Immunohistochemical identification of resistin in the uterus of ewes subjected to different diets: Preliminary results

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Abstract

Resistin is a polypeptide hormone of the adipokine-family, primarily, but not exclusively, produced by the adipose tissue. Recent studies suggested that resistin may affect the male and female reproductive activity. The study aim was to immunohistochemically evaluate the presence and distribution of resistin in the ovine uterus. Uterine samples were collected from two groups of ewes at the end of an experimental trial during which the animals of the first group (CTRL) were fed only by grazing while those of the second one (EXP) were supplemented with barley and corn. Using a monoclonal antibody against resistin, tested by Western Blot, the immunopositive reaction was identified in the cytoplasm of epithelial lining cells and uterine glands. The endogenous production of resistin seemed to be affected by different diet, as evidenced by staining differences between the CTRL and EXP groups. Our findings support the existence of a peripheral resistin system in the sheep uterus. It is possible that this system is involved in the functionality of the uterus, which is also affected by the animal's nutritional status.

Introduction

Resistin, a recently discovered polypeptide hormone that is a component of the adipokine-family, is produced primarily, but not exclusively, by adipose tissue. In this regard, it is well known that many other hormones known as adipokines are produced not only by white adipose tissue but also by other organ tissues, where they probably play different roles, always linked to the functionality of the tissue/organ where they are evidenced.[1-6] Resistin is an adipose tissue-specific secretory factor that in humans is encoded by the *RETN* gene.[7] The first study considering resistin as an adipokine was on mice, in which the molecule induced resistance to insulin (its name is most likely linked to this particular action); hence, it was perceived as a potential and dangerous link between obesity and diabetes.[8] More recent studies have shown that resistin mRNA is also present in other tissues of many species.[9-12] The influence of nutritional status was also considered in the genetic expression of resistin: chronic restriction of food markedly influenced the resistin levels with a strong down-regulation.[11] Recently, some studies have suggested that resistin could affect male and female reproductive activity.[13-15] For example, resistin mRNA and protein levels were determined in endometrial tissue from women with or without endometriosis.[16] Moreover, some research data linked the serum level of resistin to pathological situations of the genital tract, for example polycystic ovary syndrome, known to be linked to hyper-insulinemia and insulin resistance.[17,18] A recent study demonstrated the presence of resistin in ovarian granulosa cells of cows: resistin is able to modulate steroidogenesis and cellular proliferation, suggesting that it could be considered a metabolic signal for the control of reproductive activity.[19] To our knowledge, little has been published on the presence and distribution of resistin produced in the uterus of domestic animals, nor have there been studies reporting on the influence of diet in the expression of this adipokine in these animal species. Therefore, we decided to investigate the presence of this molecule in the uterus of sheep, using immunohistochemical techniques. The choice of sheep was related to the strong tradition of sheep rearing in some rural areas of central Italy, where the experimental trial was performed as part of a wider study to achieve sustainable management of grassland productive ecosystems that are subject to increasing drought stress linked to climate change, a trend that has been affecting herbage features, carrying capacity and animal welfare.[20,21] Indeed, the pasture vegetative cycle comprises two phases, the first from spring to the pasture maximum flowering (MxF), and the second during the summer as the pasture dries and reaches maximum dryness (MxD). Due to climatic change, the summer aridity has been increasing, such that the MxF moment arrives sooner and the period between MxF and MxD[20] is shortened, thus reducing the amount of food available to the animals. Therefore, being the pasture MxD moment corresponding to the mating period for the animals grazing in the studied area, summer aridity may bear negatively on the animals' reproductive performance.[22-24] To compensate the negative effects of increasing summer aridity, we explored the possible benefits of providing supplemental feed to sheep.

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Materials and Methods

In the experiment, two homogeneous groups (25 subjects for each group) of Comisana x Appenninica adult female sheep were free to graze on the pasture during the first pasture phase. Then, during the second phase the first group had grazing alone on pasture (CTRL), while the second group (EXP) also received 600 g/day/head of barley and corn (1:1). At the pasture MxD, 5 animals for each group, intended for human consumption, were slaughtered in accordance with art. 29 of the Council Regulation (EC) No. 1099/2009 on the protection of animals at the time of killing under law n.333/98. The experimental pro-

cedures were approved by the Ministry of Health (No. of approval 95/2018-PR).

