Dissertation

ENDOTHELIAL INDOLEAMINE 2,3-DIOXYGENASE-1 IN THE REGULATION OF PLACENTAL VASCULAR TONE AND ITS POTENTIAL ROLE IN INTRAUTERINE GROWTH RESTRICTION AND PREECLAMPSIA

submitted by

Lic

Pablo ZARDOYA LAGUARDIA

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Gottfried Schatz Research Center for Cell Signaling, Metabolism and Aging

under Supervision of

ao.Univ.-Prof. Dr.med.univ. Peter SEDLMAYR

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

Please note that parts of this thesis are already published in:

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Pablo Zardoya Laguardia

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Disclosures

All co-authors here presented gave their consent to re-use the data from the publication within this thesis. The following co-authors contributed to the data shown in the thesis:

- Astrid Blaschitz contributed with some immunohistochemistry staining shown in figure 31.
- Birgit Hirschmugl performed the placental perfusion experiments shown in figure 25.
- Ingrid Lang helped with knowledge in primary cell isolation of placental endothelial cells.
- Sereina A. Herzog conducted part of the statistical analysis shown in figures 9.B, 10, 12, 13, 23.A and 25.
- Liudmila Nikitina performed a quantitative PCR experiment shown in figure 6.A.
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Abbreviations and Definitions

°C:	Degree Celsius
µg:	Micrograms
µL:	Microliter
µM:	Micromolar
µm:	Micrometer
1MetDTrp:	1-methyl-D-tryptophan
1MetTrp:	1-methyl-tryptophan
3-HAA:	3-hydroxyanthranilic acid
3-HK:	3-hydroxykynurenine
ANOVA:	Analysis of variance
BMI:	Body mass index
bp:	Base pair
Ca^{2+}:	Calcium
$CaCl_2$:	Calcium chloride
cAMP:	Cyclic adenosine monophosphate
CD:	Cluster of differentiation
cDNA:	Complementary deoxyribonucleic acid
CN:	Classical normalization
CO_2:	Carbon dioxide
Ct:	Cycle threshold

DMEM: Dulbecco's Modified Eagle's medium

DMSO: Dimethyl sulfoxide

D-Trp: D-Tryptophan

EDHF: Endothelium-Derived Hyperpolarizing Factor

EDTA: Ethylenediaminetetraacetic acid

EGM-MV: Endothelial Cell Growth Medium - microvascular

ESI: Electrospray ionization

ET-1: Endothelin-1

FBS: Fetal bovine serum

FGR: Fetal growth restriction/retardation

FSA: Fibroblastic specific antigen

g: Grams

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GW: Gestational week

HBSS: Hanks' Balanced Salt solution

hCG: Human chorionic gonadotropin

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC: High-performance liquid chromatography

IDO: Indoleamine 2,3-dioxygenase

IDO1: Indoleamine 2,3-dioxygenase-1

IDO2: Indoleamine 2,3-dioxygenase-2

IFNα: Interferon alpha

IFNβ: Interferon beta

IFNγ: Interferon gamma

Ig: Immunoglobulin

IL: Interleukin

Iso: Isolation

IUGR: Intrauterine growth restriction

KA: Kynurenic acid

KCl: Potassium chloride

kDa: Kilodalton

KH_2PO_4: Potassium dihydrogen phosphate

Km: Kilometers

KP: Kynurenine pathway

KPSS: Potassium physiological salt solution

Kyn: Kynurenine

L: Liter

LCP: Length-Tension curve procedure

L-NAME: N-Nitroarginine methyl ester

LPS: Lipopolysaccharide

L-Trp: L-Tryptophan

M: Molar

mg: Miligrams

$MgSO_4$: Magnesium sulphate

mL: Milliliter

mM: Milimolar

mm: millimiters

mmHg: Millimeters of Mercury

mN: Millinewtons

MRM: Multiple reaction monitoring

NaCl: Sodium chloride

NAD: Nicotinamide adenine dinucleotide

NADP: Nicotinamide adenine dinucleotide phosphate

NaH_2PO_4: Sodium dihydrogen phosphate

$NaHCO_2$: Sodium Hydrogen Carbonite

$NaHCO_3$: Sodium bicarbonate

ng: Nanograms

nM: Nanomolar

nm: Nanometers

NO: Nitric oxyde

O_2: Oxygen

PBS: Phosphate-buffered saline

pCO_2: Partial pressure of carbon dioxide

PE: Preeclampsia

PFA: Paraformaldehyde

pH: Potential of hydrogen

PLAECs: Placental arterial endothelial cells

pO_2: Partial pressure of oxygen

QA: Quinolinic acid

qPCR: Semi-quantitative polymerase chain reaction

RIPA: Radioimmunoprecipitation assay

RNA: Ribonucleic acid

RPL30: Ribosomal protein L30

RT: Room temperature

RT-qPCR: Reverse transcription-quantitative polymerase chain reaction

SD: Standard deviation

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SGA: Small for gestational age

smA: Smooth muscle actin

TBS: Tris-buffered saline

TDO: Tryptophan 2,3-dioxygenase

TNFα: Tumor necrosis factor alpha

Trp: Tryptophan

U: UnitsVolume/volume

v/v:	Volts
V:	Von Willebrand factor
vWF:	Weight/volume
w/v:	

Abstract

A successful pregnancy requires the establishment and maintenance of an adequate feto-placental circulation. Indoleamine 2,3-dioxygenase-1 (IDO1), a L-tryptophan catabolising enzyme constitutively expressed in the endothelium of human placental chorionic vessels, has been demonstrated to modulate the vascular tone and blood pressure under systemic inflammation in mice. We asked the questions whether IDO1 may contribute to the regulation of placental vascular tone, which we supposed to have an impact on placental and fetal growth, and asked whether this mechanism is impaired in intrauterine growth restriction (IUGR) and preeclampsia (PE).

Vasorelaxation to freshly-made and stored L-Trp-solutions of *U46619*-pre-constricted chorionic plate arteries stimulated with IFNγ and TNFα as a strategy to upregulate IDO1 was assayed by myography. The back pressure of placental cotyledons in the presence and absence of U46619 was monitored in the fetal part of the placenta by *ex-vivo* perfusion during administration of Trp. IUGR, pre-eclampsia, term and gestational age matched pre-term controls (from placenta praevia) samples were assessed by Western blotting, immunohistochemistry (IHC), RT-qPCR in order to determine IDO1 mRNA and protein expression and its localization, whilst IDO1 activity was measured by LC/MS. IDO1 mRNA and protein expression in placental arterial endothelial cells (PLAECs) isolated from normal term placentas were also examined by Western blotting and qPCR.

Trp produced vasorelaxation of U46619-pre-constricted and cytokine-stimulated placental arteries, an effect that was still observed in arteries lacking the endothelium. Trp-induced relaxation was partially blocked, in the presence and absence of endothelium, by using different IDO1 competitive inhibitors. Additionally, Trp induced vasorelaxation of perfused U46619-pre-constricted and non-pre-constricted placental cotyledons without prior induction of IDO1. PLAECs from normal term placentas showed a consistently upregulated IDO1 mRNA and protein expression following stimulation with IFNγ/TNFα but also exhibited a constitutive expression (without cytokine stimulation) on the mRNA level of IDO1, whereas on the protein level this was the case only in part of the samples. IUGR and PE samples showed no differences regarding IDO1 mRNA levels when

compared with pre-term controls, however, IDO1 protein expression and activity were reduced.

These data suggest that IDO1-mediated metabolism of Trp plays a role in the regulation of the placental vascular tone, and they are consistent with our hypothesis that a deficiency in chorionic IDO1 is linked to the pathogenesis of pregnancy complications such as IUGR and PE.

Zusammenfassung

Der Aufbau und die Erhaltung eines adäquaten fetoplazentaren Kreislaufes ist für eine Schwangerschaft unabdinglich. Indoleamin 2,3-dioxygenase-1 (IDO1), ein L-Tryptophan-abbauendes Enzym, das konstitutiv im Endothel von Blutgefäßen des Chorion exprimiert wird, kann, wie früher gezeigt wurde, in Mäusen unter den Bedingungen einer systemischen Entzündung den Gefäßtonus und den Blutdruck modulieren. Wir stellten die Fragen, ob IDO1 zur Regulation des plazentaren Gefäßtonus beiträgt und möglicherweise dadurch das Wachstum von Plazenta und Fetus beeinflußt, und ob dieser Mechanismus bei intrauteriner Wachstumsretardierung (IUGR) und Präeklampsie (PE) gestört ist.

Mittels Myographie untersuchten wir die Vasorelaxation von Arterien der Chorionplatte (mit IFNg und TNFa vorbehandelt, um die Expression von IDO1 zu verstärken), die mit U46619 kontrahiert wurden, auf Anwendung einer Lösung von L-Trp hin (die frisch hergestellt oder länger gelagert worden war). Mittels ex-vivo Plazentaperfusion mit oder ohne U46619 bestimmten wir den Druck, der sich bei dieser Prozedur im arteriellen System eines Kotyledons aufbaute, und dies mit oder ohne Anwendung von L-Trp. Gewebsproben von Plazenten von IUGR, PE, Plazenten nach normaler Schwangerschaft und vom Schwangerschaftsalter zu den krankhaften passende Plazenten von vorzeitigen Geburten (von Plazenta praevia) wurden mittels Western blot, Immunhistochemie, RT-qPCR analysiert, um IDO1 mRNA und Protein zu messen und dessen Gewebslokalisation zu bestimmen. Die IDO-Aktivität wurde mittels LC/MS gemessen.

L-Trp bewirkte eine Vasorelaxation in U46619-präkonstringierten cytokin-stimulierten Arterien der Chorionplatte. Dieser Effekt verschwand auch nach Entfernung des Endothels nicht. Die L-Trp-induzerte Relaxation wurde durch Anwendung verschiedener kompetitiver Inhibitoren von IDO1 partiell blockiert. Weiters bewirkte L-Trp eine Vasorelaxation in präkonstringierten und auch in nicht präkonstringierten Gefäßen von Kotyledonen, ohne daß wir in diesem Fall eine vorhergehende Stimulation mit Zytokinen durchgeführt hätten. Aus der Chorionplatte isolierte arterielle Endothelzellen (PLAECs) von Plazenten nach normalen Schwangerschaften zeigten nach Stimulation mit IFNg/TNFa durchwegs eine gesteigerte Expression von IDO1 auf der mRNA- und der Proteinebene. Ohne Zytokinstimulation war IDO1 mRNA nachweisbar, auf der Proteinebene

jedoch bnur in einem Teil der Proben. Bezüglich IDO1 mRNA war kein Unterschied zwischen Choriongewebe von IUGR und PE einerseits und den Plazenta-praevia-Proben festzustellen, sehr wohl jedoch war der Gehalt von IDO1 -Protein und die IDO1-Aktivität bei den pathologischen Plazenten erniedrigt.

Diese Daten legen nahe, daß der IDO1-mediierte Metabolismus von L-Trp eine Rolle für die Reguilation des plazentaren Gefäßtonus spielt, und unterstützen auch unsere Hypothese, daß ein Mangel an IDO1 im Gefäßsystem des Chorion eine Rolle in der Pathogenese der Schwangerschaftskomplikationen IUGR und PE spielt.

1. Introduction

1.1. L-Tryptophan

L-Tryptophan (L-Trp), a hydrophobic amino acid with an indol ring chemical structure, is the least abundant one of the eight essential amino acids that must be incorporated from diet in humans. The main sources of L-Trp intake come from milk or dairy products, meat, fish, white bread, eggs and potatoes (1). Most of the L-Trp is metabolised through four pathways: decarboxylation, deamination, hydroxylation or oxidation. The rest of L-Trp intake (less than 1%) is utilized for protein synthesis serving as a precursor of a variety of active biomolecules such as serotonin, melatonin or tryptamine. Additionally, L-Trp can be the source of NAD and NADP or even replace nicotinic acid as an essential vitamin (1-5).

The physiological role of some of these active compounds is already well characterised. Serotonin is a neurotransmitter that functions as a trophic factor in the mammalian brain (6), controlling the neuronal information processing and a diversity of behaviours such as sexual response, aggression, impulsivity or reaction to pain (7, 8). Tryptamine enhances the activity of serotonin on neurons (9). Melatonin which is produced and released at night by the pineal gland regulates the circadian rythms and improves sleep quality (10, 11). Notably, over 95% of the Trp catabolism in mammals occurs via the kynurenine (kyn) pathway (KP) (5, 12, 13). The first step of this pathway is the oxidation of L-Trp into N-formyl-kynurenine which is further converted to kyn (14) (Fig. 1). Kyn is then degraded to diverse active metabolites of the KP such as kynurenic acid (KA) and quinolinic acid (QA) with neuroprotective and immunosuppressive properties (15-17), anthranilic acid that regulates immune modulation, 3-hydroxykynurenine (3-HK) or 3-hydroxyanthranilic acid (3-HAA) responsible of T-cell suppression and antioxidant activity (12, 17, 18). One of the last steps in the KP pathway yields the production of NAD implicated in redox reactions (19) but also in posttranslational modification of proteins (20) or even Ca^{2+} signalling pathways (21).

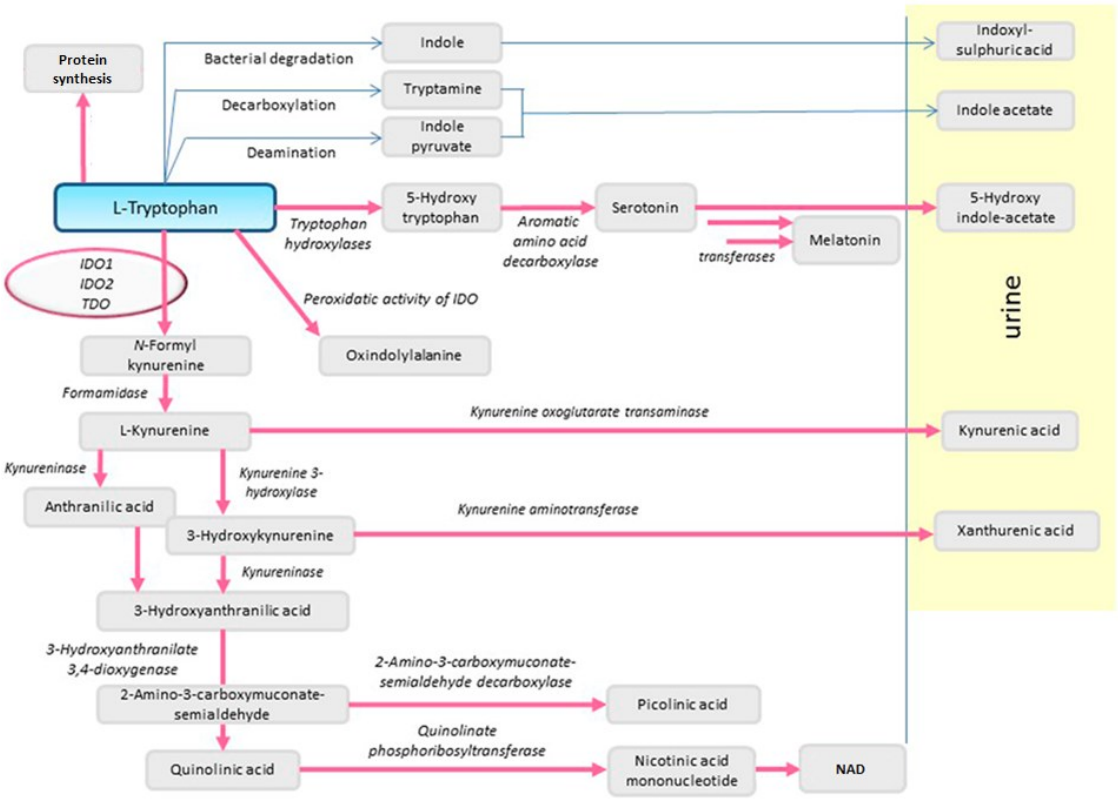


Figure 1. L-Tryptophan catabolic pathway. The different paths for L-Tryptophan utilisation in the human body. Taken and modified from (13). Permission to reproduce and modify figure 1 granted (Sedlmayr P, Blaschitz A and Stocker R)

The importance of L-Trp acquires remarkable in pregnancy is not restricted to the protein requirements but also to the role of the KP metabolites for fetal development (12, 22). The amino acid transport from the mother to the fetus is augmented in order to meet the increased requirements (23). Consequently, L-Trp depletion may be detrimental for the mother and the fetus. Conversely, it has been shown that maternal total Trp (free + albumin-bound) is elevated in early pregnancy with a subsequent decrease (24) whereas free Trp, the fraction of Trp not albumin-bound which is immediately available for tissue uptake, is in fact increased in late pregnancy (25, 26).

Therefore, a deficiency of L-Trp may jeopardise human health. For that reason, Trp-catabolising enzymes play a key role in the availability of L-Trp to the human body.

1.2. Tryptophan-catabolising enzymes

L-Trp is metabolised mainly along the KP by one of three Trp-catabolising enzymes capable of converting L-Trp into N-formylkynurenine (14, 27), namely tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO). Despite sharing a similar function, differences in both enzyme sequences suggest that they do not share a common ancestor (2, 28). A further enzyme similar to IDO in structure and function was described in 2007 (28), so we distinguish since then between indoleamine 2,3-dioxygenase-1 (IDO1) and -2 (IDO2). All these cytosolic enzymes display different expression patterns (2, 13, 29).

1.2.1. Tryptophan 2,3-dioxygenase (TDO)

TDO is the main responsible for the level of L-Trp under physiological conditions as it controls more than 95% of the Trp degradation. Despite TDO has been found in early human concepti (30), rodent brain (31), skin (32) and many tumour cells (33, 34), and is also associated with an attenuation of antitumor immune responses (34), it is primarily expressed in the liver in mammals where it is found in the cytosol of the hepatocytes (35). Encoded by the TDO2 gene, TDO is a homotetrameric enzyme with a molecular weight of 103 kDa per subunit that possesses a high substrate-specificity for L-Trp (13, 27, 36). TDO expression is upregulated in the presence of glucocorticoids (37) or L-Trp (38), not affected by pro-inflammatory stimuli (35) and specifically inhibited by the compound 680C91 (34).

1.2.2. Indoleamine 2,3-dioxygenase-2 (IDO2)

IDO2, enzyme composed of 420 amino acids and with a molecular weight of 47 kDa, exhibits 43% similarity to IDO1 (39, 40). The IDO2 gene is found adjacent to its analogous IDO1 suggesting a possible origin of the enzyme through gene duplication (41). IDO2 is found in a variety of healthy tissues such as liver, kidney, uterus, placenta, epididymis, testis and brain (39, 42) and tumor tissues such as

colon, kidney or stomach (43). IDO2 functional activity is minor when compared to its homologue (43, 44) and 50% of Caucasians and Asians together with 25% of Africans are unable to produce a functional IDO2 enzyme (45). IDO2 expression is upregulated by IFNγ in a variety of cell lines (42, 43) whereas IDO2 inhibition by the traditional IDO inhibitors such as 1-methyl-Tryptophan (1MetTrp) is as yet controversial (40). However, IDO2 is specifically inhibited by tenatroprazole (46).

1.2.3. Indoleamine 2,3-dioxygenase-1 (IDO1)

IDO1, a cytosolic enzyme with a heme group, is encoded by the INDO gene located on the chromosome 8. It is composed of 403 amino acids with a molecular weight of 45 kDa. The activity of the enzyme depends on the Fe^{2+} of the heme group (47). IDO1, unlike TDO, is ubiquitously present in human tissues. Particularly high L-Trp-degrading activity along with constitutive IDO1 expression is found in the lung, the small intestine, and the term placenta (48-50). The exact mechanism by which IDO1 converts L-Trp into N-formyl-kynurenine is still unknown (51). IDO1 exhibits a wide substrate-specificity presenting remarkable affinity for L-Trp but is able to metabolise also D-tryptophan (D-Trp), 5-hydroxytryptophan, tryptamine or even melatonin (36, 48, 52, 53). Its contribution to Trp degradation under physiological conditions is insignificant while under immune activation its activity strikingly raises assuming a major role in the control of Trp degradation (35). IDO1 expression is upregulated by IFNγ and TNFα, IFNα and IFNβ, lipopolysaccharide (LPS) and other factors in different cell types or tissues (54-60). In addition, also hormones such as human chorionic gonadotropin (hCG) or estrogen can upregulate IDO1 (61-63). Although there is still some controversy regarding IDO1 inhibitors (64), traditionally, 1MetTrp has been broadly utilized to inhibit IDO1 activity (52, 55, 57, 58, 65-68). However, a novel compound called INCB024360 already undergoing clinical trials, has been proposed to selectively inhibit IDO1 with minor impact on the other Trp-related enzymes or even the Trp-transporters (69).

IDO1, unlike the other Trp-catabolising enzymes, is known to have a wide variety of functions. It is presumed that IDO1 is induced during infections

displaying antimicrobial activity (70) or it may support the innate immunity against bacterial infection in the female reproductive tract (50). In addition, the role of IDO1 in anti-tumour responses regulating the proliferation of certain immune cells has been intensively studied (35, 69, 71, 72), a role mediated via the induction of regulatory T cells (73). However, for the last two decades IDO1 has been directly implicated in the establishment and maintenance of feto-maternal tolerance against the fetus during the pregnancy (74-78).

A novel function of IDO1 has been recently described. IDO1 acquires special importance in inflammatory processes where it is highly expressed in endothelial cells contributing to the relaxation of the vascular tension (67, 79). IDO1 is constitutively expressed in the vascular endothelium of the chorion, with a positive expression gradient towards the feto-maternal interface (49) (Fig. 2). It has been once implied a potential involvement of IDO1 with the regulation of the fetal-placental blood flow in late pregnancy (80). Therefore, a new role of IDO1 may arise regulating the vascular tone in the human placenta.

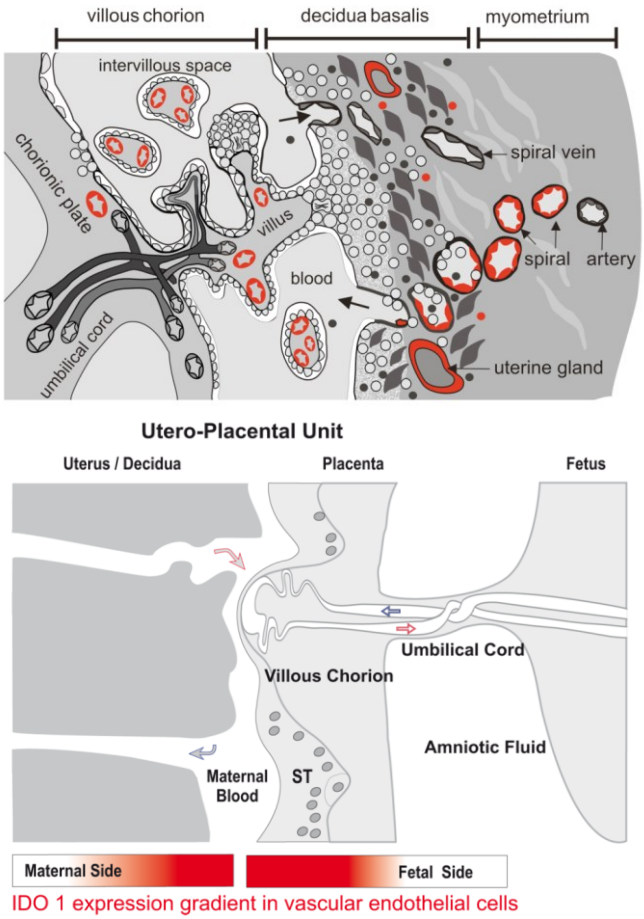


Figure 2. IDO1 expression gradient in the vascular endothelium at the utero-placental unit. The expression intensity increases close to the utero-placental materno-fetal interface. Taken from (49, 81). Permission to reproduce figure 2 granted (Copyright Clearance Center – Springer Nature)

1.3. The human placenta

The human placenta is a highly specialized transient organ exclusively formed to fulfil the demands of the fetus throughout the period of gestation. In order to satisfy these demands, the placenta and the surrounded tissue undergo maturation and functional modifications with increasing gestational age. This includes dilation of the arteries increasing the blood flow, remodelling of arterial walls, variation in the expression patterns of proteins and receptors, metabolic and hormonal changes, vasculogenesis and angiogenesis (82-84).

It provides a broad range of functions essential for a successful pregnancy. The placenta delivers an appropriate supply of oxygen and nutrients to the fetus

(83, 84) whereas it eliminates the waste products such a CO_2 or urea (85, 86). In addition, the placenta produces and secretes different growth factors, hormones and cytokines essential for the appropriate fetal development (83, 84, 87). Together with its metabolic and transporting role, this organ also offers a protective function for the fetus acting as a barrier against xenobiotics and pathogens (82, 84, 88).

Interestingly, the placenta combines two genetically different sides, the maternal side (decidua) that develops directly from the endometrium in the uterus, whereas the fetal side develops from the chorion which originates from the trophoblast and the extraembryonal mesoderm. The chorion form fingerlike protrusions named chorionic villi that will grow and sprout developing richly vascularised structures through a process called vasculogenesis which determine the exchange of nutrients, gases and waste substances between the mother and the fetus (82, 89).

1.3.1. Placental vasculogenesis

Together with all this adaptations and functional modifications during pregnancy, the placenta sustains one of the major sites of vasculogenesis and angiogenesis. Vasculogenesis is defined as the process of *de novo* formation of blood vessels from *in situ* differentiation from precursor cells whereas angiogenesis refers to the process of growing and sprouting of new vessels from pre-existing ones. Therefore in the placenta, formation of *de novo* capillaries (vasculogenesis) is always followed by expansion of the villous network (angiogenesis) (90). Placental vasculogenesis starts around 3 weeks post-conception with the differentiation *in situ* of hemangioblastic cell clumps (precursors of fetal endothelium) controlled by the release of angiogenic growth factors from the trophoblast layer located immediately beneath (91) forming tube-like structures. Further mesenchymal cells beside these endothelial structures with extensions integrated within the mesenchymal and endothelial networks will give rise to the pericytes (92). However is not until the sixth week that an effective villous circulation is established. The villous network newly-formed undergoes continuous branching

and remodelling during the whole pregnancy. This process is tightly regulated via angiogenic factors released in response to oxygen changes and shear stress (93, 94). All this changes occur in a reduced oxygen environment in comparison with maternal tissues; however, oxygen concentration increases throughout the progression of the gestation (95).

By the third month and until late pregnancy, the intermediate villi reach larger dimensions and the immature structures start to differentiate the different layers (tunica media and adventitia) into proper villous arteries and veins in the so-called stem villi. The trophoblastic capillaries from the stem villi turn into a differentiated ramified capillary network called terminal villi. Parallel to the advancing gestational age, the terminal capillaries start to dilate creating big sinusoids allowing a better exchange of gases and nutrients (96, 97). In the first and early second trimesters, the capillary network increases in number, volume and surface area allowing for an effective placental circulation (98).

1.3.2. Placental circulation

The placental circulation possesses a unique characteristic. It is the result of the combination of 2 separate circulatory systems: the maternal-placental (uteroplacental) and the fetal-placental (fetoplacental) circulations (99).

This multifaceted circulatory system begins when the maternal blood flow enters the intervillous space through the spiral arteries in the uterine wall. The terminal villi, the differentiated ramified capillary network located in the intervillous space, are continuously bathed in the maternal blood. The exchange of oxygen and nutrients between both circulations occurs at the trophoblastic surface layer of the villi. The new incoming blood drives deoxygenated blood back to the maternal circulation via uterine veins. The umbilical vein carries back to the fetus oxygen and nutrients after the exchange in the terminal villi whereas the umbilical arteries transport back the deoxygenated and nutrient-depleted blood from the fetus to the villi where a new exchange happens again, repeating this exchange cycle (99).

To fulfil the requirements of the fetus, the maternal blood volume is increased throughout the course of gestation, starting from 6-8 weeks and reaching a relatively constant volume after the 34th week of gestation (100). Due to the increased dilation and remodelling of the spiral arteries, the resistance of the uteroplacental vessels is low and therefore there is a fall in the pressure from spiral arteries (70 mmHg) to intervillous spaces (10 mmHg) that allows the maternal blood to efficiently flow around the villi without any detrimental effect. Maternal blood in the intervillous spaces is forced by the incoming arterial blood to return to the maternal circulation, allowing a complete exchange of blood 2-3 times per minute (99).

Hence, the exchange between maternal and fetal circulation is an essential and controlled process primarily modified by the regulation of the placental vascular tone through vasoactive compounds.

1.3.3. Regulation of the vascular tone

The placental circulation is characterized by low-vascular resistance and a lack of neuronal innervation, suggesting that regulation of vascular tone is determined by physical factors or by local vasoregulators (101, 102).

The placenta itself releases some of these vasoactive compounds in response to a plethora of stimuli such as transmural pressure, flow, oxygen changes or shear stress (103). On the one hand, some vasoregulators like thromboxane have a potent effect leading the vessels to a vasoconstriction state (104, 105). Some others have a more discreet effect, for instance endothelin-1 (ET-1), angiotensin, prostaglandins, oxytocin and vasopressin (105, 106). On the other hand, some vasoregulators lead to a relaxation of placental vessels such as histamine, bradykinin and nitric oxide (NO) (107, 108).

Regulation of the vascular tone may be altered in some pregnancy complications like intrauterine growth restriction (IUGR) and pre-eclampsia (PE) as implied by deficiency of uteroplacental perfusion (109, 110).

1.4. Pregnancy complications

Pregnancy complications are health problems associated to the gestation which can appear during or after pregnancy and may affect the health of the mother and the fetus separately or both simultaneously. Some important examples are: high blood pressure, gestational diabetes, IUGR, PE, preterm labor, miscarriage, low amniotic fluid (oligohydramnios), etc. Nevertheless, IUGR and PE have received considerable attention as they contribute with significant and detrimental complications for the mother and/or the fetus not only during the course of pregnancy but in later life (111).

1.4.1. Intrauterine growth restriction (IUGR)

IUGR, often referred as fetal growth restriction (FGR), is defined as the inability of the fetus to achieve its full growth potential determined by genetic and epigenetic factors due to anatomical and functional complications. IUGR is always accompanied by the sign of small for gestational age (SGA) which is defined as the weight below the 10^{th} percentile in regards to the gestational age specific for the growth rate of each population (112). It affects between 7-9 % of pregnancies worldwide, with a strong association concerning unexplained stillbirths and IUGR (113). Additionally, IUGR can be further complicated with PE leading to a more complex identification. Nevertheless, new systematic approaches have been investigated in order to improve the diagnose and reduce the clinical variability of this complication (114). The pathogenesis of IUGR remains to be completely elucidated; therefore, no effective therapies up to date can reverse IUGR.

In regards to the phenotype of this disorder, IUGR can be classified into early- and late-onset. Late-onset IUGR possesses a higher prevalence with mild complications for the newborn (114) whereas early-onset IUGR, often associated with PE, involves rapid fetal deterioration which leads to severe neurological, metabolic, cardiovascular and respiratory consequences with high mortality and morbidity rates for the newborn (111).

The major factor leading to IUGR is placental insufficiency caused by an impaired fetoplacental perfusion which causes hypoxia and acidosis (115). Some risk factors for this condition are: women with comorbidities, poor maternal nutrition, smoking, anemia or even twin pregnancies (111).

Previous reports have shown that IDO-degrading activity is reduced in IUGR placental tissue (116) or that an IDO1 knock-down pregnant mouse model causes IUGR phenotypes (117). Hence, IDO1 deficiency may play a role in the pathogenesis of IUGR.

