DOI: 10.21005/AAPZ2016.40.4.15

FOLIA POMERANAE UNIVERSITATIS TECHNOLOGIAE STETINENSIS
Folia Pomer. Univ. Technol. Stetin., Agric., Aliment., Pisc., Zootech. 2016, 330(40)4, 139–146

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ANALYSIS OF RENAL EXPRESSION OF TRPM6 AND TRPM7 IN GROWING PIGLETS FED A DIET SUPPLEMENTED WITH INULIN –TYPE FRUCTANS. A PILOT STUDY

ANALIZA EKSPRESJI TRPM6 I TRPM7 W NERKACH PROSIĄT ŻYWIONYCH PASZĄ Z DODATKIEM FRUKTANÓW TYPU INULINOWEGO. BADANIA WSTĘPNE

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Streszczenie. Spożywanie paszy wzbogaconej fruktanami typu inulinowego ma wielokierunkowe korzystne działanie prozdrowotne. Stosowanie tak zmodyfikowanej paszy m.in. zwiększa w jelitach przyswajalność wielu mikro- i makroelementów, w tym magnezu (Mg^{2+}). Ogólnie wiadomo, że Mg^{2+} bierze udział w wielu procesach biologicznych, a zaburzenia jego homeostazy szczególnie w okresie wzrostu i rozwoju mogą powodować wiele niekorzystnych zmian. W utrzymaniu właściwego bilansu Mg^{2+} zaangażowanych jest wiele mechanizmów i czynników, m.in. niedawno zidentyfikowane białka TRPM6 i TRPM7 (*transient potential melastin 6 and 7*). Ponieważ w dostępnej literaturze brakuje informacji na temat TRPM6 i TRPM7 u zwierząt gospodarskich, w tym u trzody chlewnej, podjęliśmy badania, których celem było zidentyfikowanie tych białek w nerkach prosiąt oraz analiza wpływu diety suplementowanej fruktanami typu inulinowego na ich ekspresję. Badania przeprowadzono na 16 prosiętach (samcach), krzyżówkach PIC x Penarlan P76. Zwierzęta podzielono na grupę kontrolną – prosięta żywione paszą standardową oraz na grupę prosiąt żywionych paszą suplementowaną 3-procentowym wodnym roztworem fruktanów typu inulinowego. W efekcie przeprowadzonych badań, z zastosowaniem techniki Western blot, stwierdzono, że w nerkach prosiąt występują TRPM6 i TRPM7. Stwierdzono również, że u zwierząt, którym podawano paszę wzbogaconą fruktanami typu insulinowego, wzrosła nerkowa ekspresja TRPM6, natomiast nie zmieniła się ekspresja TRMP7. Wzrost ekspresji TRMP6 u zwierząt, którym podawano wraz z paszą fruktany typu insulinowego, niewątpliwe przyczynił się do zwiększenia nerkowego zatrzymywania Mg^{2+}. Zmiany ekspresji TRPM6 wydają się korzystnym efektem suplementacji paszy fruktanami typu inulinowego.

Key words: piglets, kidney, TRPM6, TRPM7, diet, inulin, Western blot.
Słowa kluczowe: prosięta, nerki, TRPM6, TRPM7, diet, inulina, Western blot.

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INTRODUCTION

Since 2006, when the European Union introduced a total ban on antibiotic growth promoters (AGP) in animal feeds, there has been a quest for other feed additives, which could replace the previously used antibiotics (Anadón 2006). Among the many herbs and other natural feed supplements, inulin and other inulin-type fructans offer outstanding health benefits. Inulin-type fructans are polysaccharides of plant origin, with β-D-fructofuranose molecules representing their main structural units (Roberfroid 2007; Apolinario et al. 2014). In recent years, a number of studies have shown that a daily diet supplementation with inulin--type fructans has multidirectional, beneficial health effects. An addition of this type of polysaccharides in the diet primarily stimulates the growth and activity of beneficial intestinal microflora and inhibits proliferation of pathogenic microorganisms in the gut (Roberfroid 2005; Kolida and Gibson 2007; Veerman 2007; Tako et al. 2008).

