Ph.D. thesis

STUDY OF OXIDATIVE STRESS AND THE ANTIOXIDANT DEFENSE SYSTEM AFTER HEAVY METAL TREATMENT IN CARP AND IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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INTRODUCTION

Free radical research is currently at the focus of scientific interest. Extensive investigations are essential in order to determine the background of the mechanisms and relationships of changes in various pathological processes. Our experiments were related to the molecular defense mechanisms in two model systems: heavy metal exposure in carp and in streptozotocin (STZ)-induced diabetic rats, in which increased free radical production and the occurrence of oxidative stress have been demonstrated.

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism. They are continuously produced in a well-regulated manner to help maintain homeostasis at the cellular level in normal healthy tissues, and play an important role as signaling molecules. An increase in free radical formation in consequence of stress can affect the functions in biological systems. As a results of increased free radical production, all the known cellular adaptation reaction pathways may be activated, including the antioxidant defense system. Excessive free radical formation and/or a reduction in the antioxidant capacity may upset the balance between prooxidants and antioxidants, leading to oxidative stress. Oxidative stress causes damage to proteins, nucleic acids and lipids due to lipid peroxidation, which in turn leads to changes in the cellular structure and function, and ultimately cell death via apoptosis or necrosis.

To minimize the harmful effects of free radicals, aerobic organisms have evolved an antioxidant defense system, which can be activated in response to stress. During the activation, certain elements of this system can be induced, such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (GPx), affording protection by directly scavenging the superoxide anion ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2), converting them to less reactive species.

Reduced glutathione (GSH) plays an important role in the protection of cells against free radical-mediated damage. It is capable of binding metal ions in the event of heavy metal exposure, and is more readily oxidized than the cellular macromolecules in the case of oxidative stress. GSH acts as an electron donor for the GPxs, which play an important role in the reduction of inorganic and organic peroxides. Metallothioneins (MTs) are low molecular weight metal-binding proteins that contain a high proportion of cysteine. MTs additionally have important roles in heavy metal detoxification and in the neutralization of free radicals. The antioxidant defense system further includes the heme

oxygenases (HOs). The three catalytic by-products of heme catalysis by HO, i.e. bilirubin, ferritin from the sequestration of free iron and CO, all play critical roles in mediating cytoprotection against oxidative tissue injury. Although bioactive by-products possess various physiological and cytoprotective properties, in some cases they are also potentially toxic. A too high expression of HO may cause tissue injury through the generation of high levels of redox-active iron or result in bilirubinemia.

We have studied the molecular mechanisms leading to activation of the antioxidant defense system in various pathological processes, such as heavy metal exposure and STZ-induced diabetes. We have studied the effect of the stressors on the formation of reactive oxygen species (ROS), e.g. H_2O_2 and peroxynitrite ($ONOO^-$), the expression of the antioxidant system members, e.g. SOD, CAT, GSH, MTs, HOs and the extent of the damage caused by oxidative stress (lipid peroxidation, apoptosis and necrosis).

THE AIMS OF THE STUDY

A. Heavy metal exposure in fish

In earlier research, a number of protein coding genes involved in the stress response were characterized and identified by our group. In the present experiments, additional elements of the antioxidant defense were studied, i.e. the HOs, encoding genes were not yet identified in carp.

The following questions were considered:

1. Is there any dose- and time-dependent difference in the accumulation of metals between the key organs of detoxification, the liver and kidney?
2. Is there a correlation between the copy number of *ho* genes and the tetraploid chromosome set of carp?
3. Is there a correlation between the tissue distribution of heavy metals and:
    a. the changes in the expression of *ho* genes,
    b. the rate of free radical formation,
    c. the activation of the antioxidant defense system,
    d. and the extent of tissue damage?

B. STZ-induced diabetes in rat intestine

Our preliminary results indicate that the diabetes-related pathological changes in the different intestinal segments appear gradually along the colon-ileum-duodenum axis. We selected two segments, the duodenum and colon, which differ from each other in their anatomical structure and function. In the present study, hyperglycemia-induced oxidative damage was investigated in both gut segments of STZ-induced diabetic and insulin-treated diabetic rats

The following questions were posed:

1. Are there any region-specific changes in:
    a. the type or extent of tissue damage,
    b. the level of free radical formation,
    c. or the activation of certain elements of the antioxidant defense system.
2. How insulin replacement does affect the prooxidant-antioxidant balance in cells?

