Gene Expression of the Endothelin-1 in Vasospastic Flap Pedicle – an Experimental Study on a Porcine Model

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Abstract

The aim of this study was to evaluate the amount of Endothelin-1 (ET-1) gene expression in the vasospastic vessel of the flap pedicle to prove or disprove the role of ET-1 gene expression in pathogenesis of mechanically induced vasospasm. The vasospasm was induced by the tension on the pedicle of the pedicled caudal superficial epigastric flap on 8 pigs. Laser Doppler was used for peripheral blood flow measurement. Specimens from the vasospastic vessel (group of specimens B) and from the flap border with no vasospasm (control group A) were taken 2 h after the stimulus initiation. Detection of ET-1 mRNA by Quantitative Real-Time RT-PCR was performed. β-actin was selected as an acceptable reference gene. Relative gene expression data were given as the n-fold change in transcription of target genes normalized to the endogenous control. Relative gene expressions and time indicators of vasospasm were compared in both groups. No significant difference of the ET-1 gene expressions was found between groups A and B (p = 0.505). No correlation between the duration of vasospasm and ET-1 gene expression was found as well (p = 0.299). In conclusion, the expression of the ET-1 gene in the mechanically induced vasospastic vessel of the pedicled flap was not significantly increased. In this study, the causality of the vasospasm pathogenesis and gene expression of ET-1 was not proven.

Vasospasm, pathogenesis, microsurgery, pig

Free flap transfer is a routine technique in plastic surgery. Vasospasm usually develops during flap dissection (Jurell et al. 1983; Goshen et al. 1985). It is a localized and usually persistent vascular contraction that may be caused by a local injury. A prolonged vasospasm may cause a complete obstruction of the vessel and result in a flap loss (Kemp et al. 1993, Moskowitz et al. 1995, Evans et al. 1997; Gurlek et al. 1997).

Endothelial continuity is a basic condition for intact blood flow through the flap. The most frequent cause of endothelial dysfunction is the presence of arterial or venous anastomosis related to endothelial damage. With subendothelial surface denudation, the thrombus is formed and vasoactive substances that cause vasoconstriction are released. The endothelial dysfunction may also change concentrations of the nitric oxide and Endothelin-1 (ET-1) in the close tissue and aggravate vasospasm (Chong et al. 2003).

In our previous studies on the rodent model, vasospasm was provoked by the axial tension applied on the pedicle vessels and magnesium sulphate was proved as the vasospasm releasing drug (Hyza et al. 2009a,b). The aim of this study was to evaluate the amount of ET-1 gene expression in the vasospastic vessel of the flap pedicle to prove the role of ET-1gene expression in pathogenesis of the mechanically provoked vasospasm.

Material and Methods

The study was done at Veterinary Research Institute Brno and it was approved by the Commission for Animal Welfare. Eight pigs, crossbreeds of White Noble (50%) and Landrace (50%), were operated on under general anaesthesia. The average weight of the pigs was 57 kg. The surgery was conducted under standard temperature

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conditions (23 °C) in general anaesthesia using TKX (tiletamin-zolazepam + xylazine + ketamine). The specimens of the vessels were taken from the pedicles of the flaps based on caudal superficial epigastric arteries. The flaps were raised on the abdomen and the peripheral blood flow was measured using laser Doppler (Plate I, Fig. 1a). The vasospasm was induced by the tension on the pedicle using 160 g weight for 5 min and then the tension was released. On the flap saline was applied on the pedicle as a control group and the measurement of the blood perfusion was done for a minimum of 30 min. The perfusion recording signals were exported from the control software package of the laser Doppler flowmeter into ASCII format files. Two important time periods (tB and tC) were extracted from the signals with use of MATLAB (Math Works, Natick, MA, U.S.A) scripts (Fig. 1b). Time period tB represented the time to the beginning of reperfusion; tC represented the time to maximum value of reperfusion. The specimens of the vasospastic pedicle vessels of the flaps were taken 2 h after the flap elevation (Group B) and fixed in a RNA*later* RNA Stabilization Reagent (Qiagen, Germany) which immediately stabilized RNA in tissue samples to preserve the gene expression profile, and after this the samples were stored at - 80 °C. Also, specimens of the intact vessel on the periphery of the flap were taken as a control and fixed as mentioned above (Group A). The specimens were then processed in a laboratory of Department of Pathological Physiology, Faculty of Medicine at Masaryk University, Brno.

