Examination of the contractility of human placental blood vessels in vitro

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Ph.D. Thesis

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List of Publications Connected to the Thesis

1. Resch Béla Endre, Gáspár Róbert, Falkay György: A placentaerek kontraktilitásának vizsgálata elektromos téringerléssel. (2002) *Magyar Nőorvosok Lapja* 65, 161-167.
2. B. E. Resch, R. Gáspár, G. Falkay: Application of Electric Field Stimulation for Investigations of Human Placental Blood Vessels. (2002) *Obstet Gynecol* 101(2), 296-303.

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List of Abbreviations

ACE: angiotensin convertase enzyme

ANG II: angiotensin II

AR: adrenergic receptor

AT_1-receptor: angiotensin II type 1 receptor

AT_2-receptor: angiotensin II type 2 receptor

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

EFS: Electric Field Stimulation

EPO: erythropoietin

EPO-R: erythropoetin receptor

IU/ml: international unit/milliliter

M: mol/liter

mm: millimeter

mRNA: messenger ribo-nucleinic acid

ms: millisecond

rHuEPO: human recombinant erythropoietin

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

s: second

V: volt

VOCC: voltage-operated calcium channels

1. Introduction

At full term, the placenta has a discoid shape, a diameter of 15-25 cm, is approximately 3 cm thick, and has a weight about 500-600 g. At birth, it is torn from the uterine wall and, approximately 30 minutes after birth of the child, is expelled from the uterine cavity. When, after birth, the placenta is viewed from the maternal side, 15-20 slightly bulging areas, the cotyledons, covered by a thin layer of decidua basalis are clearly recognizable. Grooves between the cotyledons are formed by decidual septa. The fetal surface of the placenta is covered entirely by the chorionic plate. A number of arteries and veins, the chorionic vessels, converge toward the umbilical chord. The chorion, in turn, is covered by the amnion.

Cotyledons receive their blood through 80-100 spiral arteries that pierce the decidual plate and enter the intervillous spaces at more or less regular intervals. The lumen of the spiral artery is narrow, resulting in an increased blood pressure when entering the intervillous space. This pressure forces the blood deep into the intervillous spaces and bathes the numerous small villi of the villous tree in oxygenated blood. As the pressure decreases, blood flows back from the chorionic plate toward the decidua, where it enters the endometrial veins. Collectively, the intervillous spaces of a mature placenta contain approximately 150 ml of blood, which is replenished about 3 or 4 times/minute. This blood moves along the chorionic villi, which have a surface area varying from 4 to 14 m^2. It must be remembered, however, that placental exchange does not take place in all villi, only in those in which fetal vessels are in intimate contact with the covering syncytial membrane. In these villi, the syncytium often has a brush border consisting of numerous microvilli, thus greatly increasing the surface area and, consequently, the exchange rate between maternal and fetal circulations. Sometimes called the placental barrier, the placental membrane is not a true barrier, since many substances pass through it freely. Since the maternal blood in the intervillous spaces is separated from the fetal blood by a chorionic derivative, the human placenta is considered to be of the hemochorial type (Sadler, 1995).

The human placenta has a dual fundamental role: it connects the embryonal, and later the fetal circulation to the maternal circulation, and it isolates the conceptus from

the maternal organism. The placenta is the organ of gas exchange and nutrition between fetus and mother, and it also has important endocrine, metabolic and immunological functions. Fetal circulation in the trophoblast starts in the very early period of pregnancy: it can be detected from days 21-23 of gestation (Steven, 1975). Thus, it can be concluded that the placental circulation is decisive for the outcome of pregnancy throughout pregnancy and delivery: its appropriate circulation is indispensable for the healthy development of the fetus, for an uncomplicated delivery and for the tolerance of stress from birth.

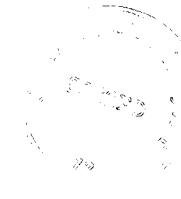
An acute insufficiency of the placental circulation results in hypoxia, acute in utero distress, preterm birth, spontaneous abortion or in utero death. A chronic insufficiency of the placental circulation can cause intrauterine growth restriction (IUGR) (Manning et al., 1981), dysmaturity and low birth weight. Oligohydramnios has frequently been reported as a further consequence of IUGR (Lin et al., 1990, Larmon and Ross, 1998). Severe placental insufficiency can cause fetal myocardial cell damage (Makikallio et al., 2000) and coronary heart disease in adulthood (Hall and McKeigue, 1999). Clinical evidence has linked an intrauterine compromise, such as a prolonged period of insufficient placental circulation during the last third of gestation, to a poor neurological outcome in the newborn, which may manifest its consequences only at the age of 5-8 years (Mallard et al., 1998). Experiments with fetal sheep and guinea-pigs (Mallard et al., 1999, Copolov et al., 2000) revealed a substantial reduction in hippocampal volume, a significant increase in the cerebral ventricles and reduced cross-sectional areas of the cerebral cortex and the striatum after a chronic insufficiency of the placental circulation. These anatomical changes resemble those found in some individuals with schizophrenia. Very low birth weight and IUGR children often have visual impairments, including reduced contrast sensitivity due to damage caused to the tyrosine hydroxylase-immunoreactive amacrine cells by a chronic insufficiency of the placental circulation (Roufail et al., 1999). A chronic placental insufficiency also increased the fetal lung surfactant-associated protein gene expression in a fetal sheep model (Gagnon et al., 1999).

Thus, it is very important to maintain the placental circulation at the best level possible, though the mechanisms responsible for the regulation of placental blood flow

are poorly understood. Vasoconstrictor drugs might cause a decrease in placental blood flow, and vasodilatator drugs might be therapeutic in an insufficient circulation of the placenta. A detailed knowledge is needed of the direct effects on the human placental vasculature of all the drugs given during pregnancy and during delivery. There are several reports on the direct placental vascular effects of different chemical substances: endothelin-1, prostaglandin F2-alfa, sodium nitroprusside (Clausen et al., 1999), histamine (Bertrand and St-Louis, 1999), ouabain, serotonin, (Sanchez-Ferrer et al., 1992, Sanchez-Ferrer et al., 1993, Okatani et al., 1996) and ketanserin (Marin et al., 1990).

Electric field stimulation (EFS) is widely used in physiological and pharmacological research, e.g. the contractility of the pregnant rat uterus (Gaspar et al., 1998, Gaspar et al., 2001). Several reports have been published on investigations of the physiological and pharmacological behaviour of different vessels by EFS: the iridial arteries of the rat (Hirst et al., 1997); the pulmonary arteries of the guinea-pig (Liu et al., 1992); the small mesenteric veins of the rabbit (Marijic et al., 1990); the dog mesenteric artery and the rabbit ear artery (Sun and Zhang, 1985, Bao et al.,1994) and the rabbit mesenteric artery and aorta (Li and Kuriyama, 1993). We have found no data on the application of EFS to study human placental blood vessels in the literature.

Thus, one of the basic aims of the present work was to develop an EFS model suitable for investigations of the contractility of placental veins and arteries, and of the direct effects of different pharmacological agents on the placental blood vessels. Comparison of the physiological and pharmacological behaviour of placental blood vessels to that of non-placental blood vessels (e.g. rat vessels) was also aimed. Most investigations of EFS are undertaken to stimulate sympathetic/adrenergic nerve terminals and to evoke local release of transmitters (Angus et al., 1988). A valid reason for determining the effects of EFS is that the placental circulation appears to be under very little or no functional sympathetic control (Khong et al., 1997; Buttery et al., 1994), e.g. there is no or only a very poor response of the vessels of this circulation to exogenous noradrenaline (Manyonda et al., 1998). Another reason for using EFS is to provide a means of constricting these vessels in a receptor-independent fashion prior to addition of dilatator/constrictor agents.



Cloning and pharmacological data have revealed that the alpha1-ARs can be classified into the three subtypes: alpha1A-, alpha1B-, and alpha1D-ARs (Hieble et al., 1995). It has been shown that the genes for each of the subtypes are expressed in discrete, tissue-specific patterns. Each of the alpha1-AR subtypes has been found to mediate distinct physiological function, e.g. glycogenolysis activation, contraction of smooth muscle, and there are responses involved in the regulation of growth-promoting (Piascik and Perez, 2001).

The adrenergic system plays an important role in the regulation of the uterine motor activity (Borda et al., 1997). Contraction is mediated by the alpha-ARs in the uterine smooth muscle (Hoffman et al., 1981; Rexroad, 1981). This provides a theoretical possibility for the use of alpha1-AR blockers as tocolytic agents, which has been verified in animal studies (Zupkó et al., 1997; Gaspar et al., 1998, Gaspar et al., 2001). Beta-ARs are also involved in uterine relaxation (Levin et al., 1980; Tanfin-Tougi et al., 1981), which is reflected in clinical obstetrics practice by the frequent application of beta2-agonists as tocolytics. Since for secure prenatal care a detailed knowledge is needed of the direct effects on the human placental vasculature of all the drugs that may be given during pregnancy and during delivery, the use of beta2-agonists and the potential use of alpha1-AR blockers as tocolytics raise the question how they influence placental circulation. We have found only limited data on the density, diversity and distribution of the alpha1- and beta2-ARs in the human placental vasculature in the literature.

Thus, a further aim of the present study was to determine the expression and pharmacological reactivity of the alpha1- and beta2-ARs in the human term placental vasculature. The receptor profile was characterized by the amount of mRNA of alpha1-AR subtypes and beta2-ARs. The mRNAs were detected by reverse trancription polymerase chain reaction (RT-PCR). EFS was applied to test the pharmacological reactivity of the human placental blood vessels.

