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Stress and adaptation to repeated stress changed the expression of two isoforms of 11β-hydroxysteroid dehydrogenase (11β-HSD1 and 11β-HSD2) in the adrenal glands of sheep

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SUMMARY

The aim of this study was to estimate the rate of cortisol release from the adrenal gland tissue of ewes and the role of 11β-HSD1 and 2 in modulating cortisol release and to determine the expression level of these two isoforms in the cortex and medulla of animals from three groups: a control (CON; n = 5); ewes subjected to a stress factor in the form of one-hour individual isolation from the flock (S1; n = 5); and ewes subjected to repetition of the stress factor three times, at 24-h intervals (S2; n = 5), when adaptive processes have taken place. After slaughter of sheep from all three groups, the adrenal glands were removed and immediately cut into smaller sections of similar weight (approx. 50 mg), covering the cortical or medullary layers. After 10 minutes of stabilization, the sections of adrenal cortex and medulla tissue were transferred to consecutive wells containing pure Eagle's Medium; the same section was transferred to consecutive incubation wells every 30 minutes. The medium harvested from the wells after 30, 60 and 90 minutes of incubation of adrenal tissue was frozen until the scheduled determination of cortisol concentration by the RIA method. Gene expression of 11β-HSD1 and 2 in the adrenal cortex and medulla were measured using Real-time qPCR. In the *in vitro* experiment, the amount of cortisol released from S1 cortex tissue into the medium during 90 minutes of incubation was significantly reduced (P < 0,01). Group S2 had a similar profile of cortisol release for the total incubation period, but the values were always lower than those

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observed in the control (CON) and S1 groups (P < 0,05-0,01). Incubation of adrenal medulla tissue from group S1 resulted in much lower cortisol release to the medium in comparison to the adrenal cortex layer, but it remained constant throughout incubation. The amount of cortisol released in group S1 was similar to that observed in the CON group. Analysis of 11β-HSD 1 expression in the adrenal cortex tissue from group S1 showed an over threefold increase (350 ± 25 RQ), while the increase in group S2 was smaller (160 ± 35 RQ). Expression of 11β-HSD 2 in the adrenal cortex tissue showed a significant reduction in groups S1 and S2 (15 ± 7 and 22 ± 3 RQ, respectively. The results most likely indicate the immanent ability of the adrenal glands to control the release of cortisol, conditioned by the presence and variable activity of the enzyme 11β-HSD in the adrenal glands, particularly isoform 1, without the need to activate all HPA axis structures during stress and adaptation processes.

KEY WORDS: 11β-HSD (1 and 2), adrenal glands; sheep; stress; adaptation to repeated stress

INTRODUCTION

Stress is the result of the impact of various factors on the body and leads to a disturbance of the body's homeostasis. Stress-inducing factors in animals stimulate both the sympathetic-adrenal system (SAS) and hypothalamus-pituitary-adrenal (HPA) axis. The release of hormones of both systems from the adrenal glands is a non-specific reaction of the body to stress, aimed at restoring the state of equilibrium. The released hormones affect both the behaviour of animals and changes in the rate and direction of metabolism aimed at overcoming stress and/or adapting to it (Herman et al., 2005; Aguilera et al., 2007; McEwen et al., 2016). Individual isolation of sheep, which are herd animals, causes a sharp increase in the concentration of glucocorticosteroids in the blood due to stimulation of the HPA axis, corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and glucocorticosteroids – cortisol and/or corticosterone (Niezgoda et al., 1993; Niezgoda et al., 1998; Grant et al., 2009). Therefore, cortisol can be treated as an indicator of the intensity of the stress response (Wrońska et al., 1990; de Kloet et al., 1998; de Kloet et al., 2005; Wrońska et al., 2007).

The blood level of glucocorticosteroids is mainly the result of their increased synthesis and secretion from the adrenal glands and their release into the bloodstream, where they reach target cells and change their activity. Large amounts of glucocorticosteroids during stress (Reul et al., 1987; Timmermans et al., 2019), especially in the adrenal glands, may act as a local mechanism for maintaining the proper balance of glucocorticosteroid levels in the body (Reul et al., 1987; Lupien et al., 2005). However, the control of glucocorticosteroid synthesis and re-synthesis involves another mechanism based on local metabolism, mediated by the microsomal enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). This enzyme is not structurally uniform, but an 'isoenzyme' for which two isoforms can be distinguished: 11β-HSD1 and 11β-HSD2. They are products of two different genes, both belonging to the dehydrogenase family, but their homology is mainly limited to conserved sites in all members of the superfamily. Expression of the genes encoding both isoforms displays tissue specificity and regulates the access of glucocorticosteroids to mineralocorticosteroid receptors (MRs) and glucocorticosteroid receptors (GRs), with 11β-HSD1 and 11β-HSD2 differing in their affinity for the substrate and the pathway of the catalysed reaction (Draper et al., 2005; Simons, 2008; Walker et al.,2015; Timmermans et al., 2019). Both types of HSD enzymes have been

shown to modulate the concentration of glucocorticosteroids in many target tissues, including those of the liver, kidney, and gonads; their presence has also been demonstrated in the adrenal glands of sheep (Yang et al., 1995; Draper et al., 2003].