Feeding animals with feeds supplemented with inulin-type fructans has a positive effect on their condition and health, reduces feed intake, increases weight gains and, in consequence, improves the viability of animal production (Pierce et al. 2006; Flickinger et al. 2010; Kjos et al. 2010). This enriched diet offered to both humans and animals increases the bioavailability of many micro- and macrominerals including magnesium, Mg^{2+} (Coudray et al. 2005; Coudray et al. 2006). It is generally known that Mg^{2+} is involved in many biological processes, and a disruption of its homeostasis, especially during the growth and development, can cause a variety of adverse changes in the skeletal, neuromuscular and cardiovascular systems (Soetan et al. 2010; Komiya et al. 2015; Yolcu et al. 2016). Maintaining the proper balance of Mg^{2+} involves many mechanisms and factors, including the recently identified proteins TRPM6 and TRPM7 (transient receptor potential melastatin 6 and 7) – Schlingmann et al. (2007).

These proteins have the ability of trans-membrane transport of magnesium ions in intestinal epithelial cells and renal tubular cells, and their expression is dependent on the supply of Mg^{2+} in the diet (Voets et al. 2004). Changes in the expression of TRPM6 and TRPM7 in the renal tubular epithelium may directly affect the excretion rate of this micro--nutrient in the urine and thus participate in the regulation of the magnesium balance of in the body. Since the available literature lacks any information about TRPM6 and TRPM7 in livestock animals, including swine, we have undertaken this study in order to (i) identify these proteins in the kidney of growing piglets and (ii) analyze the impact of a diet supplemented with inulin-type fructans on their expressions.

MATERIAL AND METHODS

Experimental animals

All experiments were performed in accordance with the principles and procedures of Local Commission of Ethics for the Care and Use of Laboratory Animals (No. 11/2012 of 23.05.2012). The study was carried out on 16 PIC x Penarlan P76 crossbred piglets (males). During the experiment, the animals were remained under unified and controlled environmental conditions. From the 10th day of life the piglets were divided into 2 nutrition groups (n = 8). Piglets from the control group were feed *ad libitum*, with the standard diet

containing: wheat (46.84%), barley (20%), corn starch (3%), full-fat soybean meal (5.9%), whey (9.7%), fish meal (4%), spray-dried blood plasma (4%), soybean oil (3.4%), calcium formate (0.3%), limestone (0.5%), dicalcium phosphate (0.6%), sodium chloride (0.07%), L-lysine (0.61%), DL-methionine (0.23%), L-threonine (0.26%), L-tryptophan (0.09%), mineral--vitamin premix (0.4%) and aroma (0.1%). The animals from the experimental group were feed *ad libitum*, with standard diet supplemented with 3% water extract of chicory root inulin--type fructans. The chemical composition of the extract of chicory root inulin-type fructans used in the present study included approximately 92% of inulin/oligofructose and 8% of other carbohydrates (glucose, fructose and sucrose). An average degree of polymerization (DP) of inulin was 10. Starch content was lowered adequately to chicory addition. Piglets were sacrificed at the age of 50 days and kidneys were dissected. Obtained material was washed twice with ice-cold 0.9% NaCl solution and subsequently twice with ice-cold Krebs-HEPES buffer (20 mM, pH 7.4). Washed tissues were dried and placed in the liquid nitrogen to protect from proteolysis.