1.4.1. Pre-eclampsia (PE)

PE is a pregnancy complication primarily characterized by new–onset of hypertension (≥140/90 mmHg) and proteinuria (≥300 mg/24 h) after the week 20 of gestation that affects about 2-8 % of pregnancies worldwide. It is one of the main causes of fetal mortality and morbidity with lifelong consequences for the newborn and the mother (118). Due to the complexity and the broad clinical presentation of PE (spectrum of conditions), the pathophysiology is not fully understood. Until now, there is no causal therapy (119).

PE is traditionally classified according to its onset in early- (appearance before the gestational week (GW) 34) or late-onset (appearance on the 34th GW and onwards). Late-onset PE (also known as mild PE) usually confers no effect on the appropriate development of the offspring and is associated with mild symptoms that generally resolve after delivery (120). Conversely, early-onset PE (also known as severe PE) is associated with multi-organ malfunction such as intracerebral haemorrhage, renal failure, placental abruption or eclampsia which implies a high rate of perinatal morbidity and mortality (121, 122).

The clinical features of severe PE begin generally during the second trimester of pregnancy, possibly as a result of an abnormal implantation which leads to an inappropriate placentation. Consequently, placental ischemia and

oxidative stress surround the placental environment leading to one of the core features of the disorder, the endothelial dysfunction, which is the main responsible of the clinical signs detected in the mother (123).

It is already known that IDO1 expression is reduced in PE (124, 125).Furthermore, IUGR and PE phenotypes are developed in pregnant mice as a consequence of disruption of the IDO1 gene.(117). Therefore, impairment of IDO1 may be one of the factors involved in the pathogenesis of this complex disorder.

2. Hypothesis and objectives

IDO1 is constitutively expressed in the endothelium of villous placental vessels. Considering the role of endothelial IDO1 in regulating the vascular tone as has been shown for inflammatory conditions and the increasing evidence that impaired placental perfusion is associated with pregnancy complications such as PE and IUGR, we hypothesized the following:

i) Constitutive IDO1 localized in the endothelium of placental vessels may regulate the vascular tone in arteries from the chorionic plate
ii) A down-regulation in IDO1 expression produces an impairment in placental perfusion, hence, in fetal growth
iii) There is a causal relationship between reduced IDO1 expression in the placental vasculature and the pathogenesis of IUGR and PE

This study aims to:

i) Determine whether IDO1 plays a role in the regulation of placental vascular tone
ii) Determine whether alterations of chorionic IDO1 expression are present and play a role for the pathogenesis of IUGR and PE.

3. Material and Methods

This chapter was taken and extended from (126)

3.1. Ethical approval

The study, including the isolation of placental arterial endothelial cells, was approved by the Ethics Committee of the Medical University of Graz, Austria (No. 25-366 ex 12/13 and No. 21-079 ex 09/10). All participants involved in the study gave written informed consent. Samples obtained from the Biobank Graz also included informed consent. Taken from (126).

3.2. Patients

IUGR was defined as the presence of signs for placental insufficiency and fetal compromise (without the characteristics of PE). These signs included asymmetric fetal biometry and estimated fetal weight below the 3rd centile (with confirmed gestational age by first trimester crown-rump-length measurement), oligohydramnios and centralized fetal circulation. From January 2016 on the Barcelona calculator was used for staging IUGR http://medicinafetalbarcelona.org/calc/ (114) and included cases with at least IUGR stage 1. PE was defined after week 20 of pregnancy as displaying a blood pressure higher than 140/90 mmHg (without pre-existing hypertension) and proteinuria. In our study we focused on early-onset PE as late onset-PE is not subject to pre-term delivery. All patients were treated at the Department of Obstetrics and Gynaecology of the Medical University of Graz. Taken from (126).

3.3. Tissue collection

Human term placental tissue (n = 64) from healthy and from pregnancy complications (IUGR and PE) was collected after vaginal delivery or caesarean section and immediately transferred from the Department of Obstetrics to the laboratory at the Institute of Molecular Biology and Biochemistry located 2 km

away. One central and one peripheral part of the placenta were washed in cold Hanks' Balanced Salt Solution (HBSS, Gibco, Invitrogen, Carlsbad, CA, USA), and either cut into multiple fragments (~0.5 g) which were thoroughly rinsed in a cold Hanks' medium, snap-frozen in liquid nitrogen and maintained at -80°C until further use, or fixed with 4% (w/v) paraformaldehyde (PFA).

3.4. Myography

Placentas were obtained from normal, IUGR and PE pregnancies within 30 min after cesarean section or vaginal delivery. Vessel rings of approximately 2 mm in length were cut from arteries of the chorionic plate using a calibrated eyepiece micrometre. The vessel rings were either used promptly or incubated in DMEM culture medium (Life Technologies, Paisley, UK) supplemented with 10% (v/v) FBS (Life Technologies), and stimulated with human TNFα (80 ng/mL, Peprotech, Vienna, Austria) and human IFNγ (80 ng/mL, Peprotech) overnight at 37 °C in 95% air/5% CO2. For some experiments, endothelium from vessel rings was previously removed by perfusing the vessel with 0.1% (v/v) Triton X-100 (Sigma, St. Louis, MO, USA). The success of the denudation was assessed by immunohistochemistry. Generally, the vessel rings were carefully mounted on two pin-supports on a myograph 620M (Danish MyoTechnology, Aarhus, Denmark) in a medium consisting of Krebs-Henseleit buffer (Sigma Aldrich, Schnelldorf, Germany) modified and made up in deionized water (6.9 g/L NaCl, 0.35 g/L KCl, 0.16 g/L KH_2PO_4, 0.141 g/L $MgSO_4$, 2.1 g/L $NaHCO_3$, 2 g/L D-glucose, 0.97 g/L EDTA•$2H_2O$ and 0.017 g/L $CaCl_2$, penicillin/streptomycin 1% (w/v), pH 7.4) and continuously aerated with 5% CO_2/95% O_2 at 37 °C. The myograph chambers were connected to force transducers for isometric tension recording (PowerLab, ADInstruments). Optimal working passive tension was calculated by the length-tension curve procedure as previously described (104, 127), and the rings were allowed to stabilize for 30 min. Krebs-Henseleit buffer modified containing 60 mM KCl (Potassium physiological salt solution: KPSS; 9.2 g/L KCl, 0,289 g/L $MgSO_4$•$7H_2O$, 0.161 g/L KH_2PO_4, 0.277 g/L $CaCl_2$, 2.1 g/L $NaHCO_3$, 0.01 g/L EDTA-Na_2•$2H_2O$, 0.991 g/L D-glucose) was used to induce a standardizing depolarising constriction of the tissue. When the developed tension attained its

peak value, the rings were relaxed by rinsing with the Krebs-Henseleit buffer. Vasoconstriction was induced with incremental doses of the thromboxane A_2 analogue U46619 (10^{-9}-10^{-6} M) (Sigma-Aldrich) to produce 50% of the maximum contraction achieved by 60 mM KCl. Following the dose-response curve, vessels were washed to the baseline with modified Krebs-Henseleit buffer. The vessel rings were pre-incubated with 10 µM indomethacin (Sigma Aldrich) and 300 µM L-NAME (Sigma Aldrich), and whenever necessary they were also pre-incubated with 1-methyl-D-tryptophan (1MetDTrp; Sigma Aldrich, Schnelldorf, Germany) or INCB024360 (30 µM, MedChem Express, New Jersey, USA) for 30 min, followed by a single-dose of U46619 to achieve a 50% maximal KPSS contraction. According to the aim of the experiment, the rings were either treated with a single dose of L-Trp (8 mM) or with increasing doses of L-Trp (0.5-32 mM) freshly-prepared on Krebs-Henseleit buffer on the day of the experiment (freshly-prepared L-Trp) or previously dissolved on Krebs-Henseleit buffer, filtrated and stored for at least 7 days at room temperature (stored L-Trp). The extent of relaxation (% relaxation) was defined as the fraction of the reversal of U46619-induced tone (see detailed protocol in appendix). Extended and modified from (126).

3.5. *Ex-vivo* perfusion of a single cotyledon of the human placenta

The ex-vivo perfusion system was adapted from Schneider et al. (128) to study changes in the vessel back pressure of a placental cotyledon. Within 20 min after delivery of the placenta, the fetal circulation was established by cannulating a corresponding chorionic artery and vein supplying one intact cotyledon. Once the circuit was established, the cotyledon was flushed with pre-warmed (37°C) perfusion medium (Earl´s buffer (6.8 g/L NaCl, 0.4 g/L KCl, 0.14 g/L NaH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L $CaCl_2$, 2.2 g/L $NaHCO_2$, all from Merck, Darmstadt, Germany), 10 g/L dextran FP40 (Serva, Heidelberg, Germany), and total concentration of 2 g/L D-glucose (Merck). The cotyledon together with surrounding tissue was cut and carefully positioned into the pre-warmed perfusion chamber. Perfusion medium kept at 37°C was connected to the fetal artery cannula where a micro-catheter pressure sensor (Millar, Houston, Texas, US) was also inserted to

monitor in real time the pressure changes during the whole experiment. The fetal circulation was gassed with 95% N_2, 5% CO_2 through a gas exchanger (Living Systems, St. Albans, VT, US). A continuous fetal artery influx of 4 mL/min was applied by a magnetic pump (Codan, Salzburg, Austria). In order to establish the maternal circulation, three rounded needles were inserted and fixed into the intervillous space of the perfused cotyledon. A constant volumetric flow rate of 8 mL/min was administered to the maternal circulation; the medium was gassed with 5% CO_2, 20% O_2 and 75% N_2. After a pre-stabilization period, (i) either 8 mM L-Trp (Sigma Aldrich, Steinheim, Germany) (ii) or pre-contraction with 10 nM U46619 (129) followed by 8 mM L-Trp were applied to the fetal reservoir so ensuing pressure changes could be studied.

Only experiments with a fetal flow recovery of at least 85% within the first 30 min were continued for analysis according to defined quality criteria (130). Perfusates from maternal and fetal circulation were collected every 30 min during the experiment in order to measure the metabolic and oxygenation status (pO_2, pCO_2, pH, lactate production and glucose consumption) of the perfused cotyledon by a blood gas analyser (Radiometer, Copenhagen, Denmark). Taken from (126).

3.6. Isolation and culture of placental arterial endothelial cells (PLAECs)

Human term placentas from uncomplicated were obtained under informed consent after vaginal delivery as already published (131). After removal of the amnion, 2 arteries and 2 veins located in the periphery of the surface of the chorionic plate were resected. Vessels were washed with Hank's Balanced Salt Solution (HBSS) (Life Technologies, Paisley, UK) to remove residual blood. Subsequently, PLAECs were isolated by perfusion of a pre-warmed solution of Graier Collagenase containing 185 U/mL Collagenase Type II (Sigma, St. Louis, MO, USA), 2 mg/mL Bovine Serum Albumin (Sigma) 0.5mM Calcium Chloride 1M (Roth, Karlsruhe, Germany), 1 Unit Aminoacids 50x (PAA, Pasching, Austria), 1 Unit Amino acids 100x (PAA) and 0.01 Unit Vitamin 1x (PAA). The perfusion time was limited to 8 minutes to avoid contamination with non-endothelial cells. Finally, the cell suspension obtained was centrifuged (200 *g* for 5 min), the pellet was

resuspended with EGM-MV medium (Lonza, Walkersville, MD, USA) and the cells were plated on culture plates pre-coated with 1% (v/v) gelatine (Sigma-Aldrich, Schnelldorf, Germany). Purity of the cultures was assessed by staining of von Willebrand factor (1:8000, A0082, Dako, Glostrup, Denmark) and the absence of Fibroblast Specific Antigen (1:2000, DIA-100, Dianova, Hamburg, Germany), Smooth Muscle Actin (1:600, M0851, Dako) and Desmin (1:1000, Dako). Only pure PLAECs cultures were used for the present study. Cells were used only between third and sixth passage. Taken from (126).

3.7. Immunohistochemistry

Tissues were fixed between 24 hours and 3 days in 4% (w/v) buffered formalin (Sanova, Vienna, Austria) at room temperature and embedded in paraffin following a protocol of a standard overnight dehydration and paraffin infiltration as previously described (49). Briefly, 5μm paraffin sections were cut on a rotation microtome, mounted on superfrost^{+} glass slides (Sanova) and dried over-night on a 50 °C hot plate. Then, slides were deparaffinised with tissue clear and rehydrated followed by heat induced antigen retrieval buffer at pH 9 (Leica Biosystems, Vienna, Austria) under pressure at 120 °C for 7 min, cooled down for 20 min and washed in distilled water. Endogenous peroxidase activity was blocked using a peroxidase-blocking solution (Dako, Carpinteria, CA, USA) for 10 min, washed and then blocked with Ultra-V block (Thermo Scientific, Cheshire, UK) for 5 min. Samples were incubated firstly with mouse anti-IDO1 antibody (IgG1 1 μg/mL, generously provided by O. Takikawa (70)) for 45 min at room temperature (RT) and then 10 min with primary antibody enhancer (Thermo Scientific). Bound antibodies were detected using a polyvalent horseradish-peroxidase polymer system (Thermo Scientific) for 15 min followed by 10 min incubation with Kaiser´s glycerol gelatine (Merk, Damstadt, Germany) aqueously mounted. All samples were counterstained with Mayer's hematoxylin. Washing steps were made with TBS/0.05% (v/v) Tween 20 (Merck). As an appropriate negative control antibody irrelevant mouse anti-aspergillus niger IgG1 (1 μg/ml, Dako) were used. Anti-CD34 class II (0.04 μg/mL, Dako) was used for staining vascular endothelium, anti-CD68 (0.025 μg/mL; Thermo Scientific) for staining macrophages. Taken from (126).

3.8. Fixation of cell pellets

PBS-washed cells were detached with 3 mL of accutase (PAA, Pasching, Austria) for 5 min at 37 °C and then resuspended in 7 mL of EGM-MV medium (Lonza, Walkersville, MD, USA). The suspension was transferred into a falcon tube and centrifuged at 630 *g* for 5 min and then, the supernatant was carefully removed. The pellet was transferred into a 1.5 mL Eppendorf tube, resuspended and fixed in 1 mL of 4% paraformaldehyde (PFA) for 30 min at RT. Suspension was washed and centrifuged twice at 630 *g* for 5 min. Pellet was resuspended and incubated with 5% gelatine (Merck, Darmstadt, Germany) for 30 min at 37 °C. Eppendorf tube was again centrifuged in a pre-warm centrifuge at 37 °C and then placed at 4 °C overnight. Pellet was finally fixed for an hour at 4 °C by adding 4% PFA on the top of the gelatine. Finally, 4% PFA was gently removed and the pellet was carefully placed in a new vessel with the help of a lancet with 4% PFA and incubated at 4 °C overnight for the final fixation. If necessary before incubation, excise the remaining gelatine that contains no cell pellet. The pellet was at last washed and embedded in paraffin as previously mentioned (see detailed protocol in Appendix). Extended and modified from (126).

3.9. Protein isolation and quantification

Protein lysates were prepared in RIPA buffer from a cell suspension or from tissue samples (Sigma Aldrich, St. Louis, MO, USA) containing protease inhibitors (Roche, Mannheim, Germany). Protein concentration was determined according to the Lowry method. Briefly, the supernant of the cell lysate collected and also protein standards with a known concentration are mixed with a reaction mixture containing mainly copper ions that under alkaline conditions and the Folin-Ciocalteau reagent will oxidize the peptide bounds of the proteins. The concentration of the reduced Folin reagent after reacting with the proteins present on the sample is measured by absorbance at 620 nm. Extended and modified from (126).

3.10. Western blotting analysis

Cell and/or tissue lysates were mixed with LDS sample buffer (4x) (Life Technologies, Carlsbad, CA, USA) and sample reducing agent (10x) (Life Technologies) and denatured at 70 °C for 10 min. Equal amounts of total protein (25 µg/well) were loaded onto 10% Bis-Tris SDS-PAGE gels (Life Technologies) and resolved at 120 V for approximately 75 min. Proteins were transferred to a nitrocellulose membrane (Life Technologies) and nonspecific binding sites were blocked for 30 min with blocking solution (Life Technologies). After blocking, the membranes were incubated with anti-IDO1 antibody (generously provided by Takikawa O., 1:1000 (70)) on a shaker overnight at 4 °C. Membranes were washed with washing solution (Life Technologies) and incubated with the appropriate secondary antibody solution conjugated with horseradish peroxidase (Life Technologies). After several washing steps, membranes were incubated for 5 min with an enhanced chemiluminescent immunodetection system (Life Technologies) and membranes were visualized using the FluoChem Q system (Biozym, Vienna, Austria). Extended and modified from (126).

Table I. Primary antibodies used for Western blotting

Primary antibody	Host	Concentration
IDO1 (provided by O. Takikawa)	Mouse	1 µg/ml
GAPDH (Novus Biologicals, NB300-221)	Mouse	50 ng/ml

3.11. Semi-quantitative polymerase chain reaction (qPCR)

Total RNA was isolated according to manufacturer's instructions using peqGOLDTriFast (VWR, Erlagen, Germany). cDNA was synthetized from 2 µg of RNA in the presence of random primers and MultiScribeTM reverse transcriptase (Applied Biosystems, Cheshire, UK). IDO1 specific fragments (297 bp) were amplified (62 °C, 35 cycles) by qPCR and detected by 2% (w/v) agarose gel electrophoresis. Taken from (126).

3.12. Real time polymerase chain reaction (RT-qPCR)

Total RNA from placental tissue was isolated using the peqGOLD TriFast reagent (VWR, Erlagen, Germany) according to the manufacturer's protocol. For RNA extraction, small tissue pieces were cut and homogenized using a tissue homogenizer and the TriFast reagent. RNA quality was assessed on 1.5% (w/v) denaturing agarose gels (Biozym, Vienna, Austria) and stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA). RNA quantity and purity were assessed using a NanoDrop1000 Spectrophotometer (Thermo Scientific). After quality check, 2 µg of total RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Reverse transcription was performed with and without reverse transcriptase to check a possible contamination with genomic DNA. cDNA was employed for quantitative real-time PCR using TaqMan Gene Expression Assays (Applied Biosystems) for human IDO1 [Hs00158027_m1], human CD34 [Hs00990732_m1], ribosomal protein L30 (RPL30) [Hs00265497_m1] and human CD68 [Hs00154355_m1] and the TaqMan Universal PCR Mastermix (Applied Biosystems). cDNA and kit compounds were mixed in a total volume of 20 µl per well (96 well plates, Thermo Scientific, UK) according to the manufacturer's instructions and amplified using a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad, Vienna, Austria). Ct values were automatically generated by the CFX Manager 2.0 Software (Bio-Rad). Relative quantification of gene expression was calculated by standard $\Delta\Delta$Ct method using the expression of CD34. Data are presented as mean of $2^{-\Delta\Delta Ct}$ values. Extended and modified from (126).

Table II. Taqman gene expression assays employed for RT-qPCR

Gene	Gene expression assay
CD34	Hs00990732_m1
RPL30	Hs00265497_m1
IDO1	Hs00158027_m1
CD68	Hs00154355_m1

3.13. IDO activity by liquid chromatography – mass spectrometry (LC-MS)

IDO activity in placental tissue homogenates was measured as the amount of kyn formed from L-Trp using the ascorbate/methylene blue assay as previously described (132) with modifications. Placental tissue homogenates were generated by placing frozen placental tissue (cut into small pieces on dry ice) in 500 μl of 100 mM phosphate buffer pH 7.4 containing 2x protease inhibitor cocktail (Roche) and homogenizing the tissue using a Heidolph homogenizer.

Homogenates were then spun at 17000 *g* for 15 min at 4 °C and the supernatant used for IDO activity assay. The standard assay mixture (in 300 μl final volume) consisted of 100 mM phosphate buffer, pH 7.4, containing 200 μl of supernatant 200 μM L-Trp, 10 mM ascorbic acid, 0.2 mg/ml catalase. The reaction was initiated by adding 15 μl of 500 μM methylene blue (25 μM final concentration to the above reaction mixture and then incubated for 30 min at 37 °C. Aliquots (100 μl) were removed at 0 min (before incubation at 37 °C) and at 30 min and the reaction stopped by the addition of cold trichloroacetic acid (4% (w/v) final concentration). Mixtures were stored on ice for 15 min and then incubated at 65 °C for a further 15 min to convert N-formyl-kynurenine to kyn before centrifugation at 17000 *g* for 15 min at 4 °C. To 25 μl of supernatant, equal volume of 1 M phosphate buffer, pH 7.4 was added, the mixture vortexed for 30 sec and then centrifuged at 17000 *g* for 5 min at 4 °C. Five microliters of the supernatant were then injected onto an Agilent 1290 UHPLC system connected to an Agilent 6490 triple-quadrupole mass spectrometer equipped with an electrospray ionization source (ESI) operating in positive ion mode. Analytes were separated on a 5 μm Luna C18 (2) column (30 × 2.10mm; phenomenex, USA) by gradient elution using 0.1% acetic (mobile phase A) and 0.1% acetic acid in acetonitrile (mobile phase B) at 0.15 mL min^{-1}. The gradient consisted of 0–5% mobile phase B from 0 to 6 min, 5–100% from 6 to 8 min. Mass spectrometer parameters were as follows: gas temperature = 250 C; gas flow = 20L/min; nebulizer pressure = 35 psi); sheath gas heater = 325 C; sheath gas flow = 12L/min; capillary voltage = 3500 V.

Detection of kyn was by multiple reaction monitoring (MRM) in positive ion mode using the above general mass spectrometry parameters with fragmentor voltage at 380 V and cell accelerator voltage at 5 V. The largest fragment ion generated by collision-induced dissociation of the $[M+H]^+$ ion were used for quantification with the following transition (parent ion $\rightarrow$ fragment ion) m/z 616.2 $\rightarrow$ 557.2 with CE = 10 V. Kynurenine was quantified against a standard curve of authentic commercial standard obtained from SigmaAldrich (USA). Taken from (126).

3.14. Statistics

Statistical analyses were performed using GraphPadPrism (version 6.01) and R (version 3.3.2). The results obtained represent at least 3 independent experiments. Data were expressed as mean ± standard deviation. After testing for normality (Kolmogorov-Smirnov and Shapiro-Wilk test), unpaired data was analysed either with one-way ANOVA and Tukey´s post-hoc test or with Dunnett´s test for comparison of at least three independent sets of data, paired non-parametric data was analysed with Wilcoxon signed-rank test and paired parametric data was analysed by paired t-test. The relaxation percentages were related to the concentration (mM) using a linear regression model including treatments, a linear and a quadratic component for the concentration, an interaction term for treatments and concentration as fixed effects and additionally intercept and concentration as random effects. P-values less than 0.05 were considered significant. Extended and modified from (126).

4. Results

4.1. IDO1 expression in isolated placental arterial endothelial cells (PLAECs) from chorionic plate arteries of normal placentas

In order to guarantee the purity of the PLAECs isolated excluding a possible contamination with fibroblast or smooth muscle cells, characterization of isolated cells was accomplished by the presence of > 90% positive staining of von Willebrand factor (vWF; 1:8000) and the absence of staining for fibroblast specific antigen (FSA; 1:2000), smooth muscle actin (smA; 1:600) and desmin (1:1000, Dako) (Fig. 3). Only 100% pure PLAECs cultures were considered for the present study.

To better characterize the stimuli that upregulate IDO1 in isolated PLAECs as a basis for subsequent functional studies utilizing chorionic plate arteries in myography, PLAECs were stimulated synergistically with IFNγ (80 ng/mL) and TNFα (80 ng/mL) for 48 hours (54) under standard culture conditions and compared with non-stimulated cells (Fig. 4). Non-stimulated PLAECs exhibited a polygonal shape forming classical cobblestone monolayers. Unexpectedly, morphology of stimulated PLAECs was changed to a more fusiform-shape cells. Characterization markers expression for endothelial cells did not show any change before and after stimulation with the cytokines (Fig. 5). Additionally, cell number and viability % were measured and compared between stimulated and non-stimulated PLAECs by the CASY® Cell Counter without any major difference (data not shown).

In order to check the IDO1 expression extent at the cellular level, we studied IDO1 mRNA and protein expression in isolated PLAECs compared to PLAECs stimulated synergistically with IFNγ (80 ng/mL) and TNFα (80 ng/mL) for 48 hours (54) (Fig.6). By qPCR, a constitutive IDO1 mRNA expression was found; following cytokine stimulation IDO1 mRNA was upregulated (Fig.4.A). Constitutive expression of IDO1 protein was detected by Western blotting in PLAECs in one case out of three, after cytokine stimulation PLAECs from 3 different isolations

were positive for IDO1 (Fig.6.B). In addition, protein IDO1 expression was assessed by immunocytochemical staining of cellular pellets of PLAECs. IDO1 was detected only after stimulation with IFNγ and TNFα (Fig.6.C). The expected molecular weight of IDO1 is 45 kDa but the band was observed at a lower level as previously described (70).

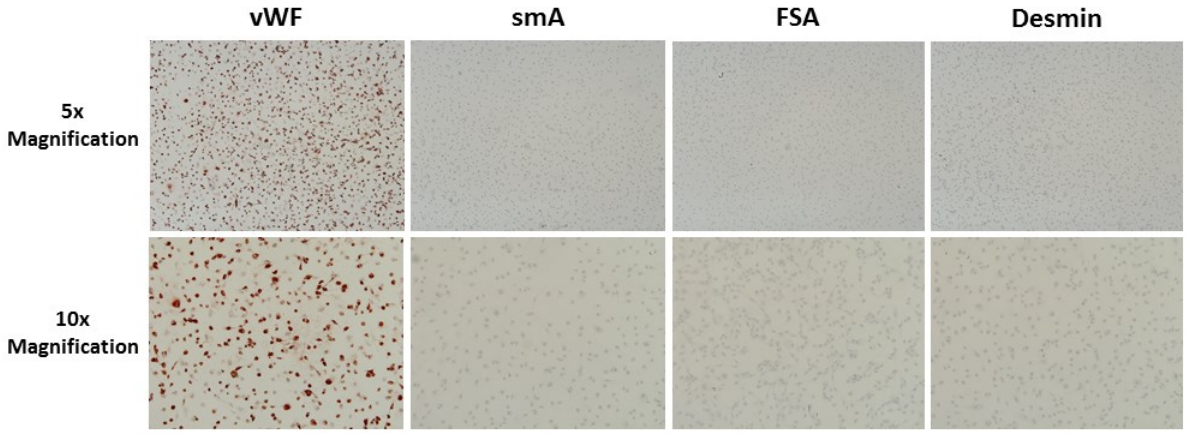


Figure 3. Immunohistochemical staining of glass chamber slides with PLAECs against characterization markers for endothelial cells. Shown are selected PLAECs staining from normal placentas at 2 different magnifications. Positive staining for vWF shown as red staining (first column) and negative staining for smA, FSA and desmin shown as blue (second, third and fourth column). The picture is representative of 3 independent isolations (n = 3). At least 2 different arteries were taken from each isolation.

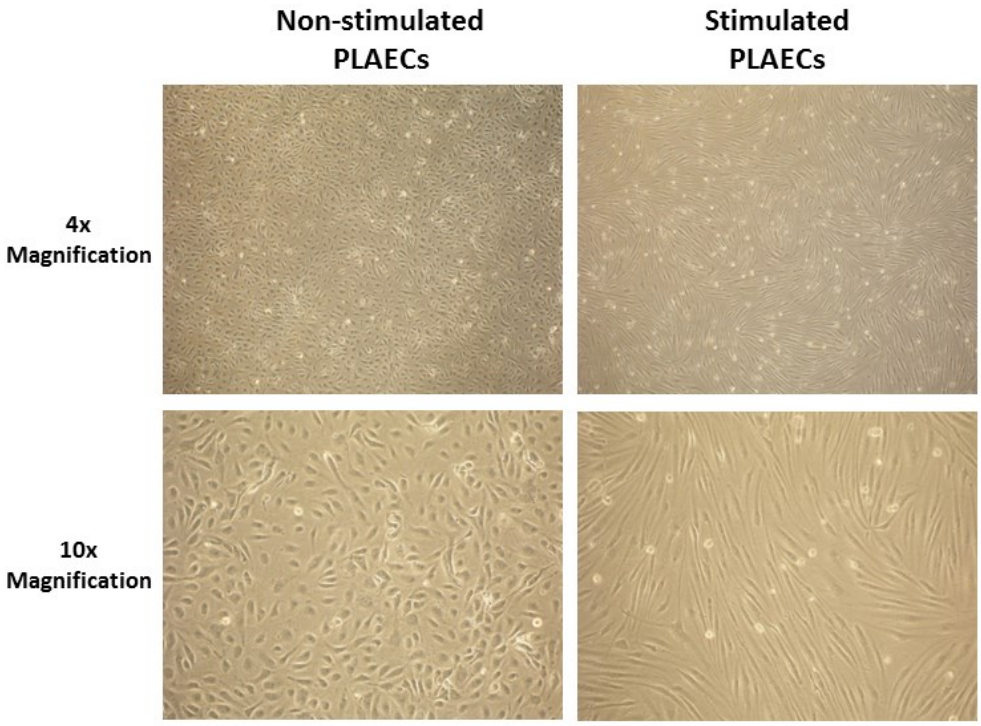


Figure 4. Picture taken of stimulated and non-stimulated PLAECs in culture. Shown are selected PLAECs in culture from normal placentas at 2 different magnifications. PLAECs in the presence of IFNγ (80 ng/mL) and TNFα (80 ng/mL) (stimulated PLAECs) or in the absence (non-stimulated PLAECs) exhibited different morphology. The picture is representative of PLAECs from 3 independent isolations (n = 3).

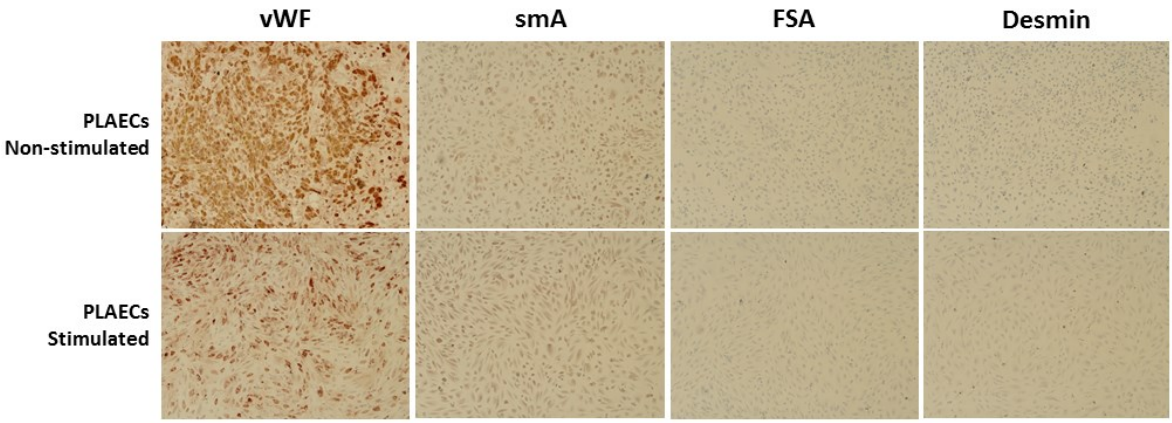


Figure 5. Immunohistochemical staining of glass chamber slides with stimulated and non-stimulated PLAECs against characterization markers for endothelial cells. Shown are selected PLAECs staining in the presence (PLAECs stimulated) or absence (PLAECs non-stimulated) of IFNγ (80 ng/mL) and TNFα (80 ng/mL) from normal placentas. Positive staining for vWF shown as red staining (first column) and negative staining for smA, FSA and desmin shown

as blue (second, third and fourth column). The picture is representative of 1 independent isolation (n = 1).

