University of Veterinary Medicine, Budapest
Doctoral School of Veterinary Sciences

Epigenetic effects of butyrate
in intestinal and extraintestinal tissues
of broiler chicken

Ph.D. thesis

Anna Kulcsár

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Supervisor and consultants:

....
Dr. Zsuzsanna Neogrády, CSc
Division of Biochemistry, Department of Physiology and Biochemistry
University of Veterinary Medicine, Budapest
supervisor

....
Dr. György Csikó, CSc
Department of Phyarmacology and Toxicology
University of Veterinary Medicine, Budapest
co-supervisor

Dr. Korinna Huber, Univ.-Prof.
Institute of Animal Science
University of Hohenheim
consultant

Dr. Orsolya Farkas, PhD
Department of Pharmacology and Toxicology
University of Veterinary Medicine, Budapest
consultant

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        Kulcsár Anna

1. Introduction

The four carbon short chain fatty acid butyrate has quite a wide range of biological activities via epigenetic and receptor-mediated pathways. As in monogastric mammals and birds it is mainly produced by bacterial fermentation in the large intestines, it can act as a sensitive messenger molecule between prokaryotic and eukaryotic organisms and maintain a symbiotic balance between the intestinal bacterial communities and the host. Besides its intestinal effects, butyrate can be also absorbed from the gastrointestinal tract, and by reaching certain organs with the portal and systemic circulation it can provoke variable effects.

Based on its epigenetic and receptor-mediated effects, butyrate can influence the expression of certain proteins. Thus it could influence the function of microsomal cytochrome P450 (CYP) enzymes, mainly involved in the oxidative phase I reactions of xenobiotic biotransformation, playing pivotal role in drug metabolism. The CYP enzymes are primarily expressed in the microsomes of hepatocytes; however, they are also localized in the intestinal mucosa to serve as a primary metabolic barrier for xenobiotics taken up orally, also influencing their bioavailability and toxicity.

The insulin homeostasis could be also greatly influenced via the epigenetic and receptor mediated modulation of its components. As besides carbohydrate and lipid metabolism, insulin is one of the most important regulator of protein synthesis and growth as well, the possible effects of butyrate on insulin production, reception and signaling are of special interest also in broilers. In both mammals and birds, pancreatic insulin release is primarily controlled by the gut-derived incretin hormones, as key members of the enteroinsular axis. The way of incretin action is mostly known from model studies with rodents, while only limited data are available with regard on domestic animal species. Since the carbohydrate metabolism of birds differs from that of mammals, studying the production and insulin regulation of incretin hormones could be of special practical and theoretical importance.

To enhance its beneficial actions, intestinal butyrate production can be stimulated by certain dietary factors, and it is also used as a feed additive in free or protected form in poultry nutrition. However, the way and the intensity of its effect could depend on the intestinal and plasma concentration of butyrate, that could be determined by the microbial butyrate production in the caecum and by the way, dose and time of oral butyrate application. Therefore, the main purpose of the present PhD work was to study the effects of butyrate in chicken from a theoretical and practical point of view as well, beginning with the absorption of butyrate of either endogenous or

exogenous origin, including its intestinal and extraintestinal influences. As an important possible intestinal effect of butyrate, the activity of the xenobiotic metabolizing cytochrome P450 (CYP) enzymes was studied, while one of the most significant extraintestinal effects, influence of butyrate on the insulin homeostasis has been also investigated. The current PhD work approaches butyrate action partly from practical point of view: to study the effects that could be manifested during the application in poultry farming, and partly from theoretical point of view: to describe the potential mechanisms of butyrate action in a model system.

To fulfill the above mentioned goals, three main experimental studies were carried out:

Long-term – feeding study: absorption and distribution of butyrate, effect of butyrate on intestinal CYP enzymes and insulin homeostasis were studied, the latter by monitoring the members of insulin signaling pathway. The investigative approach of this study was to examine butyrate action from a practical point of view, applying butyrate treatments that could be used in broiler nutrition as well.

Medium-term – multiple bolus study: insulin homeostasis was studied following multiple butyrate bolus application by monitoring the members of insulin signaling pathway.

Short-term – single bolus study: insulin homeostasis was studied following one single butyrate bolus application by assessing the production of incretin hormones. The investigative approach of this study was to examine butyrate action from a more theoretical point of view, applying a model bolus treatment system.