However, all the aforementioned objectives of ours consider the investigation of only vasodilatator agents, yet there are agents, which can be possibly dangerous during pregnancy and delivery by possessing the opposite (vasoconstrictor) characteristics on human placental blood vessels. The main side-effect of the chronic application of

recombinant human erythropoietin (rHuEPO) as therapy for hemodialyzed patients has been reported to be the development or aggravation of hypertension (Eschbach et al., 1987), which can be only partially explained by an increase in blood viscosity (Mayer et al., 1971; Raine, 1988), a diminished hypoxic vasodilatation or an enhanced cardiac output due to a better level of myocardial oxygenation (Neff et al., 1971). The development of hypertension directly after application, however, and in spite of a slow hematocrit increase, suggested that other pressor mechanisms may be involved (Jacquot et al., 1987; Edmunds and Walls, 1988). The direct vasoconstrictor effect of erythropoietin (EPO) has been reported by various authors in numerous animal studies (Heidenreich et al., 1991), but we have found no data on the direct effect of EPO on human placental blood vessels in the literature. The placental transfer of EPO in humans seems quite unlikely (Eichhorn et al., 1993), but the human placenta (Conrad et al., 1996), fetal liver, kidney, spleen, bone marrow (Dame et al., 1998) and brain (Juul et al., 1998) produce EPO during pregnancy. The EPO receptor (EPO-R) protein and its mRNA, classically found in erythroid precursor cells (Jelkmann, 1994), have been described in other cell types, including endothelial cells of the fetoplacental vasculature (Anagnostou et al., 1994; Benyo and Conrad, 1999). These novel and nonclassical sites of EPO and EPO-R expression raise the possibility of physiological roles for this hormone that are not necessarily related to erythropoiesis. To test this possibility, the direct effects of rHuEPO on isolated human placental blood vessels were examined.

Considerable interest has been focused on the potential for angiotensin convertase enzyme (ACE) inhibitors and angiotensin II (ANG II) receptor antagonists to affect the response to EPO (Macdougall, 1999). Therefore, in the present study the effects of captopril and losartan on the rHuEPO-induced contractions of isolated human placental veins and arteries were also investigated.

2. Objectives

1. To develop an EFS model suitable for investigations of the contractility of placental veins and arteries, and of the direct effects of different pharmacological agents on the human placental blood vessels. Comparison of the physiological and pharmacological behaviour of placental vessels to that of non-placental blood vessels (e.g. rat vessels) was also aimed.

2. To test the pharmacological capability of the model.

3. To determine the expression and pharmacological reactivity of the alpha1- and beta2-ARs in the human term placental vasculature.

4. To examine the direct effects of rHuEPO on isolated human placental blood vessels. To investigate the effects of captopril and losartan on the rHuEPO-induced contractions of isolated human placental veins and arteries.

3. Materials and Methods

3.1. Sampling and Preparation

Experimentation on human placentas was approved by the Institutional Review Board (permission No. 909/1998. of the Ethics Committee of the University of Szeged). Placentas were obtained immediately after birth from the Delivery Room of the Department of Obstetrics and Gynecology, University of Szeged. Achieving biassed results must be avoided by careful sampling, therefore placentas were properly examined fresh. The placental membranes were examined for both completeness and color. The chorionic vasculature was also carefully observed, and if any aberrancies or any sign of infection, inflammation or fibrosis were notable, that very placenta was dismissed from any further experimenting. Most umbilical cords insert centrally or paracentrally in the disk of the placenta. About 5% of umbilical cords insert marginally at the disk edge, and 1% to 2% of umbilical cords are velamentous and insert away from the disk (Gilbert-Barness, 2002). Velamentous cords are also associated with multiple gestations and a variety of congenital syndromes (Lewis and Benirschke, 1997). Only placentas with central or paracentral insertion of the umbilical cord were choosen to be included in the experiments. The incidence of single umbilical artery has been cited as 1% in term neonates (Heifetz, 1984; Heifetz, 2000). Edema of the umbilical cord occurs in about 3% of all deliveries (Coulter et al., 1975). If single umbilical artery or any other aberrancies in the umbilical cord was present, the placenta was dismissed from any further experimentation. Meconium evacuation is the result of intrauterine distress (Altschuler, 1997), but meconium produced physiologically may also produce ischemia (Altschuler and Hyde, 1989), therefore, in case of meconium staining of any part of the conceptus, the placenta was dismissed from any further experimenting. Noteworthy is the fact that maternal complications of pregancy carry their attendant associated placental findings. The presence of any of these pathological conditions prevented the inclusion of that very placenta in the study. All used placentas weighed in the normal range (approximately 500 g). They were transferred in 500 ml icy Krebs-Henseleit buffer (in mM: 118 NaCl, 5 KCl, 2 $CaCl_2$, 0.5 $MgSO_4$, 1 KH_2SO_4, 25 $NaHCO_3$, 10

glucose, pH = 7.4), and the experiments were begun 10-30 min after birth. All the placentas were selected randomly for study inclusion from term, singleton pregnancies of healthy Caucasian mothers that ended with uncomplicated deliveries. The ages of the women from whom the placentas were obtained ranged between 19 and 28 years, with an average of 25.3 years. The gestational age at delivery was between 37 and 40 weeks, with an average of 38.1 weeks. The vast majority (80 %) of the mothers were primiparas. After the umbilical cord had been cut off, thin polyethylene cannulae were led into the vein (with larger diameter) and the two arteries (with smaller diameters) in the stub, in order to separate the veins and arteries on the fetal surface of the placenta. The vessels were prepared for in vitro measurement according to the method outlined by Angus and Wright (2000). Rings 1-1.2 mm in diameter (Omar et al., 1992; Omar et al., 1995) were dissected from the identified veins and arteries just before their heading towards the stem villi. The precise length of the rings (4 mm) was achieved by use of a fixed double-bladed scalpel. The loose connective tissue was carefully removed under a binocular dissection microscope (10x). The rings were taken distally from the site of introduction of the cannulae, the endothelium therefore remaining intact.

Since the comparison of the physiological and pharmacological behaviour of human placental blood vessels to those of non-placental blood vessels was also aimed, comparative experiments on rat mesenterial arteries were also undertaken. The rat mesenterial artery is a widely used model for the study of peripherial resistance vessels (Heidenreich et al., 1991). The rat mesenterial arteries were dissected from 200-220 g female Sprague-Dawley rats through laparotomy after sacrifying the animals (by cervical dislocation). Rat studies were allowed by permission No.I.-74-8/2002. of the Animal Ethics Committee of the University of Szeged.

3.2. EFS Studies

The rings were mounted diametrically (as ring preparations) between two platinum electrodes in an organ bath containing 10 ml Krebs-Henseleit buffer. The organ bath was maintained at 37 °C. Carbogen gas (95% O_2 + 5% CO_2) or gas simulating the in utero conditions (Messer Ltd., Szeged, Hungary) (for veins: pO_2= 38

mmHg, pCO_2= 43 mmHg, for arteries: pO_2= 22 mmHg, pCO_2= 48 mmHg) (Longo, 1998) was bubbled through it. After mounting, the rings were equilibrated for 90 min before the experiment and the buffer was changed every 10 min. The passive force was set at approximately 3.75 g and 3.25 g for veins and arteries, respectively. The optimal degree of stretch was ascertained by determining a contraction versus passive force curve in response to an EFS stimulus with a stimulating potential of 30 V, a period time of 4 s and a pulse width of 80 ms. Using the same technique the optimal passive force for rat mesenterial arteries turned out to be approximately 2.2 g. These passive forces were similar to those used in previous EFS studies on other mammalian vascular smooth muscle preparations (Ehrreich and Clopper, 1970).

Contractions were elicited by a digital, programmable stimulator (ST-02, Experimetria Ltd., London, U.K.). The force of the vessel rings was measured with a gauge transducer (SG-02, Experimetria Ltd., London, U.K.), and recorded by an ISOSYS Data Acquisition System (Experimetria Ltd., London, U.K.).

The optimal period time (the time interval between two stimuli) was determined by decreasing the applied period time until the individual contractions fused to produce a smooth contractile response. 20 and 30 V were used as stimulating potentials (both supramaximal). The pulse width (the duration of a single stimulus) values to be used to elicit half-maximal contractions were determined by applying stimuli of different durations (pulse width: 25, 50, 100 and 200 ms) and the elicited contraction responses were registered.

The role of calcium and calcium channels was assessed by conducting experiments in Ca^{2+}-free medium and with blockers of both different ion channels (verapamil, Ni^{2+}) and the intracellular Ca^{2+}-stores (cyclopiazonic acid). To test the pharmacological capability of the model, the effects of $NaNO_2$ (nitrovasodilator) was examined. To test the pharmacological reactivity of the different AR-types terbutalin, fenoterol, ritodrine (beta2-AR agonists), WB4101 (a subtype-selective alpha1A-AR antagonist), BMY7378 (a subtype-selective alpha1D-AR antagonist) and urapidil (a non-subtype-selective alpha1-AR antagonist) were studied. These pharmacons were administered in a cumulative way. The first dose was added at half-maximal contraction, and the next ones every 5 min. Doses of human recombinant erythropoietin

(rHuEPO) ranging between 10 IU/ml and 300 IU/ml were administered in a cumulative way. Each dose of rHuEPO was given when the previous dose had exerted its maximal effect (within 2-10 min). Captopril or losartan was administered 10 min before the first dose of rHuEPO during incubation. rHuEPO and losartan were generous gifts from LaRoche (Budapest, Hungary) and Merck Sharp & Dohme (Budapest, Hungary), respectively. All other the pharmacological compounds were purchased from Sigma Aldrich Ltd. (Budapest, Hungary).

3.3. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Studies

3.3.1. Tissue isolation

After taking the rings for EFS studies more placental vessel tissues were rapidly removed for RT-PCR studies and dissected in ice-cold saline (0.9% NaCl) containing 2 units/ml of recombinant ribonuclease inhibitor (RNasin, Promega, Southampton, U.K.). The tissues were frozen in liquid nitrogen and then stored at –70 °C until the extraction of total RNA.

3.3.2. Total RNA preparation

Total cellular RNA was isolated by extraction with guanidinium thiocyanate-acid-phenol-chloroform according to the procedure of Chomczynski and Sacchi (1987). After precipitation with isopropanol, the RNA was treated with RNase–free DNase I for 30 min at 37 °C, re-extracted with phenol, precipitated with ethanol, washed with 75% ethanol and then resuspended in diethylpyrocarbonate-treated water, and the RNA concentration was determined by optical density measurements at 260 nm.