Based on these facts, it can be assumed that the concentration of glucocorticosteroids may be regulated not only systemically by HPA axis activity, but also locally at the level of the adrenal glands, through their access to target cells and the pre-receptor mechanism involving 11β-HSD. It is possible that both pathways affect the body's response to stress.

The aim of this study was to explain the role of stress and adaptation to repeated stress in modulating peripheral glucocorticosteroid release from the adrenal glands and in the activity of 11β-HSD by measuring mRNA expression of 11β-HSD1 and 11β-HSD2 in the adrenal cortex and medulla of ewes during exposure to a single and repeated stressor. To test the importance of both stress and adaptation, we used a stress factor in the form of one-hour individual isolation from the flock and the same stress factor repeated three times at one-day intervals, leading to adaptation in the sheep. This method is described in our previous works (Bobek et al., 1986; Niezgoda et al., 1987; Wrońska-Fortuna et al., 2009). A better understanding of the cellular mechanisms occurring in the adrenal gland of ewes in response to stress or during adaptation to repeated stress, determined by the activity of 11β-HSD, especially isoform 1 and/or 2, can make it possible to establish an additional modulator of the activity of this endocrine gland, particularly under stress conditions or adaptive processes, in shaping the concentration of cortisol, the most important stress hormone.

MATERIALS AND METHODS

Animals and experimental design

The experiment was conducted on 15 Polish Long-wool ewes (2-3 years old; 40,0 ± 5,0 kg, as average body weight ± SEM) outside of the reproductive period (April-May 2008). The animals were housed under a natural photoperiod and at ambient temperature in the flock; all animals had good body condition (BCS = 3 on a scale of 1 to 5) (Russel et al., 1969). Ewes were fed twice daily at 07:00 and 14:00 h with a diet formulated to provide 100% of the National Research Institute of Animal Production recommendations for maintenance (Norms, 1993). Water was available ad libitum. Before the experiment, ewes were randomly divided into three groups (n = 5 in each group) as follows:

1. Control group (CON) – sheep stayed in the flock throughout the experiment.
2. Experimental group 1 (S1) – sheep were individually isolated from the flock without visual or aural contact with the other animals. Sixty minutes after stress exposure the ewes were decapitated.
3. Experimental group 2 (S2) – sheep were individually isolated from the flock without visual or aural contact with the other animals in the flock three times, at one-day intervals. The stress factor was applied for 60 minutes each time. After the first and second isolation, the sheep were returned to the flock each time. After the third stress exposure, the ewes were decapitated.

The experiment was performed at the Experimental Station of the Department of Animal Biotechnology, University of Agriculture in Krakow. The experiment and animal procedures were approved by the Local Bioethics Committee at Jagiellonian University in Krakow (No. 75/2007). The experiment was carried out in accordance with the protocols described in previous research publications, where the influence of emotional stress in sheep was also investigated in vivo (Bobek et al., 1986; Niezgoda et al., 1987; Wrońska-Fortuna et al., 2009).

At night, the sheep were housed in a sheepfold with a straw-covered floor, and during the day they used a pasture near the sheepfold. After decapitation of the control and experimental animals, the recovered adrenal glands were placed in Petri dishes on ice and immersed in a 0,9% physiological saline solution (cat. S9888, Sigma-Aldrich, Saint Louis, USA). The adrenal glands of each of the ewes were separated into the medullary and cortical layers and cut into smaller sections of similar weight (about 50 mg). Tissues were divided into two parts and either placed in RNAlater (Sigma-Aldrich, Saint Louis, USA) for future RNA isolation or frozen in liquid nitrogen and stored at -80°C for protein extraction and measurement of cortisol levels.

