choline transporter: (a) a terminal hydroxyl group; (b) the positive charge of the nitrogen; and (c) the presence of at least two free methyl groups at the positive nitrogen. The placental choline transporter appeared to have a similar substrate specificity (Grassl, 1994). Small quaternary ammonium compounds such as TMA, acethylcholine and NMN showed affinity for the rat intestinal choline carrier (Saitoh et al., 1992), whereas TMA showed no affinity for the human

placental (Grassl, 1994) and rabbit renal choline transporter (Wright et al., 1992). The inability of large molecular quaternary ammonium compounds to interact with the choline transporter was assumed to be due to steric hindrance, viz., the masking of the positive nitrogen (Wright et al., 1992; Saitoh et al., 1992). The failure of TEA, for instance, to interact with the choline transporter was further confirmed by the finding that TEA was not able to trans-stimulate choline uptake into renal and intestinal brush-border membrane vesicles (Wright et al., 1992; Saitoh et al., 1992; Takano et al., 1993). In the present study TMA, TEA and TPrA did not or poorly inhibited placental choline transport. In spite of lack of determinants important for interaction with the choline transporter, further enlargement of the alkyl chains resulted in a significant inhibition of placental choline transport by TBA, TPeA and THA. For TPeA this was also observed in rabbit renal brush-border membranes (Wright et al., 1992), and in the study by Grassl (1994) a higher degree of inhibition of choline uptake was found in the presence of trimethylphenyl-ammonium as compared to no significant inhibition with TMA. The reduced choline uptake in the presence of the more lipophylic quaternary ammonium compounds is likely to be of noncompetitive nature (Naujokatis et al., 1982). The higher degree of alkylation probably facilitates a nonspecific interaction, e.g. solubilization into the membrane or interaction with an allosteric binding site at the transport protein, thereby interfering with the ability of the carrier to transport choline.

Cimetidine has affinity for the cation-proton antiporter (Takano et al., 1993), but to our knowledge no data concerning a possible interaction with the choline transporter are currently available. However, in renal brush-border membranes an affinity of choline for the organic cation-proton antiporter has been demonstrated, since choline inhibited TEA transport (Wright and Wunz, 1989; Miyamoto et al., 1989). Our data provide evidence that H_2-receptor antagonists inhibit choline uptake noncompetitively. The measured IC_{50} values were in the mM range and inhibitory potency of cimetidine was not dependent on choline concentration, resulting in an intersection of lines on the abscissa after Dixon transformation. It therefore seems unlikely that clinically significant interactions of these drugs with choline uptake in vivo will occur. Cimetidine is the highest dosed H_2-receptor antagonist, but maternal effective plasma concentrations (90 % inhibition of gastric acid production) are only approximately 15 µM (Somogyi and Gugler, 1983). For the lower dosed drugs, famotidine and ranitidine, IC_{50} values in the same range as for cimetidine were observed. Significant interactions in vivo of these nowadays more frequently prescribed H_2-receptor antagonists are therefore not to be expected.

The different IC_{50} values of HC-3 at different choline concentrations indicate that the inhibitory potency of HC-3 was dependent on choline concentration. The same pattern of inhibition was found for atropine, which is indicative of a competitive interaction. HC-3 is an effective inhibitor, as its affinity for the choline transprter was more than 10-fold higher than for choline itself (K_i for HC-3= 40 μM and K_m for choline= 550 μM), whereas the affinity for atropine was more than 2-fold lower (K_i= 1.2 mM). The Y-coordinates of the intersections in the Dixon plot (HC-3= 0.12, atropine= 0.07) corresponded well with the $1/V_{max}$ value of 0.10, providing further conformation that both inhibitors indeed interacted competitively (Wright and Wunz, 1989). Although Grassl did not report a K_i value for HC-3, a value of 92 μM can be calculated from his data (Grassl, 1994). In human placental fragments a K_i of 450 μM for HC-3 was reported (Welsch, 1976), which is almost 10-fold higher than the value we estimated in isolated human placental SMMV. This discrepancy is most likely due to the different experimental techniques used. An inhibition constant determined in fragments is merely a hybrid parameter from interaction with choline transporters at the microvillous and basal side of the trophoblast, whereas the inhibitory effect in purified microvillous membranes can only be ascribed to an interaction with the choline transporter at this membrane. Therefore estimating inhibitory parameters of drugs on transport proteins is more appropriate in purified membranes. However, the net effect on the transfer of choline in maternal to fetal direction should be determined in more physiologically-based models such trophoblast cell culture or cotyledon perfusion models.

We conclude that several cationic drugs are able to interact with human placental choline transport across the syncytial microvillous membrane in a competitive and noncompetitive way. Competitive inhibition was seen in the μM and low mM ranges, whereas noncompetitive inhibition was seen in the high mM ranges, indicating a nonspecific interaction with the biomembrane. Since relatively high concentrations were needed to reach half maximal inhibition, impairment of fetal choline supply in vivo due to maternal drug use during pregnancy is not to be expected.

Acknowledgements

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PART III

DRUG TRANSFER ACROSS THE ISOLATED DUALLY PERFUSED COTYLEDON

Chapter 7

DUAL PERFUSION OF THE ISOLATED COTYLEDON OF HUMAN TERM PLACENTA

- perfusion system, preparation method and viability -

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Summary

A dual perfusion system of the isolated cotyledon of human term placenta has been validated, in order to study placental drug transfer and metabolism. The recirculating (closed) perfusion system showed a good diffusibility, since the disappearance of antipyrine from the maternal circulation (CL = 2.33 ml/min) and concomitant rise in the fetal circulation, was comparable to literature. In contrast to antipyrine, the hydrophylic, membrane impermeable compound inulin did cross the placenta for only approximately 6 %, indicating the integrity of the membrane barrier. Metabolic viability of the preparation was maintained, since a net glucose/lactate conversion was observed. We conclude that our system is suitable for placental drug transfer and metabolism studies.

Introduction

Tissue cultures (Bullen et al., 1990), fragments (Welsch, 1976) and membrane vesicles (Smith et al., 1974; Kelley et al., 1983) of human term placenta have contributed greatly to the understanding of mechanisms of solute transport across the human placenta. However, for the investigation of overall transfer and the preferential direction of transport - towards the mother or towards the fetus - these techniques are not adequate enough.

In human, examination of placental transport processes is practically

impossible without severe risks for the developing fetus. Although animal studies have been used (Faber et al., 1992), preference has to be given to human data, because of the major interspecies differences in placental transport function (Page, 1993). Studies with human term placenta can overcome the extrapolation problem of animal to human data.

Attempts have been made to mimic the human in vivo situation by perfusing the whole placenta in vitro (Vermeulen, 1982; Wiegand, 1984). Since a whole placenta, completely free of major damage, is rare, this technique is difficult to perform. An alternative has been developed by Schneider and co-workers (1972), in which a single cotyledon of human term placenta is dually perfused. The advantages of this technique are obvious: the ability to select a suitable, undamaged cotyledon, the possibility to examine placental transfer in both directions and performing perfusions under steady-state conditions in a single pass mode or in a recirculating mode for drugs with a low extraction rate. Nowadays, this technique is widely used and modifications to improve its utility, resulting in a higher interexperimental consistency, have been described (Schneider and Huch, 1985; Brandes et al., 1983; Penfold et al., 1981; Contractor and Stannard, 1983).

In this chapter we describe the system, preparation method and viability of the dually perfused isolated cotyledon of human term placenta, which was set up in our laboratory based on the method of Schneider et al. (1972, 1985).

Materials and Methods

Perfusion system and preparation

Human term placentae, with clamped umbilical cord, were brought to our laboratory in 0.9 % NaCl (4°C), immediately after vaginal or caesarian delivery. After careful examination, a periferal cotyledon was selected suitable for dual perfusion. The arterial and venous chorionic vessels were canulated with metal cannulae (outer diameter: 2 mm and 3 mm, respectively), connected to a short silicon tube as an intermediate to O_2 -impermeable, TEFLON-tubing (inner diameter: 1.9 mm) in the rest of the perfusion system. The fetal arterial inflow (37°C) was started at 0.15 ml/min and gradually increased to 6 ml/min by perfusion pump 1 (MS-1 reglo/6-160, Ismatic, Zürich, Switserland). Proper connection of the fetal artery to the vein was checked by verifying that the fetal venous outflow equalled the fetal arterial inflow. The fetally perfused cotyledon was fixed in a

perspex ring and placed into the perfusion chamber (37°C) with the maternal surface faced upwards. The intervillous space, connected to the fetally perfused region, was perfused by four small metal cannulae (outer diameter: 1 mm), which were inserted by pressing them gently through the decidual plate. The maternal inflow was gradually increased to 12 ml/min by the second perfusion pump. The perfusate returning through the intervillous openings was continuously drained by the third perfusion pump. Fetal arterial perfusion pressure (TDNR transducer, Viggo Spectramed, Oxnard, CA, USA), perfusate pH (PHM 92 LABpH meter and GK2401C pH electrode, Radiometer Copenhagen, Bagsvaerd, Danmark), temperature (Thermo-probe, New Port Electronics, Amstelveen, the Netherlands) and pO_2 (Mini clark electrode 730, Diamond General dev. cor., Ann Arbor, MI, U.S.A.) and venous perfusion pressure and perfusate pO_2, were continuously monitored and stored in a computer using the program POLY (Inspector Research Systems, Amsterdam, the Netherlands). Similarly, maternal arterial perfusate temperature, pH and pO_2 were monitored throughout the experiment. Figure 1 shows a scheme of the perfusion system described above.

