The International Journal of Biochemistry & Cell Biology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

# 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

Zsanett Jancsó[a], Nikolett Bódi[b], Barbara Borsos[a], Éva Fekete[b], Edit Hermesz[a,*]

[a] Department of Biochemistry and Molecular Biology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary
[b] Department of Physiology, Anatomy and Neuroscience, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary

ARTICLE INFO

*Article history:*
Received 19 December 2014
Received in revised form 3 March 2015
Accepted 9 March 2015
Available online xxx

*Keywords:*
Apoptosis
Diabetes
Digestive tract
Peroxynitrite
Necrosis

ABSTRACT

The aim of this study was to seek possible 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 streptozotocin-induced diabetes. 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-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.
*Conclusion:* The spatially restricted vulnerability observed along the digestive tract could originate from a high level of oxidative stress *via* peroxynitrite production.



## 1. Introduction

Type 1 diabetes mellitus (T1D) results in severe metabolic imbalances and pathological changes in many tissues, and commonly affects the entire gastrointestinal (GI) tract, from the esophagus to the anorectal region (Wolosin and Edelman, 2000; Zhao et al., 2002). T1D involves a state of high oxidative stress generated as a result of hyperglycemia-induced reactive oxygen species (ROS) (Wolff, 1993). Oxidative stress is an imbalance between the production of ROS, and the ability of a biological system to achieve the ready detoxification of ROS or to repair the resulting damage. While ROS are important second messengers at low concentrations and are involved in the regulation of apoptosis and the activation of transcription factors, they can cause significant cellular damage when present in excess. They can inflict damage on all classes of cellular macromolecular components, eventually leading to tissue injury or even cell death, which can occur essentially by two mechanisms, necrosis and apoptosis (Bergamini et al., 2004). Although numerous reports have provided details of the molecular mechanisms responsible for ROS-induced apoptosis, little is known concerning the mechanisms and signal transduction pathways underlying ROS-mediated necrotic cell death. Necrosis has long been considered to be a passive mode of cell death (Kanduc et al., 2002) and much more harmful than apoptosis because it causes a robust inflammatory response (Proskuryakov et al., 2003).

To eliminate the harmful effects of reactive species, cells are equipped with an efficient antioxidant defense system, including enzymes such as superoxide dismutase (SOD), catalase (CAT), and heme oxygenases (HOs), and low-molecular weight antioxidants such as glutathione (GSH) and metallothioneins (MTs) (Kruidenier et al., 2003; Inoue et al., 2008). SOD catalyzes the reduction of the superoxide anion ($^{\bullet}O_2^-$) to hydrogen peroxide ($H_2O_2$), which is then detoxified to water by CAT in the lysosomes (Wang and Ballatori, 1998). The HOs play roles in heme degradation, yielding equimolar amounts of biliverdin, and carbon monoxide with important free radical-scavenging properties and free iron. HO-2 is expressed constitutively, contributing to cell homeostasis, whereas

* Corresponding author at: Department of Biochemistry and Molecular Biology, Faculty of Science and Informatics, University of Szeged, P.O. Box 533, H-6701 Szeged, Hungary. Tel.: +36 62 544887; fax: +36 62 544887.
*E-mail address:* hermesz@bio.u-szeged.hu (E. Hermesz).

http://dx.doi.org/10.1016/j.biocel.2015.03.005
1357-2725/

HO-1 is an inducible enzyme expressed at a relatively low level in most tissues (Maines, 1997), recently identified as an important cellular defense mechanism against oxidative stress (Abraham et al., 2009). HO-1 and HO-2 are regulated by strikingly different mechanisms, which may reflect different physiological and pathological roles (Gibbons and Farrugia, 2004).

GSH also plays a critical role in this system, as an antioxidant, enzyme cofactor and major redox buffer (Wang and Ballatori, 1998). The MTs are present in all cells throughout the body. They have a cardinal role in metal homeostasis and heavy metal detoxification through their high metal-binding capacity, they play a role in the immune function, and they are involved in a variety of GI tract functions (Thirumoorthy et al., 2011). They also play an important part in the prevention of development of T1D, the complications and the subsequent pathogenic toxicity (Cai, 2004). The overexpression of MTs in various metabolic organs has been shown to reduce hyperglycemia-induced oxidative stress, organ-specific diabetic complications, and DNA damage in experimental diabetes (Islam and Loots, 2007).

In an earlier study, we demonstrated spatially-restricted damage of the gut capillary endothelium in rats with streptozotocin (STZ)-induced diabetes in comparison with control animals (Bódi et al., 2012). Metagenomic analysis of the luminal contents of duodenum, ileum and colon of diabetic rats also furnishes evidence of the regionality of the gut microbiota (Wirth et al., 2014). The two studies are in good agreement as concerns the advantageous status of the duodenum of the diabetic rat as compared with the colon.

Those results led us to focus in the present study on the spatially-restricted differences in ROS production and activation of the antioxidant defense system in the duodenum and colon of rats with STZ-induced diabetes. The aim of the study was to characterize the possible links between the antioxidant status and the macromolecular damage in selected gut segments in the diabetic rat. We report data on the accumulation of a powerful oxidant, peroxynitrite ($ONOO^-$), the activities of antioxidant enzymes (SOD and CAT), and the expressions of a set of genes coding for members of antioxidant defense system (*mt-1*, *mt-2*, *ho-1* and *ho-2*), together with the detection of pro-apoptotic and anti-apoptotic markers (*bax, bcl-2* and *caspase-9*).

