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Heterogeneous vasomotor responses in segments from Göttingen Minipigs coronary, cerebral, and mesenteric artery: A comparative study

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\textbf{ABSTRACT}

Göttingen Minipigs (GM) are used as an important preclinical model for cardiovascular safety pharmacology and for evaluation of cardiovascular drug targets. To improve the translational value of the GM model, the current study represents a basic characterization of vascular responses to endothelial regulators and sympathetic, parasympathetic, and sensory neurotransmitters in different anatomical origins.

The aim of the current comparative and descriptive study is to use myography to characterize the vasomotor responses of coronary artery isolated from GM and compare the responses to those obtained from parallel studies using cerebral and mesenteric arteries. The selected agonists for sympathetic (norepinephrine), parasympathetic (carbachol), sensory (calcitonin gene-related peptide, CGRP), and endothelial pathways (endothelin-1, ET-1, and bradykinin) were used for comparison. Further, the robust nature of the vasomotor responses was evaluated after 24 h of cold storage of vascular tissue mimicking the situation under which human biopsies are often kept before experiments or grafting is feasible.

Results show that bradykinin and CGRP consistently dilated, and endothelin consistently contracted artery segments from coronary, cerebral, and mesenteric origin. By comparison, norepinephrine and carbachol, had responses that varied with the anatomical source of the tissues.

To support the basic characterization of GM vasomotor responses, we demonstrated the presence of mRNA encoding selected vascular receptors (CGRP- and ET\textsubscript{A}-receptors) in fresh artery segments.

In conclusion, the vasomotor responses of isolated coronary, cerebral, and mesenteric arteries to selected agonists of endothelial, sympathetic, parasympathetic, and sensory pathways are different and the phenotypes are similar to sporadic human findings.

\textbf{1. Introduction}

\textbf{1.1. Rodent and porcine models of the human cardiovascular system}

Rodent models of neurovascular and endothelial regulation of vascular tone have been thoroughly studied in vivo and in vitro and does not always offer good translation to the sparse human vascular biopsies available from surgery \cite{25,33} and autopsy \cite{51}.

While small animal models of cardiovascular physiology and disease involving mice, rats, and rabbits are widely used, they lack similarity to human physiology with regard to vascular anatomy and haemodynamics and are mainly useful for dissecting the mechanical and molecular mechanisms of tissues \cite{56}. For example, the rodent coronary left anterior descending artery (LAD) and associated branches are all embedded within the myocardium, whereas in larger mammals the LAD usually runs on the epicardial surface of the heart \cite{42}. Furthermore, rats are resistant to atherosclerosis while pigs share similar characteristics of lipoprotein metabolism including cholesterol distributions and enzymatic activities and vasculature anatomy including heart size and coronary circulation. Like humans, pigs are omnivores and the vascular

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response to the increase in fat content of the diet is similar and, elderly pigs can sponta-
nously develop atherosclerosis [22].

In current regulations for conducting preclinical trials, it is mandatory to carry out trials on non-rodents such as rabbits, dogs, cats, pigs, or primes simultaneously with rodents.

Therefore, in order to obtain reliable preclinical pharmacology data, large animal models that to a greater extent mimic human anatomy and physiology are needed.

Pigs have substantial potential as biomedical models for studying human developmental processes, congenital diseases, metabolic [47] and cardiovascular diseases [5], and pathogen response mechanisms in addition to utility as xenotransplant organ donors and tools for vaccine and drug design. The similarity of pigs to humans in anatomical size and structure, physiology, immunology, and genome enhances their potential as models for human disease. Hence, it is imperative that research is relevant and reproducible in animal models that more closely resemble humans, such as the pig [36] and as such, pigs should always be considered as a model for translational studies [40]. Specifically, Göttingen Minipigs is globally the most widely used animal model for toxicity testing [45] since it is rather small and well-defined genetically as well as microbiologically. Also, like other pigs, Göttingen Minipigs resemble humans in many aspects of anatomy and physiology, and therefore findings made in Göttingen Minipig (GM) will often be predictive of findings in humans [36,40,45].

GMs are considered superior cardiovascular animal models when compared to rodent models as their anatomy, organ size, and physiology resembles the human heart [7,24]. Indeed, the heart anatomy of GM is very similar to the human heart [50] whereas both are distinct from the more commonly studied rodent hearts. Further, the porcine genome is closer to the human genome than the rodent [59,60]. Although the GM genome is not yet completely characterized it does show a high degree of sequence homology to the porcine strain sus scrofa which is genetically characterized. Thus, probes developed for sus scrofa might be useful for detection of mRNA expression in GM.

The size of the GM organs allows us to dissect sufficient artery segments for thorough characterization and robust paired statistical comparisons using a very limited number of animals [34] and within cardiovascular disease, thus porcine models offer an obvious preference [18].

Despite the wide use of porcine disease and safety models, pharmacological studies of basic healthy porcine vasculature are relatively uncommon, and when available focusing on specific receptor agonists and limited to the study of a specific anatomical location. Indeed, a strict comparison of the pharmacology of different vascular beds in GM is lacking to further demonstrate and verify the translation of basic vascular responses in the model.

1.2. Regulation of coronary, mesenteric, and middle cerebral arteries

Vascular tone of arteries is mainly regulated through myogenic control, endothelium, locally released mediators, circulating vasoactive substances, and perivascular nerves.

