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Original article

The influence of steroids on noradrenaline-mediated contractile reactivity of the superficial nasal and facial veins in cycling gilts

W.J. Grzegorzewski[1,2], J. Muszak[1], B. Wąsowska[1], B. Jana[1], S. Stefańczyk-Krzymowska[1]

[1] Department of Local Physiological Regulation,
Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences,
Tuwima 10, 10-748 Olsztyn, Poland
[2] Department of Pharmacology and Toxicology, Faculty of Medical Sciences,
University of Warmia and Mazury, Żołnierska 14C, 10-561 Olsztyn, Poland

Abstract

The nasal venous blood may be directed through the facial vein into the systemic circulation or through the frontal vein into the venous cavernous sinus of the perihypophyseal vascular complex, where hormones and pheromones permeate from the venous blood into the arterial blood supplying the brain and hypophysis. The present study was designed to determine the effect of noradrenaline (NA) on the tension of the nasal, frontal and facial veins of cycling gilts, and influence of ovarian steroid hormones on NA-mediated contractile reactivity. Additionally, the enzyme dopamine-β-hydroxylase catalysing the conversion of dopamine to noradrenaline (DβH) was immunolocalized in these vessels. Among three studied veins, the frontal proximal vein, that fulfill a key role in the supply of the nasal venous blood into the venous cavernous sinus, reacted to NA most strongly ($P<0.001$) and this reaction was weaker in the periestrous period than in luteal phase ($P<0.001$). Inversely, the reaction to NA of the facial proximal vein, that carry blood to the peripheral circulation, was stronger in the periestrous period than in luteal phase ($P<0.05$). P_4, E_2 and T significantly lowered NA-mediated tension of the frontal proximal vein during the periestrous period ($P<0.001$), while in the luteal phase P_4 might antagonize relaxing effect of E_2 to this vessel. The result suggests that supply of the nasal venous blood into the venous cavernous sinus is greater during the periestrous period than during the luteal phase. DβH was clearly expressed in the muscular layer of the isolated superficial nasal and facial veins of gilts in both studied stages of the estrous cycle.

We suggest that the reactivity of the superficial veins of the nose and face to NA combined with the previously demonstrated reactivity of these veins to steroid ovarian hormones and male steroid pheromones may regulate the access of priming pheromone androstenol (resorebed in the nasal cavity) to the brain of gilts during periestrous period via humoral local destination transfer.

Key words: gilts, estrous cycle, noradrenaline, steroid hormones, veins reactivity

Correspondence to: J. Muszak, e-mail: j.muszak@pan.olsztyn.pl, tel.: +48 89 5393125

Introduction

The dorsal nasal vein, frontal vein and facial vein, called the superficial veins of the nose and face in several species including the pig represent an artery-like structure with a well-developed muscular layer (Godynicki and Skorupski 1979, Baker and Nijland 1993, Zezula-Szpyra and Grzegorzewski 2000). Such morphology of these veins suggested their contractile reactivity and was considered that they function as a venous sphincters (Mitchell et al. 1998). The reactivity of the superficial veins of the nose and face in female pigs to ovarian steroid hormones and steroid boar pheromones has been previously demonstrated (Grzegorzewski 2005, 2006, 2006a, Grzegorzewski et al. 2010, 2010a).

The venous blood flows out from regions of the nasal mucosa through the dorsal nasal vein and then may be directed through the frontal vein into the venous cavernous sinus of the perihypophyseal vascular complex or through the facial vein into the systemic circulation (Daniel et al. 1953). In the pig, the distribution of nasal venous blood may determine the possibility of substances absorbed in the nasal cavity, including sex pheromones, to penetrate into arterial blood supplying the brain and hypophysis (Krzymowski et al. 1999, Stefańczyk-Krzymowska et al. 2000).

The adrenoceptor mechanism is involved in the regulation of the blood flow in many areas of animal organisms but the occurrence of individual classes and subclasses of receptors varies from vascular bed to vascular bed. In most mammalian species, contraction of arterial vascular smooth muscle is predominantly mediated via α_1-adrenoceptors, e.g.: in the rat aorta (Aboud et al. 1993), in the rat carotid, mesenteric, renal and tail arteries (Han et al. 1990), in the canine femoral, mesenteric, jejunal, renal, splenic arteries (Polonia et al. 1985) and in human arteries (Flavahan et al. 1987). In veins, particularly in cutaneous veins, both α_1-and α_2-adrenoceptors contribute to vasoconstriction (Flavahan and Vanhoutte 1986). In the dog and human saphenous veins, α_2-adrenoceptors predominantly mediate the contraction (Müller-Schweinitzer 1984, Guimarães and Nunes 1990). The relaxation of vascular smooth muscle results from activation of β_1- or β_2-adrenoceptors (review see for Guimarães and Moura 2001).