Chemicals

Maternal and fetal perfusates were of the following composition (mM): NaCl (112.0), KCl (5.2), $NaHCO_3$ (20.2) KH_2PO_4 (0.3), Na_2HPO_4 (0.9), $CaCl_2$ (2.0), $MgCl_2$ (1.0), glucose (5.0), to which 2500 IU/l heparin, 2% bovine serum albumin, 1% dextran 40 and 1 % Synthamin 14 (a mixture of 15 amino acids) were added. The perfusates were equilibrated with 95% O_2 and 5% CO_2 and together with the 20.2 mM $NaHCO_3$ added in the perfusates, pH was buffered to 7.3-7.5.

Bovine serum albumin Fraction V was obtained from Boehringer Mannheim (Mannheim, Germany), heparin (Tromboliquin^R, 5000 IU/l) was purchased from Organon Teknika (Boxtel, the Netherlands) and Synthamin 14 (without electrolytes) from Travenol Laboratories Ltd. (Norfolk U.K.). All other chemicals were obtained from either Sigma Chemical Co. (St. Louis, U.S.A.) or Merck (Darmstadt, Germany) and were of analytical grade.

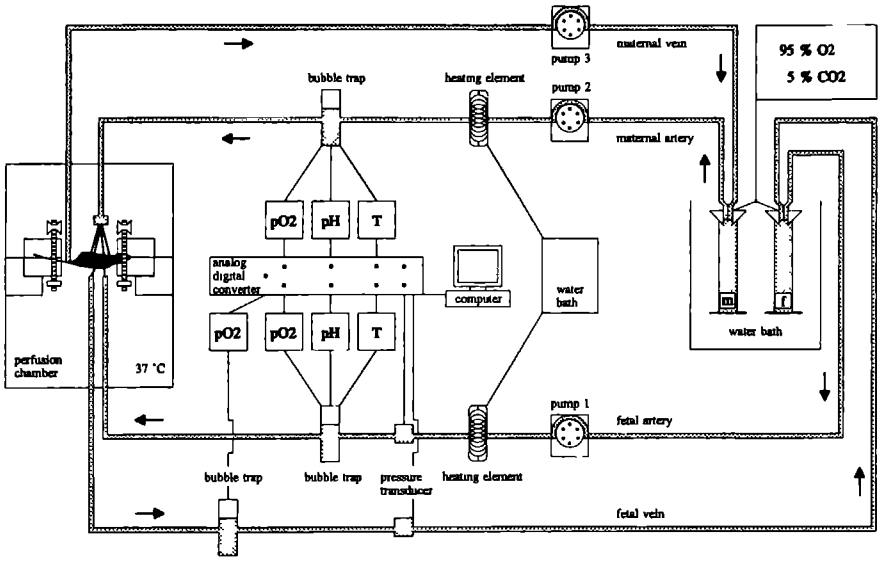

Figure 1. Scheme of the dual perfused isolated cotyledon of human term placenta (m=maternal circuit and f=fetal circuit).

Experimental design

After cannulation, perfusion experiments were started in a 30 min single pass mode (pre-phase) on both sides, to let the cotyledon stabilize and recover from the period of anoxia and to clear the cotyledon from remaining blood. The test-phase of the experiment was then started by replacing the perfusion fluid in the reservoirs and changing to the recirculating mode by closing the circuits. The perfusion fluid (175 ml for each circulation) used in this phase were the same as described above, except that antipyrine (14.1 mg) was added to the maternal perfusate, in order to validate the diffusion permeability of the placenta. In two experiments inulin (26.1 mg) was added to the maternal perfusate, to assess the integrity of the placenta. In these experiments dextran 40 was omitted from the perfusates, because of its interference with the inulin assay. During the pre- and test-phases volume loss from the reservoirs, indicative for flux into extra perfused tissue or defects in the placental barrier, were registered. When the volume loss, corrected for sampling volume, was more than 3 ml/h the experiment was terminated. Fetal inflow pressure was 21.5 ± 7.2 mm Hg. Since both reservoirs were equilibrated with carbogen no

net O_2 transfer occurs and determination of pO_2 in these experiments was unnecessary.

For determination of antipyrine, inulin, glucose and lactate concentrations, samples (2 ml) were taken from both reservoirs at every 5 min until 30 min and at 45, 60, 90 and 120 min and centrifuged (10 min, 1500·G) to eliminate erythrocytes. Volume loss due to sampling was less than 15 %. After termination of the experiment the perfused cotyledon was excised, weighed and frozen until analysis.

Analytical methods

Antipyrine was measured colorimetrically according to the method described by Brodie et al. (1949) and inulin according to the method described by Heyrovski (1956). Glucose and lactate were measured enzymatically (D-Glucose-kit and D/L-Lactic acid-kit, Boehringer Mannheim, Germany).

Data analysis

Pharmacokinetic analysis of placental antipyrine transfer was done by fitting the antipyrine maternal and fetal concentration data to equations 1 and 2, using the computer program PCNONLIN (Metzler and Weiner, 1989), where C_m and C_f are maternal and fetal concentrations, C_0 is the initial maternal concentration α_1 and α_2 are first-order rate constants, $C_{equi,m}$ and $C_{equi,f}$ are maternal and fetal concentrations at equilibrium:

$$C_m = (C_0 - C_{equi,m}) \cdot e^{-\alpha_1 \cdot t} + C_{equi,m} \quad (1)$$

$$C_f = C_{equi,f} \cdot (1 - e^{-\alpha_2 \cdot t}) \quad (2)$$

Placental clearance (CL) was calculated as D/AUC_m, where D is dosis and AUC_m is the area under the maternal concentration versus time curve from zero to infinity. AUC_m was calculated as $C_0 / \alpha_1 + C_{equi,m} \cdot t_{120}$. We assumed that equilibrium was reached at 120 min of perfusion since at least 5 times $t_{1/2}$ were passed.

Data are presented as means ± SD, where N is the number of perfusions with different placentae.

Results

Diffusion properties

Disappearance from the maternal circulation and concomitant appearance in the fetal circulation of the freely diffusionable and practically inert marker antipyrine is shown in Figure 2. Almost equal concentrations in both reservoirs were reached after 120 min ($C_{equi,m}$ = 35.8 ± 2.4 and $C_{equi,f}$ = 33.7 ± 1.6 g/l, N=4). Pharmacokinetic analysis of the drug concentration data fitted over 120 min of perfusion, revealed a placental antipyrine clearance of 2.33 ± 0.08 ml/min (N=5). First order rate constants α_1 and α_2 were 0.044 ± 0.006 and 0.034 ± 0.005 min^{-1} (N=5)

Membrane integrity

In contrast to antipyrine, only 5.7 % of the maternally administered concentration of inulin was transferred to the fetal circulation (Fig. 3). Approximately 17 % of the maternal concentration of inulin at time zero was retained in the perfused cotyledon, together with 6 % transfer to the fetal circulation, corresponding to a total disappearance of inulin from the maternal circulation of approximately 28 %.

Metabolic activity

To determine the metabolic viability of the perfused cotyledon, we investigated the glucose utilization and lactate production during 120 min of perfusion. Both were linear with time (Fig. 4), the maternal rate being twice the fetal rate, corresponding to the higher perfusion rate of the maternal circuit. Calculation of the concentrations, corrected for volume loss due to sampling and related to the weight of the perfused cotyledon, revealed values of glucose consumption and lactate production as mentioned in Table 1. As can be seen, lactate production was twice as high as glucose utilization.

