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Natural killer cell cytotoxicity, cytokine and neuroendocrine responses to opioid receptor blockade during prolonged restraint in pigs

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ABSTRACT

This study evaluated porcine natural killer cell cytotoxicity (NKCC), plasma cytokines including interleukin (IL) 1β, IL-6, IL-10, IL-12 and tumor necrosis factor-α and plasma stress-related hormones including prolactin (PRL), growth hormone (GH), β-endorphin (BEND), ACTH and cortisol (COR) during a 4 h restraint and recovery phase after saline or naloxone (1 mg/kg BW) administration. The restraint preceded with saline altered NKCC and IL-12 concentration (an early from15 to 60 min increase followed by a decrease) and increased other measured cytokines and hormones concentrations. Naloxone pretreatment blocked the suppressive effects of the restraint on NKCC and IL-12 and altered IL-10, IL-6, TNF-α, PRL and ACTH concentrations. Furthermore, in naloxone-injected pigs, a positive correlation was found between NKCC and all measured cytokines (with the exception of IL-6) and BEND, ACTH and COR. Results suggest that naloxone-sensitive opioid pathways could influence the mechanisms underlying the immune system (including NKCC) response during stress.

1. Introduction

Farm animals respond to physical stress with behavioral, physiological and immunological disturbances (Grandin, 1997; Carroll et al., 2012). Acute stress increases the neuroendocrine activity, particularly the catecholamine release and the hypothalamic–pituitary adrenal (HPA) response (Mormede et al., 2007; Goldstein and Kopin, 2008). Furthermore, acute stress has been shown to affect the release of endogenous opioid peptides (EOP), circulating inflammatory mediators (eicosanoids and cytokines) and hormones such as prolactin (PRL) and growth hormone (GH) (Estienne and Barb, 2005; Webster Marketon and Glaser, 2008; Borghetti et al., 2009). Stress-induced neuroendocrine changes affect a natural killer cell cytotoxicity (NKCC), the main innate anti-viral and anti-tumor defense system (Dhabhar, 2009; Bellavance and Rivest, 2012). A small number of studies has addressed the interactions between the EOP system, NKCC and the stress-related hormones (PRL and GH) (Gatti et al., 1993; Morrow-Tesch et al., 1993; Hale et al., 2001). Endogenous opioid peptides have been reported to modify the immune functions either directly through the immune cells opioid receptor engagement, or indirectly through the interaction with the HPA axis and the stress-related axes (e.g., lactotropic and somatotropic axes) (Bidlack et al., 2006; Berczi et al., 2009; Savino and Dardenne, 2010). Opioids are also known to modulate animal and human neuroendocrine responses during stress (Vuong et al., 2010). It seems possible that, the bidirectional communication between the neuroendocrine and immune systems is partially realized through the cytokine-mediated network (Peterson et al., 1998; Ninkovic and Roy, 2013). The functional redundancy and extensive pleiotropy of several cytokines (interleukin (IL)-1, IL-6, IL-10, tumor necrosis factor (TNF)-α) contribute to the initiation of a cascade of physiological processes resulting in the coping with stress (Elenkov, 2008). Like cytokines, opioids have been regarded as communication signals, and the potent modulators of immunological activity. The numerous studies have shown the suppressive effects of both exogenous and endogenous opioids on NKCC, but until now there is no clear explanation for the EOP engagement in the stress-induced immune response (Shavit et al., 1984; Ben-Eliyahu et al., 1990; Ben-Eliyahu, 2012).

Since to date no data exist on the stress-induced relationship between NKCC, circulating stress hormones and cytokines after opioid receptor blockade in swine. In our previous experiments performed in pigs we have observed the immunostimulatory effects of the prolonged restraint (4 h) on NKCC (Tokarski et al., 1992; Wrona et al., 2001). In the consecutive study, concerning the acute morphine administration in pigs, the long-lasting enhancement of NKCC was observed following the morphine administration in a dose of 0.5 mg/kg, and the immunostimulatory effects were reversed with opioid receptor antagonist naloxone (Borman et al., 2009). Moreover, recent findings from our group

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suggest the possible role of somatolactogenic hormones in the immunomodulatory effects of the restraint in pigs (Ciepielewski et al., 2013). Therefore, we predicted that the prolonged (4 h) restraint stress would lead to a naloxone reversible stimulation of NKCC in pigs. In order to clarify the relationship between stress, EOP and NKCC, the current study aims to determine the effects of opioid receptor blockade on stress-induced changes in pigs' NKCC during the prolonged 4 h restraint stress and in the recovery phase. Moreover, blood plasma PRL, GH, β-endorphin (BEND), ACTH, and cortisol (COR) concentrations were measured.

2. Materials and methods

All experiments were performed under the approval and guidelines of the Polish Committee on Animal Care and Use of the Local Ethical Committee, Gdansk, Poland.

2.1. Animals

Sixty crossbred intact male pigs (line 990, provided by The Pig Hybridization Centre, National Research Institute of Animal Production, Pawlowice, Poland), 12–14 weeks old and weighing 28.4 ± 2.2 kg were used. Pigs were taken from seven line 990 litters, born and raised under conventional management practices in The Pig Hybridization Centre. All pigs were housed indoor, in individual holding pens (approximately 8 m^2) with straw bedding under 12-h light:dark cycle (the light was on from 07:00 until 19:00 h) and ambient temperature 21 ± 1 °C. Twice a day (07:30 and 14:00 h) the pigs were provided a pelleted dry feed (growers–finishers diet) delivered by hand. Subjects did not consume feed prior to the experimentation period. Water was available continuously trough a nipple drinker.