The nasal mucosal vascular bed in most mammalian species is also under sympathetic nervous control, and noradrenaline (NA) is considered as classical postganglionic transmitter (Lung and Wang 1989b). It has been found that α_2-adrenoceptors predominate over α_1-adrenoceptors in the control of nasal blood flow in the pig (Lacroix and Lundberg 1989) and dog (Berridge and Roach 1986). It has been also documented that β_1- and β_2-adrenoceptors are equally important in resistance and capacitance vessels of the nasal mucosa in the pig, dog and humans (Lung et al. 1984, Lacroix et al. 1995), however, their vasodilatatory effect varied between vessels (Wang and Lung 2003).

Modulatory effect of ovarian steroids on the regional blood flow by influencing adrenoceptors function has been demonstrated in several species. Ovarian steroid hormones alter the function of uterine periarterial sympathetic nerves through changes in the number of adrenergic α_1-receptor and influence the ovarian and uterine blood supply in the pig (Ford et al. 1984). Estradiol-17β increases coronary, renal and iliac blood flow in prepubertal female pigs (Vacca et al. 1999). In ovariectomized rats, estradiol increases sensitivity of the mesenteric arteries to phenylnephrine and forces their dilatation (Berezan et al. 2008). It also increases the reaction of mouse tail arteries sensitive to cold to α_2-adrenoceptor agonist (Edi et al. 2007). Testosterone, termed a cardioprotector, increases the heart blood flow in rats by upregulating α_1-adrenoceptors (Tsang et al. 2008).

The present study was designed to determine the effect of NA on the tension of isolated superficial nasal and facial veins in cycling gilts, and the influence of ovarian steroid hormones on NA-mediated contractile reactivity. In addition, this study aimed to immunolocalization of DβH to check the presence of noradrenergic neurons in the superficial nasal and facial veins of these gilts.

Materials and Methods

Animals

All procedures were performed in accordance with protocol No. 5/2001/N approved by the Local Ethics Commission for Animal Experiments. The handling of animals complied with the standards for care and use of experimental animals. The animals were kept on a commercial farm under standard feeding conditions. The study was performed on sexually mature crossbred gilts (Polish Large White x Polish Landrance). The gilts were used after two regular estrous cycle, controlled using a vasectomized boar. The mating day was designated as Day 0 of the estrous cycle. The experimental groups comprised gilts in the luteal phase of the cycle (Days 12 to 14, n = 5) and gilts in the periestrous period (Days 18 to 20, n = 5). On the appropriate day of the estrous cycle the gilts were euthanized by electric shock (ENZ 300; Metalowiec, Bydgoszcz, Poland) and exsanguinated. The

day of the luteal phase and periestrous period was confirmed by morphological appearance of the ovaries. Both groups continued following the same procedure i.e. collection of the veins, preparation of the vascular rings, measurements of the contractile activity as well as calculation and analysis of the results.

Collection of the veins for the measurement of vascular tension and immunohistochemistry

Venous vessels were cut out directly after exsanguination. The vessels were dissected free of fat and connective tissue. The dorsal nasal, frontal and facial veins (from both sides of the head, one side for vascular tension measurement and the other side for immunolocalization) were divided into two equal parts marked as proximal or distal part of the vessel (see Grzegorzewski 2005). Based on the results of previous studies concerning the morphology and contractility of the superficial veins of the nose and face (Zezula-Szpyra and Grzegorzewski 2000, Grzegorzewski 2005, 2006) the distal part of the nasal vein, proximal part of the frontal vein and both parts of the facial vein were assigned to the measurement of reactivity to noradreanaline (NA) and immunostaining to NA. The preparation procedure and method used were described earlier in detail (Grzegorzewski 2005). Chosen parts of veins were cut into 2 cm long ring segments, and the dissected veins were put into a cold Krebs buffer solution (4ºC) (vascular tension measurement) or were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin (immunohistochemistry).