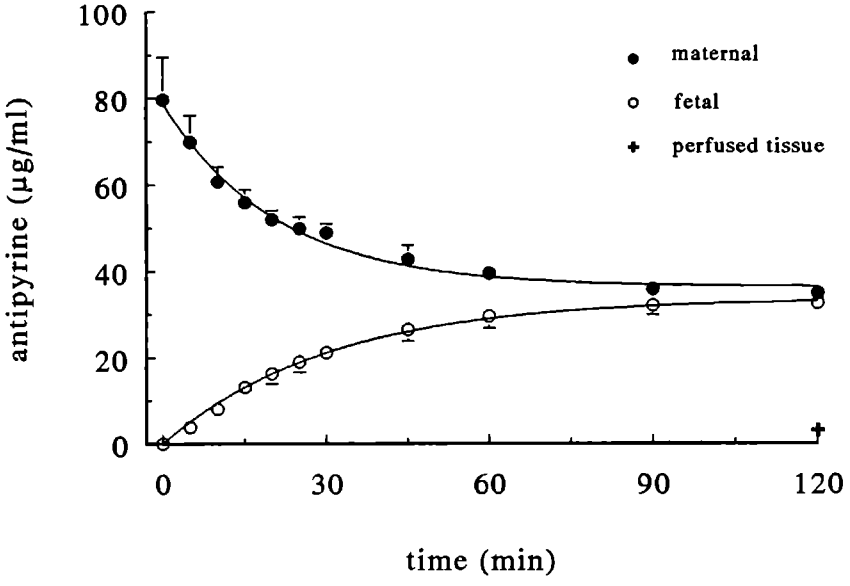

Figure 2. Time course of antipyrine disappearance from the maternal circuit and appearance in the fetal circuit Values are presented as means ± SD (N=6)

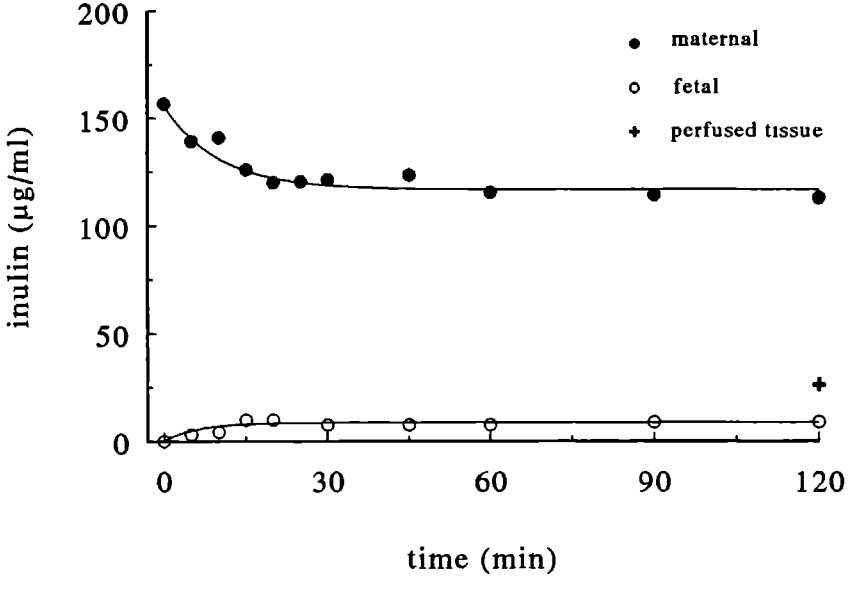

Figure 3. Time course of inulin disappearance from the maternal circuit and appearance in the fetal circuit Values are presented as means of 2 experiments

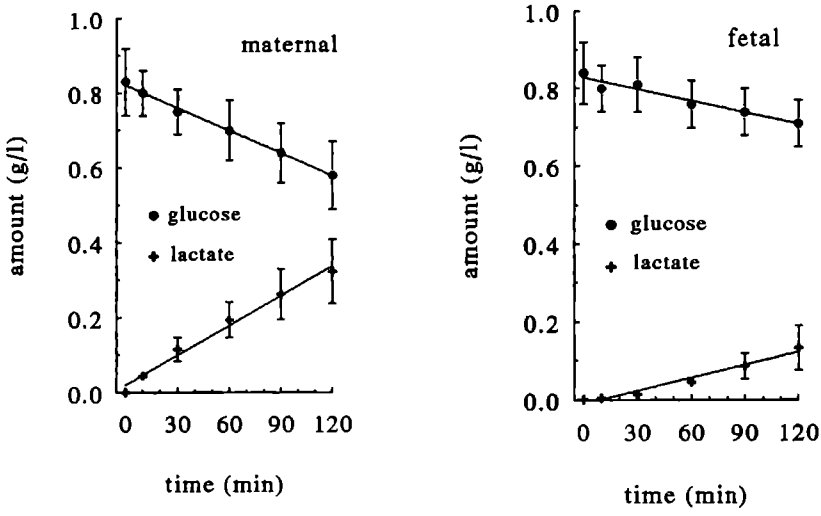

Figure 4. Glucose and lactate concentrations in maternal and fetal circuits. Values are presented as means ± SD (N=7).

Table 1. Glucose metabolism in the isolated perfused human placental cotyledon

	glucose utilization	lactate production
fetal	0.069 ± 0.040	0.095 ± 0.075
maternal	0.178 ± 0.043	0.298 ± 0.153

Values are expressed as μmol/min/g wet weight (mean ± SD) of 7 perfusions.

Discussion

In this study we describe a dual perfusion system of the isolated cotyledon of human term placenta. Our goal was to investigate the characteristics in a closed circuits model, to validate the system for future placental drug transfer and metabolism investigations. This recirculating perfusion method provides a simple and physiologically based model for in vivo placental drug transfer. To validate a perfusion system one must get insight into the variables that are of influence on

transfer processes across the placental barrier. Concentration differences can be controlled at the beginning of an experiment, however, for instance membrane characteristics are far more difficult to predict. Evaluation of flow-limited, membrane limited and metabolic marker compounds can provide more insight into the transport characteristics of the perfused cotyledon.

The rate of disappearance of antipyrine from the perfusion fluid is directly proportional to the flow delivering the compound, a mechanism which is called flow-dependent transfer (Schneider and Dancis, 1978). In our closed circuits perfusion system, maternally administered antipyrine disappeared quickly from the maternal compartment with a clearance comparable to literature values (Barzago et al., 1994). A concomitant rise into the fetal circulation, reaching equilibrium, was observed, although equal concentrations in both circulations were not completely reached. This indicates that a very small amount must have been retained in the perfused tissue, which was confirmed by the recovery of approximately 4 % of the maternally administered antipyrine concentration in the tissue sample (data not shown). Furthermore, kinetic analysis of the transfer rates from the maternal into the fetal compartment showed that the maternal disappearance rate was faster than the fetal appearance rate, possibly reflecting the difference in permeability of both sides of the trophoblast (the maternal circulation facing the microvillous membrane and the fetal circulation facing the basal membrane). The antipyrine transfer profile showed that the maternal and fetal circulations are properly connected and transfer from one compartment to the other is possible, without considerable leakage to extra-perfused tissue or significant retention into perfused tissue. The permeability of the human placenta for inulin, a water-soluble compound with a molecular weight of approximately 5500, is much lower than for antipyrine (Challier et al., 1985[a]). Indeed, in our experiments, where antipyrine did reach equilibrium at 120 min, inulin did not, indicating that the placental barrier stayed intact for at least two hours. This was also observed in a recirculating perfusion for a period up to six hours (Maguire et al., 1992).

Placental metabolism plays an important role in the transfer of certain compounds. For instance, cortisol is metabolized to cortisone during placental transfer (Dancis, 1978) and the lack of transfer of glutamic acid is assumed to be due to extensive metabolic degradation to glutamine (Malek et al., 1993). In placental fragments a net consumption of glucose and production of lactate and ammonia was observed (Holzman et al., 1979). Therefore, glucose utilization and lactate production are considered as markers of metabolic viability of the perfused cotyledon (Cannell et al., 1988). In our preparation significant glucose utilization

and lactate production was observed, maternally higher than fetally, reflecting the differences in perfusion rates. The values of this metabolic conversion were comparable to those described by Leichtweiss et al. (1985). In the term placenta, indeed glucose is mainly metabolized anaerobically, however, since lactate production is twice as high as glucose utilization other precursors than glucose must be responsible for the lactate production (Challier et al., 1985[b]).

In summary, a dual perfusion system of the isolated cotyledon of human term placenta has been validated. Maternally administered antipyrine equilibrated in both circulations, whereas inulin did not, showing the good diffusibility and integrity of the membrane barrier. Since net glucose utilization and lactate production was demonstrated, the metabolic viability of the perfused cotyledon was preserved. We conclude that our system is suitable for studies on placental drug transfer and metabolism.

Acknowledgements

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Chapter 8

CHARACTERISTICS OF SPIRAMYCIN TRANSFER IN THE ISOLATED DUALLY PERFUSED COTYLEDON OF HUMAN TERM PLACENTA

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Summary

The placental transfer of spiramycin was investigated in a dual recirculating perfusion system of the isolated cotyledon of human term placenta. Metabolic activity, measured as glucose consumption and lactate production, was maintained throughout perfusion. Maternal to fetal antipyrine transfer showed a good diffusibility of the perfused cotyledon. Spiramycin disappearance from the maternal circulation was comparable to antipyrine, indicating passive diffusion into the trophoblast. However, in contrast to antipyrine, no concomitant rise in the fetal circulation and as a result low trans-placental transfer was observed. An additional peak was observed in HPLC chromatograms of perfused tissue and to a lesser extent, but gradually increasing, also in maternal perfusate after 60 min of perfusion. Placental metabolism is assumed to be the main cause of the observed low trans-placental transfer. Due to this low transfer, the therapeutic efficacy of spiramycin in fetal Toxoplasma Gondii infection is questioned. Assuming that metabolites of spiramycin may be also effective against Toxoplasma Gondii, placental spiramycin and metabolites can act as a barrier to placental passage of this parasite, resulting in the prevention of fetal infection.