The collected uterine samples were stored at -20°C for Western Blot (WB) evaluation, or fixed by immersion in 4% formaldehyde in phosphate-buffered saline solution (pH 7.4, PBS) for 24 h at room temperature and then dehydrated and routinely embedded in paraffin for immunohistochemistry (IHC). To perform WB, total proteins were extracted from 50 mg of uterine samples and processed as previously described.[25] In the specific step, primary mouse monoclonal antibody anti-resistin (1:2000; sc-376336; Santa Cruz biotechnology, Santa Cruz, CA, USA), and a secondary biotinylated goat anti-mouse IgG (1:2000; BA-BA-9200, Vector Labs, Burlingame, CA, USA) were used. To perform IHC dewaxed and re-hydrated uterine 5 µm thick sections were microwaved three times for 5 min each at 750W in 10mM citric acid (pH 6.0). Subsequent steps were performed at room temperature in a moist chamber. Sections, after incubation with normal goat serum for 30 min were incubated overnight with the primary mouse monoclonal antibody anti-resistin (1:100 in PBS). Next day, sections were incubated with the secondary biotinylated goat anti-mouse IgG (1:200 in PBS) antibody for 30 min and then with the avidin-biotin-peroxidase complex (1:50:1 in PBS; ABC Elite Kit, PK-6200, Vector Labs) for further 30 min. Each incubation was preceded and followed by a PBS washing. Incubation with diaminobenzidine solution (DAB, SK-4100, Vector Labs) (5-10 min) evidenced the immunopositive reaction. Finally, the sections were counterstained with Mayer's haematoxylin. Sections in which the primary antibody was omitted and/or substituted by pre-immune mouse-globulin were used for the negative control of unspecific staining. All tissue analyses were carried out on randomly selected slides by two independent investigators who were unaware of the treatments, using a light microscope (Nikon Eclipse E800, Nikon Corporation, Tokyo, Japan) connected to a digital camera (Dxm 1200 Nikon digital camera). The images were processed using an image analysis system (Lucia, Laboratory Imaging Ltd., Praha, Czech Republic). The settings for image capture were standardized by subtracting the background signals obtained from the matched tissue sections which had not reacted with the primary antibodies and which were used as immunohistochemical controls. Based on these considerations, the intensity of the staining was graded in arbitrary units as follows: negative (-), weak (+), strong (++).

Results

By WB, a single band at approximately 40 kDa, was detected in uterine samples of CTRL and EXP groups (Figure 1). Immunohistochemical procedures showed specific staining for resistin in the cytoplasm of cells from the uterine glands and the epithelial lining (Figure 2 a-c). The immunopositivity involved the whole cytoplasm, though it seemed more evident in the supranuclear region of the cells (Figure 2 a,c). A positive immunoreaction for resistin was also evident in the cytoplasm of smooth muscle cells at both vascular (Figure 2d) and myometrium levels. Immuno-positive reaction was not observed in other uterine structures and in the sections used as controls for the specificity of the reaction (inserts in Figure 2 a,b). Comparing the two analysed animal groups (CTRL and EXP), in the epithelial lining samples, no difference was observed in the intensity of immunolabeling for resistin, which appeared weak. Instead, in the uterine gland samples, variations in the immunopositivity were observed. In particular, strong intensity was observed in the uterine gland samples from the EXP animals, while the immunopositivity for resistin appeared less intense in the samples from the CTRL animals. The results are summarized in Table 1.

Table 1. Resistin-immunopositivity in sheep uterine structures.

	Epithelial lining	Uterine glands	Smooth muscle
CTRL group	+	+	+
EXP group	+	++	+

Figure 1. Immunoblot showing resistin in uterine lysate of two different ewes belonging to CTRL and EXP groups. Total protein extracts were separated by SDS-PAGE and analysed by immunoblotting with an anti-resistin specific antibody.