Measurement of the vascular tone of vein segments

The method used for the measurement of the contractile activity of veins was described earlier (Grzegorzewski 2010). Briefly, each vein ring was suspended horizontally between two stainless steel parallel hooks for the measurement of isometric tension (transducer (HSE F30 type 372, March-Hugstetten, Germany) in individual organ baths containing a modified Krebs-Henseleit solution (Grzegorzewski 2005). The rings were preincubated for 60-90 min to recover a spontaneous and stable pattern of rhythmic smooth muscle contraction. The optimal passive wall tension was adjusted to 5 mN, designated as "basal tension" and was maintained throughout the experiment. After an equilibration period, the vessels were pretreated for 5-minute period with individual steroid hormones: estradiol (E_2, Estradiol-Water Soluble, Sigma, St. Louis, MO, USA), progesterone (P_4, Progesterone-Water Soluble, Sigma, USA) or testosterone (T, Sigma, USA) each added to the medium with suspended vein segments in 96% ethyl alcohol at a concentration of 10 mM, to obtain a concentration of 1 or 10 μM (Grzegorzewski et al. 2005, 2006, 2010). Then, reactivity of the vessels was tested using the noradrenaline (NA, 10^{-7} M). Changes in vascular tension of isolated vein segments were measured isometricaly and recorded on HSE-ACD software for Windows (HSE, Murch-Hugestetten Germany) for 15 min. The reactivity of the vessel not pretreated with steroid to the noradrenaline was adopted as a control. The effect of ethyl alcohol, in concentrations equivalent to those produced in the incubation medium during application of steroids solution, on the contractility of the isolated vein segments was excluded in the preliminary experiments. Measurements were performed twice for each concentration (1 and 10 μM) of each steroid.

Endothelial continuity in vein fragments was tested using acetylcholine (Ach, 10^{-5} M) and vessel reactivity was tested using potasium chloride (KCl, 40 mM) before and after treatments. Only the values of the vascular tension obtained on the vessel rings that maintained endothelial continuity and normal reactivity to KCl after treatment with noradrenaline and steroids were included in the data.

Immunohistochemistry

The vessels were cut into 5 mm sections and placed onto silanized (TESPA, Sigma, USA) slides. The tissue sections were deparaffinized, rehydrated in graded ethanol series (96%, 70%, 50%), washed in distilled water and 3 times with 0.1 M phosphate buffered saline (PBS, pH 7.4) and incubated for 30 min with 3% hydrogen peroxide in PBS to quench endogenous peroxidase activity. After washed 3 times for 10 min each in PBS they were incubated in a blocking solution (BS) containing: PBS with 10% normal goat serum (NGS, Sigma, USA), 0.1% Triton X-100 (ICN Biomedicals Inc., USA), 0.2% bovine serum albumin (BSA, ICN Biomedicals Inc, USA) and 0.05% Thimerosal (Sigma-Aldrich, USA) for 60 min. Primary antibodies raised against the dopamine-beta-hyroxylase (DβH, rabbit polyclonal, Biomol, USA; working dilution 1:100 – 1:200) or proteine gene product 9.5 (PGP 9.5, mouse, 7863-2004, Gentaur Molecular Products, Belgium; working dilution 1:300) were diluted in BS and applied to sections overnight at room temperature (RT). The optimal concentration of primary antibody was determined in

pilot experiments by several antibody dilution methods. Following subsequent rinsing in PBS (3 × 10 min), the sections were incubated for 60 min at RT with Alexa Fluor 488 goat anti-mouse for DβH (Invitrogen, Molecular Probes, USA, working dilution 1:200 – 1:400) or Alexa Fluor 594 goat anti-rabbit for PGP 9.5 (Invitrogen, Molecular Probes, USA, working dilution 1:300) in BS to visualize the anti-DβH and PGP 9.5 antibody. Next, the washed with PBS sections were coverslipped in Mounting medium for fluorescence with DAPI (Vector Laboratories, CA). Control sections were processed for each labeling reaction omitting the primary antibody and both primary and secondary antibodies to detect non-specific binding of the antibody and autofluorescence, respectively (data not shown). For the preparation of positive controls, sections with DβH- and PGP 9.5-immunoreactive perikarya of the porcine sympathetic chain ganglia (SChG) Th16-S2 and L6 were stained with the primary and secondary antibodies at the same dilution.