MATERIALS AND METHODS

1. Experimental conditions and treatment

Heavy metal treatment of carp

Induction of chronic diabetes in rat

2. Molecular biological test methods

RNA extraction from frozen samples

Reverse transcription (RT)

Polymerase chain reaction (PCR): *RT-PCR, qRT-PCR*

Primer design

Geldocumentation, densitometry

Phylogenetic analysis

3. Biochemical measurements

Quantitative determination of proteins

Determination of GSH concentration

Determination of GSSG concentration

Measurment of lipid peroxidation

Determination of H_2O_2 concentration

Determination of $ONOO^-$ concentration

Determination of SOD activity

Determination of CAT activity

Determination of heavy metal content

4. Histological analysis methods

Electron microscopy: detection of necrosis

Postembedding immunohistochemistry: HO-2, caspase-9

RESULTS AND DISCUSSION

A. Impact of acute heavy metal exposure on the expression of two *ho* genes and other antioxidant markers in common carp

1. Metal accumulation in the liver and kidney

Carp were treated with the toxic and carcinogenic heavy metals cadmium and arsenic, and their accumulation was examined in the liver and kidney, which play critical roles in detoxification. Both metals were applied in two doses (1 mg/L and 10 mg/L) and for different exposure times (6-72 h). Both metals accumulated in a tissue- and dose-dependent manner. Despite the 10-fold difference between the doses applied during the treatments, such a difference in the rate of accumulation was not detected, e.g. in the case of arsenic exposure, the difference was only 1.5-fold. Both metals accumulated to higher extents in the kidney than in the liver.

2. Identification and characterization of carp ho genes

Carp *ho* genes were first identified and characterized in this tetraploid species. The carp *ho-1* and *ho-2* sequences displayes strong homology to known *ho* genes in fish. During our study we have seen no indication of the expression of multiple *ho* genes: the amplification of the cDNAs resulted in single, well-defined products for both *ho-1* and *ho-2*, and sequencing the PCR product populations *en mase* indicated no sequence variations in the regions investigated. We therefore conclude that the *ho* genes identified by our approach are the only ones expressed in *C. carpio*. Analysis of the putative amino acid sequence revealed that the carp HO carries evolutionarily highly conserved domains and motifs. We examined the expression of *ho* genes under physiological conditions in certain organs of the carp. The basal levels of the *ho-1* transcripts were at the limit of detectability in all the examined tissues except the spleen, skin and blood. The highest *ho-1* mRNA level was found in the spleen. The *ho-2* gene was highly expressed in all of the examined tissues. The highest level was detected in the skin, and the lowest in the kidney. We observed a notably high expression of *ho-2* in the brain. Potential explanations are that the CO produced by HO has been postulated to function as a neurotransmitter, or the brain may require a greater degree of antioxidant protection due to its critical role in regulating the organ functions.

3.a. Effects of heavy metal exposure on the expression of ho genes

The acute exposure to the two metals caused different effects in the expression of the *ho* genes. The expression of *ho* genes was induced in a dose-, time- and tissue-specific manner, but these changes did not correlate with the accumulated metals in either organs.

3.b. Metal-induced free radical formation

The formation of H_2O_2 during the experiments was induced by the applied metals in a tissue-specific manner. In high concentrations, H_2O_2 plays important roles in the development of oxidative stress and damage to biomolecules. Its formation is related to the increased production of $\bullet O_2^-$. Arsenic caused an increase in the renal H_2O_2 concentration, whereas cadmium caused increased H_2O_2 production in the liver.

3.c. Activation of the antioxidant defense system

Antioxidant enzymes and low molecular weight antioxidants were tested after heavy metal treatment and in diabetic animals. Increased SOD and CAT activities were found in the kidney, where higher metal accumulations were measured. The ratio GSH/GSSG - is often used to determine cellular toxicity - varies in response to stress in a tissue- and stressor-specific manner. There was no significant change in the liver after metal treatment, but metal-specific changes were observed in the kidney. The increase in the ratio GSH/GSSG resulted from an increased level of GSH. A decrease in the ratio was caused by the elevated formation of GSSG and a slight decrease in the concentration of GSH.

3.d. A The extent of tissue damage

Enhanced lipid peroxidation is indicative of oxidative stress-related damage. We detected tissue- and dose-dependent lipid peroxidation following heavy metal exposure. Exposure to a low dose of arsenic exposure led to an increase in lipid peroxidation, whereas cadmium affected the lipid peroxidation only at high dose. We detected increased lipid peroxidation where induction of the *ho-1* gene was the most marked.