Steroid hormone determination in medium

Other pieces of tissue (about 50 mg) from the adrenal medulla and cortex of all animals from the three groups were washed with PBS (cat. P4417, Sigma-Aldrich, Saint Louis, USA) at 4°C, immediately placed in 24-well plates, and incubated in 1 mL of Eagle's medium (Laboratory of Sera and Vaccines, Lublin, Poland) supplemented with 0,05% BSA (Sigma-Aldrich, Saint Louis, USA), together with a 2 µL/mL antibiotic-antimycotic solution (10,000 U penicillin, 10 mg streptomycin and 25 µg amphotericin B/mL; Sigma-Aldrich, Saint Louis, USA). Incubation was carried out at 39°C in an atmosphere of 95% air and 5% CO_2 in a Sanyo incubator (MCO-18AIC, Tokyo, Japan). The pieces of adrenal medulla and cortex tissue were completely immersed in the incubation medium. Every 30 min they were transferred to subsequent wells containing 1 mL of pure Eagle's medium. The amount of cortisol released into the medium after 30, 60 and 90 min of incubation was measured using Cortisol-RIA-CT DIAsource kits (DIAsource ImmunoAssays S.A., Louvain-la-Neuve, Belgium) in accordance with the manufacturer's instructions. Radioactivity was measured in a Wizard gamma counter (LKB, Vienna, Austria). All samples and standards were tested in duplicate. The sensitivity of the method for cortisol was 5 nmol/L, the coefficients of variation for intra- and interassay were 3,6% and 6,7%, respectively. Cortisol levels were expressed as pg/mg protein.

Protein extraction

The collected tissues were crushed in liquid nitrogen, dissolved in cold lysis buffer (BioVision, Milpitas, USA), vortexed vigorously, sonicated at 180 watts for 1 min at an amplitude of 30%, with 3 cycles of 10 s sonication/10 s rest on ice, and centrifuged at 4°C for 20 min at 10,000 x g. The supernatants were transferred to new tubes and diluted as follows: 50 µl of tissue homogenate, 750 µl of distilled water and 200 µl of Bradford reagent containing Coomassie Brilliant Blue R-250. Then 100 µl of the mixture was placed in the wells of 96-well plates and the OD measurement was performed with a BioTek EPOCH 2 (BioTek Instruments, Inc., Winooski, VT, USA) at 595 nm. The standard curve consisted of BSA solutions with concentrations of 0, 10, 20, 50, 100 and 200 µg/ml. This method was performed according to the procedure described previously by Bradford (1976).

Total RNA isolation and RT-PCR analysis

Total RNA extraction, reverse transcription (RT), and quantitative real-time PCR were performed as previously described (Grzegorzewska et al., 2020; Sechman et al., 2020; Wolak and Hrabia, 2020). Total RNA from the adrenal medulla and cortex tissue was isolated with TRI Reagent (cat. 93289, Sigma-Aldrich, Saint Louis, USA) according to manufacturer's instructions. RNA density and quality were evaluated by measuring probes extinction at 230, 260 and 280 nm. Total RNA (2 µg) was used as a template in cDNA synthesis. Reverse transcription (RT) was performed using a Master cycler (Eppendorf, Foster City, USA) with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) according to the following profile: (i)

25°C, 10 min; (ii) 37°C, 120 min; and (iii) 85°C, 5 min. The first strand of cDNA was stored at -20°C and, after dilution (10 x), used for qPCR amplification based on 50-nuclease chemistry using TaqManTM MGB (minor groove binder) probes. Multiplex qPCR was performed in a 96-well thermocycler (StepOne Plus, Applied Biosystems, Foster City, USA). Assay-on-Demand TaqMan MGB Gene Expression Kits with specific TaqMan MGB probes designed by Applied Biosystems/Thermo Fisher Scientific (cat. 4316033; Foster City, USA) were used for analysis of mRNA expression of proteins 11β-HSD1 and 11β-HSD2 in the adrenal medulla and cortex tissue. 18S rRNA was used was used as a reference gene (Eukaryotic 18S rRNA Endogenous Control, GenBank AF173612.1; amplicon size: 187 bp; cat #4310893E Applied Biosystems/Thermo Fisher Scientific, Foster City, USA). All reactions were performed in duplicate. Amplification efficiencies for reference and tested genes were in the range of 100%. RNA expression levels within the control group of adrenal medulla and cortex tissue were arbitrarily set at 1.

Statistical analysis

Each variable was tested using the Shapiro-Wilk W-test for normality. The homogeneity of variance was assessed with Levene's test. Statistical differences were assessed using two-way ANOVA followed by Duncan's multiple range test when the data showed a normal distribution and homogeneity of variances. Differences of values were presented as mean ± SEM and considered significant at $P < 0,05$. Calculations were performed using Sigma Stat 2.03 software (SPSS Science Software GmbH, Germany). Figures were prepared using Grapher 12 (Golden Software Inc., USA).