The vascular endothelium is central to the regulation of blood flow by producing several vasoactive factors such as nitric oxide, endothelium-dependent hyperpolarization factor, eicosanoids, and endothelins. However, arteries from unique vascular beds and of difference size vary significantly in their sensitivity for endothelial, neural, hormonal, and local influences ranging from being under total control by local metabolites to dominance by sympathetic nerves [26]. For example, in resistance coronary arteries in vivo, the resting tone is high, and the blood flow is regulated by the metabolic demands of the heart tissue. Increased metabolic rate of the heart tissue normally leads to production of metabolically released vasodilators and causes a significant increase in blood flow by reducing the spontaneous myogenic resting tone of coronary artery. In contrast, changes in sympathetic activity have only minor effects on coronary artery tone [38]. In mesenteric arteries, however, the normal resting vascular tone is low leading to relatively high blood flow in this tissue. Therefore, enhanced sympathetic activity causes a significant decrease in blood flow in mesenteric arteries, whereas decreased sympathetic activity significantly increases the blood flow [17].

However, the blood flow in mesenteric arteries is relatively less affected by changes in metabolic rate [43]. This regional heterogeneity in the regulation of vascular tone may reflect the physiological demand on the vascular segments in different vascular beds. The regulation of the tone and blood flow in resistance coronary arteries are closely linked to the metabolic status of the heart, since myocardial perfusion tightly follows myocardial O2 requirements [11].

Cerebral blood flow (CBF) is also tightly regulated by cerebral metabolism. Coupling between flow and metabolism in the brain refers to the ability of cerebral arteries to adjust the blood flow to meet the metabolic requirements of the cerebral tissue, which is the least tolerant tissue to ischemia [16]. The regulation of cerebral blood flow is much more complex as perivascular nerves and astrocytes also serve to modulate cerebral blood flow, referred to as the neurovascular unit [23]. The different cell types within the neurovascular unit that contribute to neurovascular coupling include the vascular smooth muscle cells, the neuron, the astrocyte glial cells, pericytes and endothelial cells. Neurovascular coupling is a unique mechanism that controls regional cerebral blood flow (CBF) and ensures a rapid increase in the rate of CBF to activated brain structures [31].

A classical study conducted by Roy and Sherrington [49] proposed that the “chemical products of cerebral metabolism” can cause variations of the caliber of the cerebral vessels and through this the brain possesses an intrinsic mechanism by which its vascular supply can be modified locally in response to the local variations in functional and metabolic activity. Since then, a number of studies have shown that arterial and brain tissue partial pressures of CO2 and concentrations of H+ are principal factors in the regulation of cerebral blood flow [6,10]. Recent study linking the neuronal activity with regulation of local cerebral blood flow via the actions of CO2 has demonstrated that the neurovascular response in the cerebral cortex can be blocked by saturation of the brain CO2-sensitive vasodilatory mechanism (via delivery of exogenous CO2), or by inhibition of CO2/HCO3− transport and pH regulation following disruption of sodium bicarbonate cotransporter 1 expression in astrocytes [27].

1.3. Aim

The purpose of the current study is to characterize the function of healthy male GM arteries isolated from three different anatomical origins (coronary, mesenteric, and cerebral artery) and harvested from the same animals. The pharmacological characterization includes comparison of coronary, cerebral, and mesenteric response to selected agonists for sympathetic (norepinephrine), parasympathetic (carbachol), sensory (CGRP), and endothelial pathways (ET-1 and bradykinin).

The in vitro pharmacological studies were conducted using isometric myography.

Further, to model the typical time of the human tissue journey from clinic to laboratory, the vascular pharmacology of fresh and 24 h cold-stored segments are compared.

With the consistent functional effects of CGRP and ET-1 demonstrated in this study, we aimed to evaluate whether probes, developed for porcine strains different from the Göttingen Minipig, could be used for quantitative RT-PCR demonstrating CGRP- and ETA receptor mRNA in vasculature of this strain.

2. Materials and methods

2.1. Chemicals and solutions

Physiological salt solution (PSS) used for vascular studies had the
following composition (in mM): NaCl 119 (Sigma), NaHCO3 25 (Sigma), KCl 4.7 (Fluka), CaCl2 2.5 (Merck), KH2PO4 1.18 (Sigma), MgSO4·7H2O 1.17 (Sigma), ethylenediaminetetraacetic acid (EDTA) 0.027 (Sigma) and glucose 5.5 (Sigma), with pH adjusted to 7.4. Potassium enriched PSS (KPSS) was prepared by replacing all sodium chloride with an equimolar amount of potassium chloride resulting in a total K+ concentration of 125 mM. PSS and KPSS were aerated with 5% CO2 /95% O2 to maintain pH 7.4. KPSS was diluted in PSS to obtain final potassium concentrations of 30 mM (K30) and 60 mM (K60).

Selected agonists for vasomotor studies using sympathetically, parasympathetic, sensory and endothelial pathways were obtained: U46619 (preconstrictor and thromboxane A2 agonist, Cayman 16,450), Bradykinin (endothelim-dependent vasodilator and Bradykinin B2 receptor agonist, Sigma B3259), Endothelin-1 (vasoconstrictor and Endothelin ETA and ETB receptor agonist, Bachem H6995), Norepinephrine (sympathetic neurotransmitter and agonist for adrenergic α- and β receptors, Sigma A7257), Carbachol (C4382), Human alpha calcitonin gene-related peptide (αCGRP, vasodilator, sensory neuropeptide, and αCGRP-receptor agonist, Bachem). Bradykinin and CGRP were dissolved in water, endothelin-1 and norepinephrine in 1% acetic acid. All dissolved chemicals were stored in chlorinated tap water). The room temperature was kept at 22–24 °C. Lights were on between 6 a.m. and 7 p.m. (100–200 lx) and GMs were fed a SDS minipig diet and weighed monthly.