3.3.3. RT-PCR

The RNA (0.5 μg) was denatured at 70 °C for 5 min in a reaction mixture containing 20 units of RNase inhibitor (Hybaid Corp., Middlesex, U.K.), 200 μM dNTP (Sigma-Aldrich, Budapest, Hungary), 20 μM of oligo(dT) (Hybaid Corp., Middlesex, U.K.) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl and 5 mM $MgCl_2$ in a final reaction volume of 19 μl. After the mixture had been cooled to 4 °C, 20 units of M-MLV

Reverse transcriptase, RNase H Minus (Promega, Southampton, U.K.) was added, and the mixture was incubated at 37 °C for 60 min and then at 72 °C for 10 min.

PCR was carried out with 5 μl cDNA, 25 μl ReadyMix REDTaq PCR reaction mix (Sigma-Aldrich, Budapest, Hungary) and 50 pm sense and antisense primer. The primer sequences used to amplify the alpha1A-AR were 5'-ACT ACA TCG TCA ACC TGG CG-3' (for the forward primer) and 5'-TGA TCT GGC AGA TGG TCT CG-3' (for the reverse primer); to amplify the alpha1B-AR were 5'-TCG GTG GCC TGC AAC CGG CAC CTG-3' (for the forward primer) and 5'-ATG CCC AAG GTT TTG GCT GCT TTC TT-3' (for the reverse primer); and to amplify the alpha1D-AR were 5'-GTG GTG AGT GCT CAG GGC GTG-3' (for the forward primer) and 5'-GAT GAC CGC CAT GGG CAG GT-3' (for the reverse primer) (Scofield et al., 1995). The forward primer for the beta2-AR was 5'-AGT CTG TTT AGT GGT CTG-3', while the reverse primer was 5'-CCT CCT TAA CTG GTT GGG-3' (Fujii et al., 1997). A human GAPDH probe was used as an internal control in all samples (Tso et al., 1985). The PCR was performed with a PCR Sprint thermal cycler (Hybaid Corp., Middlesex, U.K.) with the following cycle parameters: after initial denaturation at 95 °C for 3 min, the reactions were taken through 35 cycles of 1 min at 94 °C, 1 min annealing at 54 °C (alpha1B- and alpha1D-AR) or 50 °C (alpha1A- and beta2-AR) and 72 °C for 2 min. PCR products were used immediately or stored at –70 °C. The PCR products were visualized by performing the electrophoresis on gel containing ethidium bromide (Sigma-Aldrich, Budapest, Hungary). Quantitative analysis was performed by densitometric scanning of the gel with the KODAK EDAS290 system (Csertex, Budapest, Hungary). An AR/GAPDH amplification ratio was calculated for each RNA pool.

3.4. Statistics

Measured or calculated points were plotted and curves were fitted to these points with Prism 2.01 software (GraphPad Software, San Diego, CA, USA). Data were statistically analyzed with Prism 2.01 and SPSS for Windows 9.0 softwares (SPSS Inc., Chicago, IL, USA). One-or two-way analysis of variance with the Newman-Keuls post

test, and two- or three-way repeated measures analysis of variance were used to evaluate the significance levels of differences. Probability values lower than 0.05 were considered significant.

4. Results

4.1. EFS Studies

EFS induced fast and reproducible contractions in the human placental blood vessel rings. The optimal period time was found to be 5 s. For both tested stimulating potentials (20 V and 30 V), the strength of the contractions was plotted against the logarithm of the pulse width, and a sigmoidal curve was fitted. The contraction versus pulse width curve was analyzed and the pulse width location at half maximal force was calculated with GraphPad Prism 2.01 software.

On stimulation at 20 V, the pulse width location at half maximal force was above 500 ms for both veins and arteries. These values were so extremely high that the

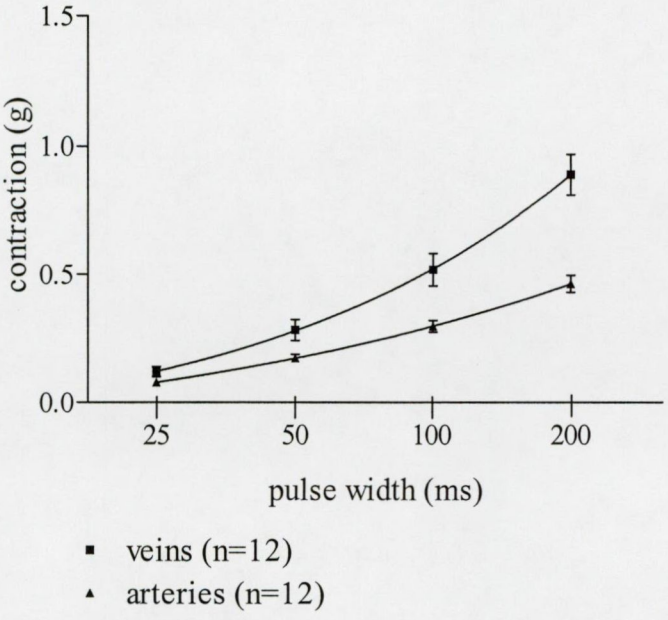


Figure 1.: The contraction versus pulse width curve of human placental blood vessels on stimulation at 20 V. The strength of the contractions was plotted against the logarithm of the pulse width, and a sigmoidal curve was fitted. The pulse width location at half maximal force was above 500 ms for both veins and arteries. These values were so extremely high that the curve was considered unsaturable. The contractions of the veins were significantly stronger than those of the arteries at all applied pulse widths ($p<0.05$).

curve was considered unsaturable. The contractions of the veins were significantly stronger than those of the arteries at all applied pulse widths ($p<0.05$) (Fig. 1).

On stimulation at 30 V, the optimal pulse width (the pulse width location at half maximum force) for veins and arteries was 100 ms and 119 ms, respectively. Bubbling through physiological in utero hypoxic gases significantly enhanced the contractile responses of both vein and artery rings to EFS at all applied pulse widths, though the optimal pulse widths were not changed significantly: 99 ms and 105 ms for veins and arteries, respectively (Fig. 2).

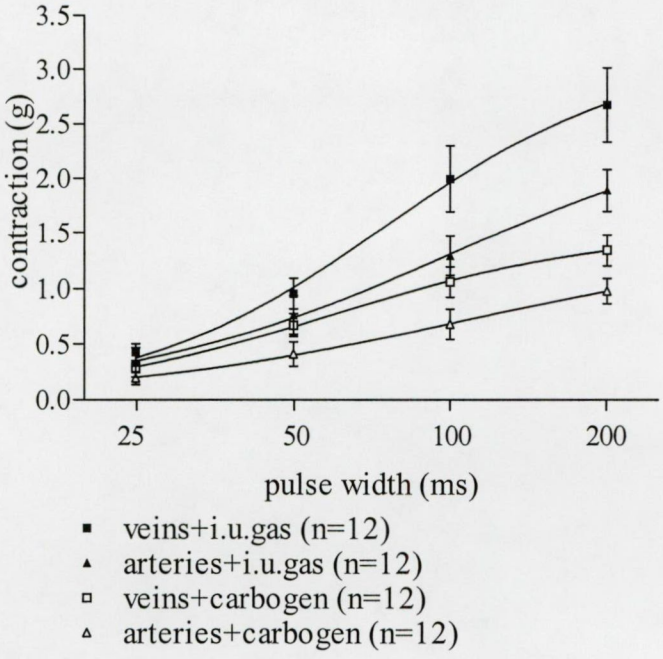


Figure 2.: The contraction versus pulse width curve of human placental blood vessels on stimulation at 30 V under different gas circumstances. The strength of the contractions was plotted against the logarithm of the pulse width, and a sigmoidal curve was fitted. The optimal pulse width (the pulse width location at half maximum force) for veins and arteries was 100 ms and 119 ms, respectively. The contractions of the veins were stronger at all applied pulse widths, but none of the differences were significant ($p>0.05$). Bubbling through physiological in utero hypoxic gases significantly enhanced the contractile responses of both vein and artery rings to EFS at all applied pulse widths, though the optimal pulse widths were not changed significantly: 99 ms and 105 ms for veins and arteries, respectively.

After reaching half-maximal contraction, the placental vessel rings exhibited a time-dependent spontaneous relaxation, despite continuous stimulation. The arteries displayed a slightly greater relaxation, but the difference between the relaxation of the veins and the arteries was not significant ($p>0.05$). The proportion of the spontaneous relaxation as a function of time after the half-maximal contraction was reached could be described by two (veins and arteries) third-degree polynomial functions ($y=A+Bx+Cx^2+Dx^3$). Bubbling through physiological in utero hypoxic gases has not altered the spontaneous relaxation of the veins, but those of the arteries has been reduced to zero (Fig. 3). According to these functions, the proportion of the spontaneous relaxation could be determined at any moment within 40 min after the half-maximal contraction.

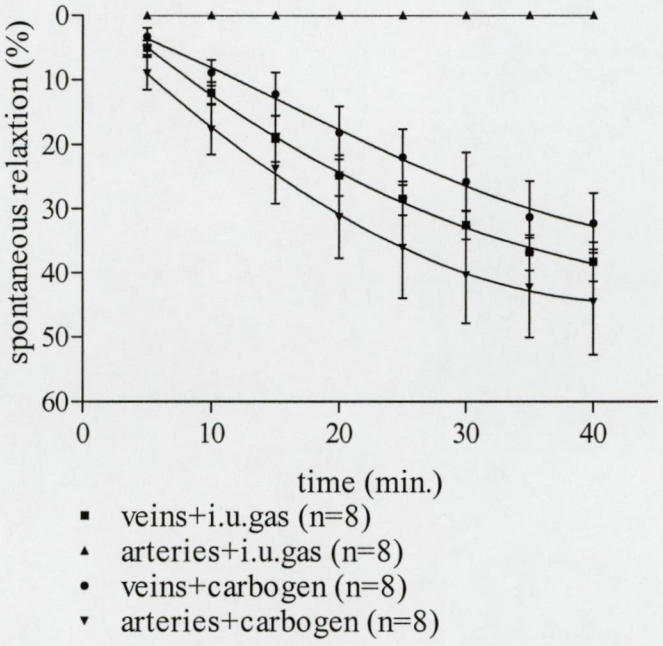


Figure 3.: The time-dependent spontaneous relaxation of human placental blood vessels under different gas circumstances. After reaching half-maximal contraction, the placental vessel rings exhibited a time-dependent spontaneous relaxation, despite continuous stimulation. The arteries displayed a slightly greater relaxation, but the difference between the relaxation of the veins and the arteries was not significant ($p>0.05$). The proportion of the spontaneous relaxation as a function of time after the half-maximal contraction was reached could be described by two third-degree polynomial functions. Bubbling through physiological in utero hypoxic gases has not altered the spontaneous relaxation of the veins, but those of the arteries has been reduced to zero.