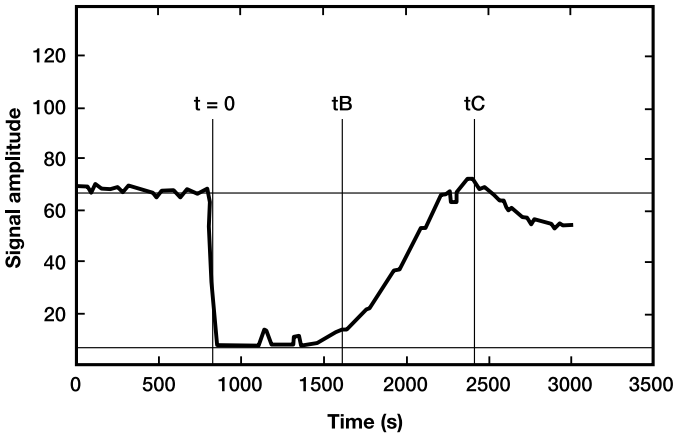

Fig. 1b. The perfusion curve obtained using laser Doppler measurement
The points t = 0, tB, tC were automatically detected by computer processing and the duration of vasospasm and reperfusion was measured.

RNA isolation and Quantitative Real-Time RT-PCR

Before RT-PCR analysis the samples were homogenized in RLT buffer (Qiagen, Germany) by Turrax T10 basic (IKA, Germany) for 2 min on ice. Subsequently, the homogenates were used for RNA isolation by Rneasy Mini kit (Qiagen, Germany) as recommended by the manufacturer. Total RNA (2 µg) was reverse transcribed to cDNAs using Omniscript RT kit (Qiagen, Germany) according to manual instructions. TaqMan® Gene Expression Assays (Applied Biosystems, Warrington, United Kingdom) were used for ET-1 detection. In order to choose an appropriate reference gene, we analyzed different pig housekeeping genes for tissue samples and elected β-actin as an acceptable reference gene for the tissue samples. Quantitative Real-Time RT-PCR was performed with TaqMan® Gene Expression Assays (Applied Biosystems) containing intron-spanning primers: Endothelin – Ss03392455_m1 and β-actin - Ss03376563_uH. Quantitative Real-Time RT-PCR was performed in an amplification mixture with a volume of 20 µl. The target gene amplification mixture contained 10 µl 2X TaqMan® Universal PCR Master Mix, 1 µl primer of the appropriate endothelin or β-actin (Gene Expression Assay), 10 ng template cDNA diluted in 5 µl nuclease free water, and 3 µl nuclease free water. PCR reaction conditions comprised 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Fluorescence generation due to TaqMan® probe cleavage by the 5'-3' exonuclease activity of the DNA polymerase was measured with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). All samples were amplified in triplicate. To cover the range of expected Values that included our amount of target mRNA, a standard curve of six serial dilutions from 50 ng to 500 pg of pooled cDNA was analyzed by Sequence Detection Software (SDS 1.9.1., Applied Biosystems). Results were imported into Microsoft Excel for further analysis. Comparable cDNA amounts in the experimental samples were calculated according to the standard curve method. Relative gene expression data were given as the n-fold change in transcription of target genes normalized to the endogenous control. The tissue sample with the lowest detectable target gene expression was arbitrarily applied as the calibrator and the results were calculated in relation to the calibrator's expression level.

Results

Table 1. Relative expression of ET-1 in groups A and B

Animal No.	Group	Relative expression	tB[s]	tC[s]
1	A	0.009562918	0.0	0.0
1	B	0.025120343	1435.2	1619.6
2	A	0.006243915	0.0	0.0
2	B	0.012216713	374.1	384.4
3	A	0.012259127	0.0	0.0
3	B	0.016250936	101.6	1183.9
4	A	0.009322935	0.0	0.0
4	B	0.010610722	979.4	1734.7
5	A	0.008608634	0.0	0.0
5	B	0.004613253	244.6	1853.8
6	A	0.006699798	0.0	0.0
6	B	0.004124209	3.5	96.4
7	A	0.010635267	0.0	0.0
7	B	0.010296753	683.0	1570.7
8	A	0.012824088	0.0	0.0
8	B	0.012473412	273.9	1708.0

The difference between ET-1 mRNA relative expression in the vasospastic vessels (Group B) and control group (Group A) vessels of the pedicle was tested using Wilcoxon paired test, because the data did not show normal distribution (Fig. 2, Table 1). The null hypothesis: "There is no difference in ET-1 mRNA relative expression between group A and B" could not be refused at $p = 0.5054$.