2. Significance and aims of the study

Summarized, the most important aims of this PhD study were:

Ad 1, to study the intestinal availability and absorption of butyrate – originated either from dietary supplementation or produced endogenously by the caecal microflora in chicken.

Ad 2, to investigate the long-term effect of butyrate as dietary supplementation and that of endogenously produced butyrate on the activity of intestinal CYP enzymes in chicken.

Ad 3, **(a)** to evaluate the long-term effect of butyrate as dietary supplementation and that of endogenously produced butyrate on the expression of certain key protein of the insulin signaling pathway.
(b) to study the medium-term effect of multiple butyrate bolus application on the expression of certain key proteins of the insulin signaling pathway in chicken.

Ad 4, to investigate the short-term effect of single butyrate bolus application on the production of incretin hormones in chicken in comparison with rabbit.

3. Materials and methods

3.1.   Long-term – feeding study

In our long-term – feeding study one-day-old male Ross 308 broiler chickens were fed with two different basal diets: a maize-based (MB) and a wheat-based (WB) diet, the latter with non-starch polysaccharide (NSP)-degrading enzyme (mixture of xylanase and glucanase) supplementation. The WB diet with higher NSP content supplemented with NSP-degrading enzymes was aimed to provide substrates for the caecal bacterial fermentation in order to enhance caecal butyrate production. Both types of basal diets (MB and WB) were supplemented with two different doses of non-protected butyrate (sodium salt, 1.5 g/kg diet – lower dose or 3.0 g/kg diet – higher dose) or with protected butyrate (micro-encapsulated form, ButiPEARL, 0.2 g/kg diet). The lower concentration of non-protected butyrate was set according to the average dose applied in poultry nutrition, while the higher concentration was administered to test the dose-dependency of butyrate activity. No butyrate was added to the diet of control MB and WB groups. Based on the feeding regime described above, animals were randomized into eight experimental groups (n=22/group): MB and WB diets with various forms and doses (lower and higher dose of non-protected butyrate; protected butyrate) or without butyrate supplementation (controls).

Animals were slaughtered on day 42.

In order to study the absorption and distribution of different application forms of butyrate, intestinal content samples from duodenum, ileum and caecum (n=10/group), and blood plasma samples from the portal circulation (*vena gastopancreaticoduodenalis, vena mesenterica communis*) and systemic circulation (*vena brachialis*) (n=6/group) were taken. Butyrate concentrations from ingesta and plasma samples were determined by gas chromatography.

To study intestinal CYP activity, duodenal mucosal samples (n=6/group) were also taken. After differential centrifugation, microsomal CYP1A4/5, CYP2H2 and CYP3A37 activities were monitored by luminescence P450-Glo assays.

To investigate insulin homeostasis, glucose and insulin concentrations from *vena brachialis* (n=6/group) were measured by colorimetric and ELISA methods, respectively. From liver, skeletal muscle and subcutaneous adipose tissue samples (n=6/group/tissue) the protein expression of key insulin signaling proteins, namely IRβ, mTOR and PKCζ was monitored applying Western Blot method.

3.2. Medium-term – multiple bolus study

In the medium-term – multiple bolus study we used a model application form of butyrate in Ross 308 broiler chicken, as they were treated once daily with orally administered intraingluvial bolus of sodium butyrate (0.25 g/kg BW – referring to the average dose used in poultry nutrition), or distilled water (control) following overnight feed deprivation for five days, on days 20-24 of life.

On day 24 plasma samples were taken from *vena brachialis* (n=10/group) to determine butyrate concentration by gas chromatography and glucose and insulin concentration by colorimetric and ELISA methods, respectively. To study certain members of the insulin signaling pathway, the protein expressions of IRβ, mTOR, PKCζ and PI3K were determined by Western Blot method from liver, gastrocnemic muscle, subcutaneous and abdominal adipose tissue samples (n=10/group/tissue).

3.3. Short-term – single bolus study

In the short-term – single bolus study we also used a model application form of butyrate in Ross 308 broiler chickens and in Pannonian giant rabbits. They were treated with a single intraingluvial or intragastric non-protected sodium butyrate bolus in two different doses (0.25 and 1.25 g/kg body weight) or with physiological saline (control group), following overnight feed deprivation on day 24 in case of chickens and on day 49 in case of rabbits.