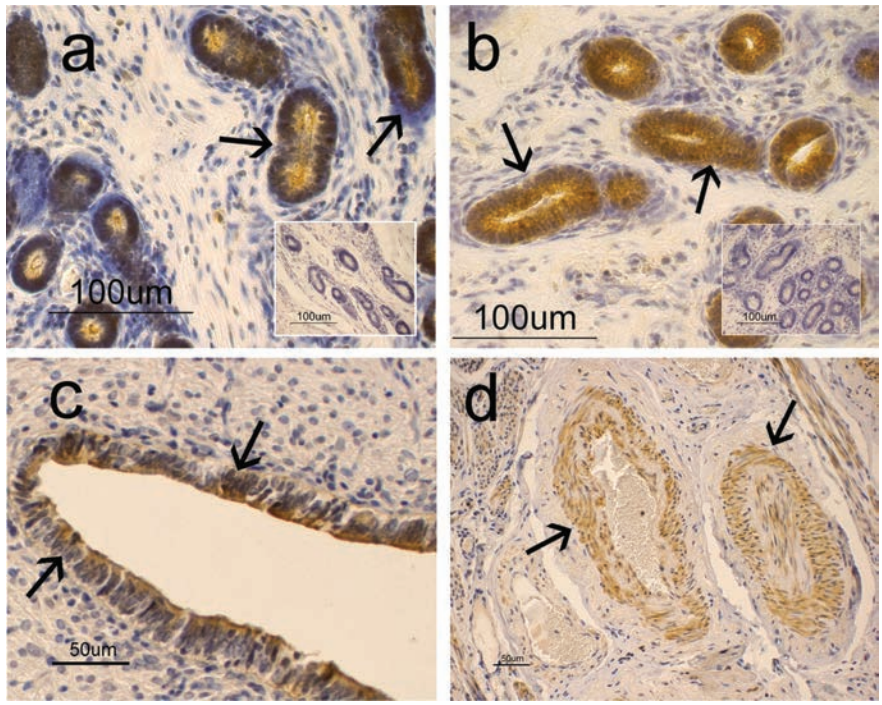

Figure 2. Resistin-immunohistochemistry aspects in the uterus of sheep: in a) and b) immunopositivity is localized in the cytoplasm of uterine glandular cells (arrows) with a stronger intensity in EXP group (b), compared to CTRL group (a). a) Immunopositivity seems to be mainly localized in the supranuclear (arrows) cytoplasm of the uterine glandular cells; c) immunopositivity is localized in the supranuclear (arrows) cytoplasm of the epithelial-lining cells; d) immunopositivity is localized in the muscle cells of arterial vessels (arrows).

Discussion

These findings suggest that the food supplementation had an effect on resistin production in the sheep uterine glands, while this does not seem in the epithelial lining. It can be speculated that food supplementation allows to a better animal body status; therefore, also resistin production by uterine glands enhances. The supranuclear location of resistin binding patterns allows us to hypothesize that resistin could have an autocrine action[26-28] stimulating the uterine gland secretory activity, in addition to a paracrine action as trophic factor towards spermatozoa and/or embryos. However, other studies will be needed to define the role of the resistin system more precisely. It is likely that resistin action is affected by the participation of other molecules, as is the case for insulin, whose action is modulated by glucagon, nesfatin, leptin and resistin.[29-32]

This immunohistochemical study identified resistin positive cells in the uterus of sheep, supporting the existence of a peripheral resistin system also in the reproductive apparatus of this animal species. There are no previous studies in this species on the resistin WB identification. The single band here detected at 40 kDa confirmed the antibody specificity used for IHC. The difference with 34 kDa weight of human resistin antibody, also considering the homology of 70% between human and sheep resistin sequence, may be ascribed to the difference between the two species. To the best of our knowledge, this is the first study to demonstrate the presence of resistin in the uterus of a species of domestic animals, showing that this adiponectin is produced by the uterus of these animals and that its production can be modified by changes in nutritional status, an observation that strengthens the link between nutritional status and reproductive activity. This finding is particularly important for the Apennine area, where the natural pastures provide the summer trophic resources needed for ewes to attain a suitable body status for sustaining pregnancy, which, in turn, is a key factor for the sustainability of local farms.[21]

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