EFS (with the same stimulating parameters as used for placental vessel rings) induced fast and reproducible contractions also on the rat mesenterial arterial rings. After reaching half-maximal contraction, the rat mesenterial rings also exhibited a time-dependent spontaneous relaxation, despite continuous stimulation. The proportion of the spontaneous relaxation as a function of time after the half-maximal contraction was reached could be described by another third-degree polynomial function. Controversially to the placental vessel rings, in utero gases significantly blunted the forces of the contractions at all applied pulse widths (Fig. 4), but it did not alter the spontaneous relaxation of the rat mesenterial arterial rings (Fig. 5).

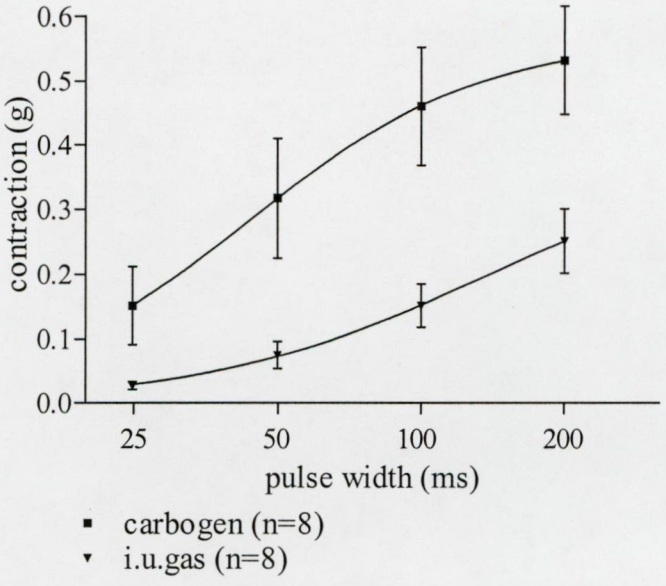


Figure 4.: The contraction versus pulse width curve of rat mesenterial artery under different gas circumstances. The strength of the contractions was plotted against the logarithm of the pulse width, and a sigmoidal curve was fitted. EFS (with the same stimulating parameters as used for placental vessel rings) induced fast and reproducible contractions also on the rat mesenterial arterial rings. Controversially to the human placental vessel rings, in utero gases significantly blunted the forces of the contractions at all applied pulse widths ($p>0.05$).

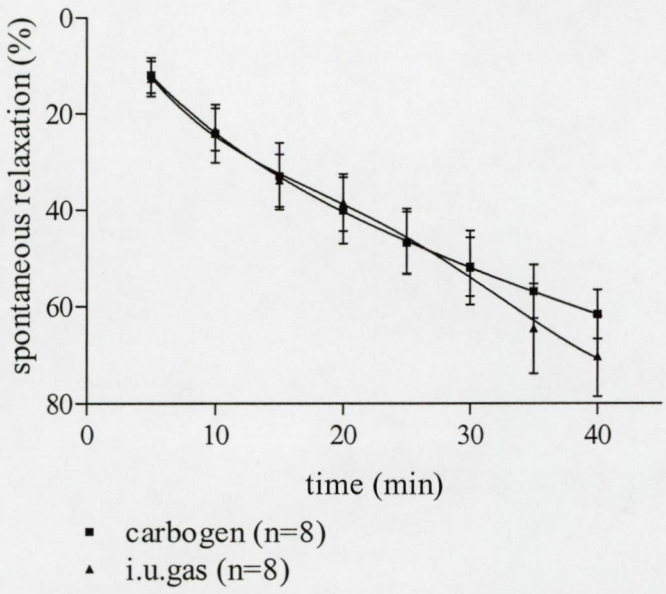


Figure 5.: The time-dependent spontaneous relaxation of rat mesenterial artery under different gas circumstances. After reaching half-maximal contraction, the rat mesenterial rings also exhibited a time-dependent spontaneous relaxation, despite continuous stimulation. The proportion of the spontaneous relaxation as a function of time after the half-maximal contraction was reached could be described by another third-degree polynomial function. In utero gases have not altered the spontaneous relaxation of the rat mesenterial arterial rings.

To test the pharmacological capability of the model, the effects of $NaNO_2$ (a nitrovasodilator) was examined. $NaNO_2$ antagonized the contractions of the placental vessel rings in a significant and dose-dependent manner, but the efficacy of $NaNO_2$ was significantly decreased by the in utero gases (Fig. 6).

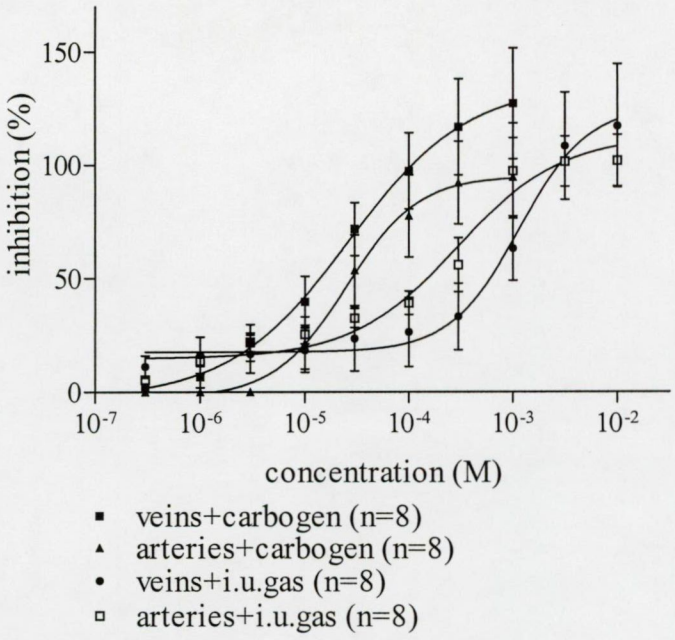
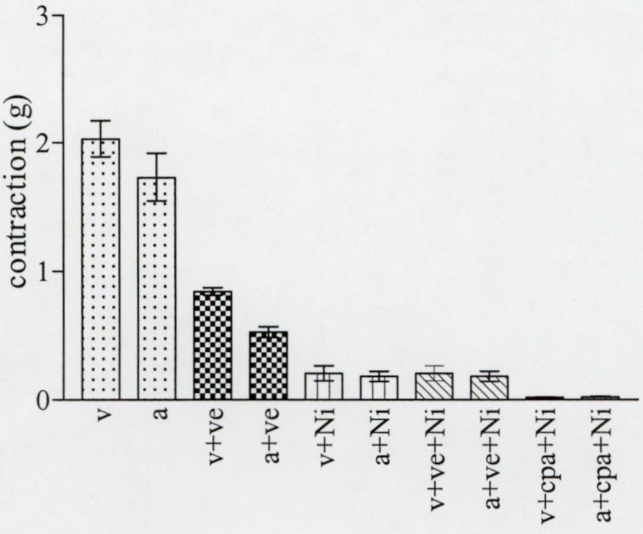


Figure 6.: The dose-response curve of $NaNO_2$ on the EFS induced contractions of human placental blood vessels under different gas circumstances. $NaNO_2$ antagonized the contractions of the placental vessel rings in a significant and dose-dependent manner, but the efficacy of $NaNO_2$ was significantly decreased by the in utero gases.

The inhibitory or stimulatory effects caused by any drug itself, besides the spontaneous relaxations, could be calculated by correcting the measured contractions by the aforementioned third-degree polynomial functions. The correction procedure was as follows: 1. The actual contractions were multiplied by the proportions of the spontaneous relaxation measured 5, 10, 15, 20, 25, 30, 35 and 40 min after addition of the first dose. The results of these multiplications were the actual spontaneous relaxations, which could have been measured without addition of the drugs. 2. Subtraction of the actual spontaneous relaxation from the actual measured relaxation, leaving the actual relaxation caused by the drug itself. 3. Division of the actual drug-caused relaxation by the actual contraction, resulting in the proportions (percentages) of the drug-caused relaxation, which were plotted against the logarithms of the concentrations (semilogarithmic dose-response curve).