Introduction

Primal maternal infections of Toxoplasma Gondii occur in approximately 6-12 of 1000 human pregnancies, resulting in fetal infections in approximately 40 %

of the cases (van der Veen, 1984; Desmonts and Couvreur, 1974). Fetal infections during pregnancy by this parasite can cause severe defects, such as microcephalus, hydrocephalus or microphtalmia, and even intra-uterine death, spontaneous abortion or partus prematurus (Desmonts and Couvreur, 1974). Visus defects and mental retardation in later life are also correlated with fetal Toxoplasma Gondii infection during pregnancy (Wilson and Remington, 1980). In pregnant women, having antibodies due to contact with the parasite before pregnancy, fetal infection is assumed to be impossible (Remington and Desmonts, 1990). Because of the severe congenitally defects, treatment is essential when infection during pregnancy does occur in women devoid of antibodies. The most prescribed treatment in case of general Toxoplasma Gondii infection is a combination of pyrimethamine and sulfadiazine. However, because of the teratogenic effects of pyrimethamine in rats (Horvath et al., 1989), this drug regimen is not the first choice in the treatment of fetal infection during pregnancy.

The macrolide antibiotic spiramycin is bacteriostatic against a variety of bacteria and also Toxoplasma Gondii in vitro (Chamberland et al., 1991) and in vivo, in mice (Araujo et al., 1991). Since no adverse effects on the fetus are observed, spiramycin might be a safe alternative to the usual treatment of Toxoplasma Gondii infections during pregnancy. In human, a reduced frequency of placental infections was observed when women were treated prophylactically (Couvreur et al., 1988). However, whether spiramycin can be also used successfully in the treatment of an already infected fetus is unknown. Recently, a limited placental transfer of spiramycin was found in rhesus monkey (Schoondermark-van de Ven et al., 1994) and human placental cotyledon (Quetin et al., 1983), suggesting that fetal therapeutic plasma concentrations will hardly be reached. The in vivo prophylactic activity might be explained by the creation of a barrier in the placenta to transmisson of the parasite, providing the prevention of trans placental passage (Schoondermark-van de Ven et al., 1994).

The cause of the low trans-placental transfer of spiramycin is unknown. We therefore investigated the characteristics of spiramycin transfer in the isolated dually perfused cotyledon of human term placenta, to gain insight into the mechanism underlying its poor placental permeability.

Materials and Methods

Perfusion system and preparation

Human term placentae, with clamped umbilical cord, were brought to our laboratory in 0.9 % NaCl (4°C), immediately after vaginal or caesarian delivery and were cannulated as described in chapter 7. The fetal arterial inflow (37°C) was started at 0.15 ml/min and gradually increased to 6 ml/min. Proper connection of the fetal artery to the fetal vein was checked by verifying that the fetal venous outflow equalled the fetal arterial inflow. The fetally perfused cotyledon was fixed in a perspex ring and placed into the perfusion chamber (37°C) with the maternal surface faced upwards. Maternal arterial inflow (37°C) was started by pressing four small metal cannulae, gently through the decidual plate, into the intervillous space connected to the fetally perfused region. The maternal arterial inflow was gradually increased to 12 ml/min. The perfusate returning through the intervillous openings was continuously drained. Temperature, pH, and fetal inflow pressure were continuously monitored as described in chapter 7. When the volume loss, corrected for sampling volume, was more than 3 ml/h the experiment was terminated. Fetal inflow pressure was 17 ± 6 mmHg. Since both reservoirs were equilibrated with carbogen no net O_2 transfer occurs and determination of pO_2 in these experiments was unnecessary.

Chemicals

Maternal and fetal perfusates were of the following composition (mM): NaCl (112.0), KCl (5.2), $NaHCO_3$ (20.2) KH_2PO_4 (0.3), Na_2HPO_4 (0.9), $CaCl_2$ (2.0), $MgCl_2$ (1.0), glucose (5.0), to which 2500 IU/l heparin, 2% bovine serum albumin, 1% dextran 40 and 1 % Synthamin 14 (a mixture of 15 amino acids) were added. The perfusates were equilibrated with 95% O_2 and 5% CO_2 and together with the 20.2 mM $NaHCO_3$ added in the perfusates, pH was buffered to 7.3-7.5.

Bovine serum albumin Fraction V was obtained from Boehringer Mannheim (Mannheim, Germany), heparin (Tromboliquin^R, 5000 IU/l) was purchased from Organon Teknika (Boxtel, the Netherlands) and Synthamin 14 (without electrolytes) from Travenol Laboratories Ltd. (Norfolk U.K.). Spiramycin (a complex of 90.0% spiramycin I, 0.5% spiramycin II and 6.3% spiramycin III) and spiramycin II were kindly provided by Rohne-Poulenc (Alfortville, France). All other chemicals were obtained from either Sigma Chemical Co. (St. Louis, U.S.A.) or Merck (Darmstadt,

Germany) and were of analytical or HPLC grade.

Experimental design

As described in chapter 7, experiments were started in a 30 min single pass mode (pre-phase) on both sides to let the cotyledon stabilize and recover from the period of anoxia and to clear it from remaining blood. The test-phase of the experiment was then started by changing the perfusates and closing the circuits, resulting in a recirculating mode of perfusion. The perfusates used in this phase (175 ml) were the same as described above, except that spiramycin I (0.88 mg) and antipyrine (14.1 mg) were added to the maternal perfusate.

For determination of spiramycin, antipyrine, glucose and lactate concentrations, samples (2 ml) were taken from both reservoirs at every 5 min until 30 min and at 45, 60, 90, 120 and 180 min and centrifuged (10 min, 1500G) to eliminate erythrocytes. Volume loss due to sampling was less than 15 %. After termination of the experiment the perfused cotyledon was excised, weighed and frozen until analysis.

Analytical methods

Antipyrine was measured colorimetrically according to the method described by Brodie et al. (1949). Glucose and lactate were measured enzymatically (D-Glucose-kit; D/L-Lactic acid-kit, Boehringer Mannheim, Germany). Spiramycin was measured by HPLC after solid phase extraction of the samples. Samples to which spiramycin II was added, as internal standard, were deproteinized with acetonitrile and centrifuged (10 min, 2800G). The supernatant diluted with Na_2HPO_4 (0.02 M) was brought onto a, with methanol and Na_2HPO_4 (0.02 M) preconditioned, solid phase extraction column (Bond Elut, C_2, Varian Sample Preparation Products, Harbor City, CA, U.S.A.). After washing the column with Na_2HPO_4 (0.02 M) and phosphate buffer (0.02 M, pH=3.0), the spiramycin with internal standard were eluted from the column with methanol, 0.02 M phosphate buffer, pH=3.0 (65:35 v/v). The chromatographic system consisted of a Spectra Physics P2000 pump (Thermo Separation Products, Breda, the Netherlands), a Lichrospher 60 RP-Select B 5 μm cartridge column (Merck, 125x4 mm), a cartridge guard column packed with the same material (4x4 mm) and an autosampler (Spectra Physics AS 3000) with a built in column heater (T = 40°C). The mobile-phase was a methanol-0.02 M phosphate buffer, pH=3.0 (39:61 v/v). The flow-rate was 1 ml/min. The column

effluent was detected with a Spectra Physics UV 1000 variable wavelength detector at 231 nm. The injection volume was 10 µl.

Data analysis

Maternal (m) and fetal (f) perfusate concentration-time curves were fitted by nonlinear regression analysis using the computer program PCNONLIN (Metzler and Weiner, 1989) according to the following equations.
For antipyrine:

$$C_m = (C_0 - C_{equi,m}) \cdot e^{-\alpha_1 \cdot t} + C_{equi,m}$$

$$C_f = C_{equi,f} \cdot (1 - e^{-\alpha_2 \cdot t})$$

and for spiramycin:

$$C_m = A \cdot e^{-\alpha_3 \cdot t} + B \cdot e^{-\beta \cdot t}$$

C_m and C_f are maternal and fetal perfusate concentrations, C_0 is the concentration at time zero, α_1, α_2 and α_3 are first-order transport rate constants, ß is first order elimination rate constant and $C_{equi,m}$ and $C_{equi,f}$ are the maternal and fetal concentrations at equilibrium. Placental clearance (CL) was calculated as D/AUC_m, where D is dose and AUC_m is the area under the maternal concentration versus time curve from zero to infinity. For antipyrine AUC_m is calculated as $C_0/\alpha_1 + C_{equi} \cdot t_{120}$ and for spiramycin as $A/\alpha+B/ß$. Half-life of elimination for spiramycin was calculated as ln2/ß.
Data are presented as means ± SD, where N is number of experiments with different placentae.

Results

Perfusion viability

Total (maternal and fetal) glucose consumption was 0.20 ± 0.05 µmol/min/g wet weight and total lactate production was 0.34 ± 0.11 µmol/min/g wet weight (N=3). Mean weight of the perfused cotyledon was 22 ± 7 g (N=3). Disappearance from the maternal circulation and concomitant appearance in the fetal circulation of the flow-dependent marker antipyrine indicated a good diffusibility of the preparation (Figure 1), comparable with literature (Maguire et al., 1992; Barzago et al., 1994). Kinetic parameters of antipyrine transfer are presented in Table 1.