2.2. Animals and organ isolation

Six healthy male GMs were kept in accordance with their natural behaviour. GMs were socially housed at the AALAC accredited barrier facility at Ellegaard Göttingen Minipigs A/S, Dalme, Denmark with straw as bedding material, various types of environmental enrichment, and unrestricted access to drinking water (non-acidified, non-chlorinated tap water). The room temperature was kept at 22–24 °C. Tissue (heart, brain, and 10 cm of the distal jejunum) from healthy male GMs (average age being 3.8 months ±0.4 and body weight being 8.6 kg ± 0.8 (mean ± SD), respectively) were obtained from six Ellegaard Göttingen Minipigs A/S over 6 weeks (Table 1).

Animals were handled, sedated, and terminated according to the animal experimentation legislation of Denmark and approved by the Danish Animal Experiments Inspectorate (license number: 2017-15-0201-01202). In brief, animals were sedated with Zoletil mixture (Zoletil 50% dry matter (50 mg/ml) + 6.25 ml xylazine (20 mg/ml) + 1.25 ml Ketamine (100 mg/ml) + 2.5 ml butorphanol (10 mg/ml)), 0.1 ml/kg i.m. After full sedation, the animals were euthanized by an i.v. overdose of pentobarbital (Exagon Vet.; pentobarbital sodium 2.3. Artery tissue dissection

A section of the left ventricle was cut out from the heart and placed in PSS buffer (4 °C). The first order branches of the left anterior descending (LAD) coronary artery (CA) were dissected free from the myocardium (Fig. 1).

The left and right middle cerebral artery (MCA) were dissected free from connective tissue, and arterial segments isolated from the second-order branches were used. The jejunum biopsy was pinned down with needles and second-order mesenteric arteries (MA) were dissected free of fat and connective tissue. Each artery branch was cut into 8 cylindrical arterial segments of 2 mm for immediate myograph vascular force measurement and 4 for 24 h cold storage (4 °C, vessel kept on a single wire) and delayed myograph studies.

2.4. Myograph studies

Vasoconstrictive and vasodilatory responses of isolated artery segments were continuously recorded using small vessel isometric wire myographs [63,64].

Briefly, arterial ring segments were mounted on two stainless steel wires (40 μm diameter) in the organ bath of a small vessel wire myograph (Danish Myo Technology, Aarhus, Denmark). One wire was connected to a micrometer screw enabling adjustments of the distance between the wires. The other wire was connected to a force displacement transducer attached to an analogue-digital converter unit (ADInstruments, Chalgrove, UK). Measurements of vascular tone were recorded using a data acquisition hardware Power Lab unit (ADInstruments). Each segment was immersed in PSS (bicarbonate-based buffer solution) kept at 37 °C, continuously aerated with 5% CO2 /95% O2 in order to maintain pH at 7.4. To ensure optimal conditions for maximal force development, each artery segment was carefully stretched to 90% of the internal circumference that the vessel would have under the passive transmural pressure of 100 mmHg. Following an equilibration period of approximately 20 min, each segment was exposed twice to K60 to confirm smooth muscle cell viability as well as the reproducibility of evoked contractions. Endothelium was mechanically removed by carefully scratching the inner surface of the artery segment with a human scalp hair.

After 15 min equilibration, a stable precontraction tone (50–80% of the K60-induced steady state contraction) was induced in arterial segments by U46619 (1 μM) or by K30 to study vasodilatory responses. The segments were then challenged by bradykinin to confirm endothelial removal and the consecutive protocol of stimuli were separated by PSS wash.

Concentration-dependent vasodilatory responses of artery segments were normalized as percentage of precontraction tone for comparison between CA, MCA and MA. Concentration-dependent vasoconstrictive responses were normalized as percentage of the contraction obtained by K60.

2.3. Artery tissue dissection

Table 1

Baseline characteristics (gender, body weight and age) of six individual healthy animals and baseline standard characteristics of blood glucose, cholesterol and triglycerides (mM) from healthy male minipigs, Ellegaard Göttingen Minipigs A/S, Denmark (https://minipigs.dk/about-gottingen-minipigs/background-data). Average values are given as mean ± SD (n).