RESULTS

In vivo influence of stress on cortisol secretion in the adrenal cortex tissue

Incubation of the adrenal cortex tissue from the CON group showed a decrease in the concentration of cortisol released, from 2,95 ± 0,41 pg·mg^{-1} of tissue after 30 min of incubation to 0,83 ± 0,11 pg·mg^{-1} of tissue after 90 min of incubation (P < 0,05; Figure 1). A similar profile of cortisol release from the adrenal cortex tissue was found in group S1; the values did not differ from those in the CON group (P > 0,05). Group S2 had a similar profile of cortisol release for the total incubation period, but the values were always lower than those observed in the CON group and group S1 (1,34 ± 0,37 to 0,30 ± 0,08 pg·mg^{-1} of tissue; P < 0,05).

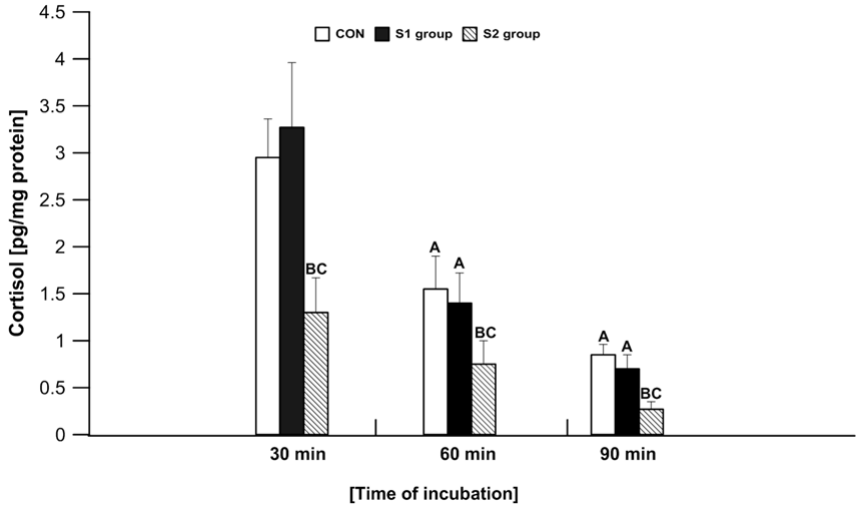

Fig. 1. Cortisol secretion from ewes' adrenal cortex tissue after single (S1) and repeated (S2) isolation stress. Each value represents mean ± SEM (n = 5); statistically significant (P < 0,05) or highly significant (P < 0,01) differences: A – compared to secretion after 30 minutes within the same group; B – value at the same time of incubation compared between groups; C – between groups S1 and S2

***In vivo* influence of stress on cortisol secretion in the adrenal medulla tissue**

Compared with the adrenal cortex tissue, the adrenal medulla tissue released less cortisol into the incubation medium ($P < 0,05$) in every group at every incubation time (Figure 2). In the CON group during the 90-min experiment, cortisol release to the medium gradually decreased from 0,35 ± 0,08 to 0,08 ± 0,02 pg·mg^{-1} of tissue ($P < 0,05$). Adrenal medullary tissue collected from group S1 released larger amounts of cortisol at each incubation time point compared to the CON group ($P < 0,05$); however the values did not differ significantly during 90 min of incubation of the adrenal medulla tissue obtained in group S1 (1,09 ± 0,31 pg·mg^{-1} of tissue after the first 30 min of incubation and 1,04 ± 0,36 pg·mg^{-1} of tissue in the next 30 min of the experiment). After 90 min of incubation the value recorded still did not differ significantly from that found at the previous measurement times (0,80 ± 0,23 pg·mg^{-1} of adrenal medulla tissue; $P > 0,05$). The adrenal medulla tissue in group S2 released significantly lower amounts of cortisol into the incubation medium compared to those obtained in group S1; the values were similar to those recorded in the CON group (0,31 ± 0,08; 0,27 ± 0,06 and 0,10 ± 0,02 pg·mg^{-1} of adrenal medulla tissue after 30, 60 and 90 min of incubation, respectively; $P > 0,05$; Figure 2).