To investigate the production of incretin hormones, blood samples from *vena brachialis* of chickens and from marginal air vein of rabbits (n=7/group) were collected before treatment, and 10, 30 and 60 min following butyrate administration. Plasma glucose, insulin, glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) concentrations were measured by colorimetric (glucose) and ELISA (insulin, GIP, GLP-1) methods.

4. Results and discussion

4.1.   Butyrate absorption and distribution

According to our results non-protected butyrate should be absorbed from the gastrointestinal tract before the small intestines, as it did not have any effect on the butyrate concentration in duodenum, ileum and caecum either in lower or in higher dose. In contrast, micro-encapsulated butyrate is protected from the early absorption, so it is released and absorbed only in the distal parts of the gastrointestinal tract. In our study, protected butyrate supplementation increased the butyrate concentration in the ileum. Butyrate concentration in the caecum was only influenced by the wheat-based diet. This is in association with the higher soluble NSP content of the wheat-based diets, which, following its degradation to oligosaccharides by the supplemented xylanase and glucanase, serves as a substrate of the intensive microbial butyrate production in the large intestine.

Regarding the portal circulation, the higher dose of non-protected butyrate supplementation and the wheat-based diet resulted in higher blood butyrate concentration in *vena gastropancreaticoduodenalis*, however, in *vena mesenterica communis* higher dose of non-protected butyrate supplementation, protected butyrate supplementation and wheat-based diet all increased blood butyrate concentration. The observed changes in butyrate concentrations in blood samples can be explained by the anatomical location of the investigated veins, draining different segments of the intestinal tract. The *vena gastropancreaticoduodenalis* collects blood mainly from the gizzard and the duodenum, the suggested site of the absorption of non-protected butyrate, while *vena mesenterica communis* collects blood mainly from the distal part of the small intestine and from the large intestine. Concentration of butyrate in systemic plasma samples (*vena brachialis*) was significantly increased only by the pronounced effect of non-protected butyrate in higher dose in the long-term – feeding study and by the multiple bolus application of non-protected butyrate in the medium-term study.

The described absorption and distribution properties of different forms of butyrate are of special relevance by highly determining the biological action of butyrate in the gastrointestinal tract and beyond the intestines as well.

4.2. Intestinal action: Effects of butyrate on intestinal CYP activity

Concerning the intestinal effects of butyrate, CYP1A4/5 and CYP2H2 activities in duodenal epithelial cells were increased by both higher dose of non-protected butyrate supplementation and wheat-based diet. These results are in connection with the absorption and distribution of butyrate. Notwithstanding that non-protected butyrate did not alter butyrate concentration in duodenum significantly, a certain amount may have reached this intestinal section and alter the activity of CYP enzymes in the mucosa layer. The higher amount of microbially produced butyrate in the caecum in wheat-based diet groups could get to the proximal parts of gastrointestinal tract through the portal circulation, thus duodenal endothelial cells could get butyrate stimulus mainly from the surrounded veins. Furthermore, several other parameters of wheat-based diet (SCFAs, amino acids, long chain fatty acids) could be also involved in the effect of this diet type. Based on the key role of duodenal CYPs as a primary metabolic barrier against orally ingested xenobiotics, all efforts altering their function could be of high importance from food safety and animal health point of view.

4.3. Extraintestinal action: Effects of butyrate on insulin signaling proteins

In the long-term – feeding study dietary cereal type had the most remarkable effect on the insulin signaling: wheat-based diet increased IRβ and mTOR expression in the liver and mTOR and PKCζ expression in the adipose tissue. IRβ expression in the liver was increased by the lower dose of non-protected butyrate as well. Regarding the investigated blood plasma parameters in the long-term – feeding study, wheat-based diet significantly decreased plasma insulin concentration, however, no significant differences were found in the blood glucose concentration among experimental groups. Although birds are known to be less insulin sensitive than mammals, our results may suggest that the increased expression levels of insulin signaling proteins in chicken kept on wheat-based diet could play a role in the maintenance of the constant blood glucose level even at the WB diet-associated decreased plasma insulin concentration.

