Region-specific molecular changes in the gut wall and in the intestinal microbiome in the streptozotocin diabetic rats

PROJEKT ZÁRÓ BESZÁMOLÓ
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In the present project, we investigated those regional molecular differences, which are thought to be responsible for the diabetes-related region-specific damage of the nitrergic myenteric neurons and mesenteric capillaries in the gut of streptozotocin (STZ)-induced chronic diabetic rats.

According to the work plan, we used STZ-induced rats to model type 1 diabetes in all three years of the present research. The rats were divided randomly into three groups: STZ-induced diabetics, insulin-treated diabetics, and sex- and age-matched controls. Hyperglycaemia was induced by a single intraperitoneal injection of STZ (60 mg/body weight kg). The animals were considered diabetic if the non-fasting blood glucose concentration was higher than 18 mM. From this time on, one group of hyperglycaemic rats received a subcutaneous injection of insulin each morning and afternoon. Non-fasting blood glucose levels were determined, and the animals were weighed weekly in each group. Equivalent volumes of saline were given subcutaneously to the rats in the other diabetic and the control groups. Ten weeks after the onset of diabetes, animals were killed by cervical dislocation and gut segments as well as the intestinal contents from duodenum, ileum and colon were collected and stored under appropriate conditions and processed for immunohistochemical, molecular and metagenomic studies.

At the end of the experimental period, the diabetic rats were characterized by a reduced body weight and an increased blood glucose concentration as compared with the age- and sex-matched controls. The insulin-treated diabetic rats did not differ significantly from the control animals in weight and immediate insulin treatment prevented the extremely high blood glucose level in all experiments.

According to our plans in the first year of the project period, we concentrated on the metagenomic analysis of the gut microbiome to assess its overall impact on the region-specific structural damage of the intestinal capillaries and diabetic enteric neuropathy. Therefore, our aim was to map the microbiota distribution along the gut and establish whether colon/faecal samples from diabetic rats adequately reflect the diabetic alterations in the microbiome. For this, total community DNA was prepared from the lumen material derived from different gut segments in each experimental group. Using next generation DNA sequencing we determined the composition of intestinal lumen-associated microbiota from phylum to genus level. No significant differences in bacterial composition were found in the luminal contents from the duodenum of the experimental animal groups. The composition of the duodenal microbiota did not indicate the development of a pathological enteric microenvironment which is in good agreement with our earlier findings that in STZ-induced diabetes the duodenum was the only gut segment in which a decrease in the number of nitrergic myenteric neurons was not accompanied by a decrease in the total number of neurons [1] and the limited diabetes-related structural alterations in the mesenteric capillaries were completely prevented by insulin treatment [2]. In the ileum and colon distinct patterns of microbiota were seen, depending on the history of the luminal samples. Ileal samples from diabetic rats exhibited particularly striking alterations, while the richness and diversity obscured some of the modifications in the colon. In the ileum and colon, where a significant decrease in the total number of myenteric neurons is accompanied by severe structural damage of the mesenteric capillaries in STZ-induced diabetic rats [1-2], the diabetes is

characterized by a massive Klebsiella invasion that cause severe intestinal inflammation results in a leaky epithelium which initiates different pathological cascades disturbing the intestinal immunology. The aberrant microbiota that develops in the diabetic ileum and colon and the characteristic upsurge of the Gram-negative Klebsiella could therefore be directly associated with development of the pathological microenvironment. After insulin treatment characteristic rearrangements in microbiome composition and diversity were detected, though the normal gut flora was not restored. The Proteobacteria were practically eliminated and the genus Klebsiella was diminished. The fact that insulin did not restore normal conditions in the capillary endothelium or in the microbiota of the ileum or the colon further indicated that the main location of hyperglycaemia-dependent events is in the ileum; therefore the luminal samples from the ileum appear more suitable for diagnostic purposes than the colon/faeces. Based on the results, the Proteobacteria should be at the focus of diagnosis and potential therapy and Klebsiella are recommended as biomarkers of type 1 diabetes (**published in PlosOne in 2014, IF: 3,234**).