Spiramycin transfer and metabolism

Figure 1 also shows the placental transfer of maternally administered spiramycin. A rapid decrease in the maternal concentration was observed, comparable to the antipyrine decrease. However, in contrast to antipyrine, no concomitant rise in the fetal circuit was observed. Kinetic parameters of placental spiramycin transfer are presented in Table 1. Since no leakage from the perfusion system occurred and maternal spiramycin decrease was not accompanied by a comparable fetal increase, spiramycin must have been retained in the placenta. At the end of the perfusion experiments the concentration of spiramycin detected in the perfused tissue was 0.12 µg/ml, which was in the same order as the concentration in the fetal circuit (0.28 µg/ml). Both concentrations cannot account for the amount of spiramycin that disappeared from the maternal circuit. HPLC chromatograms (Figure 2), however, showed an additional, earlier and higher, peak as compared to the spiramycin peak in perfused tissue samples. The peak also appeared in the maternal perfusate samples at 60 min and increased thereafter. This peak was not observed in a standard spiramycin solution maintained at 37°C for two hours, nor in samples of the maternal reservoir at t=0 and non-perfused tissue. A spiramycin solution in phosphate buffer of pH=3.0 stored at room temperature for several days showed a hydrolysis product of spiramycin, known as neospiramycin (Sander and Delphine, 1994) with the same retention time as antipyrine (large peak in chromatograms C and D in figure 2). Since the retention time of this peak differed from the retention time of the additional peak observed in placental tissue and maternal perfusate, the metabolite formed during perfusion is not the same as the degradation product formed after mild hydrolysis.

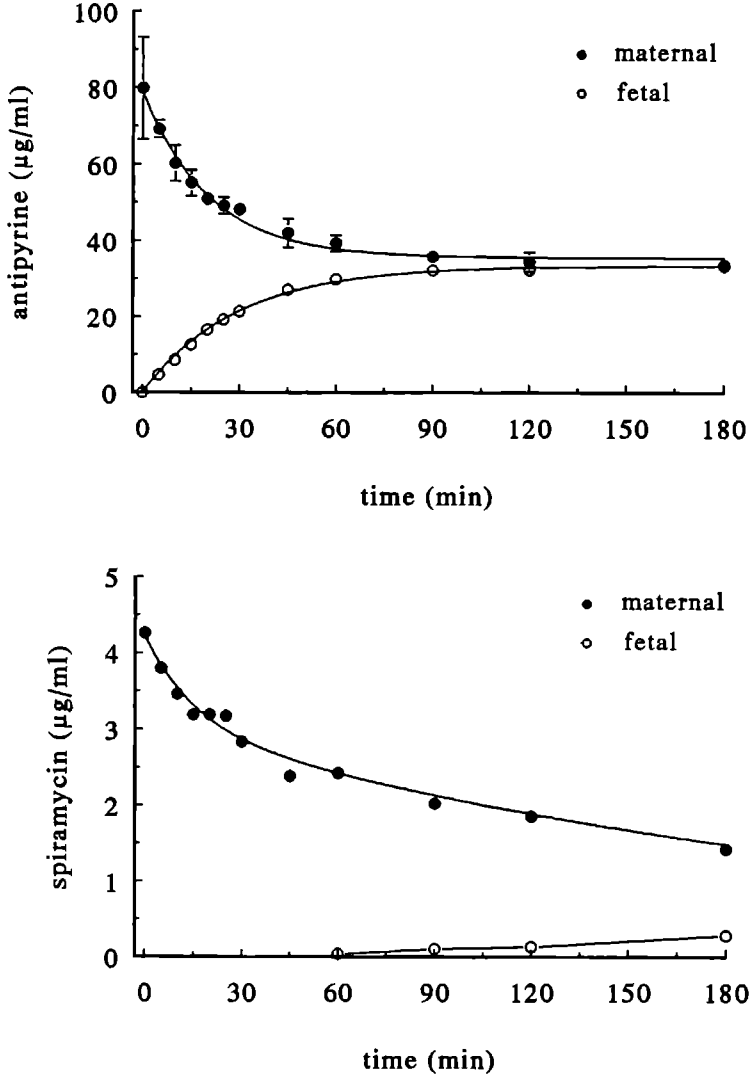

Figure 1. Time course of antipyrine and spiramycin transfer in the isolated dually recirculating perfused cotyledon of human term placenta. Values are presented as means ± SD of three experiments for antipyrine. In case of spiramycin a single experiment is shown.

Table 1. Kinetic parameters of placental antipyrine and spiramycin transfer

Parameter	Antipyrine N=3	Spiramycin N=2
C_0 (µg/ml)	75 2 ± 6 2	3 8
α_1 (min $^{-1}$)	0 043 ± 0 008	-
α_2 (min $^{-1}$)	0 035 ± 0 003	-
α_3 (min $^{-1}$)	-	0 06
ß (min $^{-1}$)	-	0 005
$C_{equi,m}$ (µg/ml)	35 2 ± 2 7	-
$C_{equi,f}$ (µg/ml)	33 5 ± 1 6	-
AUC (µg·min/ml)	6005 ± 177	565
CL (ml/min)	2 37 ± 0 06	1 3
$t_{1/2\,el}$ (min)	-	140

Values are presented as means ± SD or means

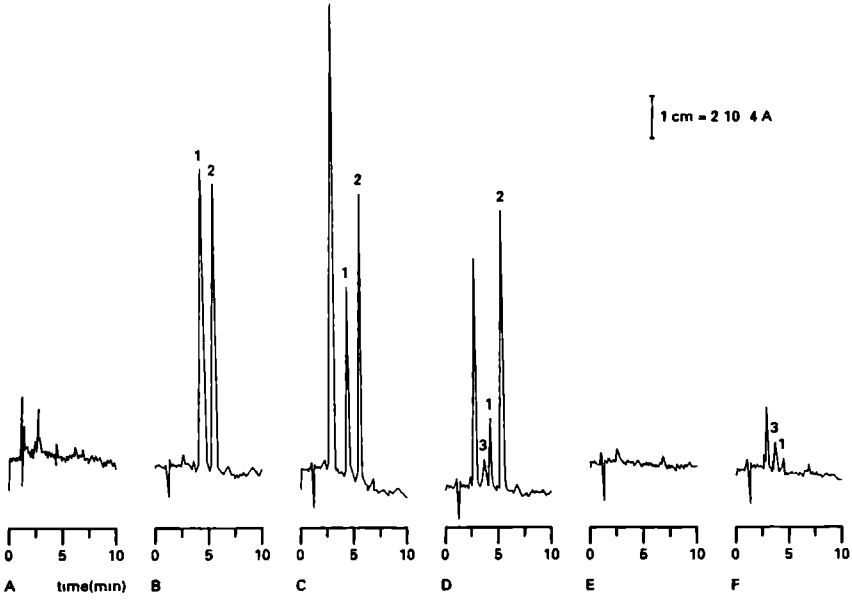

Figure 2. Chromatograms of perfusate after 30 min of open perfusion (A), perfusate spiked with 5 3 µg/ml spiramycin and 6 0 µg/ml internal standard (B), maternal perfusate plus internal standard at t=0 (C), maternal perfusate plus internal standard at t=120 min (D), non-perfused tissue (E) and perfused tissue at t=120 min (F) 1 spiramycin , 2 spiramycin II (internal standard), 3 unknown metabolite

Discussion

In this study we evaluated the characteristics of spiramycin transport across the human placental cotyledon to get more insight into the mechanisms determining its low placental permeability. From in vivo experiments in the rhesus monkey, it was concluded that the placenta may act as a clearance organ or a barrier for spiramycin, since at three hours after administration the fetal serum concentration was only 5-7% of the maternal serum concentration, and the maximal fetal-maternal serum ratio was 0.27 (Schoondermark-van de Ven et al., 1994). Furthermore, the concentration in the placenta was ten times the concentration in the maternal serum. These data corresponded well with a placental transfer rate of approximately 9% of maternally circulating spiramycin, which was reported in a preliminary study using an open perfusion system of the isolated human placental cotyledon (Quetin et al., 1983). In an open system, however, only a transfer index in comparison with antipyrine can be calculated as a measure of placental transfer. We therefore evaluated placental spiramycin transfer in a recirculating perfusion system. This system is particularly useful for studying the kinetics of slowly transferred compounds and the formation of metabolites by the placenta, which is difficult to quantify in a single pass system (Brandes et al., 1983). Since dose-dependent kinetics for spiramycin were observed in the rhesus monkey (Schoondermark-van de Ven et al., 1994), probably as a result of saturable metabolism, placental metabolism may play a role in the low placental transfer.

In our study trans-placental transfer was also low. The initial disappearance rate from the maternal circulation (α_3) was in the same range as the maternal disappearance rate of antipyrine (α_1). The calculated antipyrine and spiramycin clearances were also comparable. Therefore diffusion into the trophoblast seems the main transport mechanism governing the disappearance of spiramycin from the maternal compartment. Membrane pores are generally too small for passage of a relatively large molecule such as spiramycin. Furthermore, the lipophilicity of spiramycin is too low to explain transport in the trophoblast by simple diffusion. Facilitated diffusion seems to be the most likely mechanism, although we were not able to saturate transport in an experiment using a ten times higher concentration of spiramycin (data not shown).