Correlation between ET-1 mRNA relative expression and vasospasm duration (tB, tC) was tested using Spearman coefficient of correlation, because the data did not show normal distribution. The null hypothesis: "There is no dependence between values of mRNA relative expression and values of vasospasm duration (tB, tC)" could not be refused at $p_{tB} = 0.299$, $p_{tC} = 0.934$, $\rho_{tB} = 0.428$, $\rho_{tC} = 0.048$ (ρ -the Spearman coefficient of correlation).

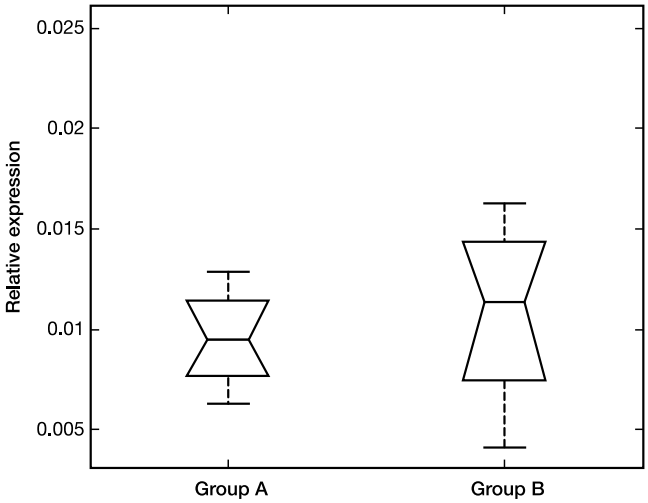

Fig. 2. Relative ET-1 mRNA expression in the vasospastic and control vessels of the pedicle
Group A (control): the specimens of the control vessels without vasospasm induction
Group B: the specimens of the vessels with induced vasospasm

Discussion

The pathogenesis of vasospasm resulting from pulling the pedicle is still not clear. In a study on pigs, Wingard et al. (1995) proved that stretching arterial smooth muscle to 1.2 × its resting length, maximal phosphorylation of myosin light chains appears and

vasospasm develops. This effect is known as a myogenic response (Wingard et al. 1995). Also, the endothelium may suffer from some functional or structural changes that can develop from either minor ruptures or hypoxia or both (Blann et al. 1993; Lantieri et al. 2003; Masaki et al. 2006). Smooth muscle contraction of skin vessels is also regulated by nervous (sympathetic neural fibres) and endocrine/paracrine system. Endothelial factors modulate the effect of norepinephrine released by sympathetic nerves and endocrine and local metabolic substances. Physiological blood flow in skin vessels is maintained by optimal balance in regulation (Table 2).

Table 2. Substances acting in regulation of the vascular tone

Vasoconstriction	Vasodilatation
Substances from vascular wall, not-coming from endothelium	
1. Eicosanoids: • $PGF_2\alpha$ (vessel myocytes) • LTC_4^* (leukocytes) 2. Superoxide radicals (ischemia/reperfusion injury)	1. Eicosanoids: • Prostaglandin PGE_2 (vessel myocytes) 2. Lactate, adenosine, kalium and H^+ ions (hypoxia, ischaemia)
Substances coming from endothelium	
Endothelial factors: • Endothelin-1	Endothelial factors: • EDRF, Nitric oxid • Prostacyclin PGI_2
Substances coming from blood	
5-HT (serotonin)	5-HT (serotonin)
Thrombin	Histamine
TXA_2 (thrombocytes)	Bradykinin

The family of endothelins consists of three closely related peptides, ET-1–ET-3, each encoded by distinct genes (Inoue et al. 1989). ET-1 is the most potent vasoconstrictor between endothelins. Peptide ET-1 is synthesized within endothelial cells from a biologically inactive precursor (big ET-1) by hydrolysis predominantly mediated by the Endothelin-Converting Enzyme (ECE-1) localised in the plasma membrane. Other enzyme ECE-2, optimally working at an acidic pH, may occur under pathophysiological conditions like hypoxia (Emoto et al. 1995; Barnes et al. 1998, Russell et al. 1999; Davenport et al. 2006).