<table>
<thead>
<tr>
<th>Animal #</th>
<th>Gender</th>
<th>Weight, kg</th>
<th>Age, months</th>
<th>Blood glucose, mmol/l</th>
<th>Triglycerides, mmol/l</th>
<th>Cholesterol, mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>9.5</td>
<td>4.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>8.8</td>
<td>4.37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>8.6</td>
<td>3.81</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>8.8</td>
<td>3.62</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>7.2</td>
<td>3.35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>8.6</td>
<td>3.58</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>Male</td>
<td>8.6</td>
<td>3.81</td>
<td>4.55</td>
<td>4.48</td>
<td>0.41</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td>0.8</td>
<td>0.38</td>
<td>0.96</td>
<td>0.91</td>
<td>0.16</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>6</td>
<td>6</td>
<td>17</td>
<td>17</td>
<td>17</td>
</tr>
</tbody>
</table>
2.5. RNA extraction

Total RNA from LAD segments (n = 3) from GM were isolated using spin columns (NucleoSpin miRNA, Mini kit for total RNA from MACHERY-NAGEL) in combination with QIAzol (Qiagen) and chloroform (Sigma) according to the manufacturer’s recommendations (version 07–2013, rev. 03). The samples were homogenized using QIAzol lysis buffer (Qiagen) and 1.4 mm ceramic beads (Lysing Matrix D, MP Biomedicals, USA) for 40 s at max speed using a FastPrep-24TM 5G instrument (MP Biomedicals, USA). The RNA concentration was measured using a Nanodrop 2000c (Thermofisher) at 260 nm.

2.6. cDNA and qRT-PCR

0.5 μg RNA from LAD were reverse transcribed using the iScript cDNA Synthesis Kit (Biorad) according to the manufacturer’s protocol. qRT-PCR was performed in a 10 μl reaction volume containing RNase-free water, 20× pre-designed TaqMan porcine specific gene expression assay (ThermoFisher Scientific, USA), 2× TaqMan universal PCR master mix (ThermoFisher Scientific, USA), and 2 μl cDNA using the QuantStudio 6 Pro Real-Time PCR system (Applied Biosystems). The thermal cycling condition included an initial denaturation step at 50 °C for 2 min and 95 °C for 10 min followed by 45 PCR cycles at 95 °C for 15 s and 60 °C for 1 min. Commercially available pre-designed (from the genome of the porcine *sus scrofa* strain) TaqMan gene expression assays used in this study: RAMP1: Ss_06942043_mL; EDNRB: Ss_03379833_u1; CALCRL: Ss_03394018_mL; β-actin: Ss03376563.

2.7. Analysis and statistics

 Vasodilation is expressed as percentage remaining contraction of the precontraction tone induced by 1 μM U46619 or K30. Active tension was determined by subtracting the passive tension (resting tension in PSS) from the total tension (during K30, K60 or U46619 challenge) and given in mN/mm.

All pharmacological data were analysed using Prism 9.04 (GraphPad Software Inc., San Diego, CA) and given as mean ± S.E.M. For all analyses, the data were unweighted and each y value (mean of 6–12 replicates for each individual experiment) was considered an individual point. All concentration-response data were analysed with a three-parameter logistic equation as described previously [29]: Y=Bottom + (Top-Bottom)/(1 + 10^((LogIC_{50}-X)*HillSlope)), where Bottom represents the y value in the absence of ligand(s), Top represents maximal stimulation in the presence of ligand, [A] is the molar concentration of ligand and IC_{50} is the molar concentration of ligand required to elicit a response halfway between Top and Bottom.

All data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology [9]. Differences in pEC_{50} and E_{max} values were analysed with paired (animal) t-tests and considered significant when P < 0.05. To evaluate whether E_{max} and pEC_{50} differed between groups and whether E_{max} values differed from a hypothetical value (0 or 100%) Extra sum-of-squares F-tests were applied and null hypothesis rejected when P < 0.05.

3. Results

The results section is divided in physical characterization (size and contractility, n = 12) (3.1) of the artery segments from coronary, cerebral, and mesenteric origins followed by characterization of responses to selected agonists (n = 6) for endothelial pathways (3.2), sensory (CGRP) (3.3), sympathetic (norepinephrine) (3.4) and parasympathetic (carbachol) (3.5). In Section 3.6 fresh and 24 h segments were compared, and in Section 3.7 spontaneous vasoactivity is compared. When vasomotor responses in different arteries were similar, efficacy and potency were compared.

The presence of mRNA encoding 2 selected receptors are demonstrated in Section 3.8.

3.1. Arterial size and contractility

The average normalized internal circumferences (IC1) for fresh experimentation of coronary, cerebral and mesenteric segments were 2442 ± 76, 2023 ± 114, and 664 ± 24 μm, respectively (mean ± SEM) and corresponding to the internal diameter of 777, 644, and 211 μm (Table 2A). The artery size as well as the constriction induced by K60, K30 and/or 1 μM U46619 differed between the coronary artery and the others. The “internal circumference” measured at passive transmural pressure of 100 mmHg (IC_{100}) and the corresponding normalized value (IC_{C}) which is 90% of the internal circumference at 100 mmHg (IC_{1} = 0.9 IC_{100}) were kept during the whole experiment with the isolated arterial segments.

Different size and contractility between different artery origins is relevant for the interpretation of the pharmacological responses described in the below sections since these basic characteristics may reflect artery sensitivity to constrictive and dilatory pharmacological stimuli.

3.2. Endothelial function

Mechanical removal of endothelium causes a reduction in both K30- and U46619-induced contraction whereas the removal of endothelium does not significantly change the maximal contractility as defined by K60-evoked vasoconstriction. While the reduction of K30- and U46619-induced contraction is significant in endothelium-denuded mesenteric and middle cerebral arteries, the coronary artery constrictions seem to be independent of endothelial removal (Table 2B).
Table 2
Baseline characteristics of coronary, cerebral, and mesenteric artery sections used for the pharmacological comparison in this study. Diameter, IC1 (normalized internal circumference) and contractility of artery segments selected from coronary, cerebral, and mesenteric locations with intact endothelium (A) and with physically removed endothelium (-EC) (B). Data are given as mean ± SEM and n is the number of artery segments.