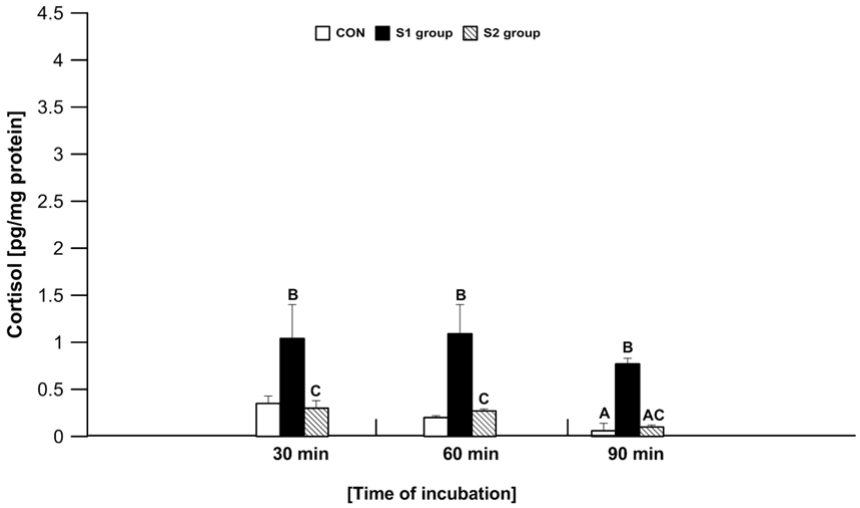

Fig. 2. Cortisol secretion from ewes' adrenal medulla tissue after single (S1) and repeated (S2) isolation stress. Each value represents mean ± SEM (n = 5); statistically significant ($P < 0,05$) or highly significant ($P < 0,01$) differences: A – within the same group; B – compared to value at the same time of incubation compared between groups; C – between groups S1 and S2

***In vivo* effect of stress on mRNA expression of 11β-HSD1 and 11β-HSD2 genes in the adrenal cortex tissue**

RT-PCR analysis indicated that in group S1 the stress factor resulted in a 3,5-fold increase in the expression of 11β-HSD1 in the adrenal cortex tissue compared to the CON group. Similarly, in group S2 there was a significant increase in 11β-HSD1 expression compared to the CON group. Expression of the 11β-HSD2 gene in the adrenal cortex tissue in group S1 and group S2 was decreased compared to the CON group ($P < 0,05$). Adrenal cortex expression of both genes was similar in groups S1 and S2 ($P > 0,05$; Figure 3).

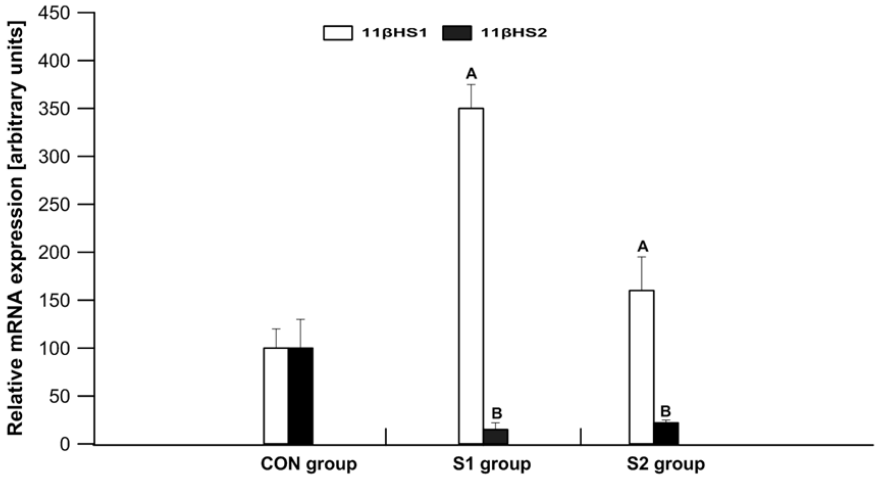

Fig. 3. Relative expression of 11β-HSD1 and 11β-HSD2 in the adrenal cortex tissue of ewes after single (S1) and repeated (S2) isolation stress. The mRNA expression levels within the control group were arbitrarily set at 1. Each value represents mean ± SEM ($n = 5$); statistically significant ($P < 0,05$) or highly significant ($P < 0,01$) differences: A – compared to control value; B – between groups S1 and S2

***In vivo* effect of stress on mRNA expression of 11β-HSD1 and 11β-HSD2 genes in the adrenal medulla tissue**

RT-PCR analysis indicated that in group S1 the stress factor had no effect on 11β-HSD1 expression in the adrenal medulla tissue compared to the CON group (P > 0,05). In contrast, in group S2 there was an increase in 11β-HSD1 expression in the adrenal medulla tissue compared to the CON group (P < 0,05). Expression of 11β-HSD2 in the adrenal medulla tissue in groups S1 and S2 was similar and not different from that observed in the CON group (P > 0,05; Figure 4).