After the investigation of lumen-associated microbiota, we continued the research work to study the composition of the mucosa-associated microbiota which may have critical role in the neuro-immune interactions under pathological conditions. Striking differences were observed in the composition of the mucosa-associated microbiota of the duodenum between the diabetic and the control rats. A significant invasion of the genus Mycoplasma was apparent in diabetes, and the abundance of the phylum Firmicutes decreased massively. It is noteworthy that the insulin treatment eliminated the Mycoplasma invasion in the duodenum. In other aspects, the richness and distribution of prokaryotes did not differ markedly in the insulin-treated diabetic rats relative to the controls. In the ileum the abundance of the phylum Firmicutes increased in the diabetic samples. Although the proportion of the phylum Proteobacteria decreased moderately, the composition of it changed significantly, and after insulin treatment the alterations were moderated. In the diabetic samples the abundance of the phylum Firmicutes decreased slightly, the relative number of the bacteria in the phylum Bacteroidetes increased strongly compared to the control values, and after insulin-treatment this increasing was more significant. In conclusion our metagenomic studies, chronic hyperglycemia had the most prominent effect on the lumen-associated microbiota in the ileum, whereas in the case of mucosa-associated microbiota the major changes were encountered in the duodenum (**manuscript is submitted to PlosOne for review**).

The links between the regionality along the digestive tract and the accumulation of reactive oxygen species, the effectiveness of the antioxidant defense system and the sensitivity to the types of demise in different gut regions of rats with STZ-induced diabetes were also demonstrated (**published in International Journal of Biochemistry and Cell Biology in 2015, IF: 4.046**). In this study, significant changes were observed in the oxidative status and in the activity of the antioxidant defense system in the diabetic colon; the peroxynitrite production was doubled, the level of hemoxygenase (HO)-2 protein was increased 11-fold and the expression of anti-apoptotic bcl-2 was also increased. The segment-specific vulnerability of the gastrointestinal tract induced by hyperglycemia was also confirmed by electron microscopy, demonstrating the presence of severe necrosis in the colon of the diabetic rats. No remarkable histopathological alterations were seen in the duodenum of the diabetic animals and there were likewise no significant changes in the production of peroxynitrite in their duodenum, whereas the level of the free radical scavenger metallothionein-2 was increased ~300-fold. In conclusion, these data suggest that the distal part of the gut is more vulnerable than the proximal to oxidative stress.

The quantitative immunohistochemical studies planned within the present project were extremely labour intensive; therefore, these were started in the first year and were continued throughout the entire project period as we planned. To investigate the role of endogenous

antioxidant system in prevention of region-specific diabetes-related neuronal injuries, we performed a detailed quantitative analysis on whole-mounts derived from different gut segments and experimental conditions using double-labelling fluorescent immunohistochemistry with primary antibodies for neuronal nitric oxide synthase and HOs (**published in Oxidative Medicine and Cellular Longevity in 2017, IF: 4.593**). In support of our earlier finding that the susceptibility of nitrergic myenteric neurons to experimentally induced diabetes is strictly regional [1], the present study provides evidence of gut segment-specific diabetes-related induction of the endogenous HO system and also intestinal region-dependent enhanced co-localization of HO1 and HO2 with nNOS in myenteric neurons. The number of both HO-immunoreactive and nNOS/HO-immunoreactive myenteric neurons was the lowest in the ileal and the highest in the colonic ganglia of controls; and it increased the most extensively in the ileum and was also elevated in the colon of diabetics. Although the total number of nitrergic neurons decreased in all segments, the proportion of nNOS-immunoreactive neurons co-localizing with HOs was enhanced robustly in the ileum and colon of diabetics. We presume that those nitrergic neurons which do not co-localize with HOs are the most seriously affected by diabetic damage. Therefore the regional induction of the HO system is strongly correlated with diabetes-related region-specific nitrergic neuropathy.

Besides to study the expression of endogenous antioxidants in the nitrergic myenteric neurons, we also investigate the intestinal region-dependent effects of diabetes and insulin replacement on nNOS- and HO-immunoreactive submucous neurons (**in press in World Journal of Gastroenterology in 2017, IF: 3.365**). Our present study provides evidence for the first time that the neurochemical character of the nitrergic submucous neurons exhibits gut region-dependent changes in diabetic and insulin-treated diabetic rats. In addition, we prove that HO1- and HO2-immunoreactive submucous neurons are present in small amounts in the small intestine, but in high abundance in the colon of control and diabetic rats, and they have segment-specific responsiveness to immediate insulin replacement.