B. Examination of cell injury and activation of antioxidant defense system in different gut segments of STZ-induced diabetic rat.

1.a. Determination of the oxidative damage caused by hyperglycemia

A The STZ treatment resulted in significantly elevated serum glucose concentrations in all animals during the 10-week experimental period. Visible changes were observed in the diabetic intestine, e.g. an enlarged cecum and a purplish discoloration that may indicate inflammation or ischemia. Signs of apoptosis and necrosis were detected by electron microscopy in diabetic rats. The expression of pro- and anti-apoptotic markers and the incidence of necrosis were region-specific during diabetes. The ratio of the pro- and anti-apoptotic markers in the duodenum may contribute to the initiation of programmed cell death. Unlike in the colon, this rate moved toward the anti-apoptotic markers. Here, we observed necrosis which may have been caused by the increased ROS levels and the lack of the activation of antioxidant defense.

1.b. Free radical formation in the gut segments

A $ONOO^-$ in high concentrations play important roles in the development of oxidative stress and damage to biomolecules, its formation is related to the increased production of superoxide anion and nitric oxide. The concentration of $ONOO^-$ also changed in a segment-specific manner along the intestine. In the colon of diabetic rats, increased $ONOO^-$ formation was observed.

1.c. Activation of the antioxidant defense system

The diabetic rat intestine also demonstrated region-specific changes; an increased concentration of $ONOO^-$ and decreased SOD activity were found in the colon. This may indicate the production of significant amounts of nitric oxide and the spontaneous formation of $ONOO^-$.

Quantitative changes in the low molecular weight antioxidant GSH and the ratio GSH/GSSG were tested. Hyperglycemia resulted in an increased ratio GSH/GSSG in the duodenum, but not in the colon. The increase in the ratio indicated elevated antioxidant protection due to the increased concentration of GSH.

MTs are low molecular weight antioxidants that play an important role in the maintenance of the thiol/redox balance and the defense against oxidative stress. The induction of *mt* genes were gut segment-specific. The results of the current study revealed that the two *mt* genes together could exert beneficial effects against harmful processes during hyperglycemia.

The HO system is induced by wide range of stressors. In a diabetic model, segment-specific changes were detected in the expression of the *ho* genes. The *ho-1* gene was induced in the duodenum, and the *ho-2* gene in the colon, where a large amount of HO-2 protein was measured. The results of gene expression and immunoanalytical measurements suggest that activation of the HO system may be involved in the stress responses in the model systems that we used.

2. Effect of Immediate insulin therapy after onset of diabetes

Immediate insulin replacement effectively reduced the hyperglycemia. Our results demonstrated show that in each case insulin maintained the levels of the tested markers above the control levels in both gut segments.

CONCLUSION

Overall, enhanced lipid peroxidation and necrosis are characteristic features of the damage caused by oxidative stress associated with the significant induction of HOs. We detected increased lipid peroxidation where the *ho-1* gene expression was the largest. The localization of necrosis corresponded with the lack of activation of the antioxidant defense, because only the HO-2 was induced in this gut segment. Under pathological conditions, ROS formation is increased. The activity of HOs may increase in consequence of the oxidative environment. HOs are cytoprotective enzymes, but the data also suggest that the increased HO activity in the higher range has a deleterious effect: the release of redox active iron before its binding to ferritin can result in enhanced radical formation. This may contribute significantly to the damage observed in our model systems.

LIST OF PUBLICATIONS

Publications related to the PhD thesis

Jancsó Zs, Bódi N, Borsos B, Fekete E, Hermesz E. 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. *International Journal of Biochemistry and Cell Biology* (Elbírálás alatt)

Zsanett Jancsó and Edit Hermesz (2014) Impact of acute arsenic and cadmium exposure on the expression of two haeme oxygenase genes and other antioxidant markers in common carp (*Cyprinus carpio*). *Journal of Applied Toxicology* 2014 April 7. **IF: 3.174**

Other papers

Bódi, Nikolett; **Jancsó, Zsanett**; Talapka, Petra; Pál, Alexandra; Poles, Marietta; Bagyánszki, Mária; Hermesz, Edit; Fekete, Éva (2014) Gut region-specific rearrangement of the cellular and subcellular compartments of nitric oxide synthase isoforms after chronic ethanol consumption in rats. *Histology and Histopathology* 29(12):1547-1555 **IF: 2.533**