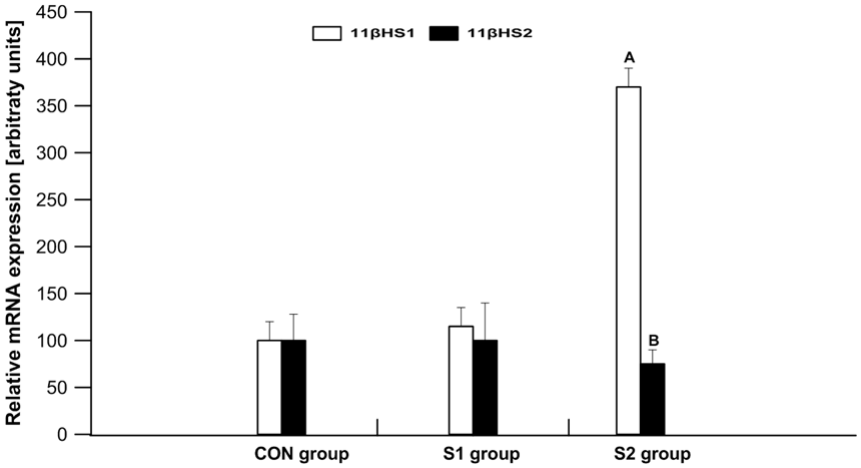

Fig. 4. Relative expression of 11β-HSD1 and 11β-HSD2 in the adrenal medulla tissue of ewes after single (S1) and repeated (S2) isolation stress. The mRNA expression levels within the control group were arbitrarily set at 1. Each value represents mean ± SEM (n = 5); statistically significant (P < 0,05) or highly significant (P < 0,01) differences: A – compared to control value; B – between groups S1 and S2

DISCUSSION

The present investigation clearly demonstrated differences in the amount of cortisol release and levels of 11β-HSD1 and 11β-HSD2 mRNA in the adrenal cortex and medulla tissue of sheep under stressful conditions and during adaptation processes to repeated stress. This experimental model has previously been used by researchers to study *in vivo* the effect of emotional stress in sheep on plasma glucocorticosteroids, glucose, thyroid hormone, vasopressin, and oxytocin levels (Bobek et al., 1986; Niezgoda et al., 1987a, 1987b; Wrońska et al., 1991; Wrońska-Fortuna et al., 2009, Wrońska et al., 2017). The results showed that separation of an individual sheep from the flock leads to activation of the HPA axis, and ultimately to a sharp increase in the blood glucocorticosteroid level, which is a specific indicator of the activity of the HPA axis in sheep. Biologically active glucorticorticosteroids are synthesized in the adrenal glands from cholesterol through steroidogenesis (Timmermans et al.,

2019), and the cortisol concentration in the blood plasma depends on the type, intensity, and duration of the stimulus and on the animal's previous experience (Buckingham, 2006).

The previously cited study by Niezgoda et al. (1987b) clearly showed that repeated emotional stress caused by the individual isolation of sheep from the flock causes a decrease in the response intensity of the HPA axis, manifested by a gradual decrease in the maximum concentration of cortisol in the blood plasma of sheep on successive days of stress of the same type and magnitude. The decrease in the activity of HPA systems indicates the ability of sheep to adapt to the stress factor and thus points to the emergence of an adaptation process. This can be explained by the negative feedback mechanism between the adrenal cortex and the hypothalamus (CRH) and between arginine-vasopressin (AVP) and the pituitary (ACTH) (Axelrod et al., 1984, Buckingham, 2006; Harno et al., 2010).

The decrease shown in the present study in the amount of cortisol secreted into the medium from ewes' adrenal tissue over the incubation time, both from the cortex and the medulla, may indicate that its *de novo* synthesis was limited, and only cortisol stored in the adrenal gland was released (Figure 1 and 2). The results pertain only to the dynamics of secretion of previously synthesized cortisol, and not to its formation in a multistage process.