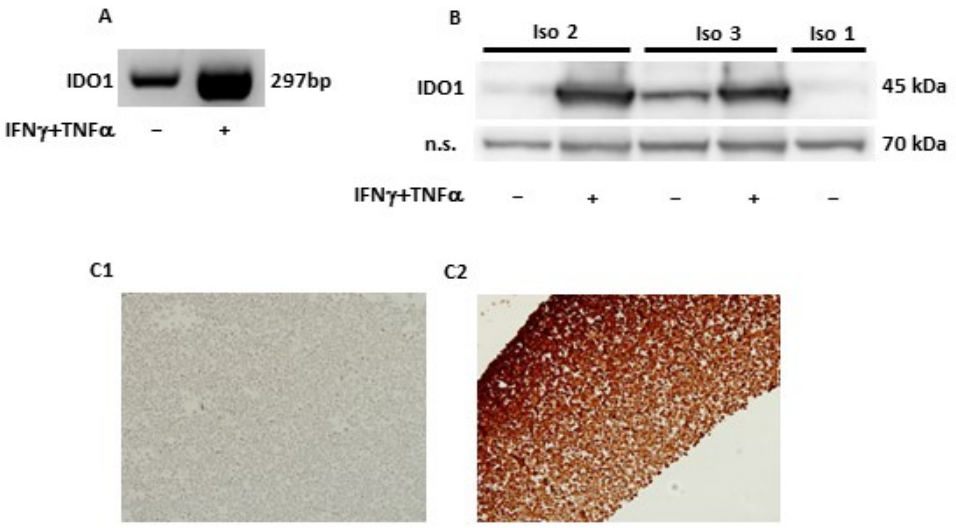


Figure 6. IDO1 expression in isolated placental endothelial cells from chorionic plate arteries. (A) IDO1 mRNA expression by qPCR in PLAECs in the absence or presence of IFNγ (80 ng/ml) and TNFα (80 ng/ml) for 48 hours. An IDO1 PCR-fragment of 297 bp was assessed by gel-electrophoresis on 2% agarose gel in a sole experiment. **(B)** IDO1 protein expression in PLAECs isolated from normal placentas was determined by Western blotting. PLAECs from 3 isolations (Iso 1-3) were incubated in the absence or the presence of IFNγ (80 ng/mL) and TNFα (80 ng/mL). **(C)** Immunohistochemical staining of cell pellets of PLAECs from 2 independent experiments in the absence **(C1)** or the presence of IFNγ (80 ng/mL) and TNFα (80 ng/mL) **(C2)**. n = 1-3. Figure taken from (126)

4.2. Establishing myography with human chorionic placental arteries

4.2.1. Defining the passive tension

The passive tension is the physiological distension of an unstimulated vessel. This passive tension defines the extent of the vascular tone response to the different agonist applied (133, 134). Defining the passive tension in a particular type of

vessel is the first essential step to perform any myography experiment with reliable results. Therefore, the first set of experiments was committed to characterize the optimal passive tension for human chorionic placental arteries.

There are two approaches to optimise the passive tension: the Classical Normalization Procedure (CN) or the Length-Tension Curve Procedure (LCP) (104). By using the CN procedure, some passive tension values are recorded after stretching the vessels (4-6 times) and using a normalization software, the system provides you the internal diameter for each specific vessel ring using Laplace´s relationship (135, 136). Through LCP, some passive tension values are recorded after reaching a plateau by applying a constrictive agonist. To accomplish full relaxation, the vessels are repeatedly washed and then stretched to a higher level and the procedure again repeated after addition of the constrictive agonist. Data produced is used to define the passive tension value as previously described (127).

Due to a problem with the software, the CN procedure was not possible to accomplish. Then we tried via LCP to achieve the point when the vessels do not constrict anymore after addition of the constrictive agonist. It seems that human placental arteries do not reach this point and can keep on contracting at high stretching points (quite distant from the physiological point). Data generated was used to determine a range of passive tension where the response to the agonist had a linear relationship. Hence, diverse relaxation percentages were obtained after applying increasing passive tensions values (mN) to arterial rings from normal term placentas with big diameter (> 600 μm, Fig.7.A), medium diameter (500-600 μm, Fig.7.B) and small diameter (250-500 μm, Fig.7.C). A force range of passive tension where the response to the agonist had a linear correlation was chosen as a final passive tension value for placental arteries. This correlation was found for big, medium and small vessels in the range of 10 to 20 mN of passive tension. Hence, the same passive tension value (13 mN) was chosen for all vessels as physiological passive tension for human placental arteries for the following experiments.

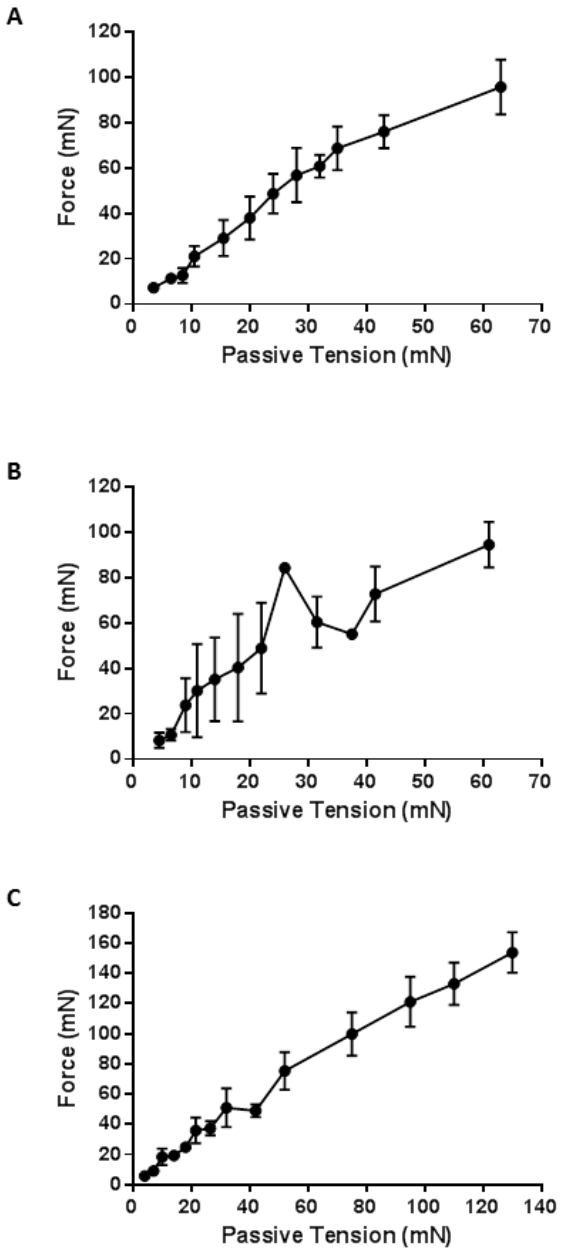


Figure 7. Force achieved by different size arteries settled to increasing passive tension values after applying a constrictive agonist. Force achieved (mN; y axis) after applying 60 mM KPSS to arterial rings settle to various passive tension values (x axis) from normal term placentas with big diameter (> 600 μm) **(A)**, medium diameter (500-600 μm) **(B)** and small diameter (250-500 μm) **(C)**. The data show the outcome of 5 **(A, B)** to 6 **(C)** independent experiments (n = 5–6); measurements at each passive tension were done at least in 2 arterial rings. Results are given as mean ± SD.

4.2.2. Selecting the better solvent for L-Trp - Response of the vascular tone of normal chorionic placental arteries

We applied increasing accumulative concentrations of L-Trp dissolved in HCl and neutralized with NaOH prior addition to the bath (Fig.8.A) or L-Trp dissolved in Krebs buffer modified (Fig.8.B) to arterial rings from normal term placentas. The relaxation of the vascular tension of the arteries was measured by myography and compared to one another. According to the linear regression model, for one unit increase in the concentration of L-Trp, there was a 0.92% (Fig.8.A) or 1.14% (Fig.8.B) increase in relaxation of the U46619-induced contraction. Stored L-Trp had a dose-dependent effect on the relaxation of U46619-induced contracted arterial rings independently of the solvent employed, with 32 mM L-Trp inducing the strongest relaxation by 28,5% dissolved in HCl (R^2 = 0.286; P ≤ 0.0001) and by 34,5% in Krebs buffer (R^2 = 0.430; P ≤ 0.0001). Focusing on the data obtained from L-Trp dissolved in Krebs buffer modified, firstly, it fits better to the linear regression model and secondly, Krebs buffer is a physiological buffer employed to maintain the vessels alive during the myography experiments (unlike HCl or NaOH which may have an effect on the vessel). Therefore, Krebs buffer was chosen as vehicle to dissolve L-Trp for all the subsequent experiments.

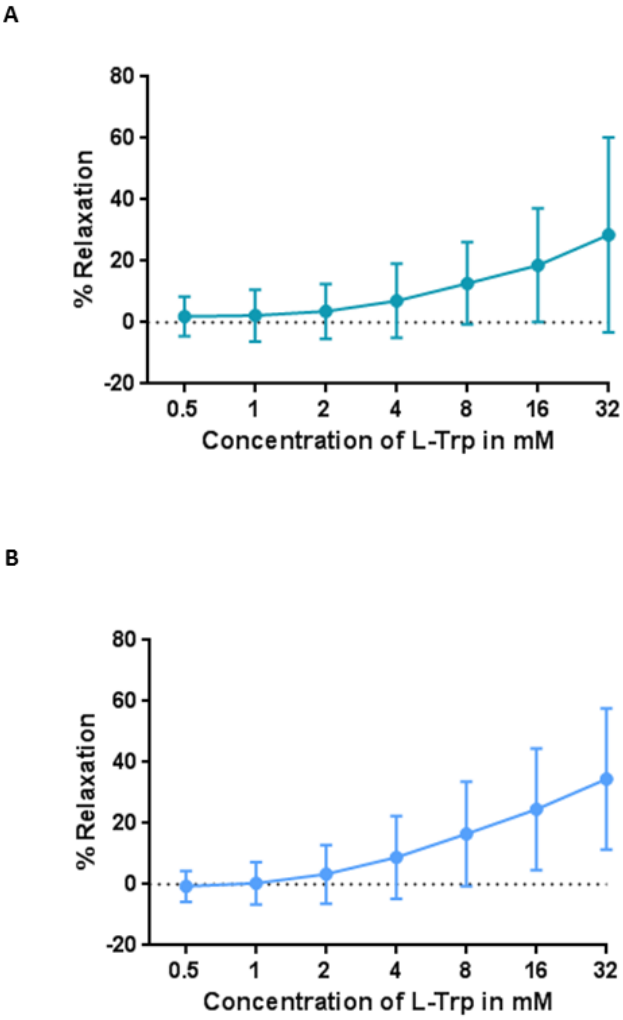


Figure 8. Relaxing effect of stored L-Trp dissolved in 2 different solvents on normal chorionic placental arteries. Percentage relaxation of the vascular tension achieved (y axis) in relationship to U46619-induced contraction after applying increasing concentrations of stored L-Trp (x axis; 0.5, 1, 2, 4, 8, 16 and 32 mM) dissolved in HCl and neutralized with NaOH prior the addition to the chamber **(A)** or dissolved in Krebs buffer modified **(B)** to arterial rings from normal term placentas represented in a logarithmic 2 scale. Fig.8.A exhibits a slope of 0.92 (95% CI 0.56-1.28; $P < 0.0001$) and Fig.8.B a slope of 1.14 (95% CI 0.80-1.47; $P < 0.0001$). The data show the outcome of 3 **(B)** and 4 **(A)** independent experiments ($n = 3–4$); measurements at each concentration were done at least in 2 arterial rings. Results are given as mean ± SD. (Linear regression model)

4.2.3. Selecting an effective IDO1 competitive inhibitor

In order to test any potential effect of the traditional IDO1 inhibitors alone on the vascular tension, increasing accumulative concentrations of Necrostatin-1 (methyl-

thiohydantoin-tryptophan (MTHT; Fig.9.A)) dissolved in DMSO (used as a vehicle control) and 1MetDTrp (Fig.9.B) dissolved in HCl and neutralized with NaOH (used as a vehicle control) were applied to arterial rings from normal term placentas (Table III). The relaxation of the vascular tension of the arteries was measured by myography and compared to the vehicle controls. The inhibitor 1MetDTrp showed a significant dose-dependent effect on the relaxation of U46619-induced contracted arterial rings for the initial concentrations ($P = 0.017$), but not for the last one ($P = 0.143$) in comparison with the control. Preliminary data using Necrostatin-1 alone suggest a possible relaxing effect on the vascular tension. Moreover, to test the effectiveness of both competitive inhibitors on IDO1 activity, arterial rings were incubated with Necrostatin-1 (Fig. 9.C) or 1MetDTrp (Fig. 9.D) with a subsequent addition of a single dose of 8 mM stored L-Trp. Preliminary results showed that Necrostatin-1 may not be a suitable inhibitor. However, 1MetDTrp preincubation seemed to either partially or totally inhibit the relaxing effect exhibited by stored L-Trp.

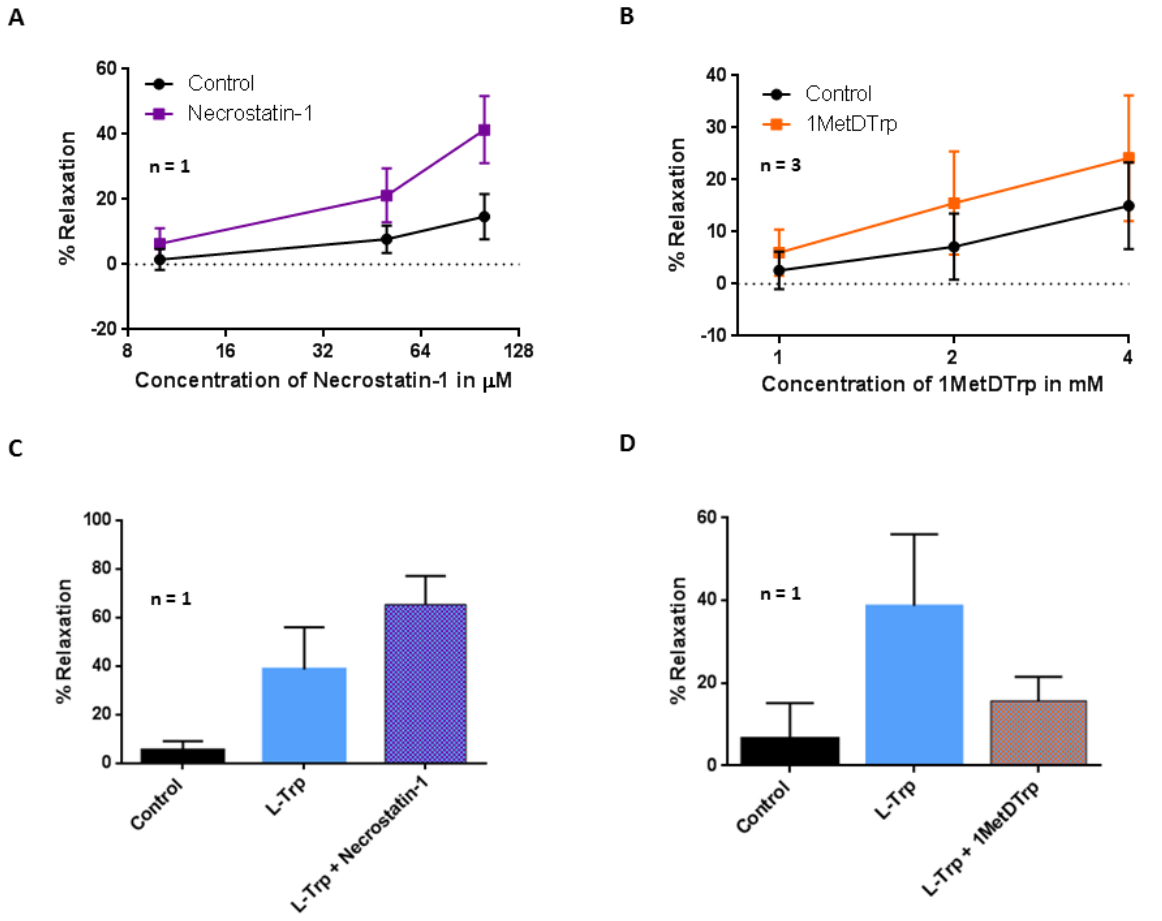


Figure 9. Effect of IDO1 competitive inhibitors alone and in the presence of stored L-Trp on the vascular tone of normal chorionic placental arteries. (A, B) Percentage relaxation of the vascular tension achieved (y axis) in relationship to U46619-induced contraction after applying increasing concentrations of Necrostatin-1 alone (x axis: 10, 50 and 100 µM) **(A)** or 1MetDTrp alone (x axis: 0.5, 1, 2 and 4 mM) **(B)** to arterial rings from normal placentas represented in a logarithmic 2 scale. **(C, D)** Percentage relaxation of the vascular tension achieved (y axis) in relationship to U46619-induced contraction after applying a single dose of 50 µM Necrostatin-1 **(C)** or 1 mM 1MetDTrp **(D)** in combination with stored L-Trp to arterial rings from normal placentas. **(B)** The linear term does differ between control and 1MetDTrp (P = 0.017), however, the quadratic term does not differ (P = 0.143). The data show the outcome of 1 **(A, B and D)** or 3 **(C)** independent experiments (n = 1; n = 3); measurements at each concentration were done at least in 2 arterial rings. Results are given as mean ± SD. (**B**: Linear regression model).

4.3. Relaxing effect of a stored L-Trp solution on arteries of the chorionic plate of normal and IUGR placentas

Increasing accumulative concentrations of stored L-Trp solution were applied to arterial rings from normal (Fig.10.A) and IUGR (Fig.10.B) placentas. The relaxation of the vascular tension of the arteries was measured by myography in comparison to the vehicle control. Stored L-Trp had a significant dose-dependent effect on the relaxation of U46619-induced contracted arterial rings on normal ($P < 0.001$) and even a stronger effect in IUGR ($P < 0.001$) arterial rings compared to its control.

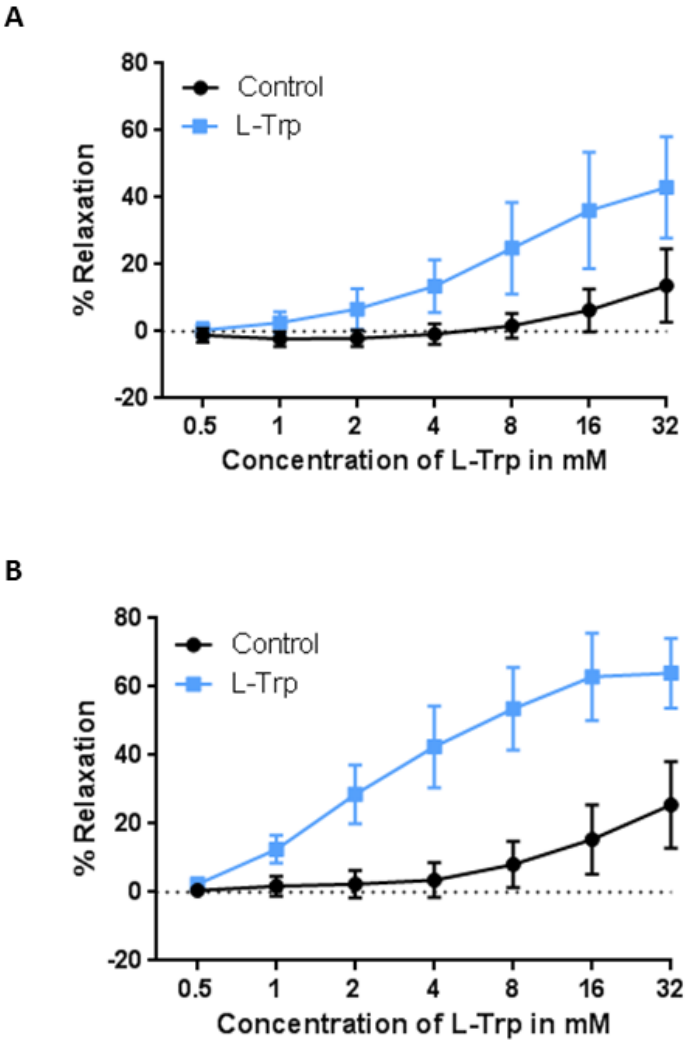


Figure 10. Relaxing effect of stored L-Trp on arteries of the chorionic plate of normal and IUGR placentas. Percentage relaxation of the vascular tension achieved (y axis) in relationship to

U46619-induced contraction after applying increasing concentrations of stored L-Trp (x axis; 0.5, 1, 2, 4, 8, 16 and 32 mM) to arterial rings from normal **(A)** and IUGR **(B)** placentas represented in a logarithmic 2 scale. The data show the outcome of 3 independent experiments (n = 3); measurements at each concentration were done at least in 2 arterial rings. Results are given as mean ± SD. (Linear regression model)

4.4. Relaxing effect of a stored L-Trp solution on arteries of the chorionic plate of normal placentas is not IDO1-dependent

In order to define whether the relaxing effect of stored L-Trp was due to the IDO1 activity, placental arterial rings were incubated with a competitive inhibitor of IDO1, 1-methyl-D-tryptophan (1MetDTrp) (Fig.11). The amount of relaxation of the vascular tension was measured following the application of 4 mM of L-Trp alone (L-Trp), of L-Trp in the presence of 4 mM 1MetDTrp (1MetDTrp+L-Trp) and 4 mM 1MetDTrp in the presence of 1MetDTrp (1MetDTrp+1MetDTrp) or of the vehicle control (control). Addition of L-Trp showed a significant relaxation in comparison with the control group ($P < 0.0001$). Preincubation with 1MetDTrp did not block the relaxing effect of L-Trp previously seen ($P > 0.05$). To test whether the relaxing effect previously observed with 1MetDTrp alone (Fig.9.B) was also IDO1-dependent, 4 mM of 1MetDTrp was applied to the arterial rings after preincubation with 4 mM of 1MetDTrp. However, addition of 1MetDTrp after preincubation with 1MetDTrp partly abolished the relaxing effect expected from 1MetDTrp ($P < 0.0001$) but not totally as compared the control with it ($P < 0.05$).

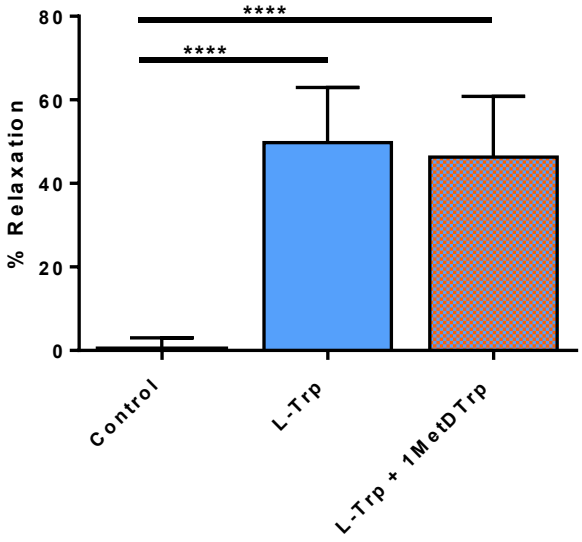


Figure 11. Relaxing effect of stored L-Trp on arteries of the chorionic plate of normal placentas is not IDO1-dependent. Percentage relaxation of the vascular tension achieved (y axis) in relationship to U46619-induced contraction after applying 4 mM of stored L-Trp (L-Trp) alone and 4 mM of stored L-Trp after preincubation for 30 min with 4 mM 1MetDTrp (L-Trp+1MetDTrp) to arterial rings from normal term placentas. The data show the outcome of 3 independent experiments (n = 3); measurements at each concentration were done at least in 2 arterial rings. Results are given as mean ± SD. ****P < 0.0001 (one-way ANOVA with Tukey´s post-hoc test)

4.5. Effect of freshly-prepared L-Trp solution on arteries of the chorionic plate of normal, IUGR and PE placentas

To assess the influence of preparing fresh L-Trp or with days in advance, increasing concentrations of freshly-prepared L-Trp were applied to normal, IUGR and PE placental arterial rings. The amount of relaxation of the vascular tension was measured by myography in comparison to the vehicle control. Freshly-prepared L-Trp did not have an effect in normal (P = 0.528; Fig.12.A) and PE (P = 0.216; Fig.12.C) arteries except for the highest L-Trp concentration (32 mM) where L-Trp exhibited a contracting effect for normal (P < 0.001) and PE (P < 0.001); however freshly-prepared L-Trp showed a relative contracting effect (p < 0.001) in IUGR arteries (Fig.12.B) for all L-Trp concentrations.

As IDO1 presence may be restricted on placental arteries of the chorionic plate (49), we upregulated IDO1 protein expression in the arterial rings by stimulation with IFNγ and TNFα overnight (Fig.13.A). We found relaxation increasing with the concentration of freshly-prepared L-Trp ("L-Trp (fresh) stimulated") and with stored L-Trp ("L-Trp (stored) stimulated") in comparison to the vehicle control with: (i) stimulated rings ("control stimulated") and (ii) non-stimulated rings ("control unstimulated", chosen in order to control for an effect due to the incubation). The non-stimulated and stimulated control groups do not show a significant difference between them ($P = 0.931$), whereas the stimulated stored and freshly-prepared L-Trp groups differ compared to stimulated control group ($P < 0.001$; $P < 0.001$). Moreover, stored L-Trp had a stronger effect on relaxation when compared with freshly-prepared L-Trp ($P < 0.001$).

In order to address the question whether the mechanism of L-Trp-dependent relaxation is functional in IUGR and PE, we then similarly studied arterial rings pre-incubated with IFNγ and TNFα on chorionic plate of IUGR (Fig.13.B) and PE (Fig.13.C) placentas. In the case of PE, we observed a significant relaxation for the L-Trp group when compared with the vehicle control ($P < 0.001$). Preliminary data in IUGR placentas seems to indicate that the L-Trp-dependent relaxation is still functional. However, more experiments need to be done with IUGR to shed some light on this matter.

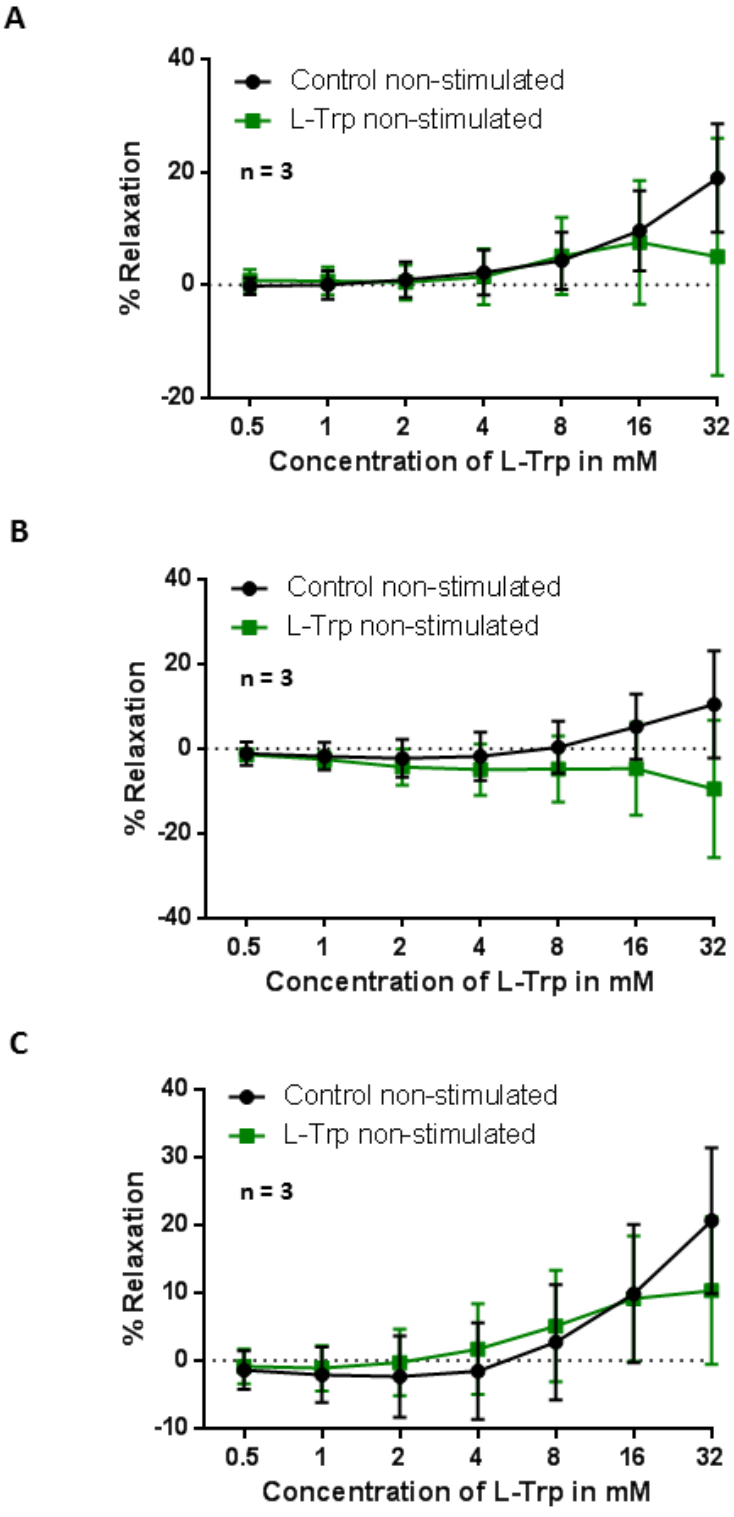


Figure 12. Effect of freshly-prepared L-Trp on non-stimulated arteries of the chorionic plate of healthy, IUGR and PE placentas. Percentage relaxation of the vascular tension achieved (y axis) in relationship to U46619-induced contraction after applying increasing concentrations of freshly-prepared L-Trp (x axis; 0.5, 1, 2, 4, 8, 16 and 32 mM) to non-stimulated arterial rings from healthy **(A)**, IUGR **(B)** and PE **(C)** placentas. Data are represented in a logarithmic 2 scale. **(A)** The linear term does not differ for freshly isolated, non-stimulated normal arteries (P = 0.528) but the

quadratic term does (P < 0.001). **(B)** For control coefficient is statistically significant different from zero (0.411, SE 0.118, P < 0.001) but for L-Trp it is not (-0.20, SE 0.11, P = 0.072). The linear term differs between control and L-Trp (P < 0.001). **(C)** The linear term does not differ between control and L-Trp (P = 0.216), however, the quadratic term differs (P < 0.001).The data show the outcome of 3 independent experiments (n = 3); measurements at each concentration were done at least in 2 arterial rings. Results are given as mean ± SD. (Linear regression model). Figure taken and modified from (126)