The role of calcium and calcium channels was assessed by conducting experiments in Ca^{2+}-free medium and with blockers of both different ion channels

(verapamil, Ni^{2+}) and the intracellular Ca^{2+}-stores (cyclopiazonic acid). The contractions were not changed by tetrodotoxine (10^{-6} M). Pretreatment with verapamil (10^{-6} M), which is a blocker of voltage operated (L-type) Ca^{2+} channels or Ni^{2+} (2 mM) (nonselective blocker of cation channels) inhibited the contractions to a magnitude of 63.81 ± 7.69 % and 88.36 ± 12.17 %, respectively. Combined verapamil and Ni^{2+} treatment inhibited the contractions to a similar magnitude as Ni^{2+} treatment in itself. In Ca^{2+}-free medium after combined cyclopiazonic acid (10^{-5} M) (depletes Ca^{2+} through



v=veins
a=arteries
v+ve=veins+verapamil
a+ve=arteries+verapamil
v+Ni=veins+Ni^{2+}
a+Ni=arteries+Ni^{2+}
v+ve+Ni=veins+verapamil+Ni^{2+}
a+ve+Ni=arteries+verapamil+Ni^{2+}
v+cpa+Ni=veins+cyclopiazonic acid+Ni^{2+}
a+cpa+Ni=arteries+cyclopiazonic acid+Ni^{2+}

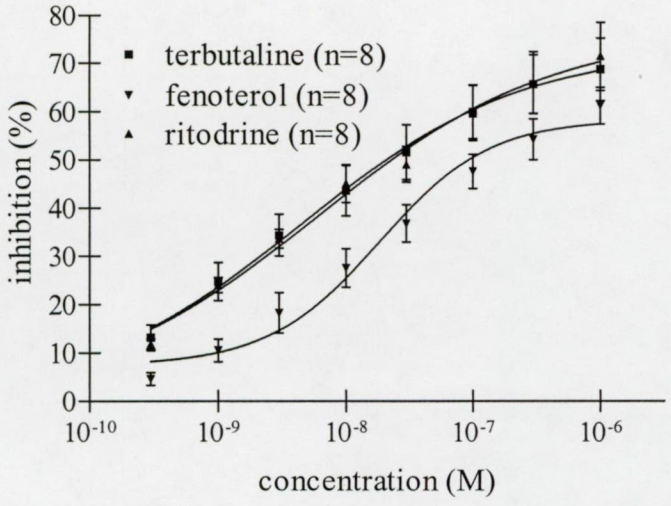
Figure 7.: The role of calcium and calcium channels in the EFS induced contractions of human placental blood vessels. Pretreatment with verapamil (10^{-6} M) or Ni^{2+} (2 mM) inhibited the contractions to a magnitude of 63.81 ± 7.69 % and 88.36 ± 12.17 %, respectively. Combined verapamil and Ni^{2+} treatment inhibited the contractions to a similar magnitude as Ni^{2+} treatment in itself. In Ca^{2+}-free medium after combined cyclopiazonic acid (10^{-5} M) and Ni^{2+} treatment it was not possible to elicit contractions with EFS.

inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase) and Ni^{2+} treatment it was not possible to elicit contractions with EFS (Fig. 7).

Beta2-mimetics antagonized the EFS induced contractions of the human placental arterial rings in a significant and dose dependent manner (Fig. 8a), but they hardly changed those of the human placental vein rings (Fig. 8b): the relaxant effects of the beta2 mimetics were significantly less marked in case of the veins at all applied doses. The differences between the inhibitory effects of the different beta2-mimetics were not significant neither in case of the arteries nor in case of the veins.

WB4101 (an alpha1A-subtype-selective adrenergic receptor blocker) and BMY7378 (an alpha1D-subtype-selective adrenergic receptor blocker) both antagonized the EFS induced contractions of the human placental arterial rings in a significant and dose dependent manner (Fig. 9a), but they hardly changed those of the human placental vein rings (Fig. 9b): the relaxant effects of WB4101 and BMY7378 were significantly less marked in case of the veins at all applied doses. Urapidil (non-subtype-selective alpha1 adrenergic receptor blocker) antagonized the EFS induced contractions of both the human placental arterial (Fig. 9a) and vein (Fig. 9b) rings in a significant and dose-dependent manner. Inhibition, however, was still lower in case of the veins at all applied doses, but none of the differences were significant.

a



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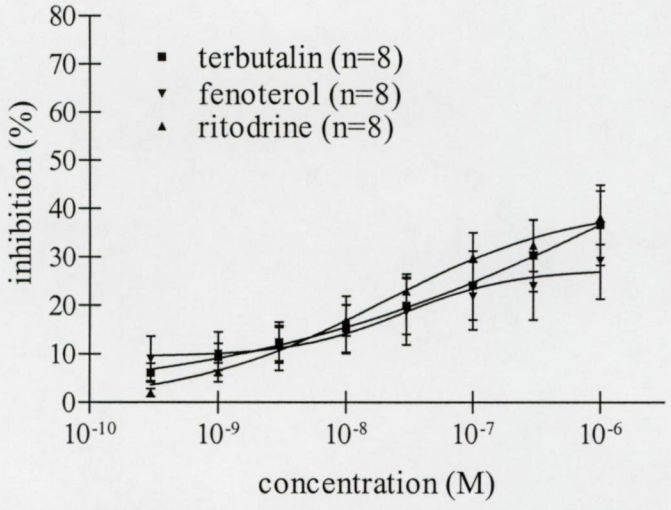
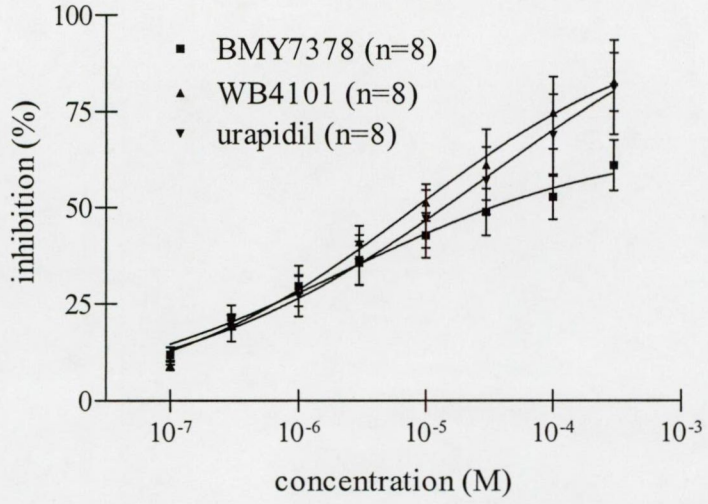


Figure 8.: The dose-response curves of different beta2-mimetics on the EFS induced contractions of human placental blood vessels. Beta2-mimetics antagonized the EFS induced contractions of the human placental arterial rings in a significant and dose dependent manner (a), but they hardly changed those of the human placental vein rings (b): the relaxant effects of the beta2 mimetics were significantly less marked in case of the veins at all applied doses. The differences between the inhibitory effects of the different beta2-mimetics were not significant neither in case of the arteries nor in case of the veins.

a



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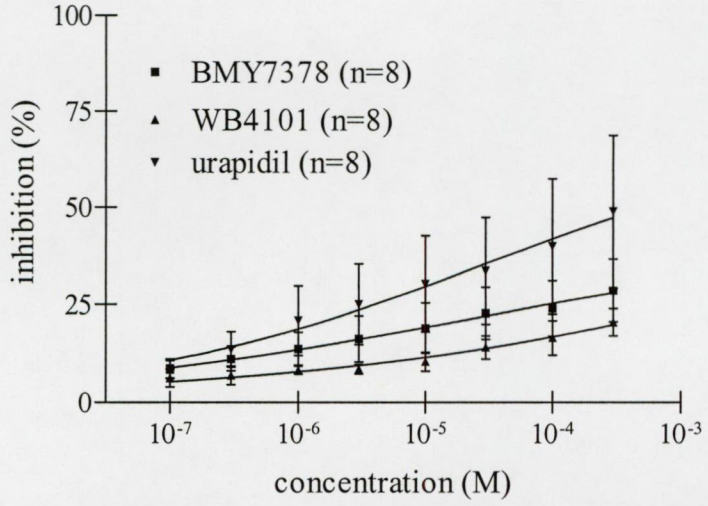

Figure 9.: The dose-response curves of different alpha1-adrenergic receptor antagonists on the EFS induced contractions of human placental blood vessels. WB4101 (an alpha1A-subtype-selective adrenergic receptor blocker) and BMY7378 (an alpha1D-subtype-selective adrenergic receptor blocker) both antagonized the EFS induced contractions of the human placental arterial rings in a significant and dose dependent manner (a), but they hardly changed those of the human placental vein rings (b): the relaxant effects of WB4101 and BMY7378 were significantly less marked in case of the veins at all applied doses. Urapidil (non-subtype-selective alpha1 adrenergic receptor blocker) antagonized the EFS induced contractions of both the human placental arterial (a) and vein (b) rings in a significant and dose-dependent manner. Inhibition, however, was still lower in case of the veins at all applied doses, but none of the differences were significant.

4.2. RT-PCR Studies

The expression of beta2-AR mRNA and all subtypes of alpha1-AR mRNA were proved in the human term placental arteries and veins. The expression of beta2-AR mRNA was significantly higher in the arteries compared to the veins (Fig. 10).

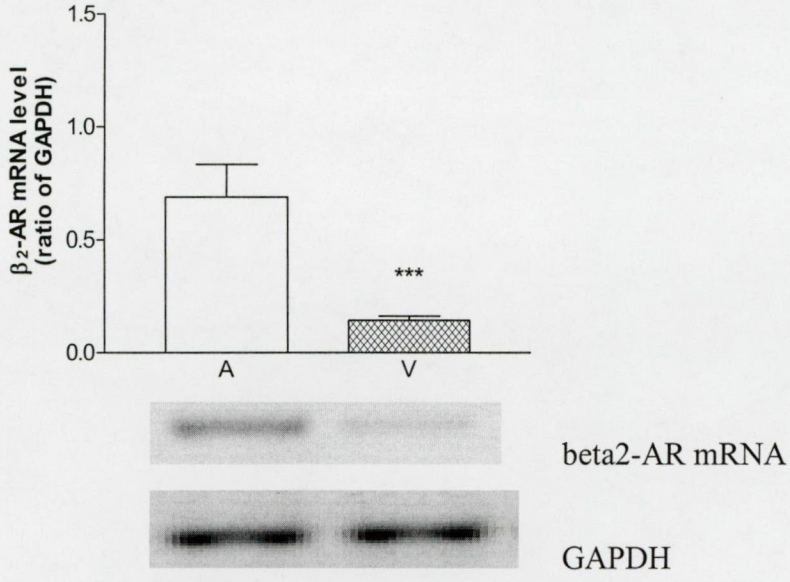

Figure 10.: The expression of beta2-AR mRNA in human term placental arteries and veins. The expression of beta2-AR mRNA was significantly higher in the arteries compared to the veins. ***=$p<0.001$

According to the results of the alpha1-AR subtypes mRNA studies we could find that the expression of alpha1A- (Fig. 11a) and alpha1B- (Fig. 11b) AR mRNA were significantly higher in the arteries compared to the veins. Significant difference in the amount of alpha1D-AR mRNA between veins and arteries was not possible to find (Fig. 11c). It could be established that the expression of ARs are predominant in the human placental arteries. Moreover the preponderance of alpha1A- and alpha1B-AR mRNA compared to the alpha1D-AR mRNA was proved both in the arteries and in the veins.