In the medium-term – multiple bolus study, in which chickens received a daily intraingluvial bolus of non-protected butyrate (0.25 g/kg body weight) on days 20-24 of life, butyrate had a more pronounced and tissue-selective impact on insulin signaling. Butyrate bolus application was associated with decreased protein expression of IRβ in liver and adipose tissues, but with elevated IRβ expression in muscle. This butyrate-triggered selective up-regulation may suggest that butyrate could act on glucose shifting among tissues by selectively increasing the glucose uptake of skeletal muscle via the stimulation of IRβ expression. Hepatic PI3K protein expression was

reduced in the butyrate-treated group, while mTOR was down-regulated by butyrate in liver and subcutaneous adipose tissue. Multiple bolus application of butyrate significantly increased both plasma insulin and plasma glucose concentration. Our results demonstrate that the application form of butyrate and the age of chicken could remarkably determine the way of butyrate action. The bolus application of butyrate had more pronounced and partly different effects on certain insulin signaling proteins compared to the long-term feeding application. Further, in the phase of intensive growth (day 20-24 of life), when insulin is mostly involved in growth regulation, broilers were more sensitive to butyrate treatment. Studying butyrate's possible influence on insulin signaling in chicken has a special importance, because the regulation of carbohydrate metabolism in birds with high fasting blood glucose level and moderate insulin sensitivity is not fully elucidated.

4.4.   Extraintestinal action: Effects of butyrate on incretins

In the short-term – single bolus study single intraingluvial bolus application of non-protected butyrate decreased plasma GIP levels in both chickens and rabbits after 30 and 60 min following butyrate ingestion. In chickens the higher dose of butyrate application (1.25 g/kg body weight), while in rabbits the lower dose (0.25 g/kg body weight) had significant effect. Plasma GLP-1, insulin and glucose concentrations remained unaffected by butyrate in both species over time. These results are in contrast to butyrate's stimulating effect on both incretin and insulin secretion in mice, indicating specific, species-dependent differences even among mammalian species. In conclusion, it can be suggested that butyrate is a potent effector of incretin production, which may provide new possibilities in the nutritional modulation of incretin and insulin homeostasis and thus influencing the efficacy of animal production.

Our results highlight that butyrate of different origin could have great importance in poultry nutrition. Different application forms can determine the site and way of its biological activity; however, we can state that both the altered caecal microbial butyrate production and butyrate as a feed additive have remarkable intestinal and extraintestinal effects in broiler chicken.

5. New scientific results

Ad 1, Different application forms of dietary butyrate can be absorbed from different sections of the gastrointestinal tract of broiler chicken. Protected butyrate supplementation (0.2 g/kg diet) elevates butyrate concentration in the ileum, while wheat-based diet with NSP degrading enzyme supplementation (associated with higher microbial butyrate production in the large intestines) increases it in the caecum. Portal plasma butyrate concentration is increased by all the higher dose of non-protected butyrate (3.0 g/kg diet), the protected butyrate supplementation and the wheat-based diet. However, systemic plasma butyrate concentration is increased by the higher dose of non-protected butyrate supplementation and daily bolus application of non-protected butyrate (0.25 g/kg body weight for 5 days).

Ad 2, Both dietary and endogenous butyrate (produced in the large intestines) alter the activity of certain duodenal cytochrome P450 (CYP) enzymes. The CYP1A4/5 and CYP2H2 activities of six-week-old broiler chickens are increased by butyrate supplementation (non-protected, 3 g/kg diet) and by wheat-based diet with NSP degrading enzyme supplementation (associated with higher microbial butyrate production).

Ad 3, Butyrate is able to influence insulin homeostasis in broiler chicken. Wheat-based diet with NSP degrading enzyme supplementation (associated with higher microbial butyrate production) increases IRβ and mTOR expression in the liver as well as mTOR and PKCζ expression in the adipose tissue of six-week-old chickens. IRβ expression in the liver is stimulated also by the lower dose of non-protected butyrate (1.5 g/kg diet) in 6-week-old chickens. At the age of 3 weeks, daily butyrate bolus application (non-protected, 0.25 g/kg body weight for 5 days) decreases IRβ, PI3K and mTOR expression in liver and IRβ and mTOR expression in the adipose tissues, but increases IRβ expression in the muscle.