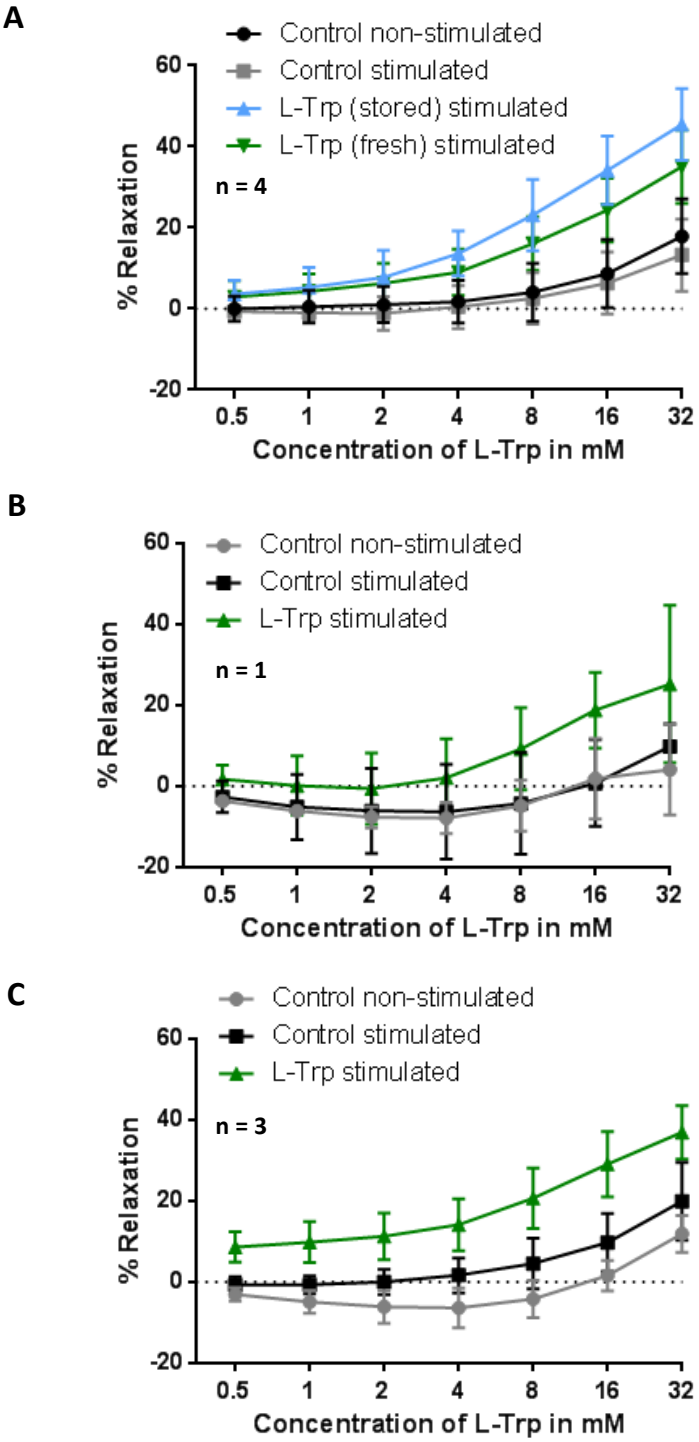


Figure 13. Effect of freshly-prepared L-Trp on stimulated arteries of the chorionic plate of healthy, IUGR and PE placentas. Percentage relaxation of the vascular tension achieved (y axis) in relationship to U46619-induced contraction after applying increasing concentrations of freshly-prepared L-Trp (x axis; 0.5, 1, 2, 4, 8, 16 and 32 mM) to IFNγ/TNFα-stimulated arterial rings from healthy **(A)**, IUGR **(B)** and PE **(C)** placentas. **(A)** Reactivity to stored and freshly-prepared L-Trp differ to both stimulated and non-stimulated controls ($P < 0.001$; $P < 0.001$); the controls do not react differently ($P = 0.931$). Data is represented in a logarithmic 2 scale. The data show the

outcome of 1 **(B)**, 3 **(C)** or 4 **(A)** independent experiments (n = 1; n = 3; n = 4); measurements at each concentration were done at least in 2 arterial rings. Results are given as mean ± SD. (Linear regression model). Figure taken and modified from (126)

4.6. Relaxing effect of freshly-prepared L-Trp solution on stimulated arteries of the chorionic plate of normal placentas is at least partly IDO1-dependent

To determine whether the relaxing effect of freshly-prepared L-Trp was due to the IDO1 activity, placental arterial rings were stimulated with IFNγ and TNFα overnight (in order to upregulate IDO1 expression) and additionally incubated either with 1MetDTrp (Fig. 14.A) or with INCB024360 (Fig. 14.B). The amount of relaxation was measured following the application of 8 mM of L-Trp alone (L-Trp), of L-Trp in the presence of the selected inhibitor, 1 mM 1MetDTrp (1MetDTrp+L-Trp) or 30 μM INCB024360 (INCB+L-Trp), or of the vehicle control (control). Addition of L-Trp showed a significant relaxation in comparison with the control group (Fig. 14.A: $P \leq 0.001$; Fig. 14.B: $P \leq 0.0001$) which was partially abolished by preincubation with 1MetDTrp ($P \leq 0.01$) or with INCB024360 ($P \leq 0.001$).

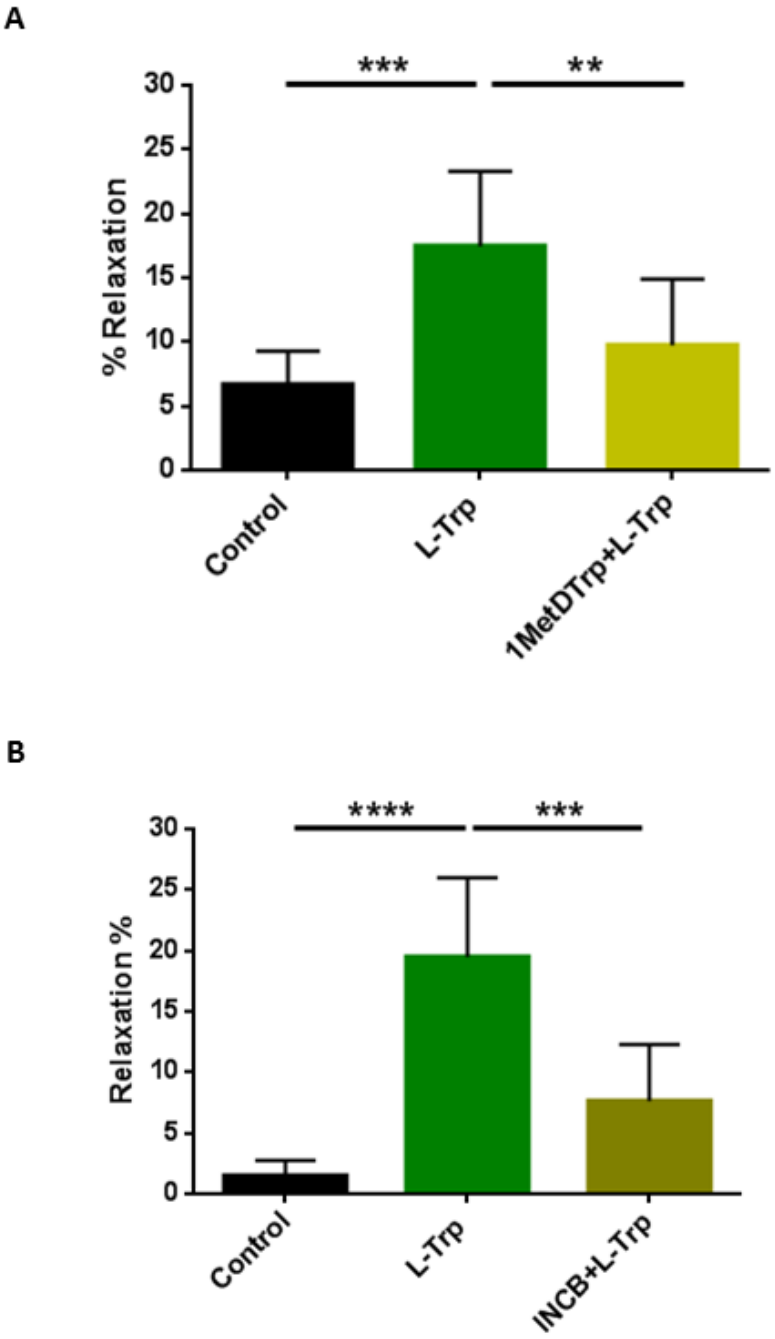


Figure 14. Relaxing effect of freshly-prepared L-Trp on arteries of the chorionic plate of normal placentas is IDO1-dependent. Percentage relaxation of the vascular tension achieved (y axis) in relationship to U46619-induced contraction after applying 8 mM of freshly-prepared L-Trp (L-Trp) or 8 mM of freshly-prepared L-Trp after preincubation for 30 min with 1 mM 1MetDTrp (**A**: 1MetDTrp+L-Trp) or 30 μM INCB024360 (**B**: INCB+L-Trp) to arterial rings from normal term placentas in comparison with the vehicle control (control). The data show the outcome of 3 independent experiments (n = 3); measurements were done at least in 2 arterial rings. Results are given as mean ± SD. **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. (Dunnett´s test)

4.7. The role of the endothelium in the relaxing effect of a stored L-Trp solution on stimulated arteries of the chorionic plate of normal placentas

4.7.1. Establishing a denudation method for human chorionic plate placental arteries - Effect on the vascular tone

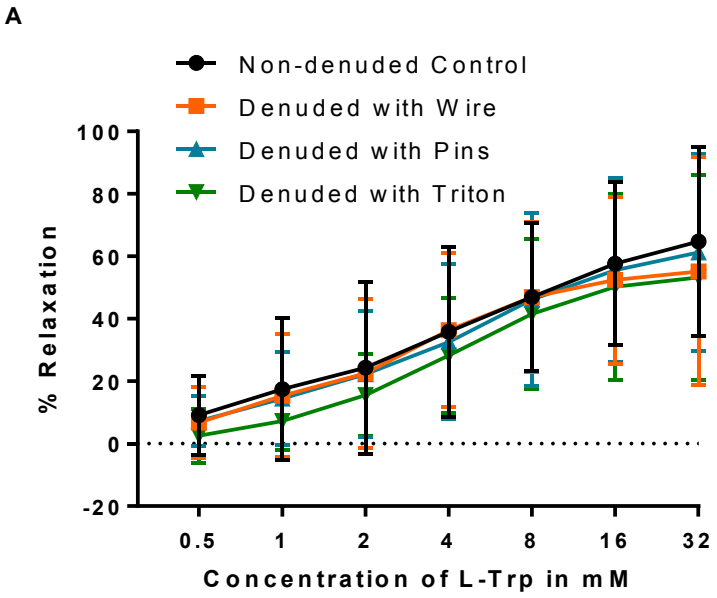
In order to determine the potential role of the endothelium in the IDO1-induced relaxation, we explore an efficient and reliable method of denudation for human chorionic arteries from normal placentas.

4.7.1.1. Comparison among several denudation methods on healthy unstimulated placental arteries from the chorionic plate

Removal of the endothelium can be accomplished mechanically (137-139), chemically (137, 140-142) or pharmacologically (143, 144). The mechanical method may be a bit traumatic for underlying smooth muscle layer, taking the risk of damaging it. There are some chemical agents (i.e. Triton X-100) that after the application, endothelial cell layer detaches. Pharmacologically, endothelial cells can be stopped by avoiding the activation of the cell layer. However, a whole variety of enzymes must be blocked depending on the research question (NOS, COX and others...) in order to remove endothelial-derived products that may influence the preparation. Therefore, we excluded a pharmacological method and we focused on (i) mechanical denudation by rubbing the lumen of the arteries against a 40 μM tungsten wire or (ii) against the pin supports within each chamber and (iii) chemical denudation by perfusing the arteries with 0.1% of Triton X-100.

Increasing concentrations of stored L-Trp solution were applied to normal placental arterial rings (Fig.15.A). The amount of relaxation of the vascular tension was measured by myography in comparison to the vehicle control. No significant differences were observed in between the different denudation methods: denudation with wire ($P = 0.970$), denudation with pins ($P = 0.851$) or denudation

with Triton X-100 (P = 0.610) in comparison with the control in response to stored L-Trp. Hence, all methods seemed equally efficient in terms of responsiveness to stored L-Trp for normal placental arteries. However, response in terms of strength (mN) was diminished for all denudation types in all experiments (Fig. 15.B), indicating that the method employed is still too aggressive to maintain an intact functionality of the placental arteries.



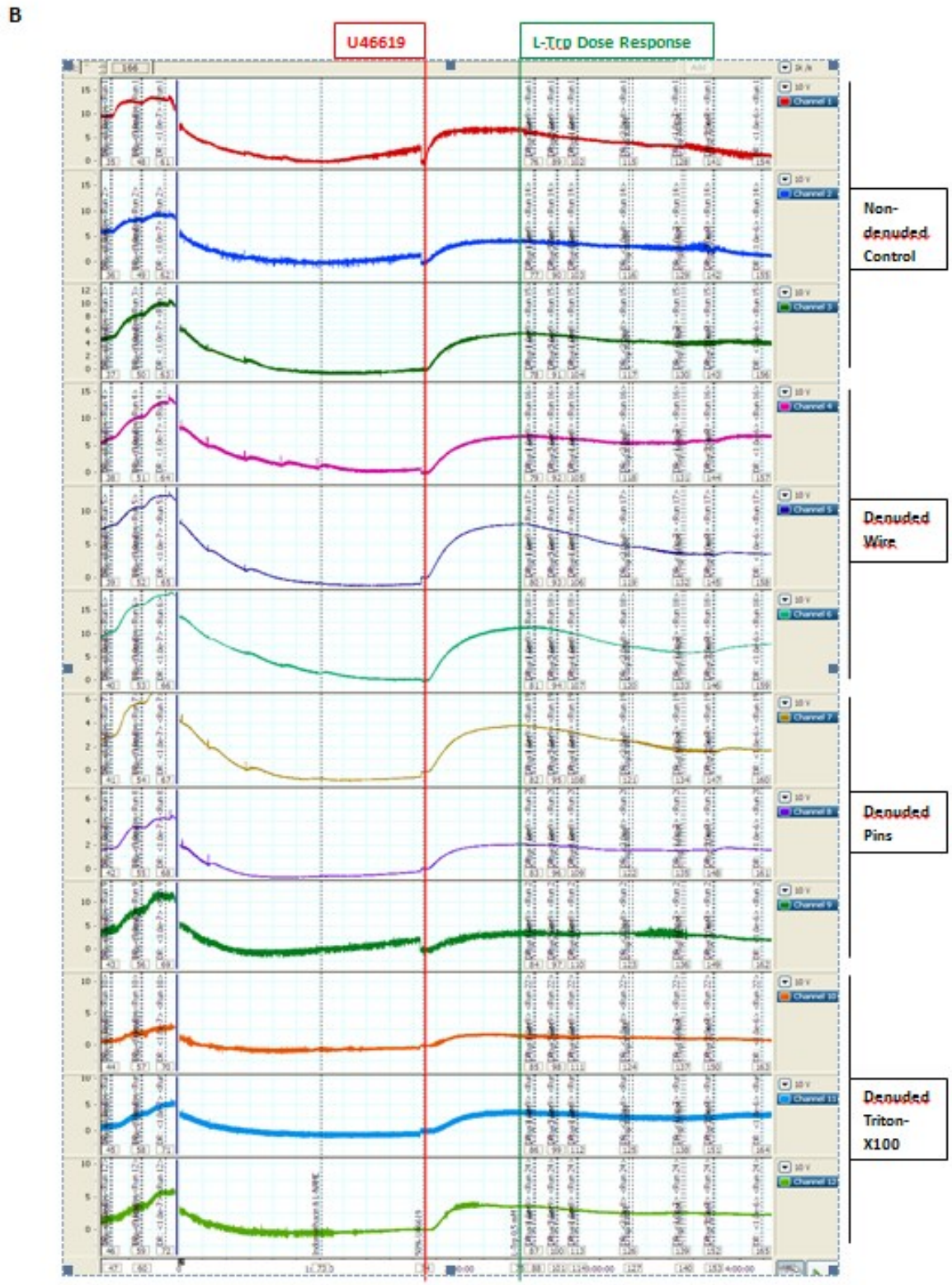


Figure 15. Effect of several denudation methods on stored L-Trp relaxation of chorionic plate arteries from normal placentas. (A) Percentage relaxation of the vascular tension achieved (y axis) in relationship to U46619-induced contraction after applying increasing concentrations of stored L-Trp solution (x axis; 0.5, 1, 2, 4, 8, 16 and 32 mM) to non-stimulated arterial rings from

normal placentas. No significant differences were observed in between the different denudation methods: denudation with wire (P = 0.970), denudation with pins (P = 0.851) or denudation with Triton X-100 (P = 0.610) in comparison with the control. Data is represented in a logarithmic 2 scale. The data show the outcome of 3 independent experiments (n = 3); measurements at each concentration were done at least in 2 arterial rings. Results are given as mean ± SD. **(B)** Original tracing (y axis: force in mN; x axis: course of time) obtained in one experiment with the ADInstruments software from the myograph illustrating tension stability of the response from placental vessels after the different denudation treatments. (Linear regression model)

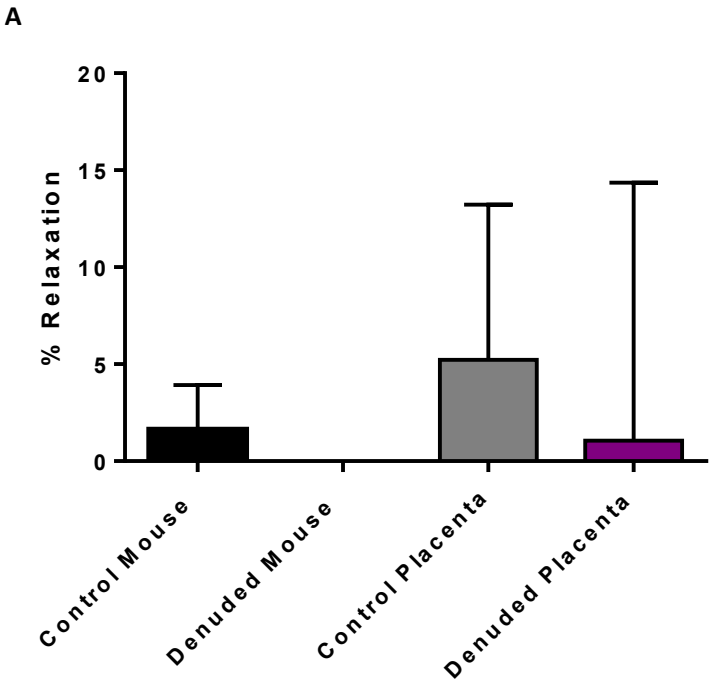
4.7.1.2. Optimizing denudation - Effect of Triton X-100 in human placental arteries vs mouse aortic arteries

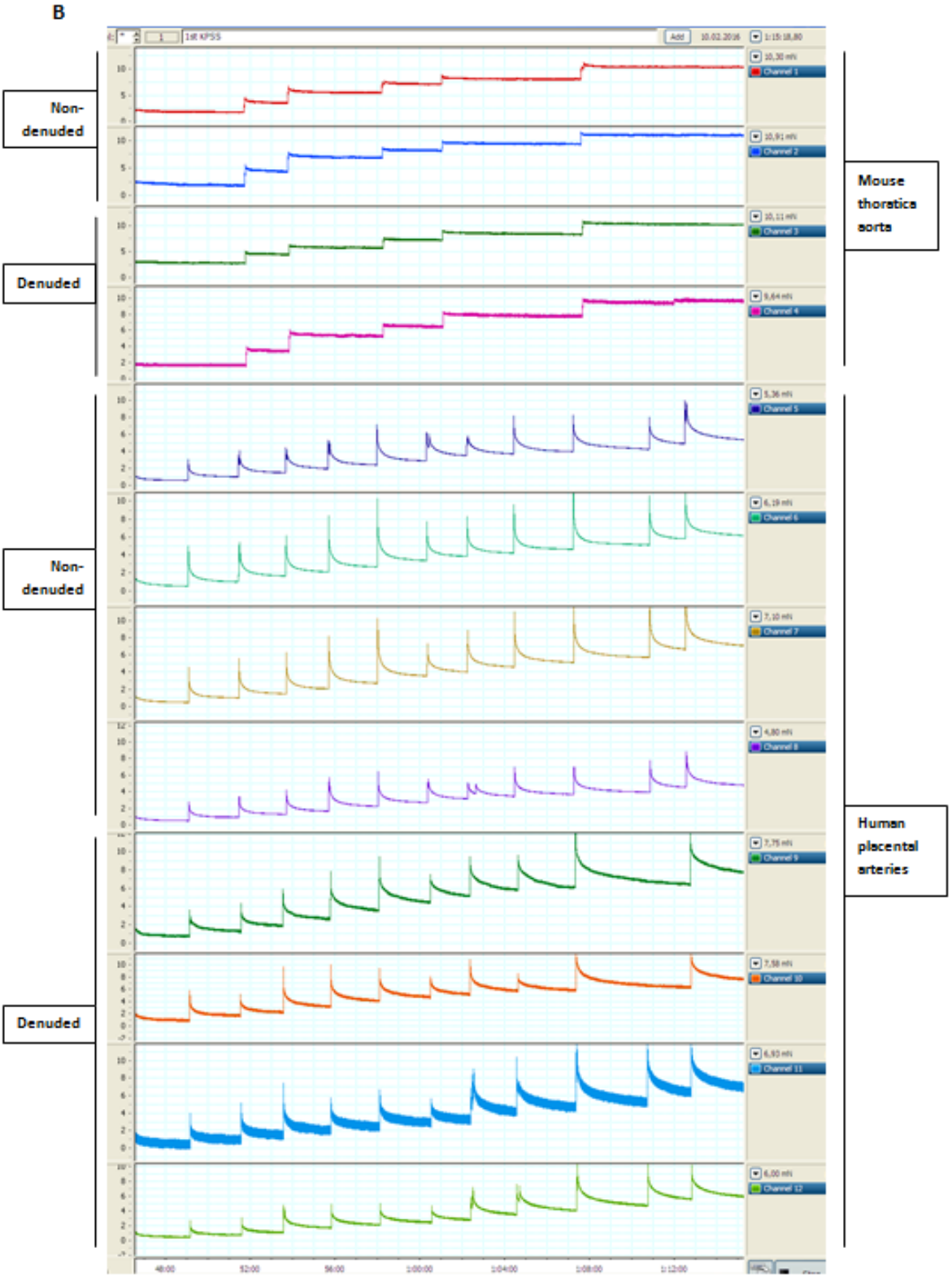
As chemical denudation can be better standardize than mechanical denudation, we focused on optimizing denudation by applying Triton X-100. Factors such as the amount or the time of perfusion with Triton X-100 can be precisely measured and better controlled than for instance the force applied while rubbing the lumen of the arteries with mechanical methods.

To evaluate how aggressive is the denudation with Triton X-100 not only in human placental arteries, and also to assess how human placental arteries perform in regards to a different species, we perform within the same experiment a comparison of the response to all compounds applied during a normal myography experiment between human placental arteries and thoracic aortas from mice (C57BL/6 mouse strain; male, 12 weeks old). Different relaxation percentages were obtained after applying 8 mM of L-Trp (Fig.16) to endothelium-denuded (using Triton X-100) and non-endothelium-denuded vessels from human placental arteries and mouse thoracic aorta.

The amount of relaxation was measured following the application of 8 mM of stored L-Trp to all arterial rings in the presence (non-denuded) or absence (denuded) of endothelium for mouse or human placental vessels. Addition of stored L-Trp solution appeared to have a weaker effect in non-denuded mouse vessels when compared to non-denuded placental vessels. More importantly, the denudation process using Triton X-100 completely abrogated any response of

mouse aorta (Fig. 16.C and D) and diminished in a strong manner the response in human placental arteries. Moreover, the response to the different agonist was reduced when the vessels were perfused with Triton X-100 not even reaching 2 mN (see y axis in figures C and D) in most of the cases (see inclusion/exclusion criteria in appendix). Thereby, Triton X-100 showed to give inconsistent results in myography and to contribute to the vessel damage independently of the species employed, indicating that the denudation process requires more optimization.

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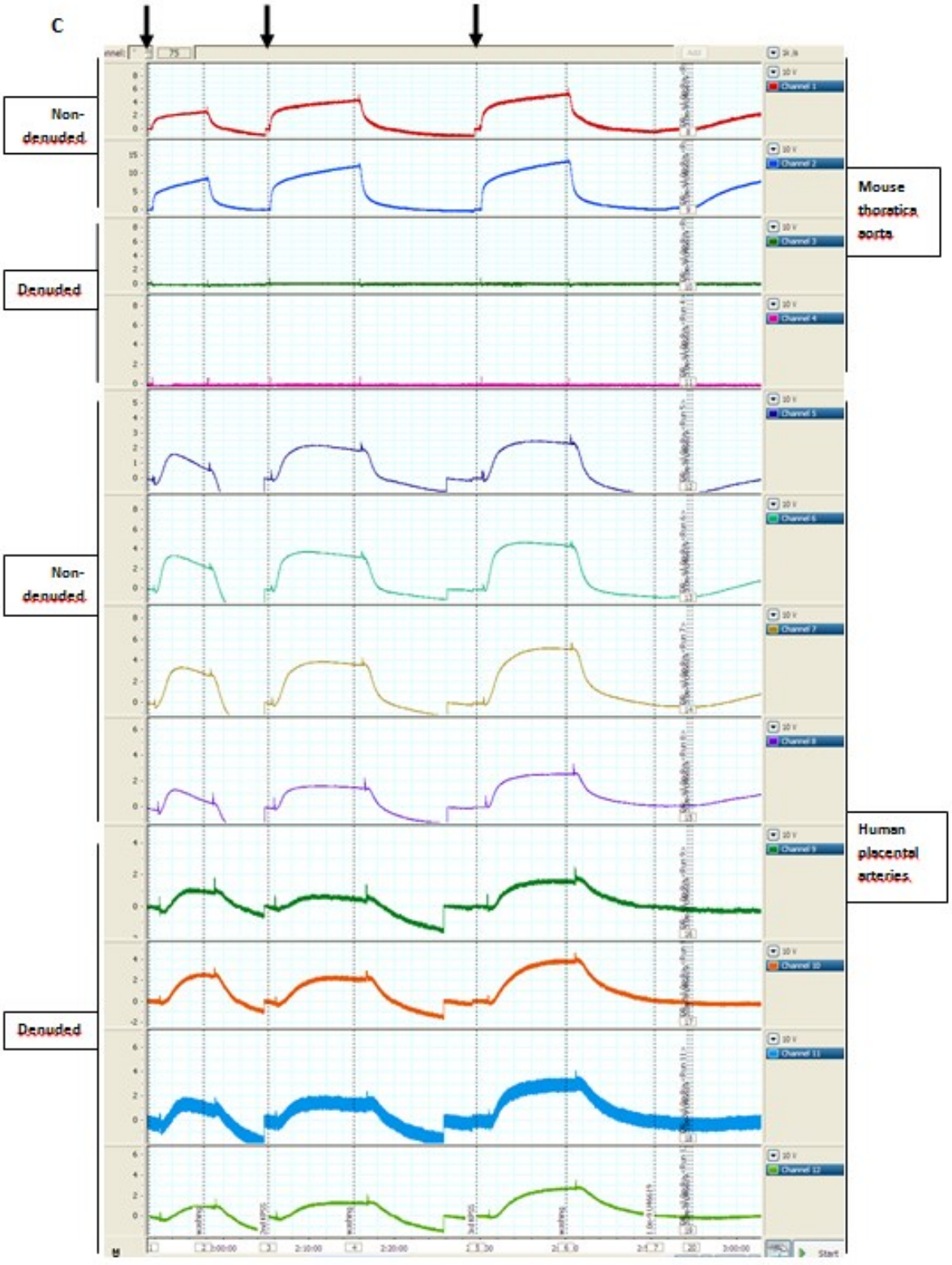
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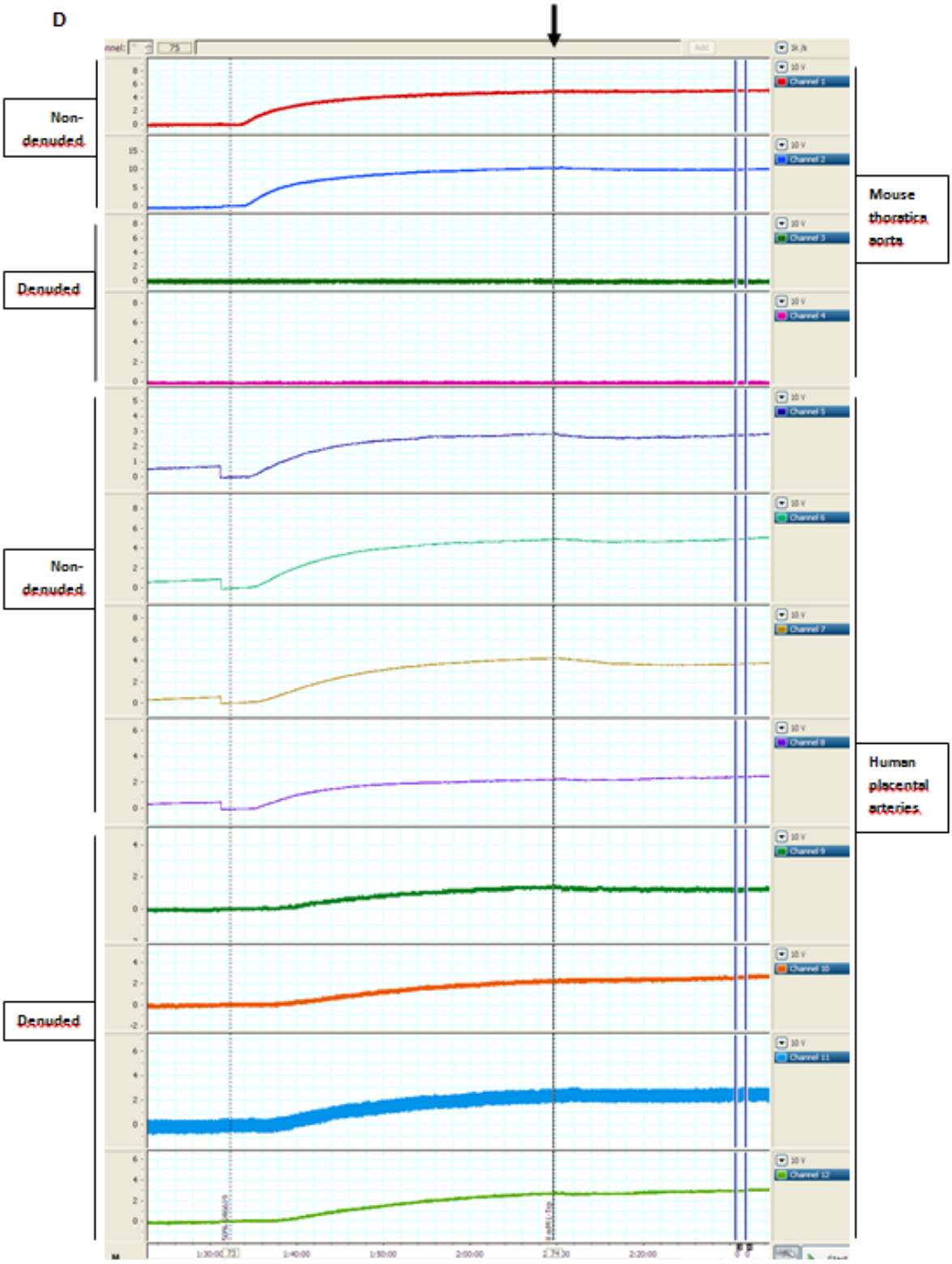


Figure 16. Effect of Triton X-100 in human placental arteries and mouse aortic arteries. (A) Percentage relaxation of the vascular tension achieved (y axis) in relationship to U46619-induced contraction after applying 8 mM of stored L-Trp solution (x axis) to unstimulated arterial rings from normal placentas. Data is represented in a logarithmic 2 scale. The data show the outcome of 1

independent experiments (n = 1); measurements at each concentration were done in at least 3 arterial rings. Results are given as mean ± SD. **(B)** Original tracing (y axis: force in mN; x axis: course of time) obtained in one experiment with the ADInstruments software from the myograph illustrating tension stability of the response from placental and mouse vessels after giving the right passive tension through the LCP method to the different denudation treatments. **(C)** Original tracing (y axis: force in mN; x axis: course of time) obtained in one experiment with the ADInstruments software from the myograph illustrating tension development to KPSS (arrows) from placental and mouse vessels after the different denudation treatments. **(D)** Original tracing (y axis: force in mN; x axis: course of time) obtained in one experiment with the ADInstruments software from the myograph illustrating tension development to stored L-Trp (arrow) from placental and mouse vessels after the different denudation treatments. Pictures are representative of 1 independent experiment (n = 1).