SDS – PAGE and Western blot

The tissue samples were placed in the lysis buffer (5M urea, 2M thiourea, 4% CHAPS, 40 mM Tris, 0.2% ampholytes pH 3–10, nuclease 1 : 1000) containing protease inhibitor cocktail 1 : 100 (Sigma-Aldrich). Afterwards, the tissue samples were frozen in liquid nitrogen and were homogenized using the Tissue Lyser, QIAGEN. The homogenates were centrifuged at 20 800 x g for 15 min at 4°C. The samples were warmed to 37°C and loaded on the 12% polyacrylamide gels and run for 120 min. at 100 V. The proteins of studied gels were then electrotransferred (17V, 15 min) to PVDF membranes. The membranes were blocked with 5% non-fat milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 0.1% Tween 20, pH 7.5) for 1 h and incubated overnight at 4°C with rabbit polyclonal antibodies anti-TRPM6 and anti-TRPM7 (Biorbyt, arb38901 and arb38899) diluted 1 : 1000, followed by incubation with secondary anti-rabbit (Serotec, STAR124P) horseradish peroxidase--conjugated antibodies. The labeling was visualized by the enhanced chemiluminescence (ECL plus) system and exposure to CCD camera (Versadoc 4000MP, Bio Rad). The densitometry values and band optical density (OD) were evaluated with Quantity One software.

Mean values and standard deviations were calculated. The resulting data were analysed by one- way ANOVA and Dunkan multiple range post hoc test (Statistyca, 10.0^{TM}) in order to test significance of differences. Expression of TRPM6 and TRPM7 was normalized against β-actin, which was used as an internal control.

RESULTS

Figure 1 show TRPM6 and TRPM7 abundance determined by Western blot. TRMP6 and TRPM7 antibodies recognized a characteristic bands in protein samples from kidneys of growing piglets. Based on the analysis of average optical density of the bands, it was found, that animals fed a diet enriched with inulin showed statistically significant ($P < 0.05$) increased expression of TRPM6 in the kidneys. Optical density of this protein in the kidney in the control group was 4563.8 ± 575.6, while in the group of the piglets feed with the

modified diet was 18734.7 ± 854.5. Expression of the TRPM 7 in the kidney in all tested animals was relatively stable. The average optical density of the TRPM7 in the control group was 17960.5 ± 1207.6, while in animals fed a diet enriched with inulin was 18362.5 ± 1358.1.

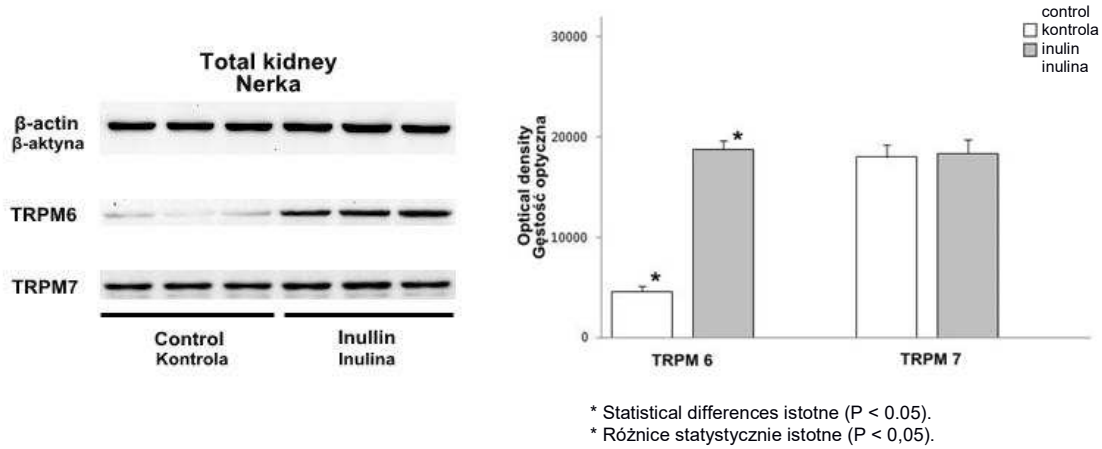

Fig. 1. Representative results of Western blot analysis of TRPM6 and TRPM7 expression in the kidney of the control group and the piglets fed diets supplemented with 3% water extract of chicory root inulin-type fructans
Ryc. 1. Reprezentatywny wynik analizy Western blot ekspresji TRPM6 i TRPM7 w nerkach prosiąt z grupy kontrolnej oraz prosiąt żywionych paszą z dodatkiem 3% wodnego roztworu fruktanów typu inulinowego z korzenia cykorii