## 2. Materials and methods

### 2.1. Animal model

Adult male Wistar rats weighing 300–400 g, kept on standard laboratory chow (Bioplan Kft., Hungary) and with free access to drinking water, were used throughout the experiments. The rats were divided randomly into three groups: STZ-induced diabetics ($n = 14$), insulin-treated diabetics ($n = 12$) and sex- and age-matched controls ($n = 6$).

Hyperglycemia was induced as described previously (Izbéki et al., 2008). The animals were considered diabetic if the non-fasting blood glucose concentration was higher than 18 mM. From this time on, one group of hyperglycemic rats received a subcutaneous injection of insulin (Humulin M3, Eli Lilly Nederland) each morning (4 U) and afternoon (2 U). The non-fasting blood glucose concentration and weight of each animal were measured weekly. The cecum size of the sacrificed rat was analyzed by means of the Image J 1.48v program (http://imagej.nih.gov/ij/). In all procedures involving experimental animals, the principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed and all the experiments received approval in advance from the Local Ethics Committee for Animal Research Studies at the University of Szeged.

### 2.2. Tissue handling

Ten weeks after the onset of diabetes, the animals were killed by cervical dislocation under chloral hydrate anesthesia (375 mg/kg i.p.). The gut segments of the control, STZ-induced diabetic and insulin-treated diabetic rats were dissected and rinsed in 0.05 M phosphate buffer, pH 7.4. Samples were taken from the duodenum (1 cm distal to the pylorus) and the middle part of the colon and processed for biochemical, molecular biological and electron microscopy study.

### 2.3. Biochemical assays

0.5 g duodenum and colon of each individual rats, tissues were homogenized in 4 volume of ice-cold 0.9% serum physiologic by means of a glass homogenizer immersed in an ice water bath, centrifuged at 17,000 × *g* for 15 min at 4 °C, and the clear supernatants used for GSH, $ONOO^-$, protein analysis, and measuring the activities of antioxidant enzymes.

Total protein levels measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. The concentrations of total and reduced GSH in the tissues were measured as described by Sedlak and Lindsay (1968). Spectrophotometric measurements were carried out by GENESYS 10S UV-Vis (Thermo Scientific) spectrophotometer.

$ONOO^-$ was assayed by diluting samples into 1 M NaOH (60:1) and measuring the increase in absorbance at 302 nm. As a control, samples were added to 100 mM potassium phosphate (pH 7.4) (60:1). The decrease in absorbance was measured at neutral pH as $ONOO^-$ decomposes (Huie and Padmaja, 1993).

Catalase activity was determined spectrophotometrically at 240 nm by the method of Beers and Sizer (1953) and expressed in Bergmeyer units (1 BU = decomposition of 1 g $H_2O_2$/min at 25 °C).

SOD activity was determined on the basis of the inhibition of epinephrine–adrenochrome autoxidation (Misra and Fridovich, 1972). Spectrophotometric measurement was carried out at 480 nm. The results were expressed in U/mg protein.

### 2.4. Post-embedding immunohistochemistry

For post-embedding immuno-electromicroscopy, small pieces (2–3 mm) of the gut segments were fixed overnight at 4 °C in 2% paraformaldehyde and 2% glutaraldehyde solution, buffered with 0.1 M PB (pH 7.4). The samples were then washed in 0.05 M PB and further fixed for 1 h in 1% $OsO_4$. After fixation, the gut segments were rinsed in 0.1 M PB, dehydrated in increasing alcohol concentrations (50, 70, 96% and absolute ethanol) and acetone, and embedded in Epon (Electron Microscopy Sciences, Hatfield, PA, USA). The Epon blocks were used to prepare ultrathin (70 nm) sections, which were mounted on Formvar-coated nickel grids and processed for immunogold labeling.

Ultrathin sections from each block were pre-incubated in 1% bovine serum albumin in TRIS-buffered saline (TBS) for 30 min, incubated overnight in the primary antibodies (heme oxygenase-2 mouse monoclonal IgG (Santa Cruz Biotechnology, USA; working dilution 1:50) and caspase-9 rabbit polyclonal IgG (Sigma–Aldrich, USA; working dilution 1:50)), followed by protein A-gold-conjugated anti-mouse (18 nm gold particles, Jackson ImmunoResearch, West Grove, PA, USA; final dilution 1:20) secondary antibodies for 3 h, with extensive washing in between. All steps were performed at room temperature. The specificity of the immunoreaction was assessed in all cases by omitting the primary antibodies from the labeling protocol and incubating the sections only in the protein A-gold-conjugated secondary antibodies. Sections were counterstained with uranyl acetate (Merck, Darmstadt, Germany) and lead citrate (Merck, Darmstadt, Germany), and were



Please cite this article in press as: Jancsó Z, et al. 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 (2015), http://dx.doi.org/10.1016/j.biocel.2015.03.005