<table>
<thead>
<tr>
<th></th>
<th>Coronary (CA)</th>
<th>Cerebral (MCA)</th>
<th>Mesenteric (MA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A With endothelium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>k60, mN/mm</td>
<td>8.8 ± 0.7</td>
<td>7.4 ± 0.8</td>
<td>4.6*** ± 0.4</td>
</tr>
<tr>
<td>U46619 (1 μM), mN/mm</td>
<td>4.0 ± 0.8</td>
<td>8.1* ± 0.9</td>
<td>6.6 ± 1.1</td>
</tr>
<tr>
<td>K30, mN/mm</td>
<td>10.4 ± 0.8</td>
<td>7.2* ± 1.0</td>
<td>5.0*** ± 1.1</td>
</tr>
<tr>
<td>IC1, μm</td>
<td>2442 ± 76</td>
<td>2023* ± 114</td>
<td>664*** ± 24</td>
</tr>
<tr>
<td>Diameter, (IC1/π), μm</td>
<td>777 ± 64</td>
<td>644 ± 211</td>
<td></td>
</tr>
<tr>
<td>B Without endothelium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA-EC</td>
<td>8.0 ± 0.8</td>
<td>8.2 ± 0.9</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>MCA-EC</td>
<td>2.6 ± 0.7</td>
<td>2.4### ± 0.5</td>
<td>0.8### ± 0.1</td>
</tr>
<tr>
<td>MA-EC</td>
<td>8.8 ± 1.2</td>
<td>3.1# ± 0.5</td>
<td>1.0## ± 0.4</td>
</tr>
<tr>
<td>IC1, μm</td>
<td>2336 ± 90</td>
<td>1939 ± 91</td>
<td>678 ± 20</td>
</tr>
<tr>
<td>Diameter, (IC1/π), μm</td>
<td>744 ± 617</td>
<td>617 ± 216</td>
<td></td>
</tr>
</tbody>
</table>

Significant differences (paired t-tests) between selected artery with intact endothelium and coronary artery with intact endothelium are indicated (*: P < 0.05; ###: P < 0.001). Significant differences between cognate characteristics of endothelium-denuded arteries and endothelium-intact arteries are given (#: P < 0.05; ##: P < 0.001).

3.2.1. Bradykinin

The vasodilatory responses to bradykinin were used to verify endothelial removal.

In all arteries bradykinin induces a significant endothelium-dependent vasodilatation (Fig. 2) with similar E\textsubscript{max} but with a higher potency (pEC\textsubscript{50}) in middle cerebral than in coronary and mesenteric arteries despite MCAs higher level of constriction by the pre-constrictor U46619 (Table 2). Physical removal of the endothelium systematically reduces E\textsubscript{max} which confirms efficient endothelial removal (Fig. 2).

3.2.2. Endothelin

Exogenous endothelin induced significant vasoconstriction in coronary, cerebral and mesenteric artery segments with intact endothelium. The ET-1 potency is similar in cerebral and mesenteric arteries (pEC\textsubscript{50} = 8.7 and 8.3, respectively), whereas it is significantly lower in coronary artery (pEC\textsubscript{50} = 7.4, P < 0.01). The efficacy is not different between CA and MA but is significantly lower in cerebral MCA (Fig. 3). The ET-1-induced constriction is given relative to the constriction induced by K60 which is lower in mesenteric than coronary and cerebral artery (Table 2) pointing towards a ET-1 relevant difference in the current comparison.

3.3. CGRP - sensory neuropeptide sensitivity

The effect of the sensory neuropeptide CGRP was assessed in coronary LAD artery (CA), middle cerebral artery (MCA), and mesenteric artery (MA) segments preconstricted with K30. Although, the level of preconstriction with KPSS30 differed between the arterial segments taken from three different vascular beds (Table 2), there was no significant difference in CGRP potency (average 5 nM) and efficacy (average 26% remaining constriction) (Fig. 4).

Further, in artery segments from all three anatomical origins, the potency and efficacy were independent of the presence of endothelium (Fig. 4).
averaging 26% of K30 constriction remaining. The efficacy is also similar comparing the three arterial segments (Fig. 4).

3.4. Norepinephrine – sympathetic sensitivity

The sympathetic neurotransmitter norepinephrine (NE) induces very different effects in cerebral, coronary, and mesenteric arteries (Fig. 5).

In U46619 (1 μM) preconstricted artery segments, NE induce additional vasoconstriction in mesenteric artery whereas it significantly dilates coronary arteries. However, in the cerebral artery, NE induce partial high potency vasodilation. In artery segments that have not been preconstricted, NE induce constriction in mesenteric and cerebral artery whereas no significant vasoactivity is observed in coronary artery (data not shown).

3.5. Carbachol – parasympathetic sensitivity

Carbachol, the metabolically stable analogue of parasympathetic neurotransmitter acetylcholine, induce opposite effects as compared to the sympathetic activation with NE. Carbachol induces different effects in CA, MCA, and MA. It induces further constriction of preconstricted coronary artery segment with an E\textsubscript{max} exceeding 200% (P < 0.001). In contrast carbachol induces dilation of preconstricted mesenteric artery with E\textsubscript{max} being not significantly different from 0% contraction but no significant effect on cerebral artery was demonstrated as E\textsubscript{max} was not significantly different from 100% contraction (P = 0.2052) (Fig. 6). Artery segments that are not preconstricted shows no significant vasoactivity to carbachol (data not shown).