DISCUSSION

TRPM6 and TRPM7 belong to a large family of transient receptor potential proteins (TRP). The proteins represent a group of voltage-independent cation-permeable channels expressed in most mammalian cells. The transient receptor potential melastin (TRPM) cations channel represent one of six subfamilies of the TRP (Hsu et al. 2007). TRPM6 and TRPM7 consist of six membrane-spanning domains that form the channel pore, and large intracellular amino-terminal and carboxy-terminal domains (Zholos 2010; van der Wijst et al. 2009; Lin et al. 2016). Both proteins share the unique feature of a C-terminal serine/threonine protein kinase domain. Both TRPM6 and TRPM7 are permeable to calcium and magnesium, although the permeability of magnesium is much higher than that of calcium (Chubanow et al. 2004).

TRPM6 and TRPM7 are localized in the gut and in the apical membrane of renal tubular epithelial cells (Voets et al. 2004; Schiffrin et al. 2005). TRPM6 plays an important role in maintaining the adequate balance of magnesium through the regulation of epithelial Mg^{2+} transport in the intestine and kidney (Schlingmann et al. 2007). TRPM6 self-contained units are not able to form a functional channel; this is due to its specific amino-acid composition, as a result of which the protein lacks sufficient amount of hydrophobic amino acids to be incorporated into the cell membrane. Therefore, it is presumed that TRPM6 and TRPM7 form a pair of co-operating proteins. TRPM7, by forming heteromultimers, allows TRPM6 to enter in the cell membrane (Monteilh-Zoller et al. 2003). To date, little is known about the mechanism of TRPM7 activity, although it is believed that the protein is responsible primarily for the maintenance of intracellular balance of magnesium (Trzeciakiewicz et al. 2005;

Schlingmann et al. 2007). Regulation of TRPM7 ion channel is complex and the intracellular level of magnesium and MgATP do influence the functioning of the channel. It has been shown that both factors tend to impair the channel activity. Presumably, an excessive increase in the concentration of magnesium in the cell blocks the channel through Mg^{2+} binding to the amino acid residues within the pore (Schmitz et al. 2003; Takezawa et al. 2004). Ion-channel blocking capability is the domain of other cations, such as Ba^{2}, Sr^{2+}, Mn^{2+} and Zn^{2+}, although the mechanism of their action is not fully understood (Monteilh-Zoller et al. 2003; Montell 2003). Also alpha-kinase has a regulatory function of the channel activity, since it can serve as a gating using its properties for channeling the phosphorylation reaction. The function and role of alpha-kinase in TRPM7 activity regulation has not been fully described (Takezawa et al. 2004; Trzeciakiewicz et al. 2005).

In homogenates of total kidney of growing piglets, we have found characteristic bands formed by TRPM6 and TRPM7 binding with specific antibodies, which confirms the presence of these proteins in the kidney of pigs. The literature lacks any data on this, both in this species and other farm animals. To our knowledge, the present study is the first report on TRPM6 and TRPM7 expression in the kidneys of growing piglets. So far, these proteins have been localized in the kidneys of the rat, mouse and human (Chubanov et al. 2004; Voets et al. 2004; Schlingmann et al. 2007). The issue of where TRPM6 and TRPM7 are localized in the particular sections of the renal tubule is currently subject to a debate.

Most authors believe, however, that TRPM6 is mainly located in the apical membrane of the cells of distal and collecting tubules, whereas TRPM7 is present in all nephron segments (Chubanow et al. 2004; Voets et al. 2004). Unfortunately, Western blot analysis does not allow determination of the specific localization of TRPM6 and TRPM7 in porcine renal tubules; however, the mere presence as these proteins and TRPM6 expression changes influenced by the diet suggest that – also in pigs – these ion channels will play an important role in the regulation of the balance of magnesium, both at the body level and inside the cells.