Glucocorticosteroids act on target cells through intracellular receptors belonging to the nuclear receptor subfamily and regulate gene transcription (Rogatsky et al., 2004; Chodankar et al., 2014; Wu et al., 2014; Lee et al., 2017). Their action is initially slow, but persists for some time after glucocorticosteroids are eliminated from the circulation (de Kloet et al., 2005). Although the plasma glucocorticosteroid level is a good indicator of HPA axis activity, the amount of glucocorticosteroids released from the adrenal glands does not determine the quantity of these steroid hormones that reaches target cells (Walker et al., 2015; Pérez et al., 2020). An important mechanism regulating the accessibility of glucocorticosteroids precedes their connection to the receptors. This process involves two 11β-HSD isoforms located in the target cells themselves (Aguilera et al., 2007; Timmermans et al., 2019; Walker et al., 2015). The first isoform, 11β-HSD1, is dependent on NADP(H) and has low substrate specificity. It has an affinity for cortisone, corticosterone, and cortisol and is present in tissues showing high sensitivity to glucocorticosteroids, such as the liver, adipose tissue, and lungs (Stewart et al., 1999; Zallocchi et al., 2004a, Draper et al., 2005), as well as the adrenal glands (Mazzocchi et al., 1998). In the presence of NADPH, it exhibits reductase activity and generates active glucocorticosteroids, increasing their local concentration (Hewitt et al., 2005; Bujalska et al., 2005). The other form of the enzyme, 11β-HSD2, acts exclusively as a dehydrogenase. It is an enzyme that is dependent on the presence of NAD+, with high substrate specificity (Yang et al., 1995; Zallocchi et al., 2004a, 2004b). It has affinity for cortisol and corticosterone, while cortisone and aldosterone are not substrates. It is present in tissues containing many MRs, including the kidneys, colon, smooth muscle, and blood vessels, as well as in cells without MRs, such as the placenta and some regions of the prenatal developing brain (Agarwal and Mirshahi, 1999; Cole, 2006; van der Laan and Meijer, 2008). MRs have a similar affinity for cortisol as well as for aldosterone (Krozowski and Funder, 1983; Condon at al., 1998; Farman and Bocchi, 2000); moreover, later research results showed that cortisol binds with high affinity to MRs and with approximately ten-fold lower affinity to GRs (de Kloet et al., 2004). The action of the enzyme 11β-HSD type 2 (11βHSD2) protects MRs from activation by glucocorticosteroids. Therefore, it is believed that the primary function of 11β-HSD2 is to inactivate cortisol and thus prevent its binding

to MRs, precluding excessive activation by glucocorticosteroids, since specificity of the target tissue depends on the enzyme and not on the receptor (Schäcke et al., 2002; Draper et al., 2003; van der Laan and Meijer, 2008).

The present study showed the presence of both types of 11β-HSD enzymes in sheep adrenal glands, although their location varies depending on the animal's situation (Figure 3 and 4). We observed an increase in 11β-HSD1 expression in the adrenal cortex tissue in group S1. The 11β-HSD2 isoform was also found in the adrenal cortex tissue in the CON group, and its expression was decreased in group S1 (Figure 3). This is consistent with previous studies demonstrating the presence of mRNA of this enzyme in the adrenal cortex of humans (Mazzocchi et al., 1998) and sheep (Yang et al., 1995), especially in the *zona fasciculata* and *reticularis*. Based on the results presented above, we suggest that the increased concentration of cortisol and the expression of 11β-HSD1 with a simultaneous decrease in expression 11β-HSD2 in the adrenal cortex in group S1 may indicate that cortisol is not only synthesized *de novo* from cholesterol by steroidogenesis. An additional source of cortisol may be cortisone conversion into cortisol, which may take place in the adrenal glands due to the activity of 11β-HSD2. Moreover, it can be concluded that the 11β-HSD2 isoform in the adrenal gland probably protects the individual layers of the cortex against the excessive influence of glucocorticosteroids, which could eventually reverse the synthesis of other steroid hormones, thus affecting the HPA axis. The results of the study showed no changes in the mRNA level of either 11β-HSD isoform in the adrenal medulla in group S1 (Figure 4). Glucocorticosteroids are synthesized in the cortex layer, so they do not need to be deactivated. On the other hand, an over two-fold increase in cortisol release from the adrenal medulla of sheep was observed in group S1 (Figure 2). At the same time, no increase in the mRNA level of 11β-HSD1 in relation to the CON group was observed in the adrenal medulla, which suggests a slight conversion of cortisone to cortisol (Figure 4). This can be assumed to be linked to the close connection of the two adrenal tissues and the specific direction of the blood flow supply. The adrenal medulla and cortex are linked by blood vessels and can interact through secreted hormone (Tokunaga, 1996; Huang et al., 2012). The adrenal medulla has a dual blood supply system – the spinal arterioles that supply the core directly and capillaries running through the cortical layers and opening into the medullary vascular bed (Breslow, 1992; Burford et al., 2017). The blood flows from the cortex towards the adrenal medulla, meaning that the blood reaching the medulla is rich in glucocorticosteroids, which are secreted in a constant circadian rhythm and, together with other adrenal cortex hormones, can be stored in the lumen of the core vessels and released in waves when the muscles of the central vein contract or relax (Breslow, 1992, Einer-Jensen and Carter, 1995). Whorwood et al. (2001) showed that the influence of stress factors on the body inhibits the activity of sheep placenta 11β-HSD2, thus enabling an increase in cortisol and/or maintaining its high levels. ACTH, the hormone that stimulates the secretion of glucocorticosteroids, has a similar effect on adrenal tissue, which indicates the need to reduce mRNA expression of this enzyme isoform during stress (Minton et al., 1992; Einer-Jensen and Carter, 1995). The results of our study did not show significant changes in the expression of 11β-HSD2 in the adrenal medulla in group S1 or S2, which suggests the need to maintain high levels of cortisol, so that the properties of this hormone fulfil their physiological role as expected in a stressful situation (Fig. 4).