4.7.1.3. Optimizing denudation - Effect of Triton-X100 in human placental arteries of the chorionic plate

To evaluate how aggressive is the denudation with Triton X-100, and also to assess how functionality of stimulated human placental arteries is affected by the treatment, we perform a comparison of the response to KPSS and to the thromboxane analogue U46619 applied during a regular myography experiment between non-endothelium-denuded and endothelium-denuded arteries. Given the previous results with the denudation process, perfusion time and volume with Triton X-100 was reduced and handling of the vessel was improved.

Functionality of non-denuded and denuded stimulated vessel rings was assessed by its response to different agonist such as KPSS or the thromboxane analogue U46619 (Fig.17). The functionality obtained was evaluated and compared to the control (endothelium-denuded arteries) and also to the response with previous experiments. Moreover, functionality was matched whether the vessels were meeting the inclusion/exclusion criteria previously established for a normal experiment. The denudation process using Triton X-100 (Fig. 17; denuded vessels) diminished in a strong manner the response to the different agonists in human placental arteries.

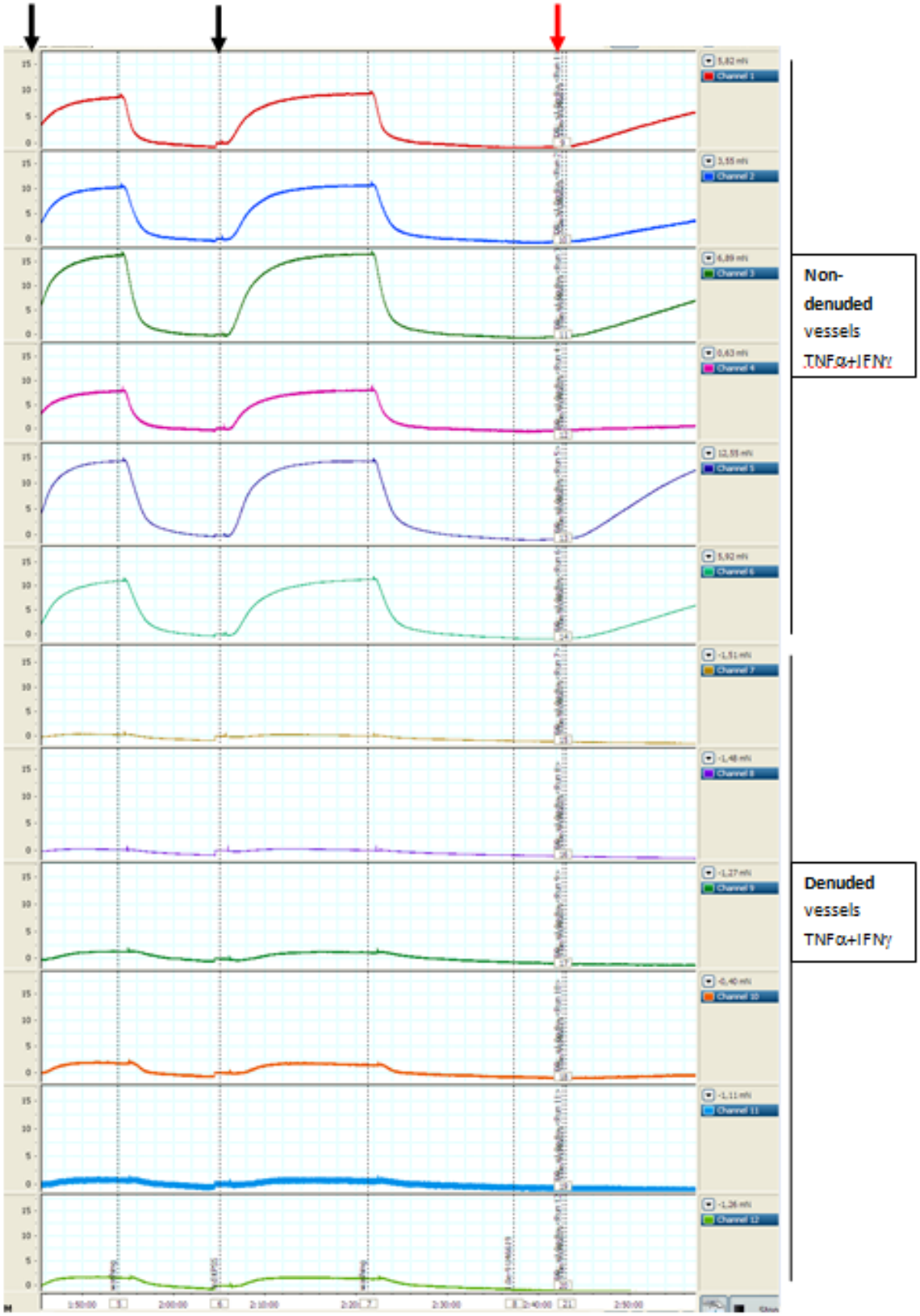


Figure 17. Non-optimized denudation - Effect of Triton X-100 in human placental arteries. Original tracing (y axis: force in mN; x axis: course of time) obtained in one experiment with the ADInstruments software from the myograph illustrating tension development to KPSS (black

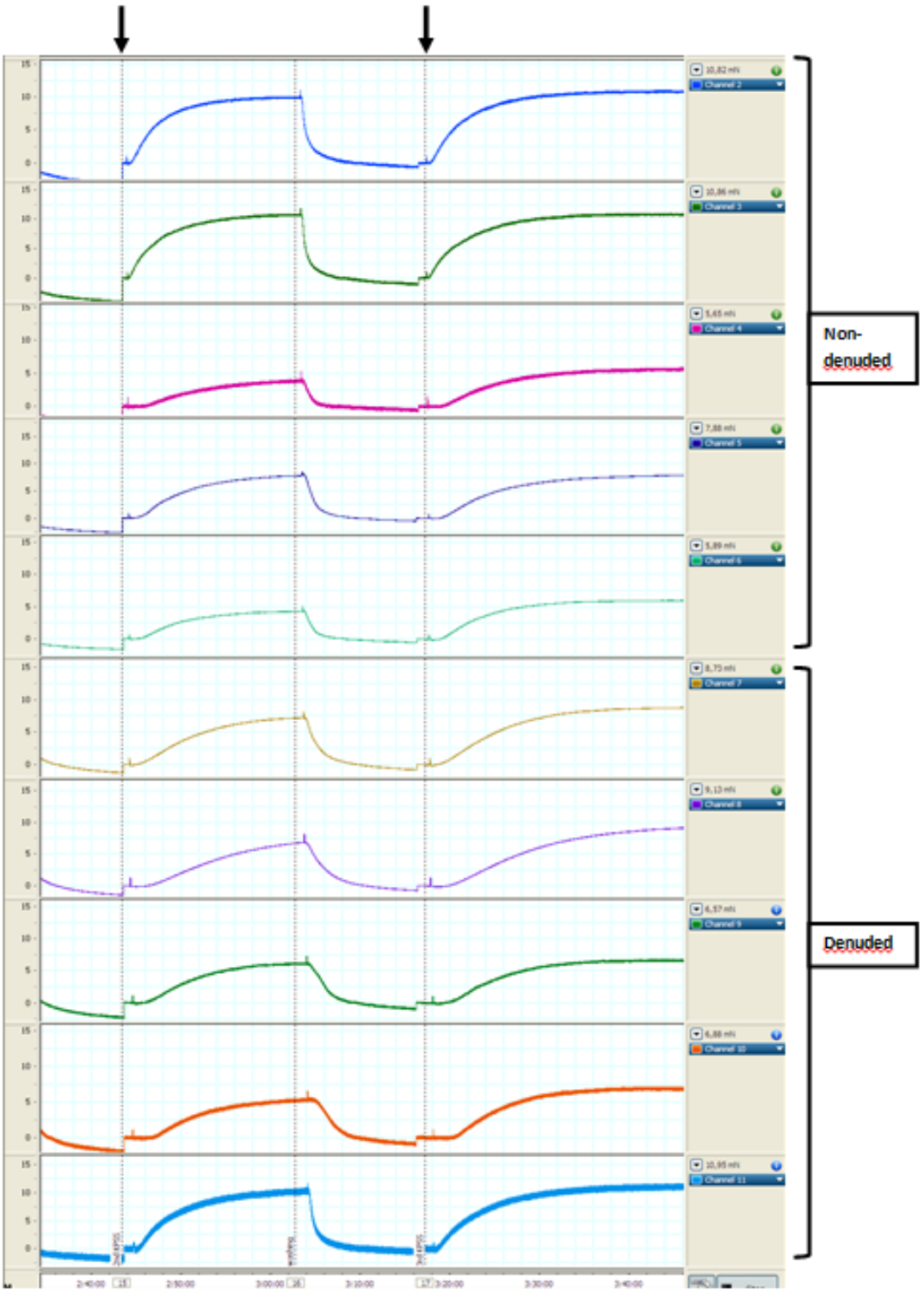
arrows) or to the thromboxane analogue U46619 (red arrow; $1e^{-9}$ - $1e^{-7}$ M) from placental arteries. The picture is representative of 4 independent experiments (n = 4).

4.7.1.4. Optimized denudation - Effect of Triton-X100 in human placental arteries of the chorionic plate

We pursued to evaluate how detrimental is the denudation with Triton X-100 for functionality of stimulated human placental arteries. As mentioned above, we perform a comparison of the response to KPSS and to U46619 between non-endothelium-denuded and endothelium-denuded arteries. Given all the previous results with the denudation process, perfusion volume with Triton X-100 was even reduced and perfusion time was increased simultaneously as the pressure applied was lowered. Furthermore, a washing step after perfusing with Triton X-100 was introduced in order to remove any possible remaining of this detergent in the lumen of the arteries during the overnight incubation time.

Functionality of non-denuded and denuded stimulated vessel rings was assessed by its response to different the agonist: KPSS and the thromboxane analogue U46619 (Fig.18). The functionality obtained was evaluated and compared to the control (non-endothelium-denuded arteries) and also to the response with previous experiments. Moreover, functionality was matched whether the vessels were meeting the inclusion/exclusion criteria previously established for a normal experiment. The denudation process using Triton X-100 (Fig. 18; denuded vessels) did not modify the response previously obtained to the different agonists in stimulated human placental arteries (see optimized detailed protocol in Appendix).

A



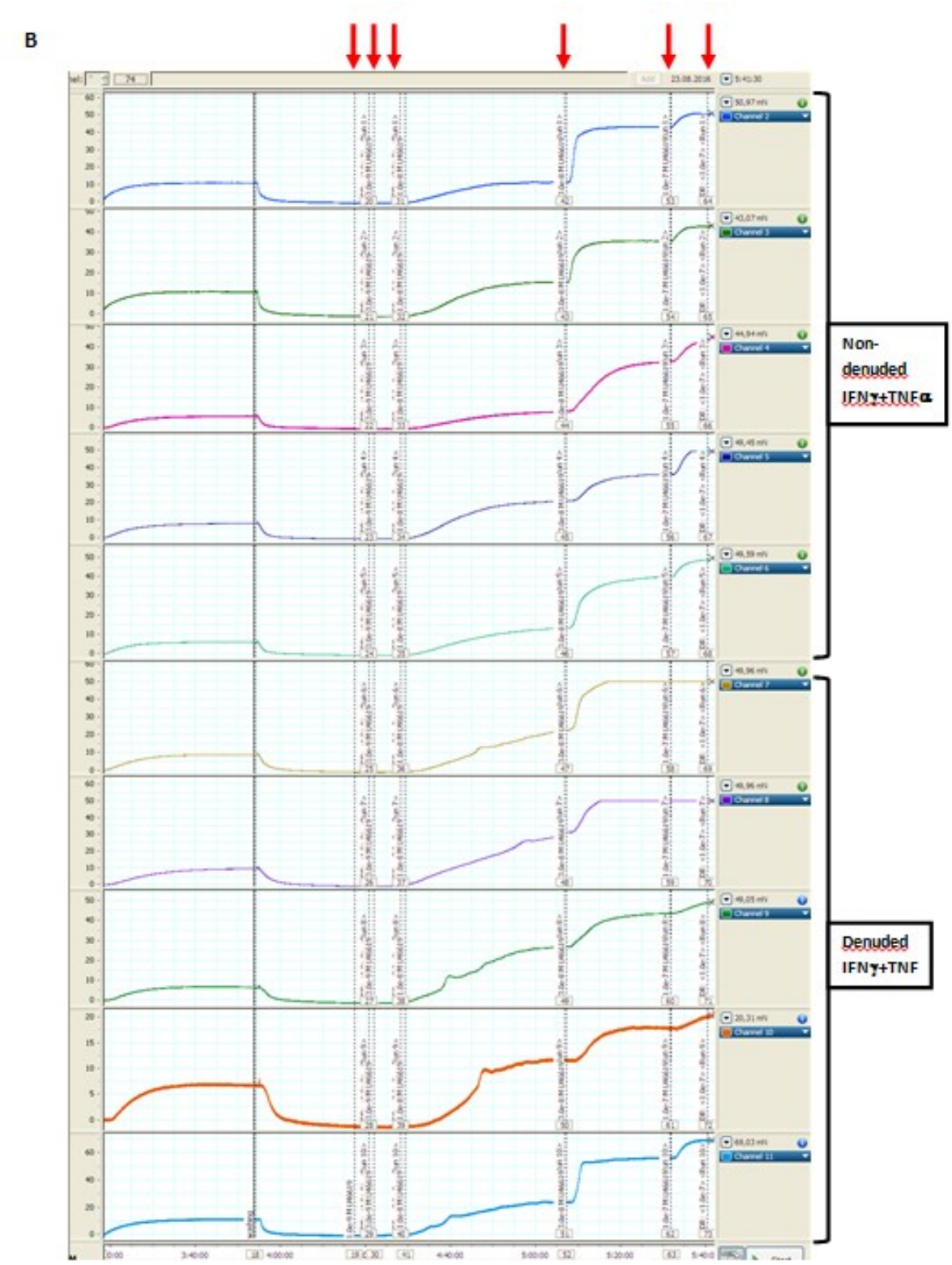


Figure 18. Optimized denudation - Effect of Triton X-100 in human placental arteries. Original tracing (y axis: force in mN; x axis: course of time) obtained in one experiment with the ADInstruments software from the myograph illustrating tension development to KPSS (**A**: black

arrows) or to the thromboxane analogue U46619 (**B**: red arrows; $1e^{-9}$ - $1e^{-7}$ M) from placental arteries. The picture is representative of 4 independent experiments (n = 4).

4.7.1.5. Verifying the success of the denudation process in human chorionic plate placental arteries

In order to confirm whether the endothelium is present and still functional in a sample, some endothelium-dependent molecules can be utilized. These molecules act directly on the endothelial cell layer producing vasoactive substances such as Nitric Oxide (NO) that will stimulate cyclic GMP, prostaglandins which trigger cAMP-dependent hyperpolarization or even endothelial-derived hyperpolarizing factors (EDHFs) that via K^+ channels or Na^+/K^+-ATPase activate vascular smooth muscle cells that at the end elicit vasodilation (145). In addition, immunohistochemistry may provide a more challenging approach to verify the presence of the endothelium on the samples.

Bradykinin ($1e^{-9}$M to $1e^{-7}$M), Histamine ($1e^{-9}$M to $1e^{-7}$M) and Acetylcholine ($1e^{-9}$M to $1e^{-7}$M) were applied to U46619-preconstricted arteries as endothelium-dependent vasodilators. No vasodilatory response was detected with any of them at any dose applied (data not shown). Moreover, calcium ionophore was also employed giving inconsistent results (Roland Stocker and co-workers - unpublished data). Thereafter, immunohistochemistry was performed in paraffin sections from normal denuded and non-denuded placental arteries (Fig. 19) against the endothelium marker CD34 II. In all cases after the denudation process the endothelium was successfully removed. In few cases the endothelium was not present in non-denuded arteries.

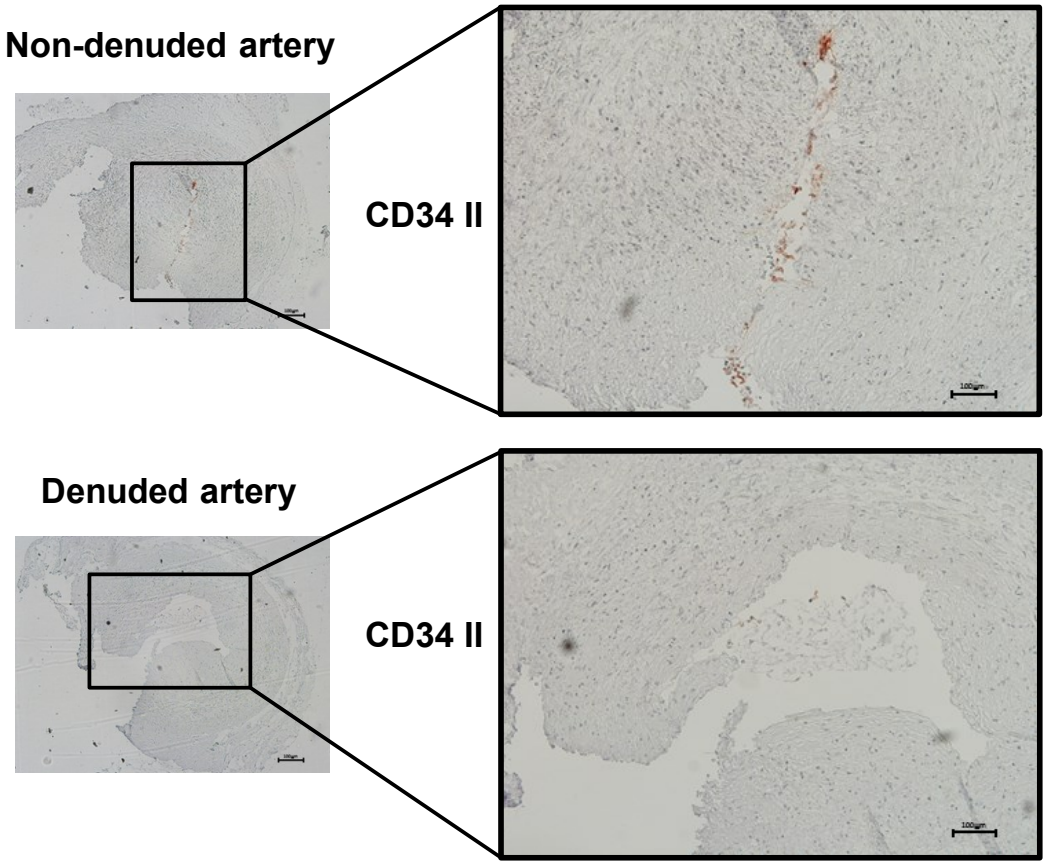


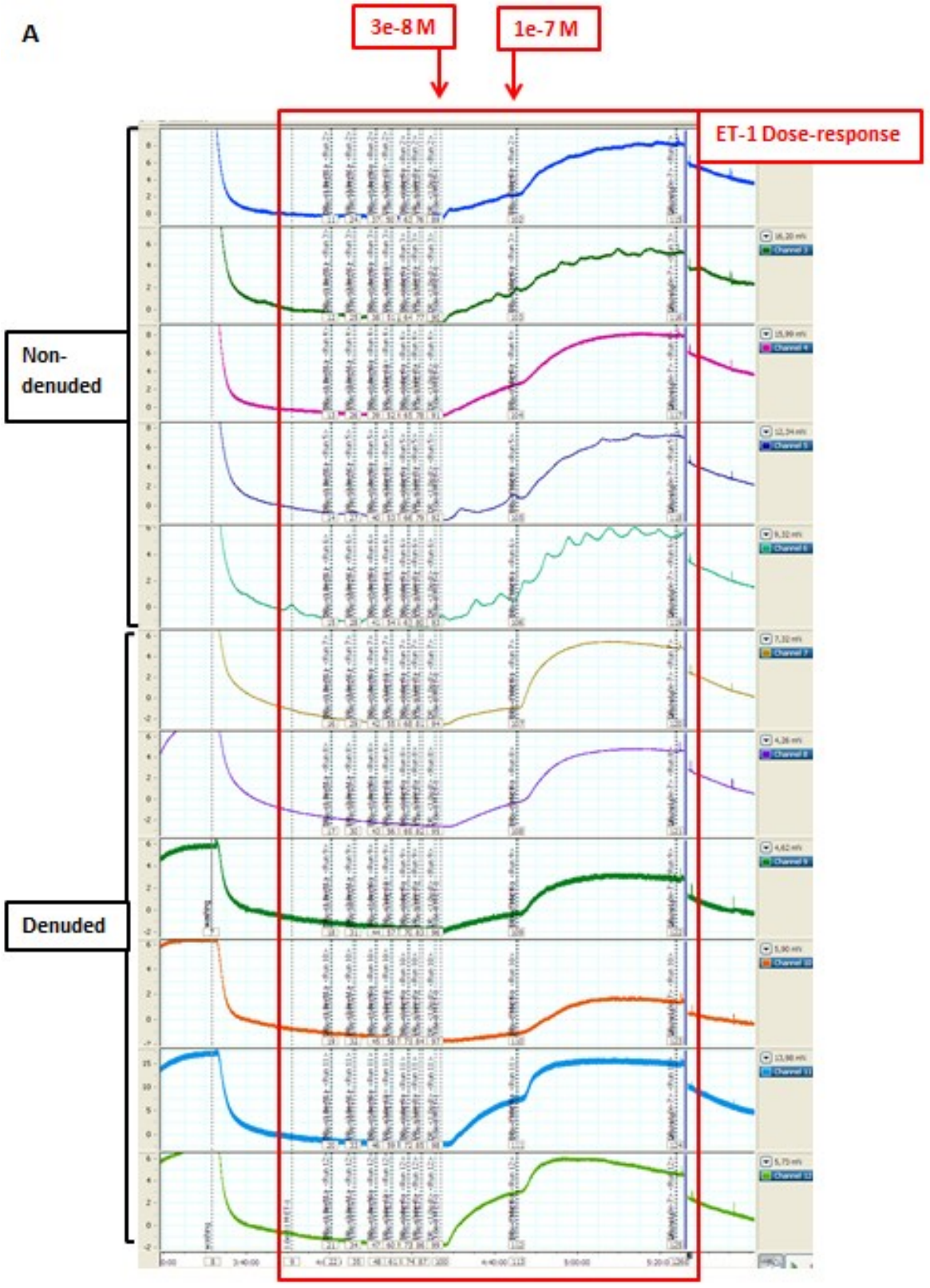
Figure 19. Immunohistochemical staining of paraffin sections of CD34 II from non-denuded and denuded arteries. Shown are selected arterial sections from normal placentas. Positive staining for CD34 II shown as red staining (non-denuded artery; upper picture). The picture is representative of different arterial sections from 3 independent experiments (n = 3). The scale bars represent 100 μm. Figure taken and modified from (126).

4.7.2. Response to several contractive agonists

To investigate the response of the vessel as a measure of relaxation in regards to a pre-constricted vessel and to completely assure that the vascular smooth muscle layer is contracting properly, a suitable agonist must be selected for the specie and the tissue type.

4.7.2.1. Endothelin-1 (ET-1)

Original tracing obtained after applying accumulative concentrations of ET-1 ($1e^{-11}$ M to $1e^{-7}$ M) to IFNγ/TNFα-stimulated (Fig. 20.A) or non-stimulated (Fig. 20.B) arterial rings from normal term placentas in the presence (non-denuded) or absence (denuded) of endothelium. The response observed was weak (most of the cases not stronger than 4 mN) and unusual (not stable response), being even more atypical for non-denuded arteries (phenomenon of vasomotion observed specially in stimulated arteries). Either the 2 last highest doses ($3e^{-8}$ M and $1e^{-7}$ M) or solely the highest dose ($1e^{-7}$ M) was active producing any response. Moreover, ET-1 added at a second time was less effective as when added for the first time showing even a weaker contraction (data not shown).


A
3e-8 M
1e-7 M
ET-1 Dose-response
Non-
denuded
Denuded

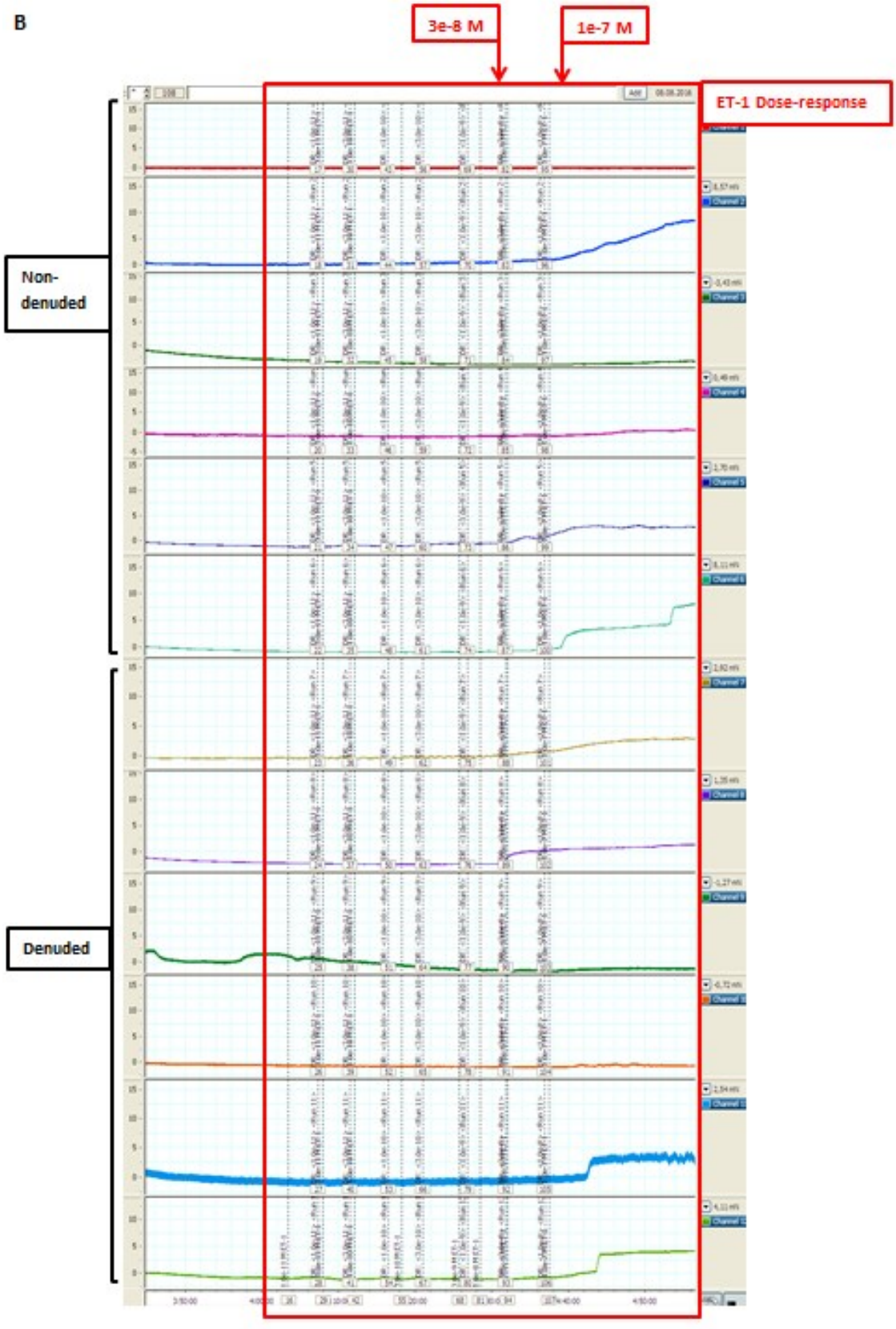


Figure 20. Response to ET-1 of stimulated and non-stimulated human placental arteries. Original tracing (y axis: force in mN; x axis: course of time) obtained in one experiment with the

ADInstruments software from the myograph illustrating tension development to the ET-1 dose-response (red square; $1e^{-11}$ M to $1e^{-7}$ M) in the presence or absence of endothelium for IFNγ/TNFα-stimulated **(A)** or unstimulated **(B)** placental arteries. Each image is representative of 1 different experiment (n = 2).

4.7.2.2. Norepinephrine (NE)

Original tracing obtained after applying a sole dose of NE (10 μM) to stimulated arterial rings from normal term placentas in the presence (non-denuded) or absence (denuded) of endothelium (Fig. 21). The response observed was remarkably weak (almost undetectable) regardless the presence or absence of the endothelium. In some cases (6 out of 11) there was no response at all.