3.6. Effect of storing artery samples for 24 h

In the current study the organs were transported in ice cooled PSS buffer to the laboratory. Here, the artery segments were dissected, kept is PSS, and mounted in myograph organ baths in PSS before the 8 h protocol was finished. To investigate the effect of 24 h cold-storage, we evaluated the effect of keeping isolated arteries in ice cooled PSS until the following day when we conducted part of the protocol.

In general, both pharmacology and potency of the pathways tested was fully maintained (data not shown). The only difference observed was related to the absolute level of contractions by K30, K60, and U46619 in coronary artery segments which was dependent on the lumen diameter of arterial segments.

For CGRP and ET-1, there was no significant difference between concentration-response curves obtained in fresh versus 24 h stored CA, MCA, or MA segments. For all dose-response curves, however, a tendency towards increased sensitivity was noted (Table 3).

For Bradykinin, a tendency of increased sensitivity from day to day was also seen although specifically in cerebral arteries, the efficacy after storage was reduced. This could be due to cerebral artery specific functional disruption of the endothelial function (see Fig. 7).

Both the quantitative and the qualitative differences of NE effects also remained (data not shown).

This is not only important for the current translational characterization of essential vascular modulators. It also strengthens the interpretation of results from human tissue from autopsy which are newer obtained fresh.

3.7. Vasospasms

Comparing fresh arteries isolated from different vascular beds or organs in one animal allows assessment of the functional distinction between arteries. Here it becomes evident that cerebral arteries behave differently compared with coronary and mesenteric artery in myograph studies (Fig. 8). They elicit periodic spontaneous vasospasms and might become hyperexcitable as most stimuli appears slightly more potent in the cerebral as compared to mesenteric and coronary artery.
3.8. RT-PCR

Since the GM is a crossbreeding of the Minnesota Minipig, the Vietnamese potbelly pig, and the German Landrace, which are genetically different from the pig, *Sus Scrofa* we set out to test if commercially available TaqMan gene expression assays could demonstrate the expression of the three selected genes (Fig. 9). We aimed to detect potential mRNA expression of three genes relating to the conserved pharmacological constrictive and dilatory actions of ET-1 and CGRP: *EDNRB* (ETB receptor gene), and *CALCR* and *RAMP1* (CGRP-receptor genes) in the coronary LAD segments (CA) from GM.

Pre-designed TaqMan assays detecting *RAMP1*, *EDNRB*, and *CALCR* specific for *Sus Scrofa* were tested, and showed that all three genes were highly expressed in CA (Ct-value = 28–34). All data was normalized to the *Sus Scrofa* specific β-actin.

4. Discussion

To the best of our knowledge, this is the first study that pairs and compares the vasomotor responses of arterial segments isolated from three different vascular origins (coronary LAD, cerebral MCA, and mesenteric branches). It is also the first study that describes and compares vascular effects of selected sensory, sympathetic, parasympathetic, and endothelial regulators in GM vasculature. We also demonstrate that commercially available gene expression probes can be used to confirm expression of CGRP and ET receptors at least.

4.1. The effect of bradykinin and ET-1

Bradykinin is an endothelium-dependent vasodilator activating endothelial B2R [28] that acts to relax vascular smooth muscle following release of endothelial nitric oxide. Here we have used it to confirm efficient and successful endothelial removal (Fig. 2).

The peptide endothelin ET-1 is established as a vasoactive peptide that is an agonist at ET<sub>A</sub> and ET<sub>B</sub> receptors which, under normal conditions...
conditions are expressed in vascular smooth muscle and vascular endothelium, respectively. In ischemic situations, ET\(_B\) receptors are induced and therefore upregulated in vascular smooth muscle mediating vasoconstriction [13]. In all arterial segments isolated from the three different vascular beds of GM, ET-1 induced a concentration-dependent contractile response. The results and statistics show that the cerebral arteries are the most sensitive (EC\(_{50}\)) with the lowest E\(_{\text{max}}\) (as percentage of K\(_{60}\)) (Fig. 3). It is noted the maximal response to the comparator KPSS is similar in CA and MCA (Table 2) which indicates that the ET-1 effect is not likely to reflect a general constrictive difference among the arteries.

4.2. The effect of CGRP

Our study clearly demonstrated that CGRP-induced concentration-dependent relaxations were consistently potent and endothelium-independent in all arterial segments isolated from three different vascular beds of the GM (Fig. 4).

The potency of 5 nM is consistent with previous studies [54] which have also shown the endothelium-independency of CGRP-induced vasodilations in specific preparations of porcine coronary, cerebral, and mesenteric arteries [15,62] and also in isolated intramural coronary arteries of both male and female rats [46,53]. When the current study is designed for the comparison between anatomical origins, the cerebral concentration-response curves were overlapping (similar potency and efficacy) in porcine mesenteric, coronary, and cerebral arteries in the nanomolar range (Fig. 4).