The sections were viewed using an automated Zeiss Axio Imager.Z1 upright microscope fitted with a Zeiss Axiocam MRm digital monochrome CCD camera (Carl Zeiss Vision GmbH) with AxioVision Rel. 4.8 program (Zeiss).

Calculation of the results and statistical analysis

The results were expressed as means ± SEM of the maximum tension in response to NA (Table 1) and as a percentage (means ± SEM) of the tension (Fig. 1). The mean basal tension was calculated for 5 min. intervals at the end of the control period lasting 15 min., and accepted as 100 per cent. The average of the periods of 5 min. were calculated using HSE-ACAD Hugo Sachs Elektronik. Changes in the tension of each vein ring recorded during a 5 min. period following steroid treatment relative to the basal tension were determined by the Student's t-test and differences between the maximum tension of individual vessel in response to NA was determined by Bonferroni's Multiple Comparison Test (GraphPad Software, San Diego, USA).

Table 1. The maximum tension of the superficial veins of the nose and face of gilts in the luteal phase of the estrous cycle and the periestrous period in response to NA at a concentration of 10^{-7} M. Values marked with asterisks differed between phases of the estrous cycle in rows. Values marked with different letters differed between vessels within phases of the estrous cycle in columns.

Vein	Vascular tension [mN]	
	luteal phase	periestrous period
Nasal distal	34.4 ± 0.7[a]	34.6 ± 0.8[e]
Frontal proximal	40.8 ± 0.6[b]	34.4 ± 1.2***[e]
Facial proximal	25.3 ± 0.3[c]	28.7 ± 0.5*[f]
Facial distal	6.6 ± 0.1[d]	10.6 ± 0.1***[g]