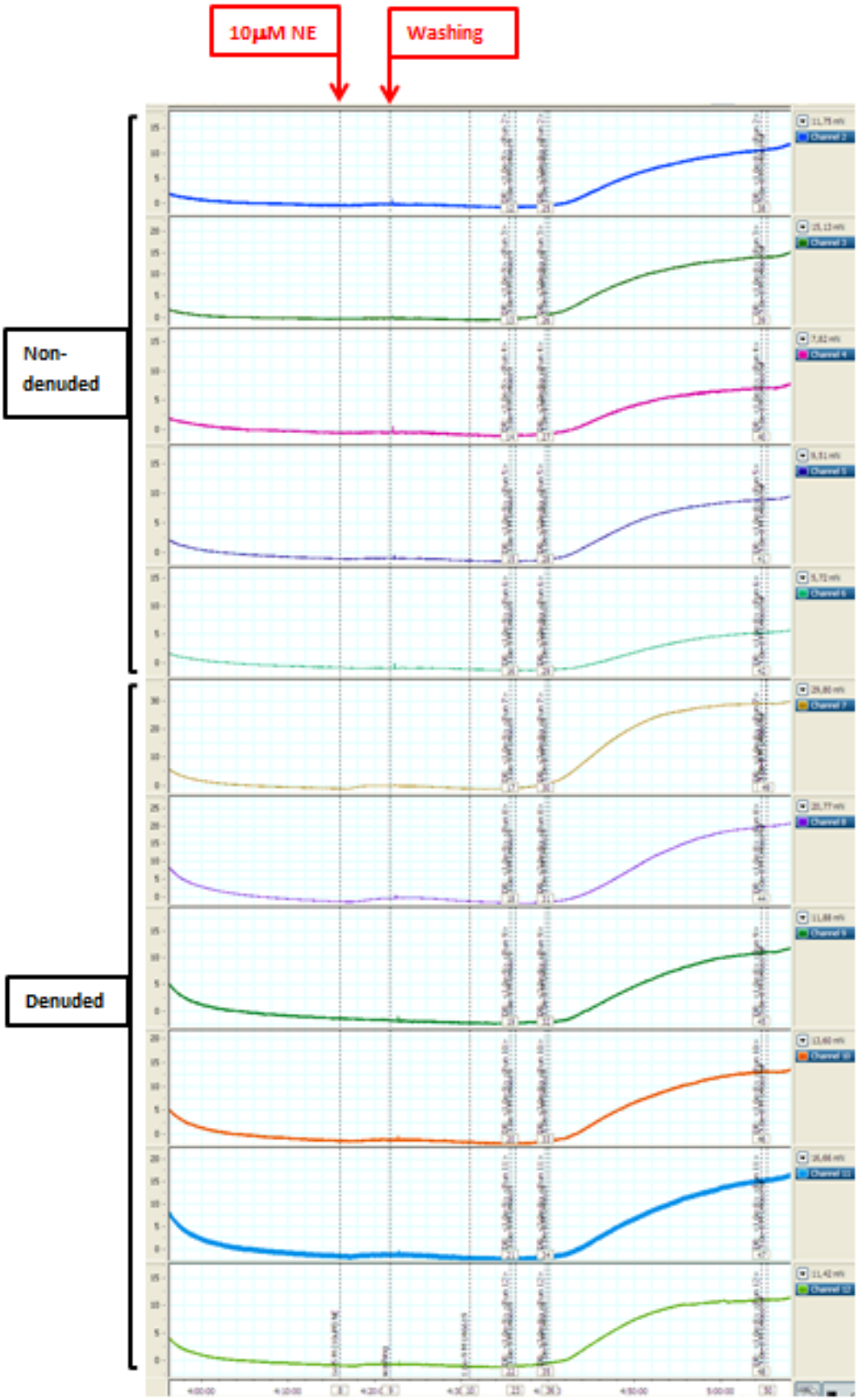
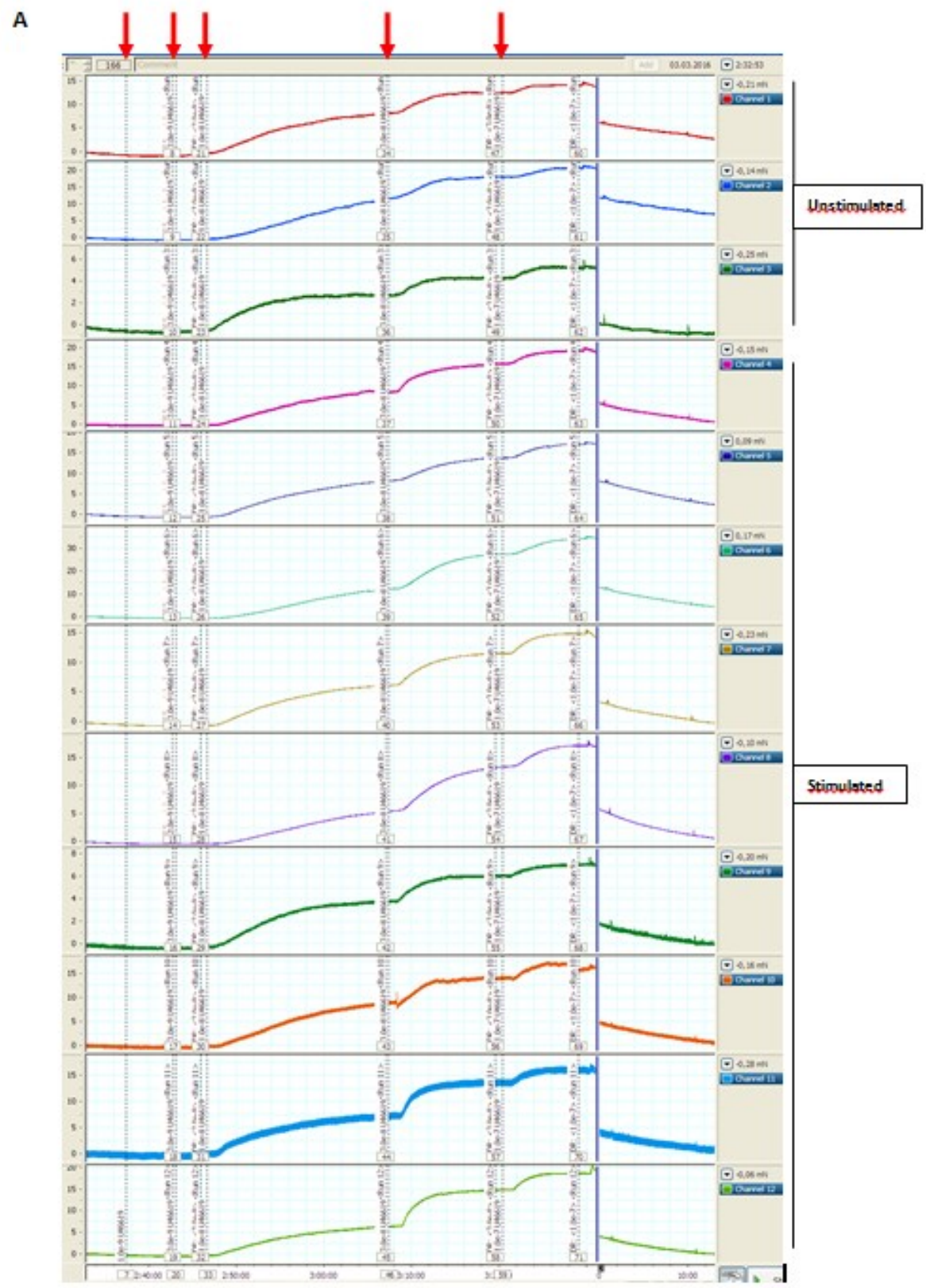


Figure 21. Response to NE of stimulated human placental arteries. Original tracing (y axis: force in mN; x axis: course of time) obtained in one experiment with the ADInstruments software

from the myograph illustrating tension development to a single dose of NE (red arrow; 10 μM NE) in the presence or absence of endothelium for stimulated placental arteries. The image is representative of 1 experiment (n = 1).

4.7.2.3. Thromboxane analogue U46619 (U46619)

Original tracing obtained after applying U46619 in a dose-response manner (red arrows; $1e^{-9}$M to $1e^{-7}$M) to IFNγ/TNFα-stimulated (Fig. 22.A) and non-stimulated (Fig. 22.B) arterial rings from normal term placentas in the presence (non-denuded) or absence (denuded) of endothelium. The response observed was always sustained, consistent and reproducible, and generally strong (reaching at least 10 mN) regardless the presence or absence of: (i) endothelium and (ii) IFNγ/TNFα.


A
Unstimulated
Stimulated

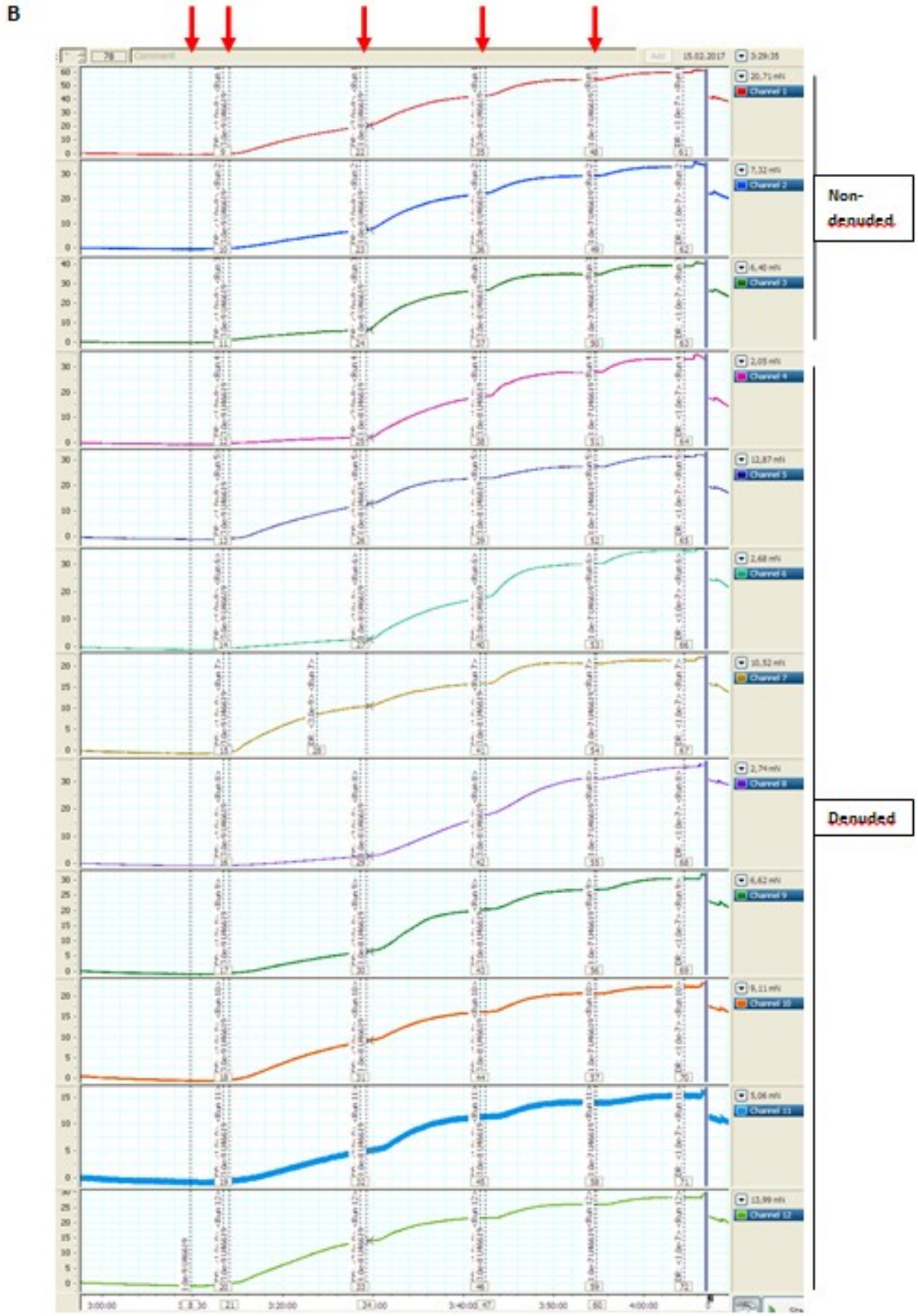


Figure 22. Response to U46619 of stimulated and non-stimulated human placental arteries. Original tracing (y axis: force in mN; x axis: course of time) obtained in one experiment with the ADInstruments software from the myograph illustrating tension development to the U46619 dose-

response (red arrows; $1e^{-9}$ M to $1e^{-7}$ M) in the presence (non-denuded) or absence (denuded) of endothelium for IFNγ/TNFα-stimulated **(A)** or non-stimulated **(B)** placental arteries. Each image is representative of at least 8 different experiment (n = 16).

4.7.3. Contribution of the endothelium to the relaxing effect of freshly-prepared L-Trp solution on denuded stimulated placental arteries

To test the role of endothelium in the relaxing effect observed by adding freshly-prepared L-Trp solution, lumen of placental arteries was perfused with 0.1% Triton X-100 (Fig. 23.A). Relaxation of the vascular tension was measured following the application of increasing doses of freshly-prepared L-Trp (Denuded L-Trp) or vehicle control (Denuded Control) in stimulated non-denuded and stimulated denuded placental arterial rings. Absence of endothelium significantly promote relaxation when compared both denuded and non-denuded controls as the linear coefficient differ ($P = 0.034$). Furthermore, despite removal of the endothelium, we still found a significant effect of freshly-prepared L-Trp in comparison to the vehicle control in the linear ($P < 0.001$) as well as in the quadratic ($P = 0.014$) component.

To elucidate whether IDO1 has a role in the relaxing effect observed on denuded vessel, we incubated denuded stimulated rings with 1MetDTrp (Fig. 23.B). Relaxation of the vascular tension was measured following the application of 8 mM freshly-prepared L-Trp solution in the presence (Denuded 1MetDTrp+L-Trp) or absence (Denuded L-Trp) of 1 mM 1MetDTrp compared with a non-denuded and denuded control in stimulated rings. Treatment with L-Trp significantly relaxed the denuded arterial rings ($P = 0.006$). Nevertheless, preincubation with 1MetDTrp did not significantly reduce the relaxing effect of L-Trp when compared with denuded control ($P = 0.076$) and with denuded L-Trp ($P = 0.094$). However, subsequent analysis with a higher number of placentas and replicates (planned at a statistical power of 90% with the calculation based on the initial set of experiments) revealed that pre-incubation with 1MetDTrp diminished L-Trp-induced relaxation ($P = 0.0259$), albeit to a small extent only (Fig. 23C). Hence, our data proves the existence of an IDO1-dependent pathway active when arteries are stimulated and free of endothelium, and additionally, the existence of a

supplementary component IDO1-independent responsible for part of the L-Trp-induced relaxation in stimulated denuded arteries.

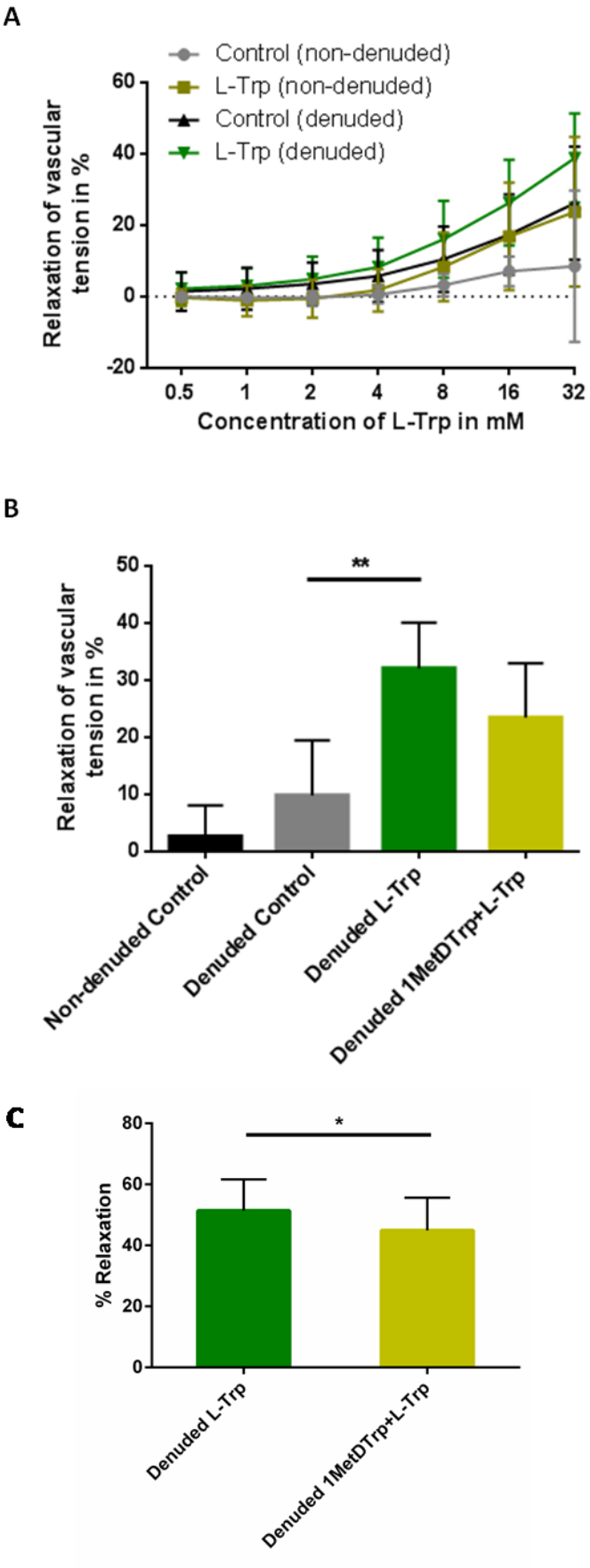

A
Control (non-denuded)
L-Trp (non-denuded)
Control (denuded)
L-Trp (denuded)
Relaxation of vascular tension in %
60
40
20
0
-20
0.5
1
2
4
8
16
32
Concentration of L-Trp in mM
B
Relaxation of vascular tension in %
50
40
30
20
10
0
**
Non-denuded Control
Denuded Control
Denuded L-Trp
Denuded 1MetDTrp+L-Trp
C
% Relaxation
80
60
40
20
0
*
Denuded L-Trp
Denuded 1MetDTrp+L-Trp

Figure 23. The effect of freshly-prepared L-Trp solution on human stimulated placental arteries is not exclusively endothelium-dependent. (A) Percentage relaxation of the vascular tension achieved (y axis) in relationship to U46619-induced contraction after applying increasing concentrations of freshly-prepared L-Trp to stimulated and non-stimulated arterial rings from normal placentas in comparison with the non-denuded and denuded controls. There is a significant difference between the two control groups (denuded versus non-denuded; P = 0.034). Reactivity of endothelium-denuded stimulated arterial rings to L-Trp differs from its control group for the linear (P < 0.001) and the quadratic term (P = 0.014). The data show the outcome of 3 independent experiments (n = 3); measurements were done at least in 2 arterial rings. **(B)** Percentage relaxation of the vascular tension achieved (y axis) in relationship to U46619-induced contraction after applying 8 mM L-Trp (L-Trp) or 8 mM L-Trp after preincubation for 30 min with 1 mM 1MetDTrp (1MetDTrp+L-Trp) to stimulated arterial rings from normal placentas in comparison with the non-denuded and denuded controls. The data show the outcome of 3 independent experiments (n = 3); measurements were done at least in 2 arterial rings. **(C)** Percentage relaxation of the vascular tension achieved (y axis) of further experiments (planned at a statistical power of 90%) after applying 8 mM L-Trp (L-Trp) or 8 mM L-Trp after preincubation for 30 min with 1 mM 1MetDTrp (1MetDTrp+L-Trp) to stimulated arterial rings from normal placentas. The data show the outcome of 4 independent experiments (n = 4); measurements were done in 6 arterial rings. Results are given as mean ± SD. *P ≤ 0.05; **P ≤ 0.01. (A: Linear regression model; B: Wilcoxon test with Bonferroni post-hoc test; C: t-test). Figure taken and modified from (126).

4.8. Effect of cytokine stimulation on IDO1 expression on the smooth muscle cell layer of arteries of the chorionic plate from normal placentas

Immunohistochemistry of a cytokine-stimulated artery ruled out a possible expression of IDO1 in smooth muscle cells that may enhance or promote a stronger L-Trp relaxing effect from a non-endothelial origin (Fig. 24). However, a few isolated cells in the vessel wall (possibly macrophages) stained positive for IDO1 and the macrophage marker CD163.

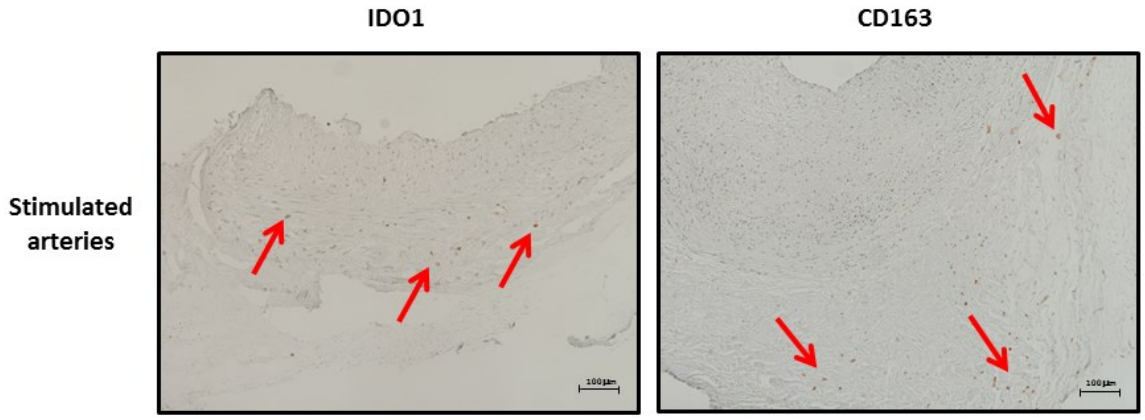


Figure 24. Immunohistochemical staining of IDO1 and CD163 in the smooth muscle cell layer of stimulated arteries from normal pregnancies. Arrows indicate either IDO1- or CD163-positive cells in cytokine-stimulated arteries from the chorionic plate of a normal placenta. The smooth muscle layer is on the whole IDO1- or CD163-negative staining. The scale bar represents 100 μm. Figure taken and modified from (126).

4.9. Effect of freshly-prepared L-Trp solution on the vessel back pressure of placental cotyledons

In order to make sure that the relaxing effect observe by L-Trp is not exclusively due to an upregulation of IDO1 and as chorionic endothelial IDO1 is preferentially located in the microvasculature rather than in big chorionic plate arteries, we then studied the fetal vessel back pressure differences as a measure for relaxation of the vascular tension in single cotyledons from normal term placentas by *ex-vivo* placental perfusion. After a stabilization period (stabilization phase), 8 mM of L-Trp was added to the perfusion medium (Fig. 25.A – Non-preconstricted). Perfusion with 8 mM L-Trp from 5 different placentas showed a median decrease of 13.53 % (range: 46.49 – 16.27%) of the back pressure measured in the artery after 40 minutes. The coefficient for the linear relationship between pressure and time was different from zero (-0.175, SE 0.020, $P < 0.001$), and the vessel pressure was significantly decreased by 0.175 mmHg every min after attaining the stable phase.

the coefficient for the linear relationship between pressure and time were different from zero.

In a second set of experiments we pre-constricted the vascular bed of normal term cotyledons from 6 different placentas with 10 nM of U46619. After the fetal back pressure reached a plateau, 8 mM L-Trp were added to the perfusion medium (still containing the same concentration of U46619 as before, Fig. 25.B - Preconstricted) resulting in a 18.51 % median decrease (range: 47.65 – 4.88%) of the back pressure measured in the artery after 40 minutes. In these data the coefficient for the linear relationship between pressure and time is statistically significant different from zero (-0.651, SE 0.059, $p<0.001$) and the vessel pressure was significantly decreased by 0.651 mmHg every min after pre-constriction with U46619.

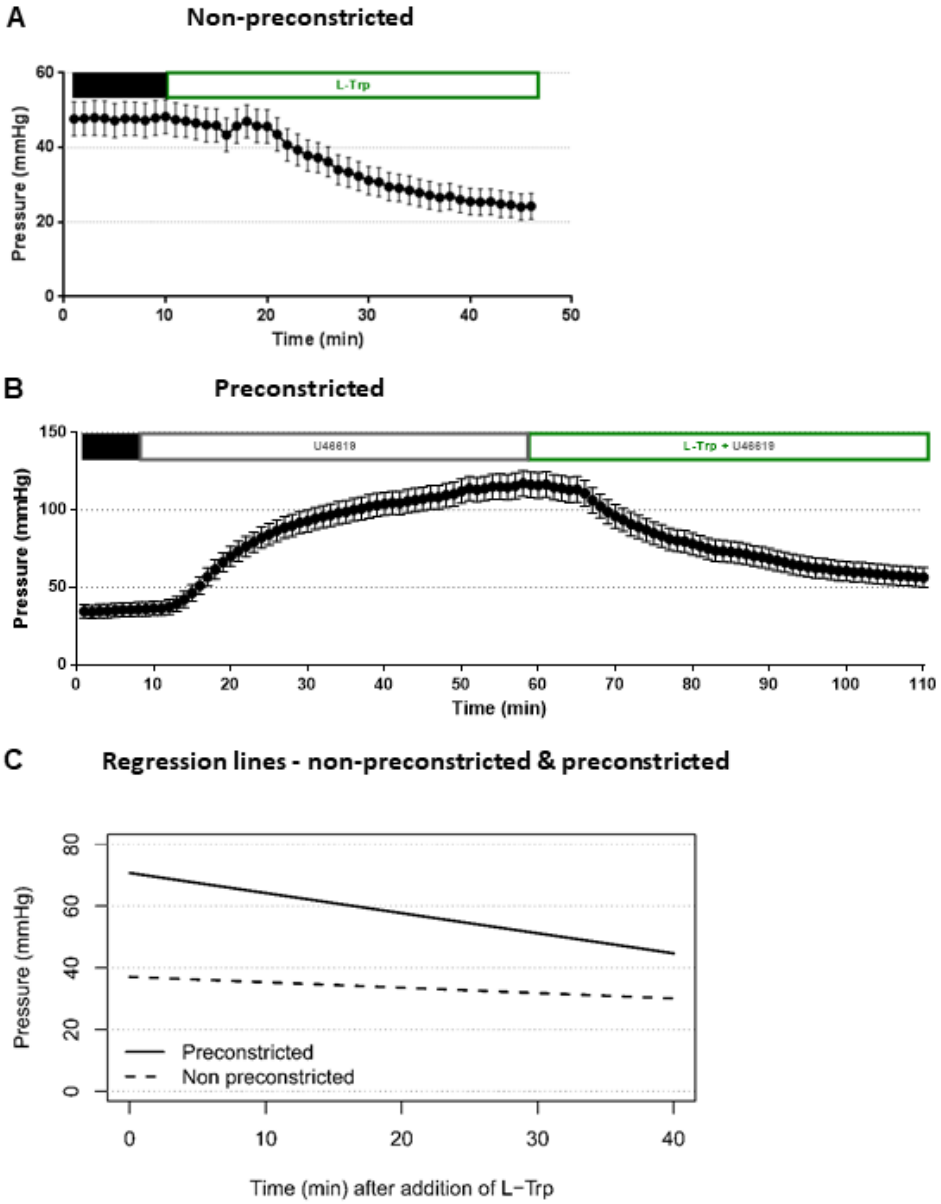


Figure 25. Effect of L-Trp on the pressure in pre-constricted arteries of placental cotyledons. (A: Non-preconstricted; n = 5) Shown is the time course of the arterial pressure during application of 8 mM L-Trp, after reaching a phase of stable pressure (black bar). The coefficient for the linear relationship between pressure and time is statistically significant (-0.175, SE 0.020, p<0.001). **(B: Preconstricted; n = 6)** Shown is the time course of the arterial pressure after reaching a phase of stable pressure (black bar), followed by the attainment of a plateau of pre-constriction with U46619 (10 nM). L-Trp (8 mM) was added as U46619 was continuously being perfused. The coefficient for the linear relationship between pressure and time is statistically significant (-0.651, SE 0.059, p<0.001). Each graph represents the measurements of a representative experiment (n = 11) and each pressure point shows the average of 60 measurements. Results are given as mean ± SD. (Linear regression model). **(C)** Linear regression model. Shown a significant decreased of the vessel back pressure (combining both sets of

experiments in one graph) during perfusion with 8 mM L-Trp. In non-preconstricted cotyledons (dashed line, 5 experiments), the coefficient for the linear relationship between pressure and time is −0.175 (SE 0.020, $P < 0.001$). In preconstricted cotyledons (continuous line, 6 experiments), the coefficient for the linear relationship between pressure and time is −0.651 (SE 0.059, $p < 0.001$). Figure taken and modified from (126).

4.10. Presence of IDO1 in non-perfused and perfused placental tissue

Immunohistochemistry was performed in paraffin sections from normal non-perfused and perfused placental tissue (Fig. 26) against the endothelium marker CD34 II and IDO1. Staining was done to find out whether the IDO1 expression is suppressed or diminished by the placental perfusion procedure. IDO1 expression does not appeared to be diminished after placental perfusion of the tissue.

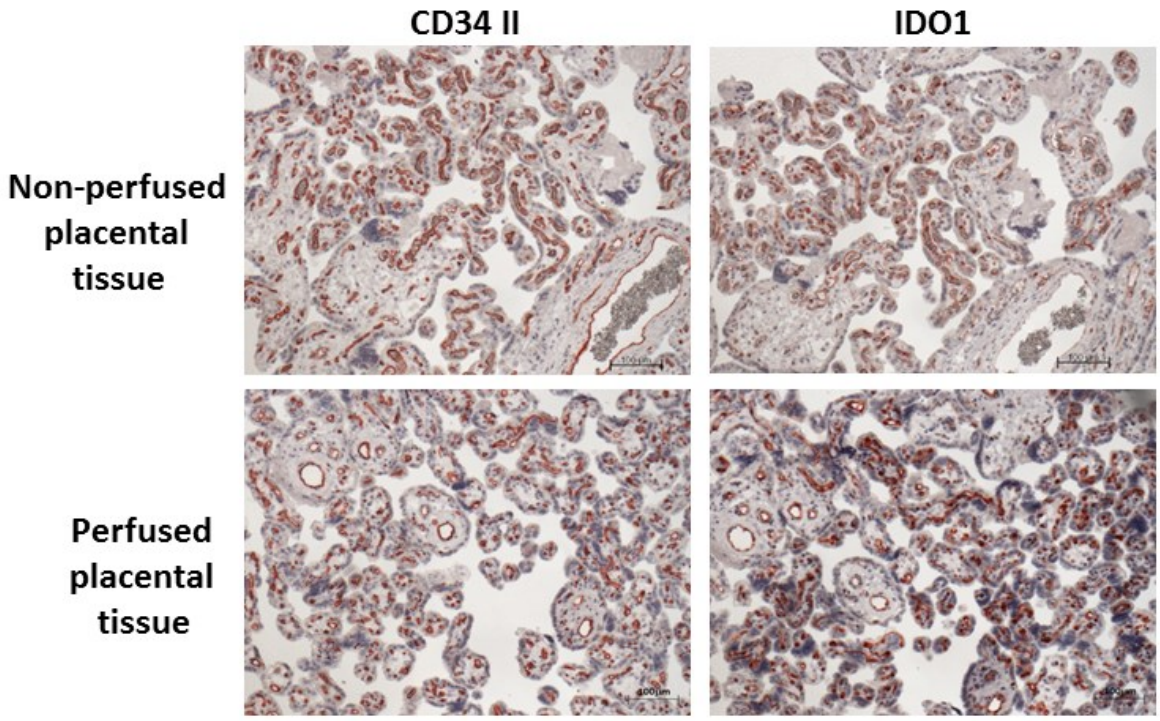


Figure 26. Immunohistochemical staining of paraffin sections of CD34 II and IDO1 from non-perfused and perfused placental tissue. Shown are selected villi sections from normal placentas collected for the placental perfusion experiments (Results section 4.9). Positive staining for CD34 II and IDO1 shown as red staining for non-perfused and perfused placental tissue. The pictures are

representative of placental tissue sections from 2 independent experiments (n = 2). The scale bars represent 100 μm.

4.11. IDO1 expression and activity in chorionic tissue of healthy and pathologic placenta

4.11.1. Ratio of IDO1 to CD34 mRNA expression in chorionic tissue of normal and pathological placentas

IDO1 mRNA levels were measured by RT-qPCR in banked chorionic tissues from pre-term controls (placenta praevia with a mean gestational age similar to the pregnancy pathology samples; n = 5), term controls (normal term placenta; n = 10), IUGR (n = 6) and PE (n = 13) (Table IV) normalised to the expression of the endothelial marker CD34. Quantitative analysis showed a difference ($P < 0.05$) between pre-term controls and term controls for the values normalised to CD34 (Fig. 27). No differences were found in IDO1 mRNA expression from IUGR and PE tissue samples compared to pre-term controls.