Interestingly there is evidence from a range of rodent studies that CGRP can act in an NO-dependent manner, for example in the rat aorta [65] and as reviewed [4]. However, the NO-independent effects of CGRP are well established, even in rodents (e.g., Argunhan et al., [66]). Here, in the porcine model that possesses anatomy that is more similar to human tissue, CGRP effects were examined in parallel in segments from three different anatomical origins showing endothelium-independency and overlapping potency. Of note is that endothelial dependency in LAD branches of coronary artery might differ depending on the location and the size of vessels [37,46,52].

The GM coronary LAD branches examined in this study demonstrates no endothelium dependency – at least when precontracted with K\(_{30}\) (Fig. 4). The similar potency and efficacy of CGRP in different artery sources found in this study indicates that previous potency differences in various artery sources as reviewed [54] might reflect differences in study design or conduction in different laboratories.

4.3. Effect of 24 h of cold storage

There is often a delay between human tissues being collected and the subsequent experiment. Here, we chose 24 h of cold storage as a maximum time of delay. This period appeared to induce a mild trend towards increased sensitivity to exogenously applied CGRP and ET-1, indicating that the vascular response to CGRP and ET-1 are preserved upon 24 h cold storage (Table 3). The only significant effect of cold storage was the middle cerebral artery-specific reduced bradykinin-induced E\(_{\text{max}}\) (Fig. 7). This might indicate that the endothelial nitric oxide pathway may be less robust in the MCA which supports previous findings that cerebral artery is the least tolerant tissue to ischemia [16].

We saw no difference in GM coronary artery endothelial responses which contrasts with previous findings in Wistar rat coronary arteries after being subjected to cold storage for up to 30 h. The study [30] showed that 15- and 30- h cold storage caused endothelial dysfunction of coronary arteries as the maximal relaxation to acetylcholine was decreased in both proximal and distal coronary arteries. Heart tissue storage time and timing of storage of isolated coronary artery segments might explain the difference of endothelial and potentially nitric oxide conservation.

4.4. The effect of norepinephrine

In our study, NE induced concentration-dependent contractions in GM mesenteric arteries while coronary arteries and cerebral arteries dilated upon NE stimulation (Fig. 5).

Contractions of most blood vessels to NE result from stimulating primarily alpha-1 adrenergic receptors, while coronary arteries relax to NE by beta-receptor stimulation [3,44]. In contrast to the rodent vessels, a previous study using a selective alpha 2-adrenoceptor agonist (B-HT 933) showed that the agonist induced contractile responses in both human and porcine mesenteric arteries, demonstrating a strong post-junctional \(\alpha_2\)-receptor component in mesenteric arteries of these species. However, this receptor was involved in NE-activated contractile responses only in human mesenteric arteries [41]. A recent study has shown that adrenergic control in mesenteric arteries is almost identical in humans and pigs. Both the evolutionary analysis of the amino acid sequences of the \(\alpha_1\) and \(\beta_2\) adrenoceptors and the quantification of the expression of these receptors revealed the presence of these receptors in the tunica media of mesenteric arteries of both humans and pigs [8].

In small, as well as large, isolated coronary arterial strips of normal pigs, norepinephrine produced relaxation which was found to be mediated through activation of \(\beta_1\) adrenoceptors. While alpha adrenoceptors were also found to be present in large coronary arteries, they were absent in the small coronary arteries [1]. A previous study also shows that isolated human coronary arterioles and small arteries dilate to NE via binding to \(\beta_2\) receptors located on smooth muscle [55] and a significant vasodilatory effect of NE in GM coronary artery thus mimics the human situation.

A previous in vivo study showed that intracarotid infusion of norepinephrine (100 ng/min) increased cerebral blood flow in new-born pigs. Norepinephrine-induced increase in cerebral blood flow was accompanied by an increase in cerebral oxygen consumption. Both the increase in cerebral oxygen consumption and the increase in cerebral blood flow were blocked by propranolol. These data show that intrarterial norepinephrine increases cerebral blood flow and cerebral oxygen consumption in new-born pigs [48] which is also supported by our data with a vasodilatory effect of NE in the GM middle cerebral artery (Fig. 5).

Another in vitro study using the large arteries at the base of the brain in the pig showed vasodilatory response to NE in these vessels which was blocked by \(\beta\) adrenoceptor antagonists [61]. It is obvious that the \(\beta\) receptor is predominant in the large cerebral arteries of the pig. However, this is different from results reported in most species [12,14], where noradrenaline induced cerebral vasoconstriction via \(\alpha_1\) adrenoceptors.
Majority of animal models indicate an increase in cerebral vasomotor responses in the three different GM vascular beds: Significant pre-constriction dependent vasodilatation was observed in GM mesenteric arteries, while contractile responses and non-significant responses were observed in GM coronary and cerebral arteries, respectively (Fig. 6). A previous study showed that about 25% of porcine cerebral arteries with intact endothelium relaxed upon application of acetylcholine at a low concentration (0.3 μM – 3 μM), whereas 61% of them constricted exclusively at both low and high concentrations of acetylcholine (0.1 μM – 3 mM) and the remaining 14% did not respond. All the pig cerebral arteries without intact endothelium (confirmed by scanning electron microscopy) constricted exclusively at both low and high concentrations of acetylcholine. These results are consistent with previous findings in other species [20] and suggest that acetylcholine-induced vasodilatation of the porcine cerebral artery is dependent on the endothelial cells, and that the direct action of acetylcholine on smooth muscle cells is constriction [32]. Cerebral vasodilatation associated with stimulation of non-adrenergic, non-cholinergic (NANC) nerve has previously been reported [57]. Following studies by the same author have shown that cerebrovascular tone is under control of small nitricergic perivascular nerves, which similarly to postganglionic parasympathetic nerves are activated by nicotinic-cholinergic receptors. It was also shown that nicotine-induced relaxation of cerebral arteries was mediated by the neuronal release of nitric oxide [58]. The lack of vasodilatation of the porcine cerebral artery with intact endothelium on application of acetylcholine may suggest a possible unusual coupling between endothelial cells and smooth muscle cells in this species which is also noted in the human situation [2]. Furthermore, differences in the density and type of innervation (i.e., NANC) can explain differences between results obtained from various studies. Another potential explanation can be that the vasoconstriction mediated via smooth muscle muscarinic receptors cancels out or repeals the vasodilatation mediated via the endothelial muscarinic receptors.