Dugmonits K, Ferencz A, **Jancsó Zs**, Juhász R, Hermesz E (2013) Major distinctions in the antioxidant responses in liver and kidney of Cd^{2+}-treated common carp (*Cyprinus carpio*). *Comparative Biochemistry and Physiology C Toxicological Pharmacology* 158(4), 225-230. **IF: 2.829**

N. Bódi, I. Battonyai, P. Talapka, E. Hermesz, **Zs. Jancsó**, Z. Katarova, F. Izbéki, T. Wittmann, É. Fekete, M. Bagyánszki (2012) Diabetes-related structural, molecular and functional alterations in capillaries supplying the myenteric plexus in the gut of the streptozotocin-induced diabetic rats. *Microcirculation* 19(4), 316–326. **IF: 2.763**

Szabolcs Ábrahám, Edit Hermesz, Andrea Szabó, Ágnes Ferencz, **Zsanett Jancsó**, Ernő Duda, Magdolna Ábrahám, György Lázár, György Lázár Jr. (2012) The effects of Kupffer cell blockade on the hepatic expression of metallothionein and heme oxygenase genes in endotoxemic rats with obstructive jaundice. *Life Sciences* 90(3–4), 140–146. **IF: 2.296**

Zsuzsanna Máté, Andrea Szabó, Edit Paulik, Edit Hermesz, **Zsanett Jancsó**, András Papp (2011) Electrophysiological and biochemical responses in rats on intratracheal instillation of manganese. *Central European Journal of Biology* 6(6), 925-932. **IF: 0.685**

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Abstracts published in conference proceedings

N. Bódi, P. Talapka, **Zs. Jancsó**, M. Poles, E. Hermesz, M. Bagyánszki, É. Fekete, F. Izbéki, T. Wittmann Gut region-specific differences in the cellular and subcellular distribution of nitric oxide synthase isoforms after chronic ethanol exposure in rats. 20th United European Gastroenterology Week Amsterdam, The Netherlands October 20-24, 2012 *Gut* Supplement No III Vol 61, A304 **IF: 10,732**

Juhasz, R., Ferencz, A., **Jancsó, Z**., Dugmonits, K., & Hermesz, E. Comparative study on the expression of glutathione peroxidase, glutathione reductase, glutathione synthetase and metallothionein genes in common carp during cadmium exposure. Society for Free Radical Research International 16th Biennial Meeting, London, 6-9 September 2012. *Free Radical Biology and Medicine* 53(1), 2. **IF: 5.271**

Zsanett Jancsó, Krisztina Dugmonits, Edit Hermesz Metal-induced alteration in the expression of two heme-oxygenase genes. FEBS 3+Meeting, Opatija, Croatia / 13 - 16 June 2012 (PIII-140)

Zsanett Jancsó, Nikolett Bódi, Éva Fekete and Edit Hermesz Oxidative stress response in different gut segments of a streptozotocin-induced diabetic rat model. 36th FEBS Congress "Biochemistry for Tomorrow's Medicine" Torino, 25-30 June 2011 (P 26.28) *FEBS Journal* 278: 74-445 **IF: 3.79**

Máté Zs, Szabó A, Hermesz E, **Jancsó Zs**, Boros I. Mangán intratracheális adagolásával előidézett funkcionális idegrendszeri eltérések patkányban. A MÉT LXXIV. Vándorgyűlése, Szeged 2010. *Acta Physiologica Hungarica* 97, 456-457 **IF: 1.226**

Máté Zs., Szabó A., Hermesz E, **Jancsó Zs**., Boros I. Alterations in the central nervous system caused by intratracheal instillation of Mn, in aspects of neurophysiology and molecular biology. The regional committee of the hungarian academy of sciences at Szeged 2010 October 13-15 ISIRR 11th international symposium interdisciplinary regional research, Szeged. p. 100. ISBN: 978-963-508-600-9

Ábrahám S., Hermesz E., Szabó A., Simonka Z., **Jancsó Zs**., Lázár G., Boros M., Lázár Jr G.: The effect of Kupffer cell blockade on the hepatic and renal expression of heme oxygenase genes after biliary obsruction combined with sepsis. The 44th Congress of the European Society for Surgical Research, Nimes, France, 20–23 May 2009 *British Journal of Surgery* 96(S5): 1-72 **IF: 4.077**