In contrast to antipyrine, spiramycin hardly appeared in the fetal circulation. HPLC chromatograms of perfused tissue samples and 60 min maternal perfusate samples showed an additional earlier and higher peak as compared to spiramycin. The gradual increase in the maternal circuit as the perfusion proceeded suggests that

this peak reflects the formation of a metabolite. Spontaneous decomposition of spiramycin, explaining the appearance of this peak, is not likely since this peak was not found when a spiramycin perfusate solution was kept at 37°C for two hours. It could also be excluded that the hydrolysis product, neospiramycin, accounted for the unknown peak. The formation of the metabolite, however, does not explain the difference between the disappearance of spiramycin from the maternal compartment and the small amount that appeared into the fetal compartment. Because no leakage was observed from both perfusate reservoirs, other metabolites must have been formed, which, due to their presumed hydrophilicity, were not extracted by the solid phase procedure we performed to selectively extract spiramycin. That more metabolites can be formed out of spiramycin was shown by Inoue et al., (1983) after adding rat plasma to a spiramycin standard. Assuming that the metabolites may be effective against Toxoplasma Gondii, placental spiramycin and its metabolites can act as a barrier to passage of the parasite, providing prevention of fetal infection. However, because of the low trans-placental transfer due to placental metabolism and the resulting low umbilical cord levels of spiramycin after maternal administration, a therapeutic effect of spiramycin against an already present fetal infection is not to be expected.

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Summary and Conclusions

The placenta is an organ which combines functions performed by separate organs in adult life. This feature indicates the extreme importance of the placenta for the embryo or fetus in utero. One of the major functions of the placenta is absorption of nutrients and excretion of waste products. This transport function of the placenta, as an intermediate between fetal and maternal circulation, is mainly performed by the syncytial microvillous membrane, facing the maternal blood and the basal membrane, facing the fetal blood. Active transport systems in these membranes have been found for nutrients, such as amino acids and minerals, ensuring an efficient transfer towards the fetus for optimal growth and development. Much less is known about the placental handling of drugs taken by the mother and the influence of maternally circulating drugs on nutrient transfer towards the fetus. Placental drug transport and interactions with nutrients can be of importance to fetal development, especially when the mother is obligated to take drugs during pregnancy, as for instance in maternal epilepsy, diabetes or hypertension. Because of the risks to the fetus it is hardly possible to investigate placental drug transfer in human in vivo. Since it has become clear that major interspecies differences exist in placental solute transfer, results from animal studies are difficult to extrapolate to the human situation. To get more insight into the mechanisms underlying human placental drug transfer and the interaction of drugs with nutrient transfer we undertook the studies described in this thesis. Two experimental models were used:

1. Syncytial microvillous membrane vesicles isolated from human term placenta. This technique provides information especially on the mechanisms of drug transport and drug-nutrient interactions, e.g. driving forces, type of transport and the kinetics of saturation and interaction with structural analogs.
2. The isolated dually perfused cotyledon of human term placenta. This physiologically based model can provide more insight into the overall placental drug transfer. Furthermore, the influence of placental metabolism on drug transfer can be evaluated.

This thesis is divided into three parts:

I Introduction, in which relevant literature data on human placental drug transfer mechanisms are reviewed.

II Studies on mechanisms of transport and interactions with nutrients of anionic and cationic drugs in isolated syncytial microvillous membrane vesicles. p-Aminohippurate, commonly used in renal studies, was chosen as a prototypic anionic compound and the H_2-receptor antagonist cimetidine as a cationic model compound. Furthermore, the inhibitory effects of various anionic and cationic drugs on the transport of the amino acid L-alanine and the endogenous organic cation choline were investigated.

III Studies on overall human placental drug transfer and metabolism in the isolated dually perfused cotyledon of human term placenta. The macrolide antibiotic spiramycin was investigated.

I Introduction

In chapter 1 we summarized the literature on human placental drug transfer mechanisms. Active drug transport mechanisms have been rarely found in cotyledon perfusion and vesicle studies. The amount or rate of transfer was mainly determined by the physico-chemical characteristics of the drug, such as molecular weight, lipophilicity, degree of ionisation at physiological pH and degree of protein binding and by physiological factors, such as maternal blood flow rate and placental metabolism. In most cases overall placental drug transport appears to be governed by simple diffusion, however, in some cases membrane transport was saturable. Especially the transport of some drugs structurally related to endogenous compounds was found to be carrier mediated. In conclusion, membranes of human term placenta possess carrier systems having affinity for certain drugs, however, in general overall human placental drug transfer will be passive.

II Drug transport and interactions in isolated membrane vesicles

In chapter 2 an improved method is described for the isolation of syncytial microvillous membrane vesicles from human term placenta according to an established procedure. The method is based on mincing and gently stirring of placental tissue to loosen the microvilli, followed by Mg^{2+}-aggregation and harvesting of the syncytial microvillous membrane vesicles by differential centrifugation. The vesicle preparation showed a high degree of purification, high yield and good functional viability. Uptake of the amino acid L-alanine was sodium-dependent, saturable (K_m= 230 μM) and susceptible to inhibition by structural analogs. However, significant interactions with anionic or cationic drugs, catecholamines and certain drugs of abuse with the alanine transporter were not found. On the other hand, a number of cationic drugs significantly inhibited the uptake of the endogenous cation choline.

In chapter 3 the mechanism of uptake of the organic anion p-aminohippurate into syncytial microvillous membrane vesicles was investigated. Uptake was pH- and Cl^--dependent, whereas a forced negative or positive membrane potential did not influence p-aminohippurate uptake. Since an anion exchange mechanism was also excluded, no driving forces seem to be present for p-aminohippurate in these membranes. However, evidence for the existence of a carrier-mediated transport mechanism was obtained since uptake was inhibited by several anionic drugs (e.g. $K_{i,probenecid}$= 2.5 mM) and saturable (K_m= 7.4 mM). The significance of this system for placental excretion of anionic drugs is difficult to value from these experiments, but will depend on the intrasyncytial drug concentration achieved through transport from the fetal circulation across the basal membrane.

The presence of a mediated transport mechanism for cationic drugs in these vesicles was investigated in chapter 4. Uptake of the cationic H_2-receptor antagonist cimetidine was stimulated by the imposition of an outwardly directed H^+-gradient (peak vs. equilibrium = 1.3). The peak uptake was decreased under voltage clamped conditions by the proton-ionophore FCCP, suggesting the presence of an organic cation-proton exchange mechanism. H^+-gradient driven cimetidine uptake was partly inhibited by structural analogs and saturable (K_m=6.5 mM). Although our results provide evidence for the presence of an active transport mechanism for cimetidine, the main part of transport occurred by simple diffusion. The barrier function of the human placenta to cimetidine transport as described in literature, is not likely to be caused by an active transport system. The degree of ionization of cimetidine at physiological pH seems to be a more plausible explanation.

In chapters 5 and 6 the uptake mechanism of the endogenous cation choline and the nature of interaction with various cationic drugs were investigated. An inside negative membrane potential and an inside-out choline-gradient stimulated choline uptake (peak vs. equilibrium = 1.8 and 2.1, respectively). Trans-stimulated uptake was saturable (K_m= 550 μM). As the maternal blood concentration of choline is approximately 20 μM, the system assures an efficient placental uptake from maternal blood driven by the negative cell interior. Several organic cations inhibited trans-stimulated choline uptake: hemicholinium-3 > atropine ≥ choline ≥ mepiperphenidol > cimetidine ≥ famotidine > tetraethylammonium. TEA was a poor inhibitor, but by enlarging the degree of alkylation of the quaternary ammonium the inhibitory effect was increased. Hemicholinium-3 and atropine inhibited choline uptake competitively (K_i = 40 μM and 1.2 mM, respectively), whereas cimetidine interacted noncompetitively (K_i = 3.4 mM). Since relatively high concentrations were needed to reach half maximal inhibition, impairment of fetal choline supply due to maternal drug use during pregnancy is not to be expected.

III Drug transfer across the isolated dually perfused cotyledon

Chapter 7 deals with the validation of a dual perfusion system of the isolated cotyledon of human term placenta. The recirculating (closed) perfusion system showed a good diffusibility, since the disappearance of antipyrine from the maternal circulation (clearance = 2.33 ml/min) and concomitant rise in the fetal circulation, was comparable to literature. In contrast with antipyrine, the hydrophilic, membrane impermeable compound inulin crossed the placenta for only approximately 6 %, indicating the integrity of the membrane barrier. Metabolic viability of the preparation was preserved, since a net glucose/lactate conversion was found. We conclude that our system is suitable for placental drug transfer and metabolism studies.

In chapter 8 we investigated the mechanism underlying the low transfer of the macrolide antibiotic spiramycin across the human placenta, as described in literature. The recirculating dual perfusion system of the isolated human placental cotyledon was used. Spiramycin disappearance from the maternal circulation was comparable to antipyrine, indicating passive diffusion into the trophoblast. However, in contrast with antipyrine, no concomitant rise in the fetal circulation and as a result low trans-placental transfer was observed. Spiramycin did not accumulate in placental tissue, but an additional peak was observed in HPLC chromatograms of

perfused tissue and to a lesser extent, but gradually increasing, also in the maternal perfusate. It seems that placental metabolism is assumed to be the main cause in the observed low placental transfer.