Our findings on the gut region-specific nitrergic neuropathy, the accumulation of ROS and the regionally distinct activation of endogenous antioxidants in the different intestinal segments of rats with STZ-induced diabetes were also summarized in a review article (**submitted to Autonomic Neuroscience: Basic and Clinical for review**).

We also finished to study the lysyl-oxidase and matrix metalloproteinase 9 density in different cellular and subcellular compartments, gut segments and experimental conditions using gold-labelling post-embedding electron microscopy and preparation of the manuscript included these results is now in progress.

In the frame of the present research, our results were demonstrated on international and Hungarian conferences, e. g. in 2017 we participated and gave presentations on 25th Congress of Hungarian Diabetes Association in Pécs, Joint Meeting of National Physiological Societies in Serbia, 59th Congress of Hungarian Society of Gastroenterology in Siófok and 25th United European Gastroenterology Week in Barcelona, Spain. (The details of the full-articles and conference abstracts included all results of the research period are listed below.)

In my opinion, the work accomplished was in accordance with our plan, and I am confident that our results provided deeper insight into the pathomechanism of the diabetes-related enteric neuropathy.

Trusting in your positive judgment,
Sincerely yours,

Nikolett Bódi, Ph.D.
principal investigator

References

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[2] Bódi N, Talapka P, Poles P, Hermesz E, Jancsó Z, et al. (2012) Gut region-specific diabetic damage to the capillary endothelium adjacent to the myenteric plexus. Microcirc 19: 316-326.

Publications of the third year of research

1. Chandrakumar L, Bagyánszki M, Szalai Z, Mezei D, Bódi N (2017) Diabetes-related induction of the heme oxygenase system and enhanced co-localization of heme oxygenase 1 and 2 with neuronal nitric oxide synthase in myenteric neurons of different intestinal segments. Oxid Med Cell Longev. 2017:1890512. https://doi.org/10.1155/2017/18905122.
IF: 4.593

2. Bódi N, Szalai Z, Chandrakumar L, Bagyánszki M (2017) Region-dependent effects of diabetes and insulin-replacement on neuronal nitric oxide synthase- and heme oxygenase-immunoreactive submucous neurons. World J Gastroenterol. (in press)
IF: 3.365

3. *Bagyánszki M, *Wirth R, Bódi N, Szalai Z, Chandrakumar L, Maróti G, Kovács KL (2017) The Composition of the Mucosa-Associated Gut Microbiota in a Changed Regionally Distinct Way in Rats with Streptozotocin-Induced Diabetes and Immediate Insulin Treatment. PLoS One. (submitted) * co-first author

4. Bódi N (2017) Impacts of imbalance between oxidative and antioxidant mechanisms on diabetic nitrergic neuropathy. Auton Neurosci. REVIEW (submitted)

Publications of the first and second year of research

5. Bódi N, Jancsó Zs, Talapka P, Pál A, Poles M, Bagyánszki M, Hermesz E, Fekete É (2014) Gut region-specific rearrangement of the cellular and subcellular compartments of nitric oxide synthase isoforms after chronic ethanol consumption in rats. Histol Histopathol. 29(12):1547-1555.
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7. Talapka P, Nagy L, Pál A, Poles M, Berkó A, Bagyánszki M, Puskás LG, Fekete É, Bódi N (2014) Alleviated mucosal and neuronal damage in a rat model of Crohn's disease. World J Gastroenterol. 20(44):16690-16697.
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8. Jancsó Zs, Bódi N, Borsos B, Fekete É, Hermesz E (2015) Gut region-specific accumulation of reactive oxygen species leads to regionally distinct activation of antioxidant

and apoptotic marker molecules in rats with STZ-induced diabetes. Int J Biochem Cell Biol. 62:125-131.
IF: 4,046