2.2. Surgery and blood sampling procedure

All pigs were surgically implanted with chronic catheters into the right external jugular vein under general pentobarbitalum sodium (Vetbutal, Biowet, Puławy, Poland, 5 mg/kg BW, infused through the marginal ear vein) anesthesia (Tokarski et al., 1992). During the next 4 post-surgery days each pig was habituated to the blood collection procedure and the catheters were flushed daily with physiological saline containing 5 IU/mL of heparin. Venous blood samples (10 mL) were collected through the jugular catheters in the sterile syringes and aliquoted into EDTA-containing tubes for peripheral blood NKCC assay (6 mL) and for plasma hormones determination (4 mL). Blood samples for ACTH, BEND, PRL, GH, COR, IL-1β, IL-6, IL-10, IL-12 and TNF-α analyses were placed immediately on ice and subsequently centrifuged (at 4000*g* for 10 min at 4 °C) to separate plasma, which stored at −20 °C until further assays. All injections (saline or naloxone) were performed through the jugular catheters immediately after the first blood sampling (Time 0) and the procedure lasted approximately 30 s.

2.3. Groups and treatment

All experiments were conducted at the housing place to avoid the novelty or transportation stress. Pigs were randomly assigned to 5 groups ($n = 12$): (i) CON group – control pigs, undisturbed except for blood sample collection; (ii) SAL group – undisturbed pigs injected i.v. with 2 mL of sterile saline; (iii) NX group – undisturbed pigs injected i.v. with 1.0 mg/kg BW of naloxone (naloxone hydrochloride, Sigma Chemical Co., St. Louis, USA) dissolved in 2 mL of sterile saline–; (iv) SAL + RS group – restrained (4 h) pigs injected i.v. with 2 mL of sterile saline; (v) NX + RS group – restrained (4 h) pigs injected i.v. with 1.0 mg/kg BW of naloxone. Naloxone in a dose of 1 mg/kg was shown to prevent the opioidergic activation in the response to acute stress and sufficient to affect the HPA axis activity in pigs (Rushen and Ladewig, 1991).

2.4. Restraint procedure and study design

The pigs were restrained for 4 h in the specially constructed hammocks, according to the procedure described previously (Tokarski et al., 1992; Ciepielewski et al., 2013). The restraint procedure was conducted in the test room located in the same building and the transfer of the pig from the pen to test room took approximately 30 s. During the restraint, each pig was placed horizontally in the hammock that was adapted to the size of the animal. The hammocks were suspended at about 0.5 m above the floor. The experiments were performed from 07:00 to 15:00 h during 6 consecutive days (10 pigs per day including 2 pigs of each group). The restraint stress protocol took place from 7:00 to 11:00 h. In the restrained groups (SAL + RS and NX + RS) blood was sampled 7 times: immediately before treatments (time 0, baseline) and then at 15, 60, 120, 180, and 240 min (the termination of the restraint), and at 480 min (240 min after the restraint termination). The control (CON group) and non-restrained (SAL group and NX group) pigs stayed in the pens located in the same building, but they could not see and hear the procedure to which the restrained pigs were subjected. In CON, SAL and NX groups pigs were sampled at the same time points as the restrained animals starting from 07:00 h.

2.5. Measurements

2.5.1. Natural killer cell cytotoxicity (NKCC) assay

Natural killer cell cytotoxicity assay was performed as previously described by Wrona et al. (2001). Briefly, porcine peripheral blood mononuclear cells (PBMC) were used as effector cells and K-562 chronic human leukemia tumor cells were used as the target cells. PBMC were separated from blood by Ficoll 400 (Pharmacia, Sweden) density centrifugation method (Boyum, 1976). Whole blood was centrifuged at 1113*g* for 30 min. The isolated cells were collected and washed 3 times with phosphate-buffered saline, counted and suspended in complete medium (RPMI 1640, Sigma, Germany) supplemented with 10% heat-inactivated bovine serum (Sigma, St. Louis, USA) and Penicillin–Streptomycin (with 10,000 units penicillin and 10 mg streptomycin/ml, suitable for cell cultures, Sigma, Germany). Cell concentration was adjusted to 1×10^7 cells/ml in complete medium and then used as effectors for NKCC determination. Cytotoxicity of NK cells was quantified using a ^{51}Cr-release assay. After washing in complete medium, K-562 target cells (3×10^6 cells/mL) were labelled with 100 μCi of $Na_2{}^{51}CrO_4$ (Radiochemical Centre, Świerk, Poland) at 37 °C for 1 h. The target cells were adjusted to 2×10^4 cells/ml in complete medium and then cultured in round bottomed micro-well plates with various concentration of effector (E) to target (T) cell ratios (E: T = 50:1, 25:1, 12:1) in triplicate. The plates were incubated for 18 h at 37 °C in a 5% CO_2 humified chamber. Supernatant was collected and percentage of specific chromium release was measured with a gamma counter (Pharmacia-LKB, Turku, Finland) All results are presented in lytic units (LU), which were calculated using the standard formula:

$$LU_{30} = E_{STD}/[(E : T_{30}) \times T_{STD}]$$

where E_{STD} (standard number of the effector cells) = 10^7, T_{STD} (specified number of the target cells) = 2×10^4 and E: T_{30} is the effector: target cell ratio needed to lyse 30% of the target cells. One LU_{30} denotes the number of effector cells necessary to kill