In conclusion, the studies described in this thesis have shown that mediated transport systems for anionic and cationic drugs are present in the syncytial microvillous membrane of human term placenta. However, their affinity and specificity appear to be low. Transport systems for the endogenous compounds alanine and choline are relatively insensitive to interaction with drugs. Important factors influencing fetal drug exposure are those that can change equilibrium of unbound drug between maternal and umbilical cord blood, viz. pH differences, differential protein binding, fetal and maternal drug elimination or placental metabolism, as was hypothesized in this thesis for spiramycin. Therefore, the results derived from the studies described in this thesis justify the conclusion that placental active drug transport mechanisms are of minor importance in fetal drug exposure.

Samenvatting en Conclusies

De placenta is een orgaan dat zorg draagt voor functies die na de geboorte door verschillende organen worden uitgevoerd. Dit betekent dat de placenta van groot belang is voor de zich ontwikkelende foetus. Een van de belangrijkste functies is de aanvoer van voedingstoffen naar de foetus en afvoer van afvalprodukten van de foetus naar de moeder. Deze transportfunctie van de placenta, als intermediair tussen de foetale en maternale bloedcirculaties, wordt met name vervuld door de syncytiale microvilleuze membraan, die in contact staat met het maternale bloed en de basale membraan, welke grenst aan het foetale bloed. Actief transport over deze membranen is aangetoond voor nutriënten, zoals aminozuren en mineralen, waardoor een efficiënte aanvoer van voedingsstoffen naar de foetus voor optimale groei en ontwikkeling is gewaarborgd. Veel minder is bekend over de placentaire transportkarakteristieken van geneesmiddelen door de moeder gebruikt tijdens de zwangerschap en de invloed die geneesmiddelen in het maternale bloed kunnen hebben op het transport van nutriënten naar de foetus. Placentair geneesmiddeltransport en interacties met nutriënten kunnen van invloed zijn op groei en ontwikkeling van de foetus, in het bijzonder wanneer de moeder tijdens haar zwangerschap niet buiten geneesmiddelen kan, zoals het geval is bij epilepsie, hypertensie of diabetes. Vanwege de risico's voor de foetus is het nagenoeg onmogelijk om het transport van geneesmiddelen door de humane placenta in vivo te bestuderen. Uit diverse onderzoeken is duidelijk geworden dat er belangrijke speciësverschillen bestaan in placentaire transportkarakteristieken. Daarom kunnen de resultaten van proefdier- onderzoek moeilijk geëxtrapoleerd worden naar de situatie in de mens. Om meer inzicht te verkrijgen in transportmechanismen van geneesmiddelen door de humane placenta en de mogelijke interacties met het placentaire transport van nutriënten, is het onderzoek opgezet en uitgevoerd waarvan de resultaten zijn beschreven in dit proefschrift. Voor het onderzoek is gebruik gemaakt van twee modellen:

1. Syncytiale microvilleuze membraanvesicles geïsoleerd uit de humane placenta a term. Deze techniek maakt het mogelijk om de mechanismen van geneesmiddeltransport en interacties met nutriënten te bestuderen, zoals drijvende krachten, aard van het transport, kinetiek van verzadiging en interacties met structuuranalogen.

2. Het geïsoleerde tweezijdig-geperfundeerde cotyledon van de humane placenta a term. Deze meer fysiologische techniek maakt het mogelijk om het totale transport van geneesmiddelen over de placenta te bestuderen, waarbij ook de invloed van metabolisme op het geneesmiddeltransport kan worden onderzocht.

Het proefschrift is opgebouwd uit drie delen:

I Een inleiding waarin de relevante literatuur over mechanismen van geneesmiddeltransport in de humane placenta wordt besproken.

II Studies naar de mechanismen van transport en interacties met nutriënten van anionische en kationische geneesmiddelen. Het in niertransportstudies veel gebruikte para-aminohippuraat werd gekozen als modelverbinding voor de anionische farmaca en de H_2-receptorantagonist cimetidine voor de kationische farmaca. In dit deel is ook onderzoek gedaan naar de invloed van diverse anionische en kationische geneesmiddelen op het transport van het aminozuur L-alanine en het endogene kation choline.

III Studies betreffende het transport en metabolisme van geneesmiddelen in het geïsoleerde tweezijdig-geperfundeerde cotyledon van de humane placenta a term. In deze studies stond het macrolide antibioticum spiramycine centraal.

I Inleiding

In hoofdstuk 1 is de literatuur betreffende transport van geneesmiddelen in de humane placenta samengevat. Actieve transportmechanismen zijn slechts een enkele keer gevonden in cotyledon-perfusiestudies en vesicle-studies. Over het algemeen wordt de mate van transport bepaald door de fysisch-chemische eigenschappen van de stof, zoals molekuulgewicht, lipofiliteit, mate van ionisatie bij fysiologische pH en mate van eiwitbinding en door fysiologische factoren zoals maternale bloedstroom of placentair metabolisme. In de meeste gevallen verloopt het totale placentaire transport via passieve diffusie, echter in enkele gevallen bleek dat de membraanpassage verzadigbaar was. Dit gold voor farmaca die qua structuur lijken op endogene verbindingen. Dit betekent dat de membranen van de humane placenta carriersystemen bezitten die affiniteit vertonen voor bepaalde geneesmiddelen, maar dat het totale placentaire transport in de regel passief verloopt.

II Geneesmiddeltransport en interacties in geïsoleerde membraanvesicles

In hoofdstuk 2 wordt de methode beschreven voor isolatie van syncytiale microvilleuze membraanvesicles uit de humane placenta a term, volgens een reeds eerder beschreven maar door ons verbeterde procedure. De methode is gebaseerd op het fijnhakken en vervolgens voorzichtig roeren van placentaweefsel, waardoor de microvilli los komen van de rest van het weefsel. Hierna worden met behulp van Mg^{2+}-aggregatie en differentiële centrifugatie de syncytiale microvilleuze membraanvesicles geïsoleerd. Het preparaat vertoonde een hoge zuiverheidsgraad, opbrengst en goede functionele eigenschappen. De opname van het aminozuur L-alanine was natrium-afhankelijk, verzadigbaar (K_m= 230 μM) en te remmen met structuuranaloga. Diverse anionische en kationische farmaca, catecholamines en bepaalde verslavende middelen gaven geen interactie met het alaninetransport. Daarentegen werd het transport van choline wel geremd door een aantal kationische farmaca.

In hoofdstuk 3 wordt het onderzoek beschreven naar de transportmechanismen van het organisch anion p-aminohippuraat in syncytiale microvilleuze membraanvesicles. De opname was afhankelijk van de pH en van de aanwezigheid van chloride. Het aanleggen van verschillende membraanpotentialen had echter geen effect op de opname. Omdat eveneens een anion-exchange-mechanisme niet werd gevonden, is het waarschijnlijk dat er geen directe drijvende kracht voor de opname van p-aminohippuraat in deze vesicles bestaat. Er werd echter wel een aanwijzing gevonden voor de aanwezigheid van een carriersysteem, omdat de opname te remmen was met structuuranaloga (bijv. $K_{i,probenecid}$= 2.5 mM) en verzadigbaar was (K_m= 7.4 mM). De bijdrage van dit systeem aan de eventuele placentaire excretie van anionische farmaca in vivo zal afhangen van de opgebouwde intrasycyntiale concentratie van deze stoffen als gevolg van transport over de basale membraan.

De eventuele aanwezigheid van gemedieerd transport voor kationische farmaca in deze membranen is onderzocht zoals beschreven in hoofdstuk 4. De opname van de kationische H_2-receptorantagonist cimetidine werd gestimuleerd door een uitwaards-gerichte H^+-gradient (piekopname versus evenwichtswaarde = 1.3). De piekopname werd verlaagd door de proton-ionofoor FCCP onder condities waarbij de membraanpotentiaal geen rol speelde. Dit is een aanwijzing voor een mechanisme dat organische kationen tegen protonen kan uitwisselen. De H^+-gradient-gestimuleerde cimetidine opname werd gedeeltelijk geremd door structuur-analoga en was verzadigbaar (K_m=6.5 mM). Ondanks dat deze resultaten bewijs

leveren voor de aanwezigheid van een actief transport-mechanisme voor cimetidine, bestond het grootste deel van het transport uit passieve diffusie. De in de literatuur beschreven barrière van de placenta voor cimetidine kan dan ook niet worden toegeschreven aan de activiteit van een dergelijk mechanisme, maar veeleer aan de relatief hoge ionisatiegraad van cimetidine bij fysiologische pH.