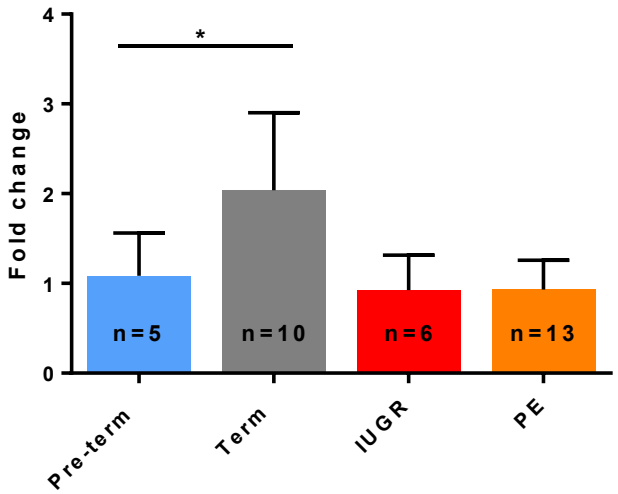


Figure 27. IDO1 mRNA levels normalised to CD34 comparing pre-term with IUGR and PE placental tissue. IDO1 mRNA expression in human placental tissue from pre-term controls (n = 5), term controls (n = 10), IUGR (n = 6) and PE (n = 13) was analysed by RT-qPCR. Results were normalized to the expression of the endothelial marker CD34 and calculated as a fold change

relative to the pre-term control; *P <0.05. Results are given as mean ± SD. (Dunnett´s test). Figure taken and modified from (126).

4.11.2. Ratio of IDO1 to RPL30 or CD68 mRNA expression in chorionic tissue of normal and pathological placentas

IDO1 mRNA levels were similarly measured by RT-qPCR in the same banked chorionic tissue samples from pre-term controls (n = 5), term controls (normal term placenta; n = 10), IUGR (n = 6) and PE (n = 13) (Table IV) normalised to the expression of the ribosomal protein L30 (RPL30; Fig. 28.A) used as a housekeeping gene or to the expression of the macrophage marker CD68 (Fig. 28.B). Quantitative analysis showed no differences in IDO1 mRNA expression from IUGR and PE tissue samples compared to pre-term controls for either the values normalised to RPL30 or to CD68.

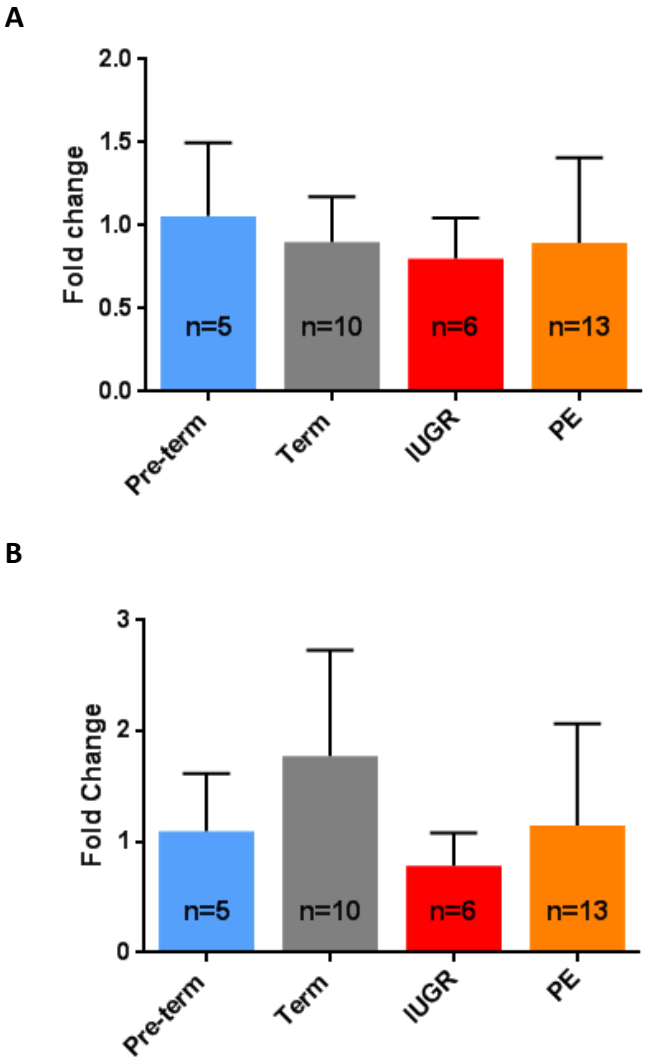


Figure 28. IDO1 mRNA levels normalised to RPL30 or CD68 comparing pre-term with IUGR and PE placental tissue. Analysis by RT-qPCR of tissue samples from pre-term controls (n = 5), term controls (n = 11), IUGR (n = 6) and PE (n = 13). Results were normalized to the expression of RPL30 **(A)** or CD68 **(B)** and calculated as a fold change relative to the pre-term control. No significant differences were found. Results are given as mean ± SD. (Dunnett´s test). Figure taken and modified from (126).

4.11.3. IDO1 protein expression in the chorion in IUGR and PE

IDO1 protein expression was also measured in banked placental tissue samples from pre-term controls (placenta praevia, n = 5), normal term controls (n = 11), IUGR (n = 6) and PE (n = 13) by Western blotting (Table IV). Densitometric analysis of the antibodies in relation to GAPDH expression showed a significant decrease in IDO1 protein expression from IUGR ($P < 0.001$) and PE ($P < 0.01$) compared to pre-term controls (Fig. 29.B). A constitutive IDO1 protein expression was found in most of the cases of pre-term and term controls. Most, but not all individual cases of IUGR and PE show this decrease (Fig. 29.A).

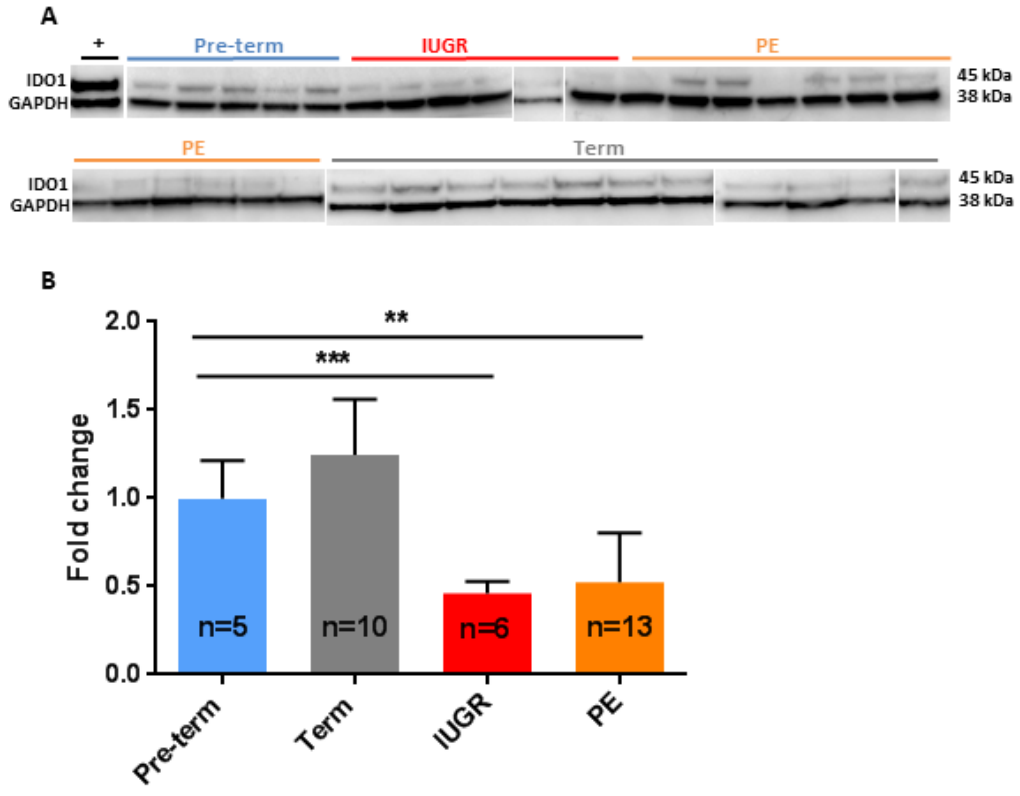


Figure 29. IDO1 protein expression in the chorion comparing pre-term with IUGR and PE placental tissue. Analysis of IDO1 protein expression in human placental tissue from pre-term controls (n = 5), term controls (n = 11), IUGR (n = 6) and PE (n = 13) by Western Blot. **(A)** The gels with the different cases are grouped in pre-term control (blue line), term control (grey line), IUGR (red line) and PE (orange line). Fibroblast stimulated with IFNγ and TNFα were used as a positive control (black line). **(B)** The results were normalized to GAPDH protein levels and calculated as

fold change relative to the pre-term control. **P ≤ 0.01, ***P ≤ 0.001. (Dunnett´s test). Figure taken and modified from (126).

4.11.4. IDO activity in normal and pathologic chorion

IDO activity was measured in banked placental tissue samples from pre-term controls (placenta praevia, n = 11), normal term controls (n = 9), IUGR (n = 7) and PE (n = 13) by LC-MS (Table V). Quantitative analysis showed a significant decrease in IDO activity from term controls (P < 0.05), IUGR (P < 0.01) and PE (P < 0.05) compared to pre-term controls (Fig.30) whereas it was higher in term placental tissue (P < 0.05).

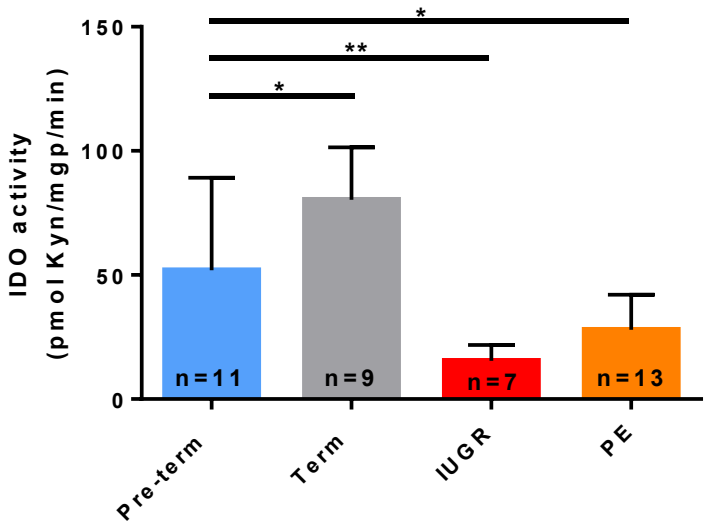
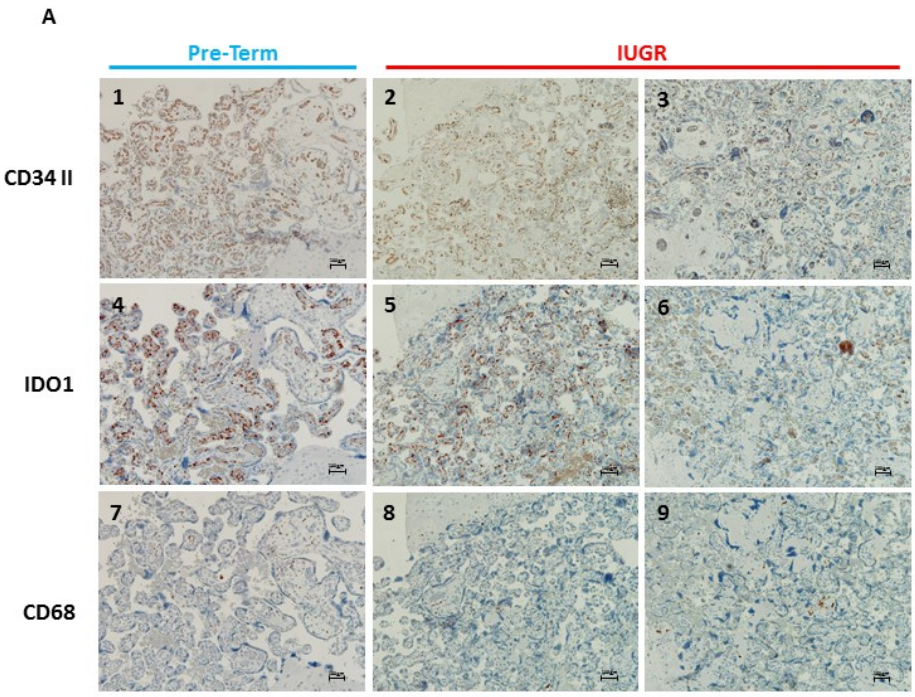


Figure 30. IDO activity comparing pre-term with IUGR and PE placental tissue. Measurement was done by LC/MS in tissue of pre-term controls (n = 11), term controls (n = 9), IUGR (n = 7) and PE (n = 13). IDO activity is given as kyn formed per mg protein per minute. Results are given as mean ± SD. *P ≤ 0.05, **P ≤ 0.01. (Dunnett´s test). Figure taken and modified from (126).

4.11.5. Immunohistochemical staining of serial paraffin sections from IUGR and PE placental tissue.

In order to assess whether the IDO1 protein localization and distribution is different in the chorion of IUGR and PE as compared to pre-term controls, we performed immunohistochemistry in serial paraffin sections from banked placental tissues (Fig. 31.A and 31.B; Table IV). Vascular endothelial expression of IDO1 was reduced in some cases of IUGR (Fig. 31.A6; 3 cases out of 5) and PE (Fig. 31.B6; 4 cases out of 11) in comparison with pre-term controls (Fig. 31.A4 and Fig. 31.B5; 1 out of 5). Unexpectedly, Non-endothelial localisation of IDO1 was found in maternal macrophages aggregated within the intervillous spaces in areas affected by chronic villitis and intervillositis. These were found mostly in the PE group (Fig. 31.B8; 3 cases out of 11) and in only one case in pre-term controls.



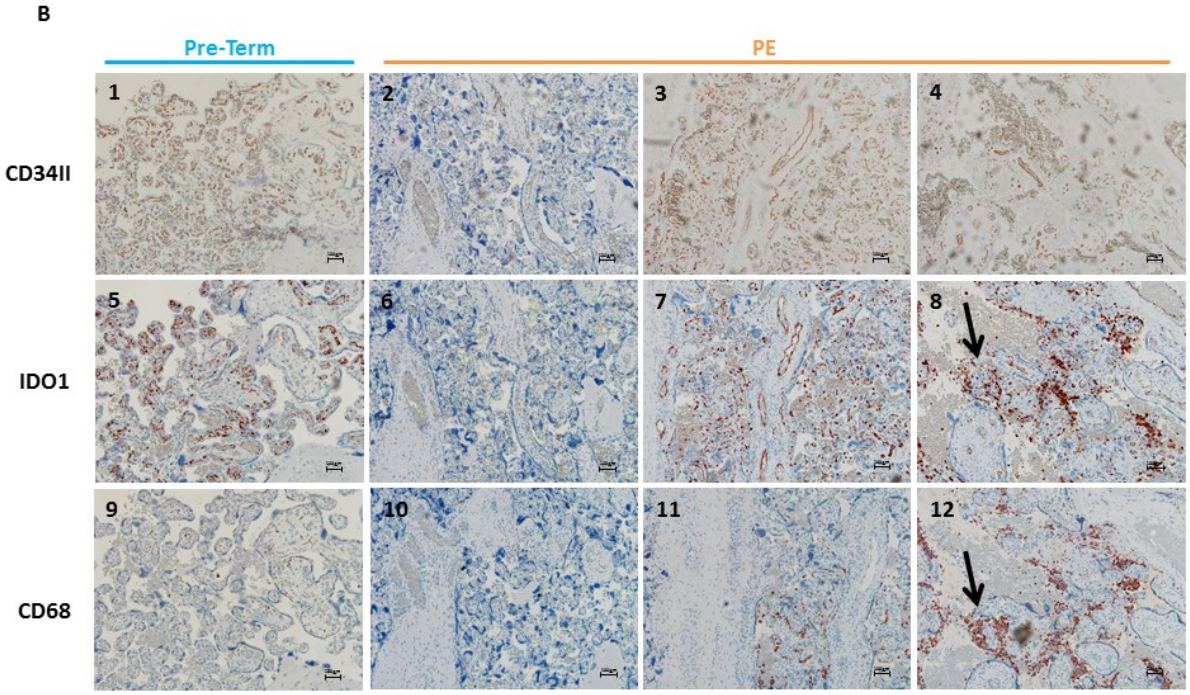


Figure 31. Immunohistochemical staining of serial paraffin sections comparing pre-term with IUGR and PE placental tissue. Shown are selected tissue samples. One case of PE chorionic tissue shows an area of chronic villitis and intervillositis with macrophages expressing IDO1 (arrows). The scale bars represent 100 μm. Figure taken and modified from (126).

Table III. Clinical data of the patients from fresh placental samples used for myography and placental perfusion experiments. Abbreviations: V, vaginal; C, cesarean; F, female; M, male; Y, yes; N, no. Data are mean ± SD.

	Term Control	IUGR	PE
Number of samples	54	6	4
Maternal age (years)	31.4 ± 5.2	31.4 ± 3.7	34 ± 4.2
Mode of delivery (V:C)	17:36*	0:6	0:4
Gestational age (weeks)	39.4 ± 1.1	34.6 ± 3.2	32.3 ± 3
Newborn sex (F:M)	21:32*	3:3	2:2
Newborn weight (grams)	3458.7 ± 471.9	1646.7 ± 697.6	1436.2 ± 541.6
Placenta weight (grams)	668.2 ± 156.0	378.3 ± 109.4	325 ± 75.9
Asymmetric biometry (Y:N)		4:2	
Estimated fetal weight < 3rd percentile (Y:N)		6:0	
Arrest of growth/weeks (Y:N)		4:2	
Oligohydramnion (Y:N)		2:4	

*In one case the mode of delivery and the fetal sex are unknown

Table IV. Clinical data of the patients from biobanked placental samples used for mRNA, Western blotting and immunohistochemistry analysis. Abbreviations: V, vaginal; C, cesarean; F, female; M, male; Y, yes; N, no. Data are mean ± SD.

	Pre-term Control	Term Control	IUGR	PE
Number of samples	5	11	6	13
Maternal age (years)	34 ± 5	29.9 ± 7.2	31.3 ± 5.6	31.8 ± 8.2
Mode of delivery (V:C)	0:5	2:9	0:7	2:11
Gestational age (weeks)	33.6 ± 2	39 ± 1.6	35.2 ± 2.2	33.9 ± 2.1
Newborn sex (F:M)	2:3	4:7	4:2	11:2
Newborn weight (grams)	2069.2 ± 327	3209.1 ± 555.8	1823.3 ± 157.6	1809.7 ± 435.6
Placenta weight (grams)	498 ± 68.3	621.8 ± 127.4	355.8 ± 74.5	393.3 ± 93.9
Asymmetric biometry (Y:N)			3:3	
Estimated fetal weight < 3rd percentile (Y:N)			6:0	
Arrest of growth/weeks (Y:N)			6:0	
Oligohydramnion (Y:N)			2:4	
centralization (Y:N)			4:2	

Table V. Clinical data of the patients from biobanked placental samples used for LC/MS analysis. Abbreviations: V, vaginal; C, cesarean; F, female; M, male; Y, yes; N, no. Data are mean ± SD.

	Pre-term Control	Term Control	IUGR	PE
Number of samples	11	9	7	13
Maternal age (years)	33.1 ± 4.5	31. ± 6.7	32.3 ± 5.7	31.9 ± 7.3
Mode of delivery (V:C)	1:10	1:8	0:7	3:10
Gestational age (weeks)	33.6 ± 1.8	39.1 ± 0.9	34.8 ± 2.3	34.1 ± 2.3
Newborn sex (F:M)	4:7	4:5	4:3	10:3
Newborn weight (grams)	2070.2 ± 292.5	3232.2 ± 346.7	1738.3 ± 267.1	1783 ± 443.7
Placenta weight (grams)	500.9 ± 61.9	614.4 ± 126.7	355 ± 74.5	403.1 ± 96.6
Asymmetric biometry (Y:N)			3:4	
Estimated fetal weight < 3rd percentile (Y:N)			7:0	
Arrest of growth/weeks (Y:N)			6:1	
Oligohydramnion (Y:N)			2:5	
centralization (Y:N)			2:5	

5. Discussion

5.1. IDO1 expression in isolated placental arterial endothelial cells (PLAECs)

Although IDO1 expression has been well described in a variety of cell lines, primary cells and animal models (54, 55, 57, 60, 69, 74, 146) and due to its role in the human placenta that has recently emerged, little is known about its expression in isolated placental arterial endothelial cells (PLAECs).

Here, we investigated what is the extent of IDO1 expression with different methods in PLAECs isolated from the chorionic plate of normal placentas, and also what stimuli better upregulate IDO1 expression in this cell type.

IDO1 placental expression varies depending on the pregnancy stage and cell type. In first trimester of pregnancy, on the endothelium of chorionic vessels, IDO1 expression is exclusively detected in capillaries directly below the trophoblastic layer (49, 77). Additionally, increasing IDO1 expression was found with advancing pregnancy within vessels from villi trees and some arteries and veins on the chorionic plate at term. In full-term pregnancy however, IDO1 expression is generally increased, present in most endothelia of the small chorionic vessels but also in bigger vessel of the stem villi being restricted in bigger vessels of the chorionic plate (49, 77). Previously, it has been shown that isolated PLAECs from the chorionic plate of the normal term placenta have a constitutive expression of IDO1 mRNA in contrast with those isolated from umbilical vein, iliac vein or aorta (49).

We confirmed a constitutive IDO1 mRNA expression in PLAECs from normal term placentas. Moreover we have also shown that after synergistic stimulation with IFNγ and TNFα (147), IDO1 mRNA levels are significantly higher. IDO1 protein expression was also assessed on isolated PLAECs in the presence and absence of IFNγ and TNFα. Constitutive IDO1 protein expression in the absence of cytokines was different depending on the isolation (IDO1 detected in

one isolation out of three) whereas there was a markedly increased expression upon cytokine stimulation. IDO1 staining of PLAECs pellets did not show any constitutive IDO1 expression which was only detected after cytokine stimulation. Considering that PLAECs were isolated from big arteries of the chorionic plate, one possible explanation for this different constitutive IDO1 protein expression found may correlate with the fact that IDO1 presence is reduced in big vessels of the chorionic plate in normal term placentas compared to the microvasculature (49). Besides, isolated PLAECs have been passaged to keep them in culture and therefore, they have been susceptible to change (148-151) suggesting that they do not mimic the *in vivo* situation. Furthermore, one additional factor contributing to this situation might be that after certain passage, cells in culture lose the capacity to express IDO1 in a constitutive manner.

We also tested whether the treatment with IFNγ and TNFα was detrimental to some degree in PLAECs in culture by checking cell number, viability percentage or even the characterization markers for endothelial cells. Despite no major alterations were detected or visualized, cell morphology was unequivocally altered from a polygonal shape without any stimulation to an elongated-fusiform shape. It may be possible that cytokine stimulation in PLAECs activates cytoskeleton pathways that modify the regular shape in culture of this cell type (152-154) or that PLAECs after stimulation secrete some other cytokines that could sustain some morphological changes (155).

Our data suggest that in isolated PLAECs from arteries of the chorionic plate from normal term placentas, constitutive IDO1 expression may depend on the artery chosen for isolation. Besides, cell culture conditions might have an impact on its constitutive expression. Moreover, we also revealed that upregulation of IDO1 expression can be achieved by stimulation with IFNγ and TNFα for up to 48 h. Furthermore, this cytokine stimulation did not reveal any detrimental effect on the cell culture for this cell type. However, a cell-shape morphology change was observed after cytokine stimulation.

5.2. L-Trp regulates the placental vascular tone via IDO1

The role of IDO1 in pregnancy has caught considerable attention recently. Many studies have been focused on how IDO1 supresses the immune system preventing immunological rejection of the fetus during pregnancy (74, 75, 77, 147). To date, a minor focus has been taken about the role of IDO1 in the control of the blood pressure which acquires a major role under inflammatory conditions in several animal models (67) and supports hypotension in human sepsis (79); however its importance for the regulation of the human placental vascular tone has not been described previously.

In this study, we first established the *in vitro* myography technique with human placental arteries. Then, we explored what is the role of IDO1 using stored and freshly-prepared L-Trp solution (the substrate of the enzyme prepared in 2 different manners) on regulating the vascular tone by *in vitro* wire myography in human placental arteries isolated from the chorionic plate of healthy, PE and IUGR donnors; and the role of freshly-made L-Trp solution on a human placental cotyledon through the *ex vivo* placental perfusion.

The feto-placental circulation is mainly characterized by low vascular resistance (101, 156) and an absence of neuronal innervation (102), indicating that the control of vascular tone is governed by physical factors and/or local vasoregulators (108, 157, 158).We focused our investigation on the physiological relevance of IDO1 expression in the endothelia of the chorionic vasculature described earlier (49) according to our hypothesis that IDO1 may play an important role in the local control of the feto-placental circulation.

Wire myography is a well-known technique traditionally used in mouse and rat models to measure vascular responses to different agonists (135, 159). We compared the vascular responses from human placental arteries with thoracic aortas from mouse showing that stability of the response to the different agonist is much more stable in mice than human placenta. We also established an efficient method to remove the endothelium from human placental arteries without harming

the functionality and response of the smooth muscle cell layer. The contractive agonists employed for myography studies are essential in order to get a reliable relaxation (160). In accordance with M. Wareing (104), we observed that norepinephrine has no effect on human placental arteries whereas endothelin-1 displays a slight contraction as described previously (105). Endothelin-1 may induce a process of desensitization in human placental arteries as its effectiveness was markedly reduced when added to placental arteries for a second time (161, 162). According to previous reports (104, 105, 109), we found that the thromboxane analogue U46619 is the most suitable and reliable contractive agonist employed for the study of relaxation responses in human placental vessels.

We tested the effect of a stored L-Trp solution *in vitro* on normal human placental arteries taken from the chorionic plate. This yielded a robust relaxative effect in a dose-dependent manner. Moreover, the relaxing effect exhibited by the stored L-Trp solution was even higher in the case of IUGR arteries compared to normal. There are many IDO1 inhibitors already described in the literature which have been used to inhibit IDO1 in a competitive mode (57, 58, 65). Two of these traditional inhibitors, necrostatin-1 and 1MetDTrp, were tested in the presence and absence of its competitor, L-Trp. Both inhibitors showed a dose-dependent relaxing effect when added alone on normal human placental arteries. It is already known that IDO1 has a broad substrate specificity (52) and its binding site can accommodate a wide variety of structures, meaning that in the absence of L-Trp, 1MetDTrp can behave like a substrate (163) which may explain the relaxing effect previously mentioned. Preliminary data on simultaneous addition of a stored solution of L-Trp and 1MetDTrp show that 1MetDTrp competes for the binding site with stored L-Trp causing a real competitive effect. Nevertheless, preincubation with 1MetDTrp before addition of a stored L-Trp solution revealed that 1MetDTrp does not abolish the relaxing effect observed with the L-Trp solution. Former studies based on commercial preparations originally reported that kynurenine, one of the Trp-catabolism products in the IDO1 pathway, is a key factor regulating the relaxation of the vascular tone (67). However, HPLC-purified kynurenine was detected to be ineffective in regulating arterial relaxation which was related to a yet

non-identified Trp metabolite (Proceedings of the British Pharmacological Society at http://www.pa2online.org/abstract/abstract.jsp?abid=31322). We speculate that preparation and storage of an L-Trp solution may lead to a spontaneous conversion of L-Trp into this non-identified Trp metabolite. Therefore, stored L-Trp solutions may contain already this vasorelaxant metabolite explaining the great and instant effect of stored L-Trp on relaxation of the human arteries from the chorionic plate in normal and IUGR placentas. Moreover, if the vasorelaxant compound is already present within the stored L-Trp solution this could also explain why no inhibition of the relaxing effect was observed after preincubation with 1MetDTrp, as the novel compound would produce the relaxation directly on the arteries without the need of the enzyme.

We also checked the effect of freshly-made L-Trp *in vitro* of normal, IUGR and early-onset PE human placental arteries from the chorionic plate. Freshly-prepared L-Trp did not display any effect in normal or PE placentas except for the highest concentrations where it showed a slight contraction. In contrast, freshly-prepared L-Trp showed a relative contraction in IUGR arteries which we could not explain. The localization of IDO1 expression within the endothelia of the placenta displays a positive gradient on the maternal and the fetal side towards the feto-maternal interface (49). Considering this finding, only a lesser number of big arteries of the chorionic plate which present a lumen wide enough to be placed on myography pin sets, expresses endothelial IDO1 protein constitutively. Therefore, the lack of constitutive IDO1 expression may explain why freshly-prepared L-Trp did not exhibit any relaxation in the placental vascular tone.

We already proved in PLAECs that synergistic induction of IDO1 with IFNγ and TNFα stimulates IDO1 expression without any detrimental effect on the cells. We assessed similarly the effect of freshly-prepared L-Trp solution on cytokine stimulated human placental arteries (with induced endothelial IDO1 expression). Cytokine stimulation in normal placental arteries changed the response to freshly-prepared L-Trp solution into displaying a relaxing effect which was even more pronounced in the case of stored L-Trp solution (although similar to the relaxation observed without any stimulation). It is already known that IFNγ stimulates IDO1

activity in different cells lines (55) or even in placental explants (56) whereas an induction with the combination of IFNγ and TNFα increases IDO1 expression even further than with either cytokine alone (54) which may explain the sudden susceptibility to freshly-prepared L-Trp solution after stimulation. Uptake of L-Trp is the pre-requisite to evidence any effect of IDO1 which is found in the cytosol (52). IDO1 has been shown to upregulate the expression of a novel L-Trp transporter system with high affinity and selectivity in human tumor cells (164). It has been reported recently in human cell lines and mouse models that IFNγ stimulation augments the expression of L-Trp transporters (60) which, may lead to making L-Trp available to the already upregulated endothelial IDO1. We also observed that early-onset PE cytokine-stimulated chorionic arteries relaxed in response to freshly-prepared L-Trp after stimulation (as already detected with normal placental arteries). In accordance to this, increased IDO activity was found in both PE and normal human villous explants after IFNγ stimulation (59). Remarkably, PE arteries were more sensitive compared to normal for all concentrations of L-Trp with marked importance in the lowest concentration (0.5 mM L-Trp) possibly due to an already higher constitutive expression of L-Trp transporters (165) or even to a higher concentration of TNFα present in PE (166) that may sustain this higher transporter expression. Preliminary data in IUGR on chorionic arteries from one placenta (measurements in quadruplicates) suggest a similar behaviour than the one observed in PE, but more samples are required in order to make reliable conclusions. These data with the pregnancy complications samples must be interpreted with prudence, however, as proper gestational age–matched vessels were unaccessible to our study.

We also proved the involvement of IDO1 in the relaxation previously obtained by preincubating the cytokine stimulated arteries with 1MetDTrp or INCB024360. The fact that either 1MetDTrp or INCB024360 abrogated the relaxing effect of L-Trp involves unequivocally IDO1 in the formation of a vasoactive L-Trp metabolite implicated in the regulation of the placental vascular tone in intact stimulated arteries (57, 58, 65).