* P<0.05; *** P<0.001; a/b,c,d; b/c,d; c/d; e/f,g; f/g P<0.001

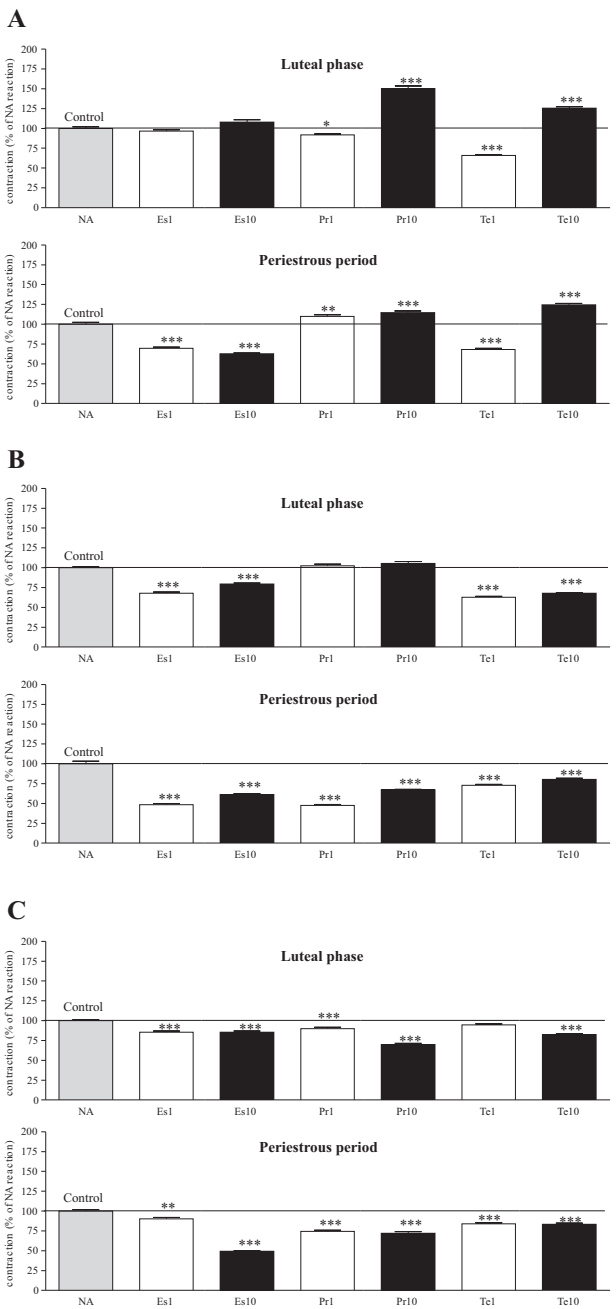

Fig 1. The effect of E_2, P_4 and T on NA (10^{-7}M)-mediated isometric tension of segments of the nasal distal vein (A), frontal proximal vein (B) and facial proximal vein (C) collected from the gilts in the luteal phase of the estrous cycle (days 10-12, n=5) and the periestrous period (days 18-20, n=5). Values (mean ±SEM) are presented in percentage in relation to the tension produced by NA alone.
* P<0.05; ** P<0.01; *** P<0.001.

Results

Noradrenaline used in the present study at the dose of 10^{-7} M caused a strong increase in the tension of sections of the three tested veins, i.e. the nasal distal vein, frontal proximal vein and facial proximal vein, while the reaction of the facial distal vein to NA was weak (Table 1). The tension caused by NA in individual sections of veins differed between vessels within phases of the estrous cycle ($P<0.001$). Moreover, in all tested vessels, with the exception of the nasal distal vein, this tension differed between phases of the cycle ($P<0.05$) (Table 1). The effect of preincubation with steroid ovarian hormones on NA-mediated vascular tension of the isolated superficial nasal and facial veins of cycling gilts is presented in Fig. 1. In general, preincubation with steroids changed tension of these veins produced by NA. The effect of steroid hormones on this tension was dependent on the phase of the estrous cycle and the type of vessel. The doses of steroids had considerable impact only in case of the nasal distal vein (Fig. 1A).

The tension caused by NA in the nasal distal vein during the luteal phase was not changed by preincubation with E_2, but both T and P_4 affected this tension in dose-dependent manner (Fig. 1A). The lower dose of P_4 and T lowered NA-mediated tension of this vessel ($P<0.05$ and $P<0.001$, respectively), while the higher dose of both hormones resulted in the increase of this tension ($P<0.001$). In the periestrous period, NA-mediated tension of the nasal distal vein was lowered by E_2 ($P<0.001$), but increased by both doses of P_4 ($P<0.01$). The effect of T was dose dependent and the lower dose decreased, while the higher dose increased this tension ($P<0.001$).

The tension of the frontal proximal vein caused by NA in the luteal phase was lowered by incubation with E_2 and T ($P<0.001$), while P_4 did not affect this tension. In the periestrous period all the hormones used lowered NA-mediated tension of this vessel ($P<0.001$) (Fig. 1B).

All doses of steroid hormones used, with the exception of T lower dose, lowered the tension of the facial proximal vein caused by NA in the luteal phase of the estrous cycle ($P<0.001$). In the periestrous period, all steroids lowered the NA-mediated tension of this vessel ($P<0.01$) (Fig. 1C). Because of low sensitivity of the facial distal vein to NA (Table 1) the influence of steroid hormones to NA-mediated tension of this vessel is not presented.

In all studied veins, both, in the luteal phase and in the periestrous period nerve fibers exhibited DβH-immunoreactivity (IR). Immunostaining with antibody against DβH was observed in the muscular layer, especially around smooth muscle fibres of the frontal (Fig. 2A), nasal (Fig. 2B) and facial (Fig. 2C) veins. In addition, immunoreactivity to PGP 9.5, a specific marker of neuronal cell bodies and axons was clearly observed in the smooth muscle layer of veins (Fig. 2D).

Discussion

The results of the present study is the first to demonstrate a reactivity of the nasal distal vein, frontal proximal vein and facial proximal vein of gilts to NA. The enzyme DβH catalysing the conversion of dopamine to noradrenaline was localized in the muscle layer of the superficial veins of the nose and face for the first time. This study demonstrated also the influence of preincubation of vessels with ovarian steroid hormones on their NA-mediated tension. The above data suggest the participation of adrenergic system in the regulation the function of veins draining nasal venous blood and in the distribution of this blood as well as modulatory effect of ovarian steroids earlier action on this adrenergic regulation.