In de hoofdstukken 5 en 6 is het onderzoek beschreven naar het mechanisme van transport van het endogene kation choline en de interactie met verscheidene organische kationen in syncytiale microvilleuze membraanvesicles. Een negatieve membraanpotentiaal aan de binnenkant van de membraan en een uitwaardse choline-gradient stimuleerden de opname van choline (piekopname versus evenwichtswaarde waren respectievelijk 1.8 en 2.1). De trans-gestimuleerde opname was verzadigbaar (K_m= 550 μM). Omdat de maternale bloedconcentratie van choline ongeveer 20 μM is, is in vivo een efficiënte placentaire cholineopname via dit systeem verzekerd. Verscheidene organische kationen waren in staat het trans-gestimuleerde transport van choline te remmen: hemicholinium-3 > atropine ≥ choline ≥ mepiperphenidol > cimetidine ≥ famotidine > tetraethylammonium. Hemicholinium-3 en atropine remden de choline-opname competitief (K_i respectievelijk 40 μM en 1.2 mM), terwijl cimetidine een noncompetitieve interactie liet zien (K_i = 3.4 mM). Omdat relatief hoge concentraties nodig waren om half-maximale inhibitie te bewerkstelligen, zal een nadelige invloed van dergelijke farmaca op de placentaire cholineopname in vivo niet te verwachten zijn.

III Geneesmiddeltransport in het geïsoleerde tweezijdig-geperfundeerde cotyledon

Hoofdstuk 7 behandelt de validatie van het geïsoleerde tweezijdig-geperfundeerde cotyledon van de human placenta a term. Het recirculerende (gesloten) perfusiesysteem liet een goede diffusie van antipyrine zien, omdat de verdwijning uit de maternale circulatie (klaring = 2.33 ml/min) en de gelijktijdige verschijning in de foetale circulatie goed overeen kwamen met data uit de literatuur. In tegenstelling tot antipyrine passeerde het hydrofiele, membraanondoorgankelijke inuline het cotyledon voor slechts ongeveer 6 %, hetgeen een indicatie vormt voor de intactheid van de placentabarrière. De metabole activiteit van het preparaat is eveneens goed bewaard gebleven, omdat een netto glucose/lactaat omzetting kon worden aangetoond. Op basis van deze gegevens hebben we geconcludeerd dat ons systeem geschikt is voor het doen van studies naar placentaire passage en metabolisme van geneesmiddelen.

In hoofdstuk 8 is het onderzoek beschreven naar het onderliggende mechanisme van het lage trans-placentaire transport van het macrolide antibioticum spiramycine, zoals in de literatuur is gerapporteerd. Er is gebruik gemaakt van het hierboven beschreven perfusiesysteem van het cotyledon uit de humane placenta a term. De verdwijning van spiramycine uit de maternale circulatie was vergelijkbaar met die van antipyrine, hetgeen een aanwijzing vormt voor passief transport de placenta in. Echter, in tegenstelling tot antipyrine werd de maternale verdwijning van spiramycine niet gevolgd door een gelijktijdige foetale verschijning, waardoor netto een lage transplacentaire passage werd gevonden. Spiramycine accumuleerde niet in de placenta, maar op HPLC-chromatogrammen van monsters van geperfundeerd weefsel werd een additionele piek gevonden, die eveneens - maar in mindere mate en geleidelijk in hoogte stijgend - ook in monsters van het maternale perfusaat vanaf 60 min werd waargenomen. Deze piek geeft aan dat placentair metabolisme waarschijnlijk als oorzaak van het waargenomen lage trans-placentaire transport van spiramycine kan worden aangemerkt.

Geconcludeerd kan worden dat de studies zoals beschreven in dit proefschrift aangeven dat gemediëerd transport van anionische en kationische farmaca aanwezig is in de syncytiale microvilleuze membraan van de humane placenta a term, maar dat er sprake is van een lage affiniteit en lage specificiteit van de systemen. De actieve transportsystemen voor de endogene verbindingen alanine en choline blijken relatief ongevoelig te zijn voor remming met anionische en/of kationische farmaca. De belangrijkste factoren die de foetale blootstelling aan geneesmiddelen kunnen beïnvloeden zijn die welke het evenwicht van het vrije farmacon tussen het maternale en navelstrengbloed bepalen, zoals pH-verschillen, verschillen in eiwit-binding, maternale en foetale eliminatie of placentair metabolisme, zoals is gevonden voor spiramycine. Daarom rechtvaardigen de resultaten beschreven in dit proefschrift de conclusie dat actieve transportmechanismen voor geneesmiddelen in de placenta een ondergeschikte rol spelen bij de expositie van farmaca aan de foetus.

Dankwoord

' TEAMWORK '. Van onderzoekers verbonden aan een universiteit wordt vaak gedacht dat ze, als een eilandje, geïsoleerd op hun kamertje naarstig op zoek zijn naar een wereldontdekking. Niets is minder waar. Samenwerking is van essentieel belang voor het welslagen van een onderzoeksproject. Ook voor ons project deed dit laatste opgang. Iedereen die meegewerkt heeft aan het placentaonderzoek heeft een belangrijk steentje bijgedragen. In dit dankwoord wil ik echter even stilstaan bij diegenen die rotsblokken verzet hebben.

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 26 oktober 1965 te Bodegraven. In 1985 legde hij met goed gevolg het examen gymnasium-ß af aan het Coornhert Gymnasium te Gouda. Het zelfde jaar begon hij met de studie Biomedische Gezondheidswetenschappen (toen nog Algemene Geneeskunde geheten) aan de Faculteit der Medische Wetenschappen van de Katholieke Universiteit Nijmegen. Tijdens deze studie koos hij voor de afstudeerrichting Toxicologie en werd tevens het examen voor erkend proefdieronderzoeker (Artikel 9 functionaris) met goed gevolg afgelegd. In het kader van twee stageperioden verrichtte hij onderzoek bij de Afdeling Farmacologie van dezelfde Faculteit alwaar een begin werd gemaakt met de isolatie van membraanvesicles uit de humane placenta (o.l.v. Prof. Dr C.A.M. van Ginneken en Dr F.G.M. Russel) en bij de Afdelingen Genetische en Reproductie Toxicologie van de Drug Safety R&D Labs van Organon International te Schaijk, waar hij meewerkte aan de validatie van een in vitro teratogeniteitstest (o.l.v. Dr H.F.P. Joosten en Dr T.D. Yih). In 1990 werd met goed gevolg het Doctoraalexamen afgelegd en bleef hij als Junior Research Fellow tijdelijk werkzaam bij de Drug Safety R&D Labs. Van februari 1991 tot februari 1995 werkte hij als Onderzoeker in Opleiding, in dienst van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek, bij de Afdelingen Farmacologie en Toxicologie van de Faculteit der Medische Wetenschappen van de Katholieke Universiteit Nijmegen alwaar hij, onder directe begeleiding van Dr F.G.M. Russel, dit promotie-onderzoek uitvoerde. Tijdens deze onderzoeksperiode werd een vijftal modulen van de Postdoctorale Opleiding Toxicologie gevolgd, welke tezamen met de gevolgde keuzemodulen tijdens de studie Biomedische Gezondheidswetenschappen moeten leiden tot SMBWO erkenning als Toxicologisch Onderzoeker. Sinds 1 mei 1995 is hij werkzaam als projectmanager FTO/Formularia bij Organon Nederland.

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STELLINGEN

Behorende bij het proefschrift
Drug transport and drug-nutrient interactions in the human placenta
E M van der Aa, 15 november 1995

1 Het transport van geneesmiddelen door de humane placenta wordt in de regel bepaald door diffusie
dit proefschrift

2 Bij de beschrijving van het placentaire transport van geneesmiddelen is het begrip 'zeef' een betere benaming voor de humane placenta dan het begrip 'barrière'
Prof Dr C A M van Ginneken, 1989

3 De transportmechanismen van organische anionen en kationen over de microvilleuze membraan van de humane placenta komen niet overeen met de mechanismen die beschreven zijn voor de brush-border en basolaterale membraan van de niertubulus
dit proefschrift

4 De choline-carrier in de microvilleuze membraan van de humane placenta verzekert een efficiente foetale cholinevoorziening
dit proefschrift

5 In tegenstelling tot wat soms in de literatuur wordt verondersteld, betekent interactie van een geneesmiddel met een transporteiwit nog niet dat het eiwit het geneesmiddel ook daadwerkelijk transporteert

6 Niet alleen een diepgevroren moederkoek kan nog heel wat opleveren
de Volkskrant, 9 februari 1993

7 De op een stoplicht gelijkende kleurverandering van de humane placenta van rood naar groen, die kan optreden bij meconiumhoudend vruchtwater, is niet indicatief voor een mogelijke transportregulerende functie van de placenta
eigen waarneming

8 Stageprojecten voor studenten, als onderdeel van promotieonderzoek dat gebruik maakt van organen van het vrouwelijk lichaam, wekken vooral interesse bij de vrouwelijke studentenpopulatie

9 Het vóórkomen van de humane placenta op de menukaart van een restaurant in Wusban (Centraal China) kan de wetenschappelijke vooruitgang van chinees placentaonderzoek ernstig schaden
de Gelderlander, 15 januari 1994

10 Farmacotherapie-beleid in Nederland dreigt een aangelegenheid te worden van de zorgverzekeraars in plaats van de daarvoor terdege opgeleide beroepsgroepen van apotheker en arts