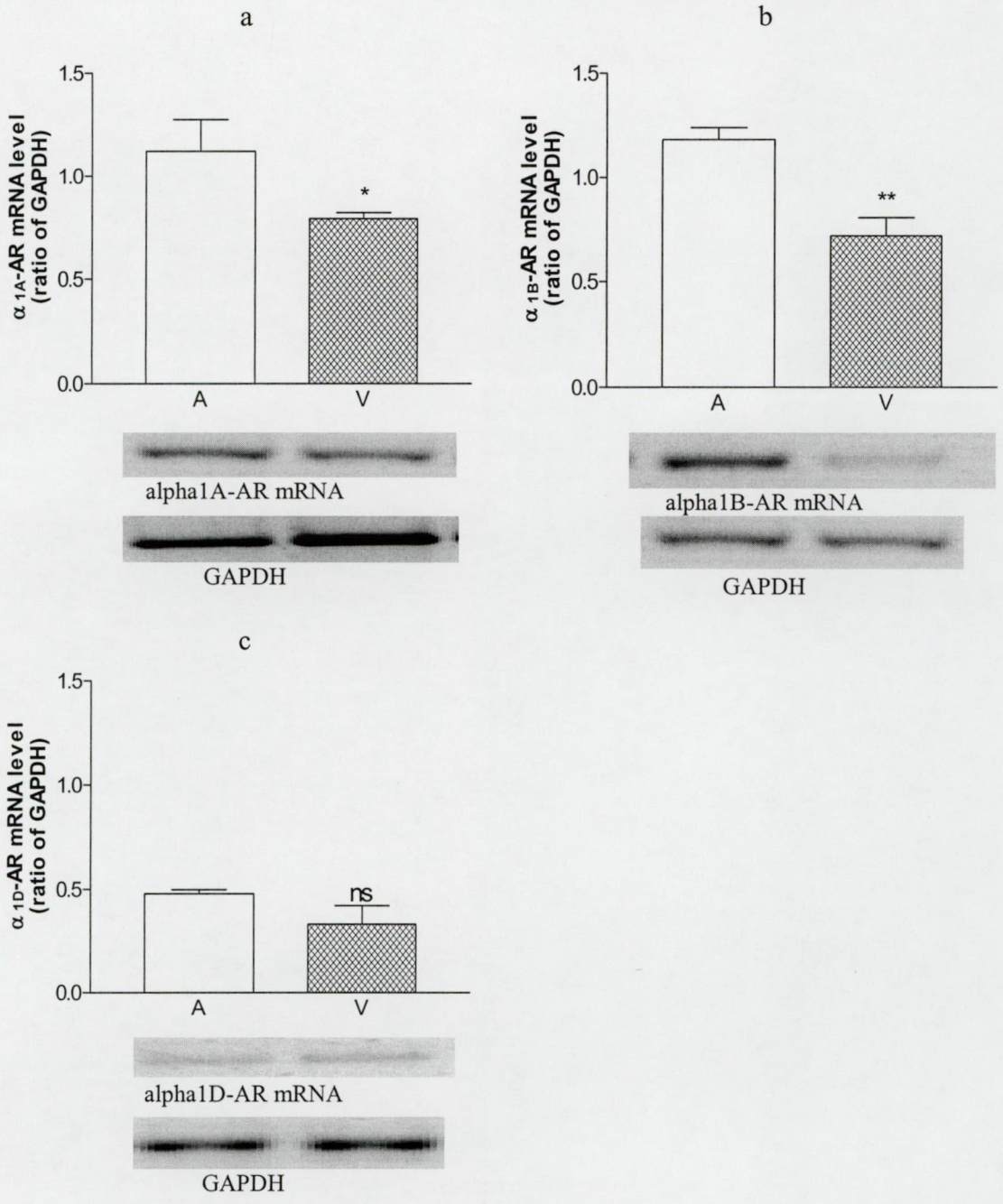

Figure 11.: The expression of different alpha1-AR mRNAs in human term placental arteries and veins. The expression of alpha1A- (a) and alpha1B- (b) AR mRNA were significantly higher in the arteries compared to the veins. Significant difference in the amount of alpha1D-AR mRNA between veins and arteries was not possible to find (c). The expression of ARs are predominant in the human placental arteries. The expressions of alpha1A- and alpha1B-AR mRNA were much higher both in the arteries and in the veins compared to that of the alpha1D-AR mRNA. *=$p<0.05$, **=$p<0.01$, ns=difference not approaching significance level

4.3. EPO Studies

rHuEPO evoked reproducible contractions in human placental blood vessel rings in a significant and dose-dependent way. At all applied doses the effect of rHuEPO on the placental veins was significantly more marked, as compared to that on the arteries. The vasocontractions in the case of the 10 IU/ml were 0.21 ± 0.03469 g and 0.05273 ± 0.01415 g for human placental veins and arteries, respectively. At the 300 IU/ml dose, the contractions were 1.088 ± 0.1296 g and 0.4509 ± 0.05017 g for the veins and arteries, respectively.

Incubation of the vessel rings with captopril (10^{-5} M) did not affect their contractile response to rHuEPO. In contrast, losartan, at a concentration of 10^{-5} M, completely abolished the contractile responses of the vessel rings at 10 IU/ml rHuEPO and significantly blunted them at 300 IU/ml rHuEPO: 0.11 ± 0.01528 g and 0.1575 ± 0.03119 g for arteries (Fig. 12a) and veins (Fig. 12b), respectively.

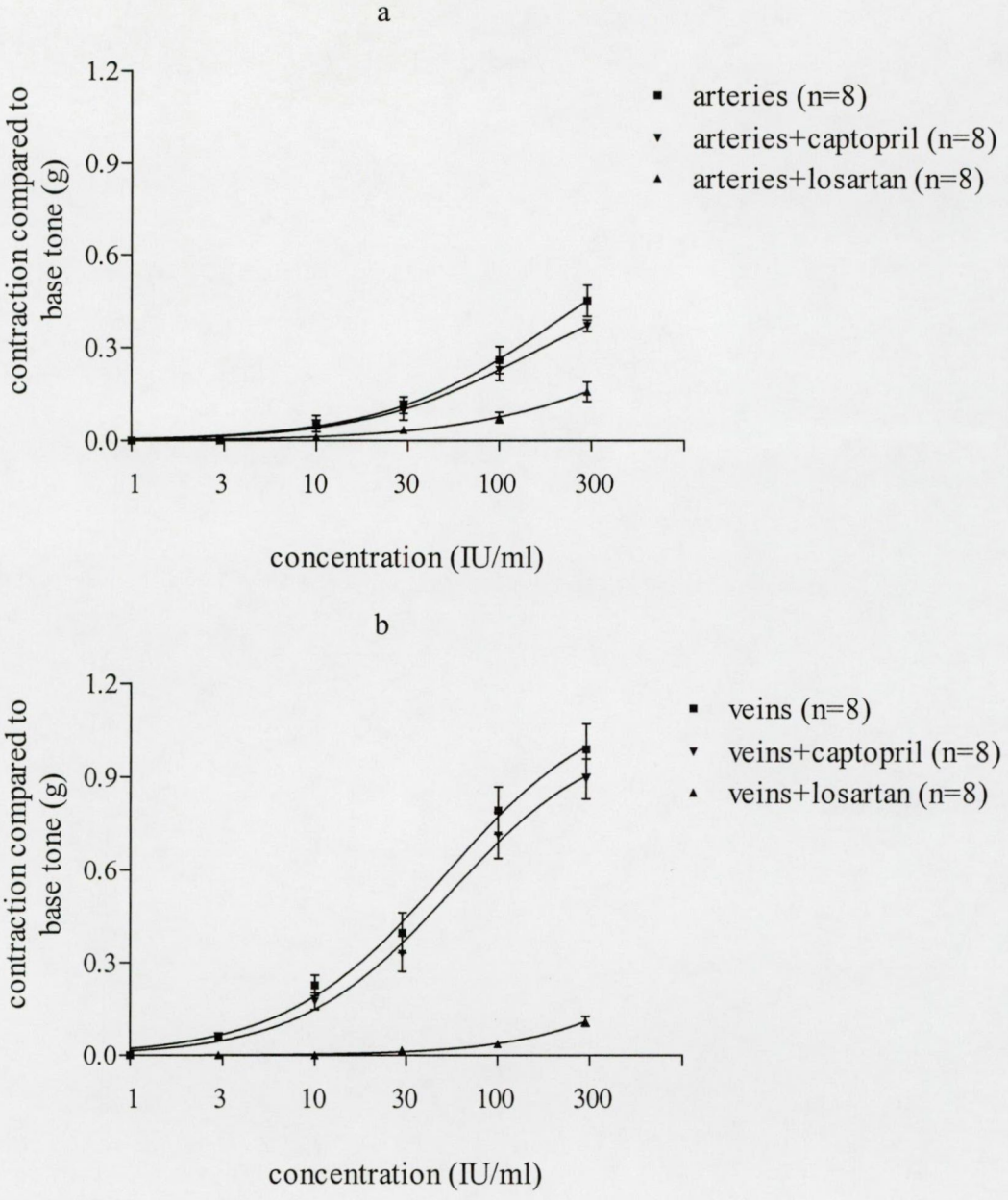

Figure 12.: The dose-response curve of rHuEPO on human placental blood vessels and the effect of captopril and losartan on the rHuEPO induced contactions. rHuEPO evoked reproducible contractions in human placental blood vessel rings in a significant and dose-dependent way. At all applied doses the effect of rHuEPO was significantly more marked on the placental veins (b), as compared to that on the arteries (a). Incubation of the vessel rings with captopril (10^{-5} M) did not affect their contractile response to rHuEPO (a-b). In contrast, losartan, at a concentration of 10^{-5} M, completely abolished the contractile responses of the vessel rings at 10 IU/ml rHuEPO and significantly blunted them at 300 IU/ml rHuEPO (a-b).