Endothelium plays a key role in the regulation of the vascular responses of any tissue (167). We therefore tested the role of the endothelium in the relaxing effect of freshly-prepared L-Trp. Interestingly, denudation of the arterial endothelium and treatment with 1MetDTrp did partly abort vascular relaxation in response to L-Trp of stimulated arteries, suggesting the existence of an IDO1-dependent pathway active when arteries are stimulated and free of endothelium, and additionally, the existence of a supplementary component IDO1-independent responsible for part of the L-Trp-induced relaxation in stimulated denuded arteries. The scattered cells (probably activated macrophages) within the smooth muscle cell layer that express IDO1, may in part explain the relaxation produced by freshly-prepared L-Trp solution on stimulated arteries. Apart from this, the IDO-independent mechanism by which L-Trp relaxes the placental arteries through the smooth muscle layer remains unclear. Additionally to our knowledge of IDO1 localization within the endothelium of villous trees very early on in pregnancy (49), it would be intriguing to investigate the involvement of other cell types such as pericytes in the regulation of the placental vascular tone.

Ex vivo placental perfusion is a complex technique that allows to explore the physiological mechanisms governing the feto-placental circulation by re-establishing a closed-circulation system in a small part the placenta. Therefore, placental perfusion is a valuable method for our research question that allows us to assess the effect of L-Trp in the placental microvasculature where endothelial IDO1 is expressed constitutively (49). Perfusion with L-Trp consistently exhibited relaxation in the vessel back pressure, either by U44619-pre-constricted vasculature (four cases out of five) or not (five cases out of six) even in the absence of cytokine stimulation. In one experiment in each group (U44619-pre-constricted vasculature or not) we observed a slight contraction that we were unable to explain. Additionally, IDO1 staining on villous tissue before and after placental perfusion confirmed that the enzyme is constitutively present within the endothelium of the microvasculature. Interestingly, the intensity of the IHC staining appeared to be increased after placental perfusion compared with the same tissue before placental perfusion. Continuous perfusion with U46619 may induce Ca^{2+} flow or activation of several pathways (168-170) that in the last instance might influence IDO1 expression (as Ca^{2+} is required for its activation). Our data

obtained by *ex vivo* placental perfusion which is consistent with the vascular responses measured by myography using INCB024360 (a specific competitive IDO1 inhibitor that does not affect L-Trp transporters in order to inhibit IDO1 activity), make it improbable that the relaxing effect of L-Trp on cytokine-stimulated arteries might rely exclusively on a possible upregulation of an endothelial L-Trp transporter.

Can we exclude that any of the other two Trp-catabolising enzymes contribute to the vasorelaxing effect of L-Trp in cytokine-stimulated chorionic plate arteries? Due to 1MetDTrp or INCB024360 are capable of abolishing the relaxing effect of L-Trp (57, 58, 65, 69) exclude the option that TDO might be involved in the relaxing activity described (34). In regards to IDO2, the catabolic Trp-activity of the enzyme has been reported to be quite low in comparison with IDO1 (69, 171), joined to the fact that IDO2 functional alleles are absent already in 50% of Caucasians (45). Therefore, any contribution of IDO2 to the L-Trp relaxing effect is rather improbable.

Altogether, these results suggest that stored L-Trp may contain a novel vasoactive compound capable of relaxing the placental vascular tone. Moreover, freshly-prepared L-Trp solution produces relaxation of the placental vascular tone upon IDO1 upregulation with IFNγ and TNFα, an effect that is IDO1-dependent in non-denuded arteries but may be not exclusively endothelium-dependent. Furthermore, perfusion with freshly-prepared L-Trp solution leads to a relaxation of the microvasculature on normal non-pre-constricted and pre-constricted cotyledons without IDO1 upregulation.

5.3. IDO1 expression and activity in chorionic tissue of healthy and pathologic placenta

In humans, IDO constitutive expression has been well characterized in the lung, the intestine and particularly in the term placenta (48). Focusing on IDO1 placental expression, it varies depending on the pregnancy stage and cell type. In first

trimester of pregnancy, on the endothelium of chorionic vessels, IDO1 expression is exclusively detected in capillaries directly below the trophoblastic layer (49, 77). Additionally, increasing IDO1 expression was found with advancing pregnancy within vessels from villous trees and some arteries and veins on the chorionic plate at term (49, 50) which correlates with a decreasing Trp levels from early to late pregnancy (24). In full-term pregnancy however, IDO1 expression is generally augmented, present in most endothelia of the small chorionic vessels but also in bigger vessel of the stem villi being restricted in some bigger vessels of the chorionic plate (49, 77). Consistent with this, the increase of IDO1 expression was also correlated earlier with the activity of the enzyme measured in the chorionic plate as kyn/Trp ratio (49). Similar results for IDO1 expression were found in rhesus monkeys and common marmosets in term placentas (146). It has been shown that IDO1 can be present on villous stromal macrophages (76, 80) in the placental tissue. Diverging data regarding placental IDO1 localization (76, 77, 80) are reviewed extensively elsewhere (49).

Regarding placental pathology, it is well known already that fetoplacental circulation (109, 172) and oxygenation (173) are altered in IUGR and PE. Earlier reports (59, 116) indicate a decrease in L-Trp catabolism by placental IDO1 in IUGR and PE. Consistent with this, we observed a significant decrease in IDO1 activity (determined as kyn/Trp ratio) in IUGR and PE when compared with pre-term controls, as measured by LC/MS. Based on immunohistochemistry analysis, a recent study correlated a reduction of IDO1 in villous stromal endothelial cells with PE (174) In accordance with this, we found a decreased in IDO1 protein expression in comparison with our pre-term controls (gestational age-matched normal placentas). Earlier reports have also shown a reduction in IDO1 expression in PE placentas (124, 125). Even though they reported a reduction in comparison with term controls which are an inappropriate control for PE as these are subject to pre-term delivery that cannot exclusively explain the decreased in IDO1 expression reported. In fact, we proved that IDO1 activity and expression are reduced in early-onset PE and IUGR when compared with pre-term controls. Although IUGR samples did not perfectly matched the gestational week (IUGR with older gestational week) with our pre-term controls (the older the gestational

week the higher the IDO1 expression) and considering that some PE placental samples were highly expressing IDO1 localized not only in endothelia but in macrophages in areas affected by chronic villitis and intervillositis, the significant differences in protein IDO1 expression and activity become even more striking taking into account that placental IDO1 expression increases during the course of normal pregnancy.

IDO1 mRNA levels remained stable after normalizing with the endothelium marker CD34 (or even using the housekeeping gene RPL30 or the macrophage marker CD68) between pre-term controls and IUGR and PE. However, striking differences were found in IDO1 protein expression and activity. We presume that either post-transcriptional or post-translational modifications may influence IDO1 protein stability differentially in pre-term in comparison with IUGR and PE (175-177). As already underlined the role of deficient IDO1 in developing PE/IUGR phenotype in mice (117), our data point towards a possible pathogenic role for IUGR and PE of a deficiency in IDO1-mediated Trp catabolism. Additional experiments of *ex vivo* placental perfusion need to be conducted in pathological placental in order to shed some light on this issue.

Overall, the data presented indicates despite IDO1 mRNA levels remained stable regarding the pregnancy complications, IDO1 protein expression and activity are clearly reduced in IUGR and PE in comparison with age-matched controls, suggesting a possible contribution of the IDO1 downregulation in the pathogenesis of IUGR and PE.

6. References

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7. Appendix

7.1. Embedding of Cell Pellets

Preparation beforehand:

- Pre-warm accutase at 37 °C
- Pre-warm PBS at 37 °C
- Pre-warm ECM media at 37 °C

Gelatine / PFA / Paraffin Method:

- Suck off old medium
- Optional: Wash 1x with PBS (37 °C)
- Put 3 ml of acctuase to the cells flask (T75)
- Incubate at 37 °C for ~ 5 min
- Put 7 ml of medium to the flask and resuspend cell suspension well
- Take out the whole suspension and put it in a 50 ml falcon
- Centrifuge (1700 RPM / 5 min)
- Suck off the supernatant (be careful with the cell pellet)
- Put 1 ml 4% PFA to the pellet, resuspend well and transfer it to a 1.5 ml eppi vial (= fixation)
- Incubate 30 min at RT
- Centrifuge at (1700 RPM / 5 min)
- Wash 2 x in PBS at RT (1700 RPM / 5 min) (resuspend well)
- Put 5% warm (37 °C) gelatin (same size as pellet) on the pellet (resuspend well)

  Preparation of the 5% gelatine:

  Dissolve 5 g gelatine in 100 ml PBS, put the mix on the magnetic stirrer and warm it to 37 °C. Let it stir until the gelatine is completely in solution.

  Storage: 1 week at 4 °C or long term storage at -20 °C

- Incubate 30 min / 37 °C in the incubator
- Pre-warm the centrifuge in the cell culture to 37 °C
- Centrifuge the vial (1700 RPM / 5 min)
- Put the vial for 30 min (for better results increase till 24 h) / 4 °C in the fridge
- Put 1 ml 4% PFA on top of the gelatine and fix it for 1h / 4 °C in the fridge
- Suck of the supernatant of 4% PFA carefully
- Cut the eppi with a razor blade over the gelatine border
- Use a needle to untighten the pellet/gelatine block from the wall of the eppi

- If the pellet is too big, you can cut it into pieces, cut off the waste gelatine
- Put the pellet in 4% PFA / overnight / 4 °C

NEXT DAY:

- Wash the pellets in PBS
- Imbed the pellets in cassettes with the VIP 5

NEXT DAY:

Embedding: put the pellets in a mold

7.2. Protocol - Regulation of the placental vascular tone

Medical University of Graz

Institute for Cell Biology, Histology and Embryology	Ref: SOP-001
Regulation of Placental Vascular Tone	Pages: 1 – 9
Written by: Pablo Zardoya Laguardia	**Myography**

1. Notes

Regulation of placental vascular tone is conducted on placental arteries from the chorionic plate harvested from fresh placentas at term within the next hour after collection

2. Equipment/material

a. Myograph 610M
b. Waterbath
c. Dissecting instruments (non-sterile)
d. Krebs-Henseleit buffer (Sigma K3753)
e. Milli-Q water
f. $NaHCO_3$
g. EDTA-$Na_2.2H_2O$
h. $CaCl_2$
i. KCl
j. $MgSO_4.7H_2O$
k. KH_2PO_4
l. Thromboxane analog U-46619 (D8174-1MG, Sigma)
m. NG-Nitro-L-Arginine Methyl Ester hydrochloride – L-NAME (N5751, Sigma)
n. Indomethacin (I7378, Sigma)
o. L-Tryptophan (T0254, Sigma)
p. 1-Methyl-L-Tryptophan (447439, Sigma)
q. 1-Methyl-D-Tryptophan (452483, Sigma)
r. Necrostatin-1 (N9037, Sigma)

3. Reagents to be prepared before beginning the procedure

a) 0.15mM $CaCl_2$ Krebs buffer 2 litres

Add 2 bottles of Krebs-Henseleit buffer modified to 2 L of Milli-Q water. Add 4.2 g of $NaHCO_3$ (Fach 1) and 20 mL of 2.6 mM EDTA$Na_2.2H_2O$ (Titriplex Fach 5)(final concentration: 26 μM). Aerate solution with 95% O_2 + 5% CO_2 for 20 min. Add 0.6 mL of 0.5 M $CaCl_2$ (Fach 4) (final concentration: 0.15 mM $CaCl_2$).

Add 4 tubes (5 mL/tube) penicillin/streptomycin antibiotics to bottle (1%: 5 mL antibiotics/500 mL solution) and filter buffer with 0.2 μm filters. Store it in 4°C for up to 1 month.

- In the literature a 2.5 mM $CaCl_2$ is used to make the Krebs Buffer. The risk of using such a low concentration is that when the membrane is depolarize by adding agonist or high K^+, the Ca^{2+} influx may not be enough to enable the vessel rings to contract. However, strong and stable contractions have been observed in every experiment conducted. The only difference is that 2.5 mM $CaCl_2$ gives a faster contraction and a slower plateau than 0.5 mM $CaCl_2$.

b) 120 mM KPSS (High-Potassium physiological saline solution) 1 Litres

Solution 1: 9.2 g KCl, 0.289 g $MgSO_4.7H_2O$, 0.161 g KH_2PO_4 in 100 mL of Milli-Q water

Solution 2: 0.277 g of $CaCl_2$ (final concentration: 2.5 mM $CaCl_2$) in 100mL of Milli-Q water

Solution 3: 2.1 g $NaHCO_3$, 0.01 g EDTA-$Na_2.2H_2O$, 0.991 g Glucose in 100 mL of Milli-Q water

Add solution 1 to a graduated bottle, fill with Milli-Q water to 500 mL, add solution 3 and fill up to 850 mL with Milli-Q water. Aerate solution with 95% O_2 + 5% CO2 for 20 min. Solution 2 is added to graduated bottle and filled to 1000 mL with Milli-Q water. Continue to aerate the solution until pH reaches 7.4

c) 60 mM KPSS solution

Mix the same volume of Krebs Buffer and 120 mM KPSS (125mL Krebs Buffer + 125 mL 120 mM KPSS).

d) 1 mM and 1 mM Thromboxane U-46619 (MW: 350.49 g/mol)

1mg of U-46619 is already dissolved in 100μL of methyl acetate. To make 1 mM stock solution add 2.76 mL of methyl acetate (final volume = 2,85 mL methyl acetate). Aliquot and store it at -20°C. Aliquots can be stored at -20°C in the freezer for up to one year. Avoid more than 3 cycles of thawing-freezing. Keep solutions on ice during the course of the day.

e) 30 mM NG-Nitro-L-Arginine Methyl Ester hydrochloride (MW: 269.7 g/mol)

Dissolve 0.202 g in 250 mL dH_2O to give 30mM stock solution. Store the solution as 10 mL aliquots in -20°C freezer for up to 6 months. Add 50 μL/chamber (final concentration: 300 μM)

f) 1 mg/mL (2,8 mM) Indomethacin (MW: 357.79 g/mol)

Dissolve 5 mg of Indomethacin in 810 μL 0,5 M Na_2HPO_4 + 190 μL 0,5 M NaH_2PO_4 + 4 mL dH_2O. Store it in -20°C for up to 6 months. Add 18 μL/chamber required (final concentration of 10 μM)

g) L-Tryptophan (MW: 204.23 g/mol)

Weight 245 mg out of L-Tryptophan and dissolve it in 15 mL Krebs buffer making a stock solution of 80mM. In order to dissolve the compound, warm the solution up (~40-50°C). Add 500 μL/chamber required (final concentration of 8 mM)

h) 100 mM 1-Methyl-D-Tryptophan (MW: 218.25 g/mol)

Dissolve 1g of 1-Methyl-L-Tryptophan in 46 mL 0,5 M HCl creating a stock solution of 100mM. Add 100 μL/chamber required (final concentration of 1 mM)

i) 5 mM Necrostatin-1 (MW: 259.33 g/mol)

Dissolve 10 mg in 7.72 mL DMSO creating a stock solution of 5 mM. Aliquot it and store at -20°C. Add 50 μL/chamber required (final concentration of 50 μM)

4. Procedure

a) <u>Dissection and mounting</u>

1) Isolate the artery of choice from the chorionic plate. Try to isolate it as clean as possible without damaging the vessel. **Avoid stretching the vessel** as much as possible.
2) Transfer the vessel in a dissection dish for cleaning, **containing cold Krebs buffer**. **The petri dish should be placed on ice**. Cleaning is best done under a dissection microscope in a petri dish coated with some kind material such as Sylgard so as to be able to pin the vessel down without causing major damage to the vessels in general. Discard the parts of the vessel that has been damaged by the pins or forceps.
3) Cut the vessel to an appropriate length (between 1.5 – 2.5 mm). Use to cut the vessels up an eye piece micrometre under the dissecting microscope as a reference for length.
4) Fill the chambers with 5 mL of **cold Krebs buffer** in each.
5) Transfer a single ring into each chamber.
6) Mount the vessel on the wire (25 μm tungsten or 40 μm stainless steel) or the pins (depending on the internal diameter of the vessel). For placental arteries the pins (200 μm) fit better than the wire.
7) **Take note of the diameter** (appendix 3 – reading a millimetre micrometre) **and length of each vessel** (with eyepiece micrometre).
8) Place the chambers with the mounted vessels on the interface and plug the transducer cables into the black of the interface.

b) <u>Equilibration</u>

Once the vessel has been mounted the vessel needs to equilibrate in the myograph chamber before reliable and reproducible results can be obtained. The equilibration period allows the vascular ring preparation to heat up to experimental temperature slowly while giving the preparation time to reset ions gradients that may have been disturbed during dissection and cleaning. This period also allows the preparation time to achieve a stable level of passive tension (tension produced by un-stimulated muscle). Typically, equilibration takes no more than one hour.

1) Turn on your gas to bubble your chambers with 95% O_2 + 5% CO_2.
2) Turn on the heat, which should be preset to 37°C. Place the sensor to measure the temperature into the chamber.
3) **Wait 20 min without touching the vessels or system**. This allows the vessels to slowly heat up.
4) Perform your normalization to determine the optimal passive tension (see normalization protocol). If using larger vessels, a length-tension relationship will need to be performed to determine the optimal passive tension to conduct the reactivity at. For human placental arteries the **basal tension** is between 10 and 20 mN, but optimally it works better around **13 mN.**
5) **Increase the tension steadily** with the sleeve for each chamber until you reach the passive tension already calculated. The tension hold by each vessel will dramatically fall with every manual tension increase. That is normal for

placental arteries. **Make a note of what the passive tension is** for each chamber.

6) Wash the vessels with bubbled, warmed (37°C) Krebs buffer by pressing the "all channels" button in the front of the interface to evacuate the chambers of buffer.
7) Immediately replace the buffer (5 mL) with **fresh, bubbled, warmed (37°C) Krebs buffer.**
8) Allow the artery to continue equilibrating for another 30 min until the passive tension established is stable.
9) During the next equilibration period, if the tension slips or changes, continue to adjust the passive tension so that it remains at the noted passive tension in step 5.
10) After 20 min from the first wash, (now 40 min since the start of the equilibration), do another single wash with warmed, bubbled Krebs buffer.
11) Once a total of at least 60 min have passed, the wake-up protocol can be initiated.

c) <u>The wake-up protocol</u>

The purpose of this protocol is to:

1. Re-activate the mechanical, functional, and signalling properties of the vessel segment.
2. Check that responses to different types of stimuli are normal and verify that the functionality of the vascular ring preparation was not damaged during the dissection or mounting procedures.
3. Check whether the vessel ring meets inclusion/exclusion criteria to be used for experimentation. It is up to the end-user to determine appropriate inclusion/exclusion criteria for the vessel being used for their particular experiments

The wake-up protocol is performed after the vessel segment has been heated, equilibrated and stretched with the appropriate passive tension.

The wake-up protocol consists of a series of stimuli and washout periods. The stimuli chosen will depend on the mounted vessel.

KPSS (high potassium physiological salt solution) should always be a part of the wake-up protocol. The number of stimuli, the order, and the length of time for each stimulus may need to be optimized, depending on the vessel chosen for observation.

1) **Reset the baseline values to "Zero"** by using the "Zero" function menu on the interface.
2) Use 60mM KPSS (1 volume Krebs buffer + 1 volume 120mM KPSS) for the wake up protocol.
3) Start the protocol by stimulating the vessel with 60mM KPSS. Stimulate the vessels until a stable plateau is reached.
4) Once a stable plateau is reached in every chamber, wash the KPSS out.

5) Repeat this procedure (stimulation with 60 mM KPSS and washing) at least 2 more times.
6) Wait until the vessels returned to baseline/plateau after every wash.

Final vascular function assessment

One of the last components of the wake-up protocol is an agonist-mediated contraction and test for endothelial function. The contraction should be a contraction elicited by a submaximal concentration of a vasopressor of choice. This contraction can be used as a final stimulus in the wake-up protocol. Moreover, this stimulus can be used in two ways:

- To assess whether the vascular smooth muscle is contracting properly
- It can be used as the contraction needed to test an endothelium-dependent relaxation, if intact endothelial experiments are to be performed.

It is recommended that endothelial function is evaluated. Stimulating a vessel segment with bradykinin causes the release of nitric oxide (NO) from the endothelial cells. If the endothelium is undamaged after dissection and mounting, a substantial relaxation will occur. If the endothelium is removed or damaged during dissection and mounting, a partial relaxation or no relaxation to bradykinin will be observed.

The purpose of checking endothelial function, therefore, is:

- To check whether the endothelium is intact.
- To verify that endothelium was sufficiently removed (denuded) if an experiment requires removal of the endothelium.

These two parameters can be used as inclusion/exclusion criteria. If the vessel does not contract or relax according to the end-user´s acceptable criteria, new vessels can be mounted if desired.

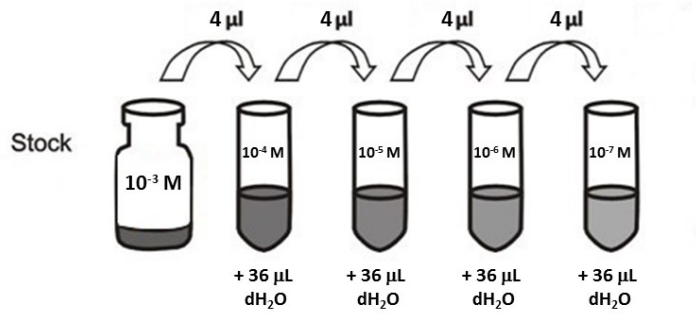
- **How to check an endothelium-dependent response?** Bradykinin (regarding the literature it should lead to a minimal relaxation), Histamine (regarding the literature it should lead to a modest relaxation) or Acetylcholine (Ach; regarding the literature it should lead to a minimal relaxation) did not show any response on human placental arteries [1, 2]. Calcium Ionophore A23187 (regarding the literature it should lead to a minimal relaxation as well) has been employed by some collaborations partners (Roland Stocker group from Victor Chang Cardiac Research Institute, Sydney, Australia) in mouse, and it does not seem to be a good marker either. Immunohistochemistry seems to be the best method to check the presence/absence of the endothelium
- Sarafotoxin 6c (ED50 values: 165 ± 19 pmol) could be a possibility to check the presence/absence of endothelium while doing myography. Suggested concentrations to use for this are: 50, 100, 200 and 400 pM.

1) Add cumulatively U-46619 (agonist concentration response) as follows:

5uL of 10^{-6} M to get 10^{-9} M in the 5 mL bath (0,1nM)
1uL of 10^{-5} M to get $3x10^{-9}$ M in the 5 mL bath (0,3nM)
3.5uL of 10^{-5} M to get 10^{-8} M in the 5 mL bath (1nM)
1uL of 10^{-4} M to get $3x10^{-8}$ M in the 5 mL bath (3nM)
3.5uL of 10^{-4} M to get 10^{-7} M in the 5 mL bath (10nM)
1uL of 10^{-3} M to get $3x10^{-7}$ M in the 5 mL bath (30nM)
3.5uL of 10^{-3} M to get 10^{-6} M in the 5 mL bath (100nM)
1uL of 10^{-2} M to get $3x10^{-6}$ M in the 5 mL bath (300nM)
3.5uL of 10^{-2} M to get 10^{-5} M in the 5 mL bath (1μM)

You can skip the last 2 dilutions

Stock dilutions for 8 chambers:



Each concentration should be added cumulatively without washing the previous addition out. Each concentration is applied until a steady response has been reached. The next concentration is added once the plateau is reached.

- Other agonist such a Norepinephrine (NE), Endothelin-1 (ET-1), arginine vasopressin, angiotensin II, prostaglandin E2 and prostaglandin F2 alpha have been described in the literature for human placental vessels. U46619 (thromboxane analogue) was the most potent constrictor agonist studied in all of them, giving a strong, sustainable and reproducible constriction [1, 2, 3, 4]. Experiments with ET-1 (response weak and odd, with only 2 effective doses: 30 and 100 nM) and NE (the response is barely visible and in some cases there is no response) were conducted without satisfactory results. Hence, U46619 should be used in the study of constrictive responses to placental chorionic plate arteries.

2) Wash out U-46619 from the vessels for one hour (approx. 7 times).
3) Calculate EC_{50} for each vessel in the meantime while washing the U-46619.
4) Constrict each vessel with the EC_{50} using the U-46619 compound. Wait until the constriction is stable at a plateau. This will take more than 5 min, and can take up to 10-15 min, maybe longer depending on the vessel.

5) Wash U-46619 from the vessel preparation. Wash the vessels at least 5 to 6 times over at least 60 min to completely eliminate the thromboxane analogue.
6) Start preparation of 80 mM L-Trp stock solution while washing U46619 (see section d)
7) Once the baseline has been reached **incubate the vessels for at least 30 min with 300 μM L-NAME** (50 μL/chamber) **and 10 μM Indomethacin** (18 μL/chamber). Hence, we can exclude any effect coming from eNOS or COX
8) Constrict with EC_{50} concentration of U-46619.
9) Preparation is now ready for experiments.

d) Actual experiment: regulation of placental vascular tone

After completion of the wake-up protocol, the actual experiment can be conducted. A typical experiment would consist of concentration-response curves to one or several agonists in the presence or absence of antagonists. Depending on the agonists, multiple concentration-response curves protocols can be performed in the same vessel segment. If the agonists used are water-soluble and their effects are easy to wash out, then animals can be preserved and date collection can be maximized by performing several concentration-responses to different agonists.

1) Start preparing the stock solution of 80 mM L-Trp 10 min before reaching a plateau with the EC_{50} concentration. Prepare the stock solution as follows:

   Weight 245 mg out of L-Tryptophan and dissolve it in 15 mL Krebs buffer making a stock solution of 80mM. In order to better dissolve the L-Trp, warm the solution up (~40-50°C).

2) Once the stock solution is ready, place it into the waterbath until necessary for the experiment.
3) Once the plateau in each chamber is reached, start the L-Trp dose response:

   31.25 μL of 80 mM to get 0.5 mM in the 5 mL bath (1 μM)
   31.65 μL of 80 mM to get 1 mM in the 5 mL bath (3 μM)
   63.7 μL of 80 mM to get 2 mM in the 5 mL bath (10 μM)
   129.73 μL of 80 mM to get 4 mM in the 5 mL bath (30 μM)
   256.33 μL of 80 mM to get 8 mM in the 5 mL bath (100 μM)
   579.5 μL of 80 mM to get 16 mM in the 5 mL bath (300 μM)
   1336.9 μL of 80 mM to get 32 mM in the 5 mL bath (1 mM)

   Each concentration should be added cumulatively without washing the previous addition out. Each concentration is applied until a steady response has been reached. The next concentration is added once the plateau is reached.

4) Wash out U-46619 from the vessels for at least one hour (approx. 8 times).
5) Once the baseline has been reached, constrict with EC_{50} concentration of U46619 again.
6) Add 1 μM of Sodium Nitroprusside (SNP) to check the functionality of the smooth muscle cell layer. Wait until a relaxation response is seen (maximum 1 min).
7) Collect the vessels rings either in liquid nitrogen or in paraformaldehyde 4% for further analysis

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7.3. Embedding of Cell Pellets

1. Once you place the vessels into the myograph, the response to the KPSS goes always down. Could the vessels be naturally pre-contracted? → after adding 10-7 M SNP to all vessels 4 out of 6 relaxed but extremely little meaning that the vessels are not really pre-contracted
2. The relaxing effect of 1MetDTrp alone is reversible with time (within 10 min)
3. L-Trp maximum concentrated solution possible is 100 mM. However, better make a 80 mM stock as with 100 mM sometimes you can see some precipitates as T° decreases
4. L-Trp freshly-prepared from 100 mM stock after 2 days exposed to:
    a. Light and "higher T° → L-Trp changes colour and smell (from transparent to yellow/stronger smell)
    b. No light and "lower" T° → no yellow colour but some of them precipitated
    c. Kept at 4 °C → all precipitated
5. pH measurements of solutions used:
    a. Not bubbled Krebs Buffer = 8.30
    b. Freshly-prepared L-Trp not bubbled = 8.30
    c. Stored L-Trp less yellow (36 days old) = 8.22
    d. Stored L-Trp dark yellow (42 days old) = 8.40
    e. 1MetDTrp (stored up to 5 months) and not bubbled = 7.53
    f. Freshly-prepared L-Trp Bubbled = 7.45
6. IUGR placentas → in general passive tension is lower as it keeps on returning to the previous mN value as soon as you stretch the vessel. Dose-response to U46619 is also somestimes odd in terms of stability but very strong. Weaker

response obtained generally to KPSS (in compared with normal term placentas) and longer time to reach a plateau. Stronger response to the vehicle control than usual.

7. Once the vessel rings are left in the incubator with medium or medium + cytokines, the length of the vessel is shorter and the lumen is more occluded (a bit pre-contracted?). They have a weaker response then in general to KPSS but stronger to U46619
8. Preincubation of the vessel rings with 1MetDTrp (plus L-NAME and Indomethacin as well) always give contraction over time while the incubation period
9. Stimulated vessels show always a slight relaxation and then a contraction when applying 1MetDTrp alone
10. Denuded vessels seem to be more sensitive to the first concentration (0.5 mM) of L-Trp

7.4. Myography – Inclusion & exclusion criteria

Inclusion/Exclusion criteria

Inclusion criteria:

1. All vessels rings belong to the same vessel – long vessel required

Exclusion criteria:

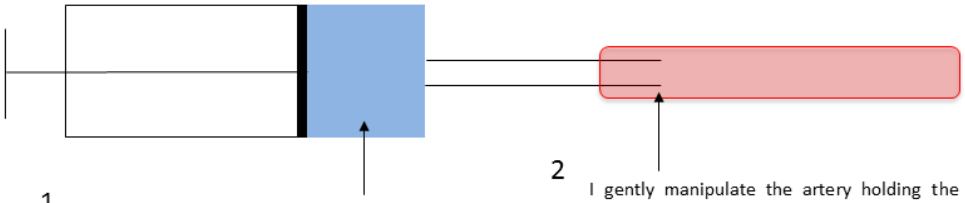
1. Physical examination
   a. Vessel ring is damaged or has been squeezed with the forceps
   b. Vessel ring has another branch/hole
   c. Vessel ring is smaller than 15 mm
   d. Vessel rings is larger than 25 mm
2. Performance during experiment
   a. Vessel ring does not show stronger contraction after KPSS than 2mN
      i. Additionally, vessel ring does not respond to dose response with U46619 (stronger than 3mN)
   b. Vessel ring exhibits strong and constant vasomotion (cyclic oscilation)
   c. Vessel ring precontracts stronger than 5 mN after applying L-NAME & Indomethacin
   d. Vessel ring does not show stronger contraction after EC_{50} of U46619 than 3mN
   e. Vessel ring does not reach a plateau after adding EC_{50} of U46619

Medical University of Graz Pablo Zardoya-Laguardia

7.5. Myography – Denudation procedure

Medical University of Graz

Institute for Cell Biology, Histology and Embryology	**Ref: SOP-003**
Denudation of Human Placental Arteries	Pages: 1
Written by: Chris Stanley	Modified by: Pablo Zardoya-Laguardia
Authorised by: Peter Sedlmayr	Myography



1

Triton X-100 (Sigma, product code T9284) made 10µL in 10mL Krebs buffer. **When making the mixture make sure the Krebs is at room temp**, if the Krebs is just out of the fridge the Triton X-100 will not mix properly.

2

I gently manipulate the artery holding the syringe with forceps into the tip of the vessel, the syringe tip is smaller than the artery. Break the end of the Syringe to prevent piercing the vessel. Hold the vessel in a vertical position with the syringe in the top of the vessel.

3 I perfuse between 50-100µL through the vessel lumen over a ten second period (~4 to 6 droplets normally). After doing this I then perfuse at least 3000-5000 µL Krebs only through the vessel over a minute period (~50 droplets). I then transfer the arteries to a clean dissection plate with only Krebs present.