Ad 4, Butyrate can be a potent effector of incretin hormones in both chicken and rabbit. Single bolus application of non-protected butyrate decreases plasma GIP concentration of 3-week-old broiler chickens in higher dose (1.25 g/kg body weight), and of 6-week-old rabbits in lower dose (0.25 g/kg body weight).

6. Own scientific publications

6.1. Full text papers in peer-reviewed journals related to the topic of the present dissertation:

Gábor Mátis, Anna Kulcsár, Máté Mackei, Janka Petrilla, Zsuzsanna Neogrády
>Comparative study on the modulation of insulin and incretin homeostasis by butyrate in chickens and rabbits
>
>**PLOS ONE** 13(10): e0205512, 2018.

Anna Kulcsár, Gábor Mátis, Andor Molnár, Janka Petrilla, László Wágner, Hedvig Fébel, Ferenc Husvéth, Károly Dublec Zsuzsanna Neogrády
>Nutritional modulation of intestinal drug-metabolizing cytochrome P450 by butyrate of different origin in chicken
>
>**RESEARCH IN VETERINARY SCIENCE** 113 pp 25-32, 2017.

Anna Kulcsár, Gábor Mátis, Andor Molnár, Janka Petrilla, Ferenc Husvéth, Korinna Huber, Károly Dublecz, Zsuzsanna Neogrády
>Effects of butyrate on the insulin homeostasis of chickens kept on maize- or wheat- based diets
>
>**ACTA VETERINARIA HUNGARICA** 64:(4) pp. 482-496, 2016.

Kulcsár Anna, Mátis Gábor, Kulcsárné Petrilla Janka és Neogrády Zsuzsanna
>A bélnyálkahártya szerepe a xenobiotikumok metabolizmusában, különös tekintettel a citokróm P450 enzimrendszerre. Irodalmi áttekintés (The role of intestinal mucosa in the metabolism of xenobiotics with particular regard to the cytochrome P450 enzyme system. Literature review)
>
>**MAGYAR ÁLLATORVOSOK LAPJA** 138:(4) pp. 243–250, 2016.

Gábor Mátis, Anna Kulcsár, Vanessa Turowski, Hedvig Fébel, Zsuzsanna Neogrády, Korinna Huber
>Effects of oral butyrate application on insulin signaling in various tissues of chickens
>
>**DOMESTIC ANIMAL ENDOCRINOLOGY** 50: pp. 26-31, 2015.

Gábor Mátis, Péter Lengyel, Anna Kulcsár, Janka Kulcsárné Petrilla, Zsuzsanna Neogrády
>A szénhidrát-anyagcsere és az inzulin-homeosztázis sajátosságai csirkében. Irodalmi összefoglaló (Special characteristics of carbohydrate metabolism and insulin homeostasis in chicken. Literature review)
>
>**MAGYAR ÁLLATORVOSOK LAPJA** 136:(6) pp. 342–349, 2014.

6.2. Full text papers in peer-reviewed journals not related to the topic of the present dissertation:

Janka Petrilla, Gábor Mátis, <u>Anna Kulcsár</u>, Petra Talapka, Enikő Bíró, Máté Mackei, Hedvig Fébel, Zsuzsanna Neogrády

Effect of dietary cereal type, crude protein and butyrate supplementation on metabolic parameters of broilers.

ACTA VETERINARIA HUNGARICA, 66(3):408-52, 2018.

Kurucz Ádám, Nagy Csaba, <u>Kulcsár Anna</u>, Neogrády Zsuzsanna, Mátis Gábor

Méregtelenítő folyamatok vizsgálata vadon élő állatfajokban. Investigations of detoxifying processes in wild animal species

MAGYAR ÁLLATORVOSOK LAPJA, 140:(9) pp. 551-562, 2017.

Gábor Mátis, <u>Anna Kulcsár</u>, Janka Petrilla, Petra Talapka, Zsuzsanna Neogrády

Porcine hepatocyte-Kupffer cell co-culture as an in vitro model for testing the efficacy of anti-inflammatory substances

JOURNAL OF ANIMAL PHYSIOLOGY AND ANIMAL NUTRITION, 101, pp. 201-207, 2017.

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I**NFLAMMATION** 37:(1) pp. 247-260, 2014.

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