5. Discussion

5.1. The optimal parameters of EFS and the role of calcium and calcium-channels

The EFS method has the advantage that no other pharmacological agent is needed to elicit contractions. First we examined the effects of EFS on human placental blood vessels obtained from uncomplicated term pregnancies. Small diameter resistance vessels, compared to conduit vessels, probably contribute more to the hemodynamics of placental bed perfusion, though the question of the location and the diameter of resistance vessels is in general unsolved and the feed arteries can be as active in flow control as the microvasculature (Christensen and Mulvany, 2001). According to these recent data the vessel rings used in these studies can be considered both as conduit and resistance vessels. In addition, evidence, that larger or smaller segments contribute to a lesser or greater degree to changes in the blood flow of human organs (including the placenta) is not available (Christensen and Mulvany, 2001). The optimal parameters of EFS were then determined. It has been proved that in oxygenated Krebs-Henseleit buffer EFS does not generate substances that change the contractile state of the smooth muscle (Jongejan et al., 1989). To investigate both the dilatator and the constrictor effects of the pharmacons, half-maximal contractions were needed. The pulse width location at half maximal force of these contraction versus pulse width curves were considered to be the optimal pulse widths. On stimulation at 20 V, the contraction versus pulse width curve proved to be unsaturable, and it was therefore impossible to determine the optimal pulse widths. On stimulation at 30 V, the contraction versus pulse width curve was saturable; accordingly, 30 V was chosen as optimal stimulating potential in our further experiments. The parameters needed for half-maximal contractions of the veins and arteries were slightly different, but the difference was not significant. The spontaneous relaxation after the half-maximal contractions were reached did not display a significant difference either. The assessed optimal pulse width parameters conform to the theory, that the use of a short pulse width (0.7 to 5 ms) is thought to selectively stimulate nerves, but not smooth muscle, which requires a much

longer pulse width (60 to 133 ms) for direct excitation (Tomita, 1970). Though with the high pulse widths and voltages applied responses to EFS were not likely due to stimulation of nerves, the non-neurogenic nature of the contractions was checked functionally by blocking the nerve action potentials conduction with tetrodotoxine (10^{-6} M). These results are therefore in agreement with previous studies which suggested an absolute lack of sympathetic innervation in the placental circulation (Khong et al. 1997; Buttery et al., 1994).

The involvement of extracellular and/or intracellular calcium and a possible subsequent opening of voltage-operated calcium channels (VOCC) was assessed by conducting experiments in Ca^{2+}-free medium and with blockers of both VOCC and the intracellular Ca^{2+}-stores. The results of these experiments suggested that the direct, non-neurogenic contractile effect of EFS on isolated human placental blood vessel rings mainly depends on the influx of extracellular Ca^{2+} via voltage operated Ca^{2+}-channels, partly on the mobilization of intracellular Ca^{2+}-stores, and on a mechanism independent of intracellular Ca^{2+}-concentration elevation. Because of the approximately 25 % difference between the inhibitory effects of verapamil and Ni^{2+} this mechanism independent of intracellular Ca^{2+}-concentration elevation is likely to be the influx of other extracellular cations besides Ca^{2+}.

In our experiments, veins gave stronger contractile responses than arteries. These functional results confirmed the morphological properties of the walls of the placental veins and arteries. Force-producing smooth muscle cells lie within the media (Mulvany, 1984), and the smooth muscle containing tunica media is thicker in placental veins than in arteries (Tanaka et al., 1999). The clinical relevance of the differences between the contractions of the veins and the arteries in the testing of future drugs is difficult to predict, because the response of the vessels to drug actions depends not only on the muscle layer thickness, but also on the densities of different receptors, the activities of second messenger systems, etc. These parameters differ for every pharmacon. Otherwise, in general it might be stated that drugs which can alter the diameter either of the veins or the arteries may change the placental blood flow. Increased blood flow may be beneficial in preeclampsia and intrauterine growth restriction.

Since the placenta is a hypoxic organ, the effects of in utero gases on human placental blood vessel rings was examined. In addition, as a comparison, the effect of the same gases was also investigated on rat mesenterial arterial rings. Interestingly, the results of these experiments suggested that in utero physiological hypoxic circumstances has a stimulatory/enhancing effect on the contractility of human placental vessels controversially to non-placental vessels. These results also suggest that during EFS studies on human placental blood vessels in utero gases should be used instead of carbogen gas.

To test the pharmacological capability of our model, the effects of $NaNO_2$ was examined. $NaNO_2$ is a well-known NO donor. As might be expected, it antagonized the EFS contractions of the placental vessel rings in a significant and dose-dependent way. This was in agreement with literature data that placental vessels obtained from normotensive pregnancies are sensitive to the relaxant effect of nitrovasodilatators (glyceryl trinitrate, sodium nitroprusside and S-nitroso-N-acetylpenicillamine) (Gonzalez et al., 1997). The efficacy of $NaNO_2$ was significantly decreased by the in utero gases leading us to the conclusion that the contractions of human placental blood vessel rings are stronger under physiologically hypoxic in utero conditions. The relaxant effect of $NaNO_2$ was more pronounced on placental veins than on arteries at all applied doses, but none of the differences reached the level of significance. These results also confirm the benefit of using in utero gases during human placental blood vessel EFS studies.

From our present findings, it may be concluded that we have successfully applied EFS for the study of human placental vessels, which is therefore a new experimental possibility for investigations of the direct placental vascular effects of different pharmacological agents.

5.2. Adrenergic Studies

The alpha1A,- alpha1B-, alpha1D- and beta2-AR mRNA expressions in term human placental blood vessels has been demonstrated with the RT-PCR technique and their pharmacological reactivity was investigated with the EFS technique. To the best of

our knowledge this is the first study to prove the presence of alpha1-ARs in human placental blood vessels. The EFS and RT-PCR findings were in harmony concerning the mRNA expression and the pharmacological reactivity of the beta2-ARs. This is partly in agreement with previous literature data, that the human placental tissue contains an AR of beta2 subtype (Falkay and Kovacs, 1983). The beta2-ARs seem to play an important role in the regulation of the contractility of the human term placental blood vessels, mainly in the arteries. Terbutalin, used at therapeutic concentrations (2.3±1.8 ng/ml) (Borgström et al., 1989), was found to exert its maximal relaxant effect, increasing the placental blood flow, which is an additional benefit during the management of imminent abortion or preterm labor.

The EFS and RT-PCR findings were also in harmony concerning the mRNA expression and the pharmacological reactivity of the alpha1-AR subtypes. The alpha1A-AR subtypes seem to play the most important role in the regulation of the contractility of both the human term placental arteries and veins. According to the RT-PCR results alpha1B-ARs also play a significant role in the regulation of the contractility of both the human term placental arteries and veins. Though because of the lack of a subtype-selective alpha1B-AR antagonist (Robinson and Hudson, 1998) the direct role of the alpha1B-ARs in the contractility of human placental blood vessels have not been investigated, it was, however, possible to assess some indirect information about that by examining the effects of urapidil (a non-subtype-selective alpha1-AR antagonist). The EFS results with urapidil also suggest a significant role for the alpha1B-ARs in the regulation of the contractility of both the human term placental arteries and veins, though not that important as the RT-PCR result would suggest it.

Taken together, as regards the AR-subtypes the most important subtypes in the cases of both veins and arteries seem to be the alpha1A- and the beta2-ARs, nevertheless alpha1B-ARs also seem to play a considerable role in this respect. The role of the alpha1D-ARs seem to be moderate compared to the alpha1A-, alpha1B- and beta2-ARs in the regulation of the contractility of both the human term placental arteries and veins. It may also be concluded that in general in the regulation of the contractility of the human term placental vessels the importance of the alpha1- and the beta2-ARs is significantly more marked in case of the arteries compared to the veins. Beta2- and

alpha1A-ARs seem to influence the contractility of human term placental vessels to a similar magnitude.

Our findings may also provide some additional insight into the patho-genesis of preeclampsia, where the pregnancy-induced adaptions in vascular tone seem to be impaired (Anumba et al., 1999; Anim-Nyame et al., 2000). Pregnancy may diminish alpha- and beta-adrenergic vascular responses, and the attenuation of responses to these receptor systems is an important vasoregulatory mechanism during pregnancy (Landau et al., 2002). Much of the changes in cardiovascular parameters occurs by eight weeks of gestation, suggesting that hormonal or receptor-mediated alterations rather than gross anatomical changes may be the predominant cause. The precise nature of the molecular alterations that cause or allow these hemodynamic changes to occur is still unclear. Recently, another study described an increased fraction of receptors in high-affinity state with an unchanged total density in normal pregnancy, whereas preeclampsia seemed to reduce the number of functional beta2-receptors attributable to a decreased total receptor number with an unaltered fraction of high-affinity receptors. (Aune et al., 2000). Thus, we plan to undertake further experiments on the expression and pharmacological reactivity of ARs in human placental blood vessels originating from preeclamptic pregnancies.

In the light of the aforementioned facts the use of beta2-mimetics and the potential use of alpha1-blockers as tocolytics can be considered secure or even beneficial concerning placental blood flow. Moreover, the side-effects of alpha1-AR antagonist might be possibly moderated or even advantageous (e.g. pregnancy induced hypertension).

