Effect of experimental hyperglycemia on oxidative transformations of endogenous and exogenous compounds in the rat

Doctoral (Ph.D.) Thesis

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1. Introduction

Diabetes mellitus (DM) is a common disease with a complex metabolic and endocrine system influencing a large proportion of the global population. DM is described by its high blood glucose level because of insufficient action and secretion of insulin from beta-cells of the pancreas. Hyperglycemia is considered a source of the development of diabetic complications *via* altering a variety of signaling pathways, leading to the induction of reactive oxygen species, oxidative stress, and cell death. A high rate of reactive oxygen species (ROS) generated from the inducted oxidative stress is considered to contribute to the pathogenesis of diabetic patients.

In addition, besides their insulin injection and/or consumption of diabetic medications, patients are supposed to use a variety of other medicines. However, how and to what degree diabetes affects the metabolism of drugs has not been well studied. Pathophysiological changes during diabetes can affect various drugs' absorption, distribution, metabolism, and excretion. However, previous studies have provided inconsistent data for multiple drugs, possibly due to variations in patient characteristics or control of patients' diabetes at the time of data collection.

Therefore, research on the effect of hyperglycemia using experimental animals is of great importance in better understanding the changes in the fate of drugs in diabetic individuals. Streptozoticin (STZ) induced hyperglycemia in the rat is a frequently used animal model for such investigations. There have been a series of previous investigations in the Institute of Pharmaceutical Chemistry studying the effect of experimental hyperglycemia on the pharmacokinetics of various xenobiotics. As a continuation of these previous studies, the present work aims to investigate the change in the pharmacokinetics (metabolism and elimination) of ibuprofen, a widely used non-steroid anti-inflammatory drug. Furthermore, chemical characterization of oxidative damage of lipids, proteins, and selected peptides (glutathione) and amino acids (phenylalanine) was to be performed under our regularly used animal model. The research also seeks information on whether oxidized ibuprofen metabolites could indicate the hyperglycemia-induced oxidative stress in the small intestine and the liver.

1.1. Oxidative stress and hyperglycemia

Investigations are shown the existence of a connotation between hyperglycemia and oxidative stress. Hyperglycemia enhances the production number of reactive oxygen species (ROS) and reduces antioxidant defense activities. The enzymatic source of ROS formation is enzymes of the mitochondrial respiratory chain, xanthine oxidases, cyclooxygenases, lipoxygenases, peroxidases, and nitric oxide synthases. Together, hyperglycemia and oxidative stress participate in the onset of diabetes and are considered the source of the pathogenesis of diabetic complications. Hyperglycemia upsets the ordinary mitochondria and non-mitochondrial ancestries *via* metabolic disorders producing extra superoxide. This step triggers several oxidative stress pathways causing diabetic complications.

Several metabolic and signaling pathways are proposed to promote oxidative stress, oxidative damage, and diabetic complications in diabetes, generally correlating to the metabolism of carbohydrates and lipids. In addition, extra-mitochondrial superoxide production is central to these metabolic mechanisms and the development of disease complications. The proposed pathways are glucose oxidation, glyceraldehyde-3-P dehydrogenase (GAPDH) or (PARP) pathway, polyol pathway, hexosamine pathway, diacylglycerol (DAG) formation, and protein kinase C (PKC) activation, glyceraldehyde autoxidation, advanced glycation end-products (AGEs), and stress-sensitive signaling pathways.

1.2. Biotransformation of ibuprofen

Ibuprofen is one of the non-steroidal anti-inflammatory drugs (NSAIDs) and belongs to the group of 2-arylpropionic acid derivatives with a molecular formula of $C_{13}H_{18}O_2$. The main structural feature of its 2-arylpropionic acid side is an sp^3-hybridized chiral carbon atom. Oral intake of ibuprofen is the usual therapeutic route. The high proportion of ingested ibuprofen will undergo biotransformation, with the bit of remaining amount of the parent drug unchanged. The first metabolism step of ibuprofen is oxidation, to inactivate the molecule through CYP isoform enzymes. In humans, the main CYP isoform responsible for the clearance of ibuprofen *via* hydroxylation is extensively CYP2C9, with the minor role of CYP2C8, CYP3A4, and CYP2C19. 2-hydroxyibuprofen (2-HO-IBP) and carboxy-ibuprofen (HOOC-IBP) are the most common metabolites of ibuprofen after oral

intake compared to the rest oxidized metabolites. Conjugation metabolism of ibuprofen and its hydroxylated metabolites will be followed, primarily with glucuronic acid, to form acyl-glucuronides. Marginally, ibuprofen will conjugate