Some amount of endothelin is secreted into the circulation, but the majority of endothelin is secreted by a paracrine way and contributes to maintenance of basal vascular tone (Haynes et al. 1994). Big endothelins, ET-1 and ET-3 are detectable in blood, but their concentrations are low and represent about 25–30% of endothelin formed by the endothelial cells. The major part of this amount is formed by big endothelin. ET-1 is a powerful vasocostrictor. Recent studies proved that ischemia induces an increase of ET-1 mRNA expression and consequently this elevation may cause vasospasm (Tsui et al. 2004; Khalil et al. 2006).

Based on the above, we may assume that vasospasm originates by combination of myogenic response of the vessel to mechanical irritation during dissection and effect of paracrine substances released mainly by damaged or dysfunctional endothelial cells and degranulated thrombocytes (Jurell et al. 1983; Goshen et al. 1985; Pang et al 1993; Vans et al. 1997; Chong et al. 2003). In this study, the results have not confirmed expected higher expression of the ET-1 gene in the vessels, where vasospasm was mechanically induced. This could be due to a small number of cases in the study or the origin of mechanically induced vasospasm could have been related mainly to other regulatory pathways. Even though we did not prove increased ET-1 gene expression at the vasospastic vessel, we still can expect some effect of ET-1 stored and released from Weibel-Palade bodies after an external pathophysiological stimulus. We can anticipate two possible ways of ET-1 release: degranulation initiated by regulated or constituted pathway or by cell destruction. Proportion of ET-1 to big ET-1 is strongly influenced by ECE-1 and ECE-2 activities. From this viewpoint, the therapeutic effect of non-selective ET-1 antagonists in combination with local use of magnesium sulphate may be anticipated (Vachieri et al. 2009). Increased

expression of ET-1 in the vessel with vasospasm induced by mechanical stimulation of the flap pedicle was neither confirmed nor disproved. Indirect evidence of participation of ET-1 in the vasospasm could be further studied by testing non-selective ET-1 inhibitors.

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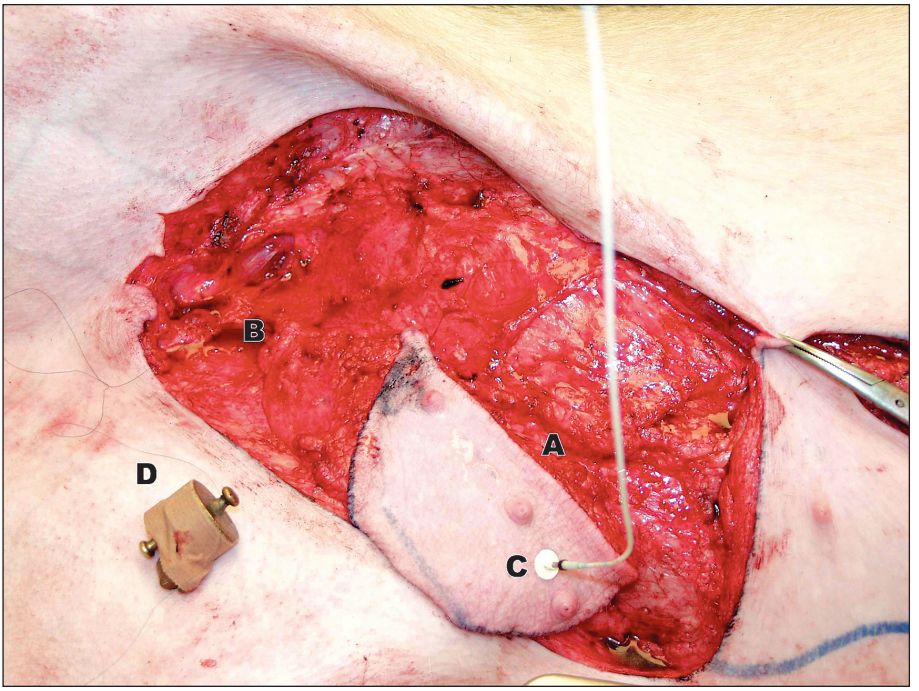

Fig. 1a. The pedicled flap based on the caudal superficial epigastric artery
A – control group, undamaged vessels were taken from the opposite side of the flapthe fat tissue of the flap; B – the pedicle vessel of the flap – study group; C – the laser Doppler probe attached to the flap, peripheral blood flow was measured; D – the weight and thread attached to the adventitia of the flap pedicle vessels to be used for vasospasm stimulation.