It has been earlier demonstrated the role of adrenoceptor mechanism in the control of blood flow in the porcine nasal mucosa (Lacroix and Lundberg 1989). The present study revealed a strong increase in the tension of the nasal distal vein, draining the venous blood from the nasal cavity, in response to NA. This reaction of the superficial veins of the nose and face to NA suggests operation via α_1-and/or α_2-adrenoceptors (Müller-Schweinitzer 1984, Flavahan and Vanhoutte 1986, Guimarães and Nunes 1990). The tension of the nasal distal vein produced by NA was similar during the luteal phase and periestrous period. However, the effect of earlier action of steroid hormones differed between phases of the cycle. During the periestrous period NA-mediated tension was significantly lowered by E_2 and T, which were dominant hormones in this stage of the estrous cycle. Although higher dose of T caused an increase in this tension, the systemic concentration of this hormone was relatively low in gilts. Also P_4 could not have a significant impact on venous tension at this stage of the cycle. During the luteal phase tension of the nasal vein was very strongly increased by earlier action of progesterone. The above data suggest easier outflow of blood from the nasal area during the periestrous period than during the luteal phase.

A key role in supply of nasal venous blood into the venous cavernous sinus of the perihypophyseal vascular complex is fulfilled by the contractile activity of the frontal proximal vein. As shown in Table 1, the reaction of this vessel to NA differed between phases of the cycle and was stronger in the luteal phase than in