Adaptation to a repeated stress factor should also be treated as a form of learning, which explains the need for activation of the limbic structures, especially the hippocampus (Krozowski and Funder,

1983; Reul et al., 1985, Reul et al., 1987). The results of the present research support the conclusion from earlier studies that a repetitive stress factor leads to adaptation in sheep and to a gradual decrease in the maximum cortisol concentration in successive sessions of repeated stress (Bobek et al., 1986; Niezgoda et al., 1987; Wrońska-Fortuna et al., 2009). In group S2, a twofold increase in 11β-HSD1 mRNA expression in the adrenal cortex was observed in comparison with the CON group as well as group S1 (Figure 4). As a result, the conversion of inactive glucocorticosteroids into active ones was sustained at an increased level, but it was less than half of the value in the case of a single stress event, which suggests a decrease in HPA axis activity. Our own research found that the amount of cortisol secreted from the adrenal cortex tissue after three repetitions of isolation stress was half of the level recorded in the control group and in sheep exposed to a single stress factor (Figure 1).

The results additionally confirm the role of 11β-HSD1 in the body's adaptation to recurring stress factors through modulation of the availability of glucocorticosteroids. The presence of 11β-HSD1 in the adrenal medulla, demonstrated in the experiment, suggests an additional mechanism allowing for the release of appropriate amounts of active glucocorticosteroids, depending on the organism's needs (Figure 4).

After the stress has ceased, and thus after the accumulated cortisol has been used, there is a need to replenish its reserves so that, in the event of the reactivation of stress-generating factors, the organism can fully mobilize again. The need to restore an appropriate level of cortisol in the adrenal glands is also evidenced by the decrease in the mRNA expression of 11β-HSD2 in the adrenal cortex of sheep in group S2, which limited the conversion of cortisol into inactive cortisone (Figure 3). Similar results were obtained in the adrenal medulla, where a decrease in the 11β-HSD2 mRNA level was also observed in group S2 (Figure 4). Apart from the influence of the second isoform on restoring the appropriate concentration of cortisol in the adrenal glands, the results of this study also indicate that this form of the enzyme at the adrenal level is not involved in stress response or the body's adaptation to repeated stress.

The high level of 11β-HSD1 mRNA in the adrenal medulla demonstrated in our experiment (Figure 4) may be caused by the need to maintain an adequate level of glucocorticosteroids for catecholamine biosynthesis in the adrenal medullary. In group S2, an over three-fold increase in the mRNA expression of 11β-HSD1 in the adrenal medulla was obtained in relation to both the CON group and in group S1 (Figure 4). Therefore, we conclude that some amounts of cortisol can be stored in the form of inactive cortisone. The results of the experiment clearly indicate the existence of additional mechanisms regulating the cortisol level, other than the activity of the HPA axis and the SAS system. In response to stress-inducing factors, the immanent properties of the activity of the adrenal glands themselves are as important as the proper functioning of the HPA axis. The role of 11β-HSD was demonstrated in our research. The adrenal cortex is the only source of glucocorticosteroids, whose effect becomes visible after they reach the target tissues via the circulatory system, when their biological role becomes apparent. The presence and variable activity of both isoforms of the 11βHSD enzyme in the adrenal gland allow for a faster and more efficient way to control the amount of cortisol released, which indicates an inherent property of these endocrine glands, without the need to activate all structures of the HPA axis.

CONCLUSIONS

The experiment demonstrated changes in the mRNA expression of both enzyme isoforms of 11β-HSD in sheep adrenal glands under stress and adaptation to stress. Changes found in 11β-HSD1 at

the mRNA level in the adrenal cortex of sheep during stress indicate that the activity of this enzyme is required to maintain an increased cortisol concentration. It was shown that the 11β-HSD2 present in the adrenal medulla of sheep is not involved in the stress response or adaptation processes. The lack of clear antagonism between the mRNA level of both isoforms of the 11β-HSD found in the adrenal medulla of sheep under stress and during adaptation processes indicates that this isoenzyme may play an unexplained role in the endocrine function of this part of the adrenal gland, which suggests the need for further research on this topic.

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