One of the main factors influencing the changes in the renal expression of TRPM6 and TRPM7 is the supply of Mg^{2+} with the diet and its intestinal absorption. An increased level of supply and absorption of Mg^{2+} will cause increased urinary excretion of this micromineral, and thus decreased expression of mainly TRPM6. As mentioned before, ingestion of dietary inulin-type fructans increases intestinal absorption of magnesium. Currently, the available literature brings only one report whose authors describe the effect of inulin supply on the expression of TRPM6 and TRPM7. Rondón et al. (2008) have shown that mice fed a diet supplemented with long-chain inulin demonstrated reduced renal expression of TRPM6, at a constant level of TRPM7 expression. According to the authors, increased Mg^{2+} urinary excretion, resulting from reduced expression of TRPM6, was a homeostatic response of the kidneys to an increased intestinal absorption of Mg^{2+} with its reduced fecal excretion level. Namely, in order to maintain the proper balance of magnesium, including plasma Mg^{2+} level, its renal excretion increases. Like the data reported by Rondón et al. (2008), this study revealed no changes in renal TRPM7 expression in growing piglets fed a diet enriched with inulin-type fructans. However, in contrast to these authors, we observed a significant increase in the expression of TRPM6, and thus elevated renal retention of Mg^{2+}. The increase in renal expression of this protein in growing piglets was probably linked with the fact that the animals

had a low level of magnesium in the blood plasma (unpublished data). Plasma concentration of Mg^{2+} in all the porcine subjects averaged only 0.49 mmol/l. We presume that the addition of inulin to the diet of growing piglets not only contributed to an increase in the intestinal retention of magnesium, but also – with the low magnesium status – to the renal retention of this element. The mechanism of this efect is difficult to explain though. This issue certainly requires further, more detailed studies.

CONCLUSION

In conclusion, the results of our experiment confirm the presence of TRPM6 and TRPM7 proteins in the kidneys of growing piglets. It was also found that the animals fed a diet supplemented with 3% aqueous extract of inulin-type fructans showed an increased renal expression of TRPM6, as compared with the control. Expression of TRPM7, on the other hand, has not changed. In both control animals and those fed a modified diet the level of this protein in the kidneys was relatively stable. The increase in TRPM6 expression in animals treated with inulin-type fructans supplemented diet undoubtedly contributed to increased renal retention of Mg^{2+}. Changes in the expression of TRPM6 seem to be a positive effect of the dietary supplementation of inulin-type fructans.

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Abstract. A diet enriched with inulin-type fructans has multidirectional, beneficial health effects for both humans and animals. The benefits of such a modified diet include increased intestinal absorption of micro- and macrominerals, including magnesium (Mg^{2+}). It is generally known that Mg^{2+} is involved in many biological processes, and a disruption of its homeostasis during the growth and development may result in a number of adverse changes. Maintaining the proper balance of Mg^{2+} involves many mechanisms and factors, among them the recently identified protein TRPM6 and TRPM7 (transient receptor potential melastin 6 and 7). Since the available literature lacks any information about TRPM6 and TRPM7 in farm animals, including swine, we have undertaken this research aimed at identification of these proteins in the kidney of growing piglets and analysis of the impact of diets supplemented with inulin-type fructans on their expressions. The study was performed on 16 male, PIC x Penarlan P76 crossbred piglets.

Animals were divided into two groups: the control was fed a standard diet and the treatment group was fed a diet supplemented with 3% aqueous solution of inulin-type fructans. As a result of the study, using Western blotting, we found TRPM6 and TRPM7 in the kidneys of growing piglets. We also found that renal expression of TRPM6 increased in the animals treated with a diet supplemented with inulin-type fructans. Expression of TRPM7, on the other hand, did not change. The increase in TRPM6 expression in the supplement-treated animals presumably contributed to an increased renal retention of Mg^{2+}. The changes in the expression of TRPM6 seem to be a positive effect of the dietary supplementation with inulin-type fructans.