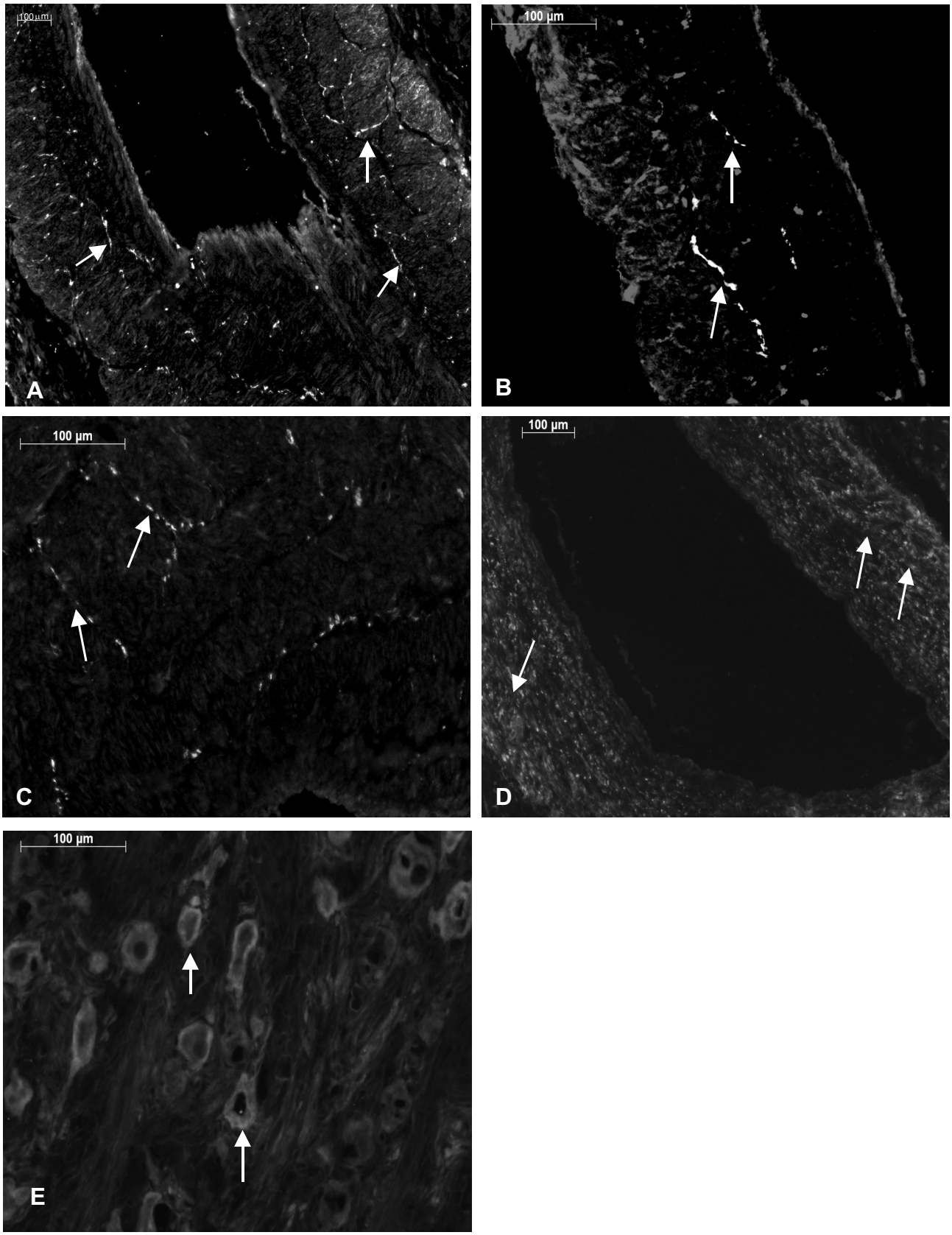

Fig. 2. Representative sections showing DβH- (A, B, C, E) and PGP 9.5 (D) -immunoreactivity. Specific immunostaining was observed in the muscle layer of frontal (A), nasal (B) and facial (C) veins – arrows show nerve fibers. Also in the muscle layer, numerous PGP 9.5-IR nerves were observed. In the SChG some DβH-IR perikarya are visible (E, positive control). Magnification ×200: B, C, E; mag. ×100: A, D.

the periestrous period. It was previously demonstrated that P_4 produced an increase in the number of α_1-adrenoceptors in the uterine artery during the luteal phase of the porcine estrous cycle (Ford et al. 1984). Similar effect of P_4 on the frontal proximal vein could be the cause of a stronger reaction of this vessel to NA in the luteal phase than in the periestrous period observed in the present study. Moreover, it was demonstrated that an increase in the number of α_1-adrenoceptors produced by P_4 antagonized the relaxing effect of other steroids on the uterine artery (Ford et al. 1984). This might explain a relatively weak effect of E_2 on NA-mediated tension of the frontal proximal vein in the luteal phase of the estrous cycle. In the periestrous period all the steroids lowered the tension of the facial proximal vein produced by NA. This data suggest the existence in the gilts favourable conditions for the supply of the nasal venous blood into the venous cavernous sinus of the peri-hypophyseal vascular complex during the periestrous period. The supply of the nasal venous blood through this vein into the venous cavernous sinus determines the function of humoral pathway for the priming action of sex male pheromone androstenol in the female pig (Krzymowski et al. 2001). The influence of androstenol via humoral pathway on hormonal regulation of the periestrous period in gilts was earlier demonstrated (Stefańczyk-Krzymowska et al. 2002, 2003, 2005).

The facial proximal vein, through which blood goes directly to the systemic circulation, responded to NA weaker than other superficial veins of the nose and face. Moreover, the effect of NA on this vessel was stronger in the periestrous period than in luteal phase of the estrous cyle, in contrast to the frontal proximal vein. In both phases of the cycle, preincubation of the vessel with all steroid hormones applied lowered this tension to a similar extent. Therefore, it may be supposed that outflow of venous blood through this vessel into the systemic circulation may be less effective during the periestrous period than during the luteal phase.

The differences in the contractility of individual veins of the superficial vein system of the nose and face determines the distribution of blood outflowing from the nasal cavity and enables the nasal venous blood to reach the cavernous sinus of the peri-hypophyseal vascular complex. This in turn allows substances absorbed in nasal mucosa, including male pheromones, to penetrate into the arterial blood supplying the brain and pituitary in gilts (Krzymowski et al. 1992, 1999, Stefańczyk-Krzymowska et al. 2000, Skipor et al. 2003, Krzymowski and Stefańczyk-Krzymowska 2012). It has been demonstrated the absorbtion of primer boar pheromone androstenol in the porcine nasal cavity and its destination transfer into the hypophysis and brain structures participating in the hormonal regulation of female reproductive processes (Krzymowski et al. 1999, Stefańczyk-Krzymowska et al. 2000, Krzymowski and Stefańczyk-Krzymowska 2012). This humoral pathway seems to be particularly suited to the priming action of male sex pheromones which prepare the female organism to the estrus (Krzymowski et al. 2001, Stefańczyk-Krzymowska et al. 2003).

We suggest that the reactivity of the superficial veins of the nose and face to NA combined with the previously demonstrated reactivity of these veins to steroid ovarian hormones and male steroid pheromones may regulate the access of priming pheromone androstenol (resorebed in the nasal cavity) to the brain of gilts during periestrous period via humoral local destination transfer.

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