9. Bagyánszki M, Bódi N (2015) Gut region-dependent alterations of nitrergic myenteric neurons after chronic alcohol consumption. World J Gastrointest Pathophysiol. 6(3):51-57.
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10. Rázga Zs, Kovács G, Bódi N, Talapka P (2015) Upregulation of the L–type calcium channel in renin-positive smooth muscle cells of arterioles in the kidneys of rats with streptozotocin-induced diabetes. Anal Quant Cytopathol Histpathol. 37(4):214-220.
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11. P. Talapka*, A. Berkó*, L. I. Nagy, L. Chandrakumar, M. Bagyánszki, L. G. Puskás, É. Fekete, N. Bódi (2016) Structural and molecular features of intestinal strictures in rats with Crohn's-like disease. World J Gastroenterol. 22(22):5154-5164. * co-first author
IF: 3.365

Hungarian and international conference presentations in the third year of research:

1. Bagyánszki M, Wirth R, Maróti G, Chandrakumar L, Szalai Z, Bódi N, Kovács K L (2017) A béltartalomban élő és a nyálkahártya-asszociált mikrobióta bélszakasz-specifikus változása streptozotocin-indukált diabéteszes patkánymodellben. Magyar Diabetes Társaság XXV. Jubileumi Kongresszusa, Pécs, Magyarország

2. Chandrakumar L, Mezei D, Barta B P, Szalai Z, Bódi N, Bagyánszki M (2017) Diabetes-related expressional changes of the inflammatory cytokine, tumor necrosis factor alpha in the myenteric ganglia and its microenvironment of different intestinal segments. Joint Meeting of National Physiological Societies, Szabadka, Szerbia

3. Bódi N, Chandrakumar L, Szalai Z, Bagyánszki M (2017) Diabetes-related induction of heme oxygenase system and enhanced co-localization of heme oxygenase 1 and 2 with neuronal nitric oxide synthase in myenteric neurons of different intestinal segments. Magyar Gasztroenterológiai Társaság 59. Nagygyűlése, Siófok, Magyarország

4. Chandrakumar L, Mezei D, Barta B P, Szalai Z, Bódi N, Bagyánszki M (2017) Diabetes-related alterations in the expression of the inflammatory cytokines, tumor necrosis factor alpha and interleukin 6 in the myenteric ganglia and its microenvironment of different intestinal segments. 25th United European Gastroenterology Week, 2017. október 28-november 1., Barcelona, Spanyolország. (in press)

Hungarian and international conference presentations in the first and second year of research:

5. Bódi N, Jancsó Zs, Giricz Zs, Pál A, Talapka P, Poles M, Bagyánszki M, Hermesz E, Fekete É (2013) Changes of lysyl-oxidase expression in different gut segments of streptozotocin-induced untreated and insulin-treated diabetic rats. Biomedica Minikonferencia, Szeged, Magyarország

6. Bódi N, Jancsó Zs, Giricz Zs, Pál A, Talapka P, Poles M, Bagyánszki M, Hermesz E, Fekete É (2014) Changes of lysyl-oxidase expression in myenteric ganglia in different gut segments of streptozotocin-induced untreated and insulin-treated diabetic rats. IBRO Workshop, Debrecen, Hungary

7. Bódi N, Wirth R, Maróti G, Talapka P, Giricz Zs, Fekete É, Kovács LK (2014) A bél baktériumflórájának bélszakasz-specifikus változása streptozotocinnal indukált diabéteszes patkányokban. 15. Kolozsvári Biológus Napok, Kolozsvár, Románia

8. Bódi N, Wirth R, Maróti G, Talapka P, Giricz Zs, Fekete É, Kovács LK (2014) Gut region-specific alterations in the composition of the intestinal microbiota in streptozotocin-induced diabetic rats. 22nd United European Gastroenterology Week, Vienna, Austria

9. Giricz Zs, Talapka P, Pál A, Fekete É, Bódi N (2015) A lizil-oxidáz enzim bélszakasz-specifikus expressziója streptozotocinnal indukált diabéteszes patkányokban. XX. Korányi Frigyes Tudományos Fórum, Budapest, Magyarország

10. Giricz Zs, Talapka P, Pál A, Fekete É, Bódi N (2015) A lizil-oxidáz expressziójának változása streptozotocinnal indukált diabéteszes patkányok különböző bélszakaszaiban. 16. Kolozsvári Biológus Napok, Kolozsvár, Románia