As far as the GM coronary arteries in our study is concerned, carbachol induced concentration-dependent contraction in these vessels, which is supported by previous observation [39] that acetylcholine produced a very powerful contractile response in isolated porcine intramyocardial resistance arteries (≈ 223 μm). The same observation for acetylcholine is made in isolated human coronary arteries [21]. In addition to species variation, there is also evidence for significant differences in vascular reactivity occurring as a function of concentration/dose of vasoactive compound, vessel size and anatomical location (i.e., distal vs. proximal) [46]. This can be attributed to differences in receptor type or –subtype, intracellular signalling (i.e., endothelial dependency) and to heterogeneous receptor and innervation density, reflecting the physiological demand on each vascular bed.

Most studies have been concerned with adrenergic and cholinergic responses of large epicardial arteries, although it is generally accepted that the major component of blood flow resistance and, hence, control of blood flow resides in smaller arteries and arterioles. From the viewpoints of both size and pharmacological reactivity, porcine coronary arteries appear to be more similar to those of humans than to the canine arteries (non-rodent mammal).

4.6. Cerebral artery vasospasm

It has previously been noted that vasospasms are common in cerebral artery. Since this is the first study to conduct a direct comparison of cerebral, mesenteric, and coronary artery the notion is confirmed (Fig. 8). Vasospasm after stretch was recently evidenced in murine basilar artery of young age after inhibition of endogenous NO or in senescence without any pharmacological treatment [35] and cerebral artery might be less robust to the stretch which is applied during normalization of the artery segment than non-cerebral artery.

4.7. PCR

We have demonstrated mRNA encoding two vascular abundant receptors, CGRP and ET₄ with commercially available TagMan gene expression assays (Fig. 9). Here, probes from the typical pig (sus scrofa) were utilised and our results indicates that in future studies profiling can be established using these techniques.

In this and other studies the presence of CGRP- and ET₄ receptors in vasculature has consistently been demonstrated by functional studies, PCR, immunohistochemistry and – to a limited extend - by western blots where antibody suitability for CGRP and ET₄ receptors have been debated.

4.8. Strengths and limitations of the study

To the best of our knowledge, the current study is the first study comparing three distinct vascular beds isolated from the same animal (GM), which has previously shown to have many similarities to human physiology and anatomy. Therefore, the primary strength of our study is the use of in vitro methodology in a large animal model that provides translational impact. The choice of vascular beds in our study is also highly relevant as these vascular beds are supplying the key body organs with blood. Furthermore, our study sheds light on the regional differences in vascular response to different endogenous vasoactive compounds.

Having said that, the current study is limited by presenting only a basic characterization of vascular responses in healthy 3–4 months male GMs and underscores the importance of further examining these findings in female and aged animals as well as in human artery segments to increase the translational value.

5. Conclusion

In the current study we paired and compared in vitro vasoactivity of coronary, cerebral, and mesenteric artery segments isolated from healthy 7–10 kg male GM. The results support previous experiments and allow us to emphasise the importance of optimal pairing of animals and tissue from different locations in the same animals.

When established agonists for sympathetic (norepinephrine), parasympathetic (carbachol), sensory (CGRP), and endothelial pathways (ET-1 and bradykinin) were used for comparison; bradykinin and CGRP consistently dilated, and ET-1 consistently contracted artery segments from coronary, cerebral, and mesenteric origin. Norepinephrine and carbachol, induced tissue-specific constriction or dilatation of arteries isolated from the different anatomical locations, presumably relevant to the receptors expressed in these differing sites.

An important finding is that the responses were largely maintained up to 24 h afterwards, except for a trend for loss of the endothelium-dependent response in cerebral artery.

It is also noted that the cerebral arteries display a special spasmogenic feature as compared to coronary and mesenteric artery segments. These findings add on to previous findings of cerebral artery being less...
robust than others. This study supports the translational strengths of using GM as a model for safety and pharmacological studies in drug discovery.

CRediT authorship contribution statement

Anette Sams: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. Kristian Agmund Haanes: Data curation, Formal analysis, Methodology, Software, Supervision, Visualization, Writing – review & editing. Anja Holm: Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. Spyridoula Kazantzi: Methodology, Data curation. Lars Friis Mikkelsen: Conceptualization, Writing – review & editing. Lars Edvinsson: Funding acquisition, Writing – review & editing. Susan Brain: Conceptualization, Data curation, Writing – review & editing. Majid Sheykhzade: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Lars Friis Mikkelsen is a former employee at Ellegaard Göttingen Minipigs.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2023.107231.

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