5.3. EPO Studies

rHuEPO was found to have a direct and dose-dependent contractile effect on human placental blood vessels. As the fetoplacental vasculature lacks autonomic innervation (Reilly and Russel, 1977), the control of this vascular bed must involve humoral mechanisms: EPO might participate in one of these mechanisms. At all applied doses, the contractions elicited by EPO were significantly more marked on veins than

on arteries. These functional results also confirmed again the morphological properties of the walls of the placental veins and arteries (Mulvany, 1984; Tanaka et al., 1999). The contractile effect of rHuEPO on human placental blood vessels was abolished by losartan (a selective angiotensin II type 1 (AT_1) receptor antagonist), but was not blunted by captopril (an ACE inhibitor). Similarly, the contractile effect of EPO on the rat mesenteric artery can be abolished by losartan, but cannot be blunted by captopril (Heidenreich et al., 1991). There is a theoretical basis for this effect in view of the known close links between the renin-angiotensin system and erythropoiesis. It has been known for some time that the renin-angiotensin system is intricately linked with the production of endogenous EPO in the peritubular fibroblasts of the kidney. Activation of this system will enhance EPO production (Vlahakos et al., 1995). Similarly, suppression of ANG II production by ACE inhibitors may inhibit EPO synthesis, reducing circulating levels of the hormone, and so exacerbating anemia (Kamper and Nielsen, 1990), but there are also findings refuting this hypothesis (Conlon et al., 1994). Additionally, renin substrate (angiotensinogen) has chemical similarities to EPO (Fyhrquist et al., 1984). All the components of the renin-angiotensin system have been shown to be present in the human term placenta (Cooper et al., 1999). The human term placenta contains predominantly AT_1 receptors with low levels of AT_2 receptors (Kingdom et al., 1993). When placental membrane preparations were used, the angiotensin II type 2 (AT_2) receptor antagonist PD123177 failed to compete for [^{3}H]ANG II binding at relevant concentrations, whereas the AT_1 receptor antagonist losartan competed in a monophasic manner (Li et al., 1998). Specific receptor binding sites for ANG II have also been identified in placental vascular smooth muscle cells (McQueen et al., 1990). Other experiments with the AT_1 receptor-selective antagonist losartan indicate that this subtype is responsible for the vast majority of the hemodynamic and cardiovascular effects of ANG II (Wong et al., 1992). In the rat, rHuEPO exerts its primary action on vascular smooth muscle cells via an increase in angiotensin receptor messenger RNA, resulting in a parallel increase in ANG II receptor expression (Barrett et al., 1998). Thus, it may be concluded that AT_1 receptors are needed to mediate the contractile response of human placental blood vessels to rHuEPO.

The elucidation of these potential EPO binding sites and the potential transport of EPO across the placental barrier (Widness et al., 1995) are of clinical significance as concerns assessment of the safety to the fetus of rHuEPO administration to anemic pregnant women. However, cases have been presented in which no maternal and perinatal complications attributable to rHuEPO were registered (Braga et al., 1996). Since AT_1 receptor activation may play a role in preeclampsia (Doering et al., 1998) and AT_1 receptor expression is reduced in intrauterine growth restriction (Li et al. 1998), EPO might also be involved, in part, in the pathogenesis of these disorders. Our findings add to the growing list of nonhematopoietic roles of EPO during human development.

6. Summary

From our findings we have concluded the following:

1. EFS has been successfully applied for the study of human placental vessels, which is therefore a new experimental possibility for investigations of the direct placental vascular effects of different pharmacological agents. The optimal parameters of our EFS model for human placental blood vessels are as follows: gases simulating physiological intrauterine hypoxia bubbled Krebs-Henseleit buffer; passive forces are 3.25 g and 3.75 g for arteries and veins, respectively; stimulating potential: 30 V, period time: 5 s; pulse widths are 105 ms and 99 ms for arteries and veins, respectively.

2. Controversially to other, non-placental vessels, in utero physiological hypoxic circumstances has a stimulatory/enhancing effect on the contractility of human placental vessels.

3. The direct, non-neurogenic contractile effect of EFS on isolated human placental blood vessel rings mainly depends on the influx of extracellular Ca^{2+} via voltage operated Ca^{2+}-channels, partly on the mobilization of intracellular Ca^{2+}-stores, and on a mechanism independent of intracellular Ca^{2+}-concentration elevation. This mechanism independent of intracellular Ca^{2+}-concentration elevation is likely to be the influx of other extracellular cations besides Ca^{2+}.

4. Beta2-, alpha1A-, alpha1B- and alpha1D-ARs are all involved in the regulation of human term placental vessels' contractility. This involvement seems significantly more marked in the case of the arteries compared to the veins. There is relationship between the mRNA expression and the pharmacological reactivity for ARs. The alpha1A- and the beta2-ARs seem to play the major role in the regulation of both the human term placental arteries and veins as regards the AR-subtypes, nevertheless alpha1B-ARs also seem to play a considerable role in this respect. The role of the alpha1D-ARs seems to

be moderate compared to the alpha1A-, alpha1B- and beta2-ARs in the regulation of the contractility of both the human term placental arteries and veins.

5. On the basis of our in vitro studies the use of beta2-mimetics and the potential use of alpha1-blockers as tocolytics can be considered secure or even beneficial concerning placental blood flow.

6. rHuEPO has a direct and dose-dependent contractile effect on human placental blood vessels. EPO might participate in one of the humoral mechanisms involved int he control of the placental vascular bed. AT_1 receptors are needed to mediate the contractile response of human placental blood vessels to rHuEPO.

7. EPO might also be involved, in part, in the pathogenesis of preeclampsia and intrauterine growth restriction.

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9. Appendix

9.1. List of publications:

1. B. E. Resch, R. Gáspár, G. Falkay: Electric Field Stimulation: a New Method for the Investigation of Human Placental Vessels' Contractility. (2000) *Placenta* 21(7),A.24.
   14th Rochester Trophoblast Conference In Association with the 6th Meeting of The International Federation of Placenta Associations And The Society for the Investigation of Early Pregnancy, Rochester, New York, USA, 3-8 October 2000.

2. Resch Béla Endre: Beszámoló a 14th Rochester Trophoblast Konferenciáról (Rochester, NY, USA, 2000. október 3-8) (2001) *Magyar Nőorvosok Lapja* 64(2), 177.

3. S. Sonkodi, B. E. Resch, A. Letoha, G. Ábrahám, G. Falkay: Vasoactive effects of recombinant human erythropoietin (RHUEPO) on the mesenteric artery in normal, hypertensiv and uraemic rats. *In vivo* and *in vitro* studies. (2001) *Am J Hypertension,*14(4),156-7.
   Sixteenth Annual Scientific Meeting of the American Society of Hypertension, May 15-19, 2001., San Francisco, California.

4. Resch Béla Endre, Gáspár Róbert, Falkay György: Humán placentaerek kontraktilitásának vizsgálata elektromos téringerléssel.
   A Magyar Élettani Társaság (MÉT) LXVI. Vándorgyűlése, 2001. június 6-8., Szeged.

5. B. E. Resch, A. Letoha, S. Sonkodi, G. Falkay: Erythropoietin induces vasoconstriction in rat mesenteric and human blood vessels. (2001) *Nephrol Dial Transplant,*16(6),A8.

XXXVIII Congress of the European Renal Association European Dialysis and Transplant Association, June 24-27, 2001, Vienna, Austria.

6. E. Ducza, B. E. Resch, R Gáspár, G. Falkay: Identification and pharmacological characterisation of adrenergic of receptors in human placental vessels. (2001) *Placenta,*22(7),A40.
   7th Meeting International Federation of Placenta Associations 9th Meeting European Placenta Group The Placenta and Fetal Growth September 19-23, 2001, Sorrento, Italy.

7. B. E. Resch, R. Gáspár, S. Sonkodi, G. Falkay: Investigation of the effects of erythropoietin on human placental vessels in vitro. (2001) *Placenta* 22(7), A50.
   7th Meeting International Federation of Placenta Associations 9th Meeting European Placenta Group The Placenta and Fetal Growth September 19-23, 2001, Sorrento, Italy.

8. Resch Béla Endre, Gáspár Róbert, Falkay György: A placentaerek kontraktilitásának vizsgálata elektromos téringerléssel. (2002) *Magyar Nőorvosok Lapja* 65, 161-167.

9. Ducza Eszter, Resch Béla Endre, Falkay György: Adrenerg receptor altípusok változásának vizsgálata humán uteruszban.
   Magyar Szülészeti és Nőgyógyászati Endokrinológiai Társaság II. Nemzeti Kongresszusa, 2002. április 11-13., Kecskemét

10. Resch Béla Endre, Gáspár Róbert, Sonkodi Sándor, Falkay György: Az erythropoietin közvetlen hatásának vizsgálata humán méhlelpény ereken in vitro.
    Magyar Szülészeti és Nőgyógyászati Endokrinológiai Társaság II. Nemzeti Kongresszusa, 2002. április 11-13., Kecskemét

11. M. Bagyánszki, É.G. Kovács, B.Á. Resch, V. Román, B.E. Resch and E. Fekete: Computer-aided morphometric analysis of the developing concentric structure of the human fetal intestinal tube. (2002) *Histology and Histopathology* 17, 731-737.

12. Resch Béla Endre, Gáspár Róbert, Sonkodi Sándor, Falkay György: Az Erythropoietin Humán Placentaerekre Gyakorolt Hatásának Vizsgálata in vitro (2002) *Magyar Nõorvosok Lapja* 65, 407-410.

13. B. E. Resch, R. Gáspár, G. Falkay: Application of Electric Field Stimulation for Investigations of Human Placental Blood Vessels. (2002) *Obstet Gynecol* 101(2), 296-303.

14. B. E. Resch, R. Gaspar, G. Falkay, S. Sonkodi: Vasoactive Effects of Erythropoietin on Human Placental Blood Vessels in vitro. (2002) *Am J Obstet Gynecol,* 188(4), 993-996.

15. Resch Béla Endre, Ducza Eszter, Gáspár Róbert, Falkay György. 2002. Adrenerg receptor altípusok vizsgálata humán méhlepény erekben RT-PCR és elektromos téringerlés módszerekkel
Magyar Kísérletes és Klinikai Farmakológiai Társaság V. Kongresszusa, 2002. december 12-14.

16. V. Román, M. Bagyánszki, B. E. Resch, É. Fekete (2002) Individual distribution and colocalization of VIP, nitric oxide synthase, GABA, glutamate and NMDA receptors in the developing human enteric nervous system.
III. Magyar Sejtanalitikai Konferencia, Budapest, május 16-18.

17. B. E. Resch, E. Ducza, R. Gáspár, G. Falkay: Role of Adrenergic Receptor Subtypes in the Control of Human Placental Blood Vessels. in press at *Mol Rep Dev.*

9.2. Copies of all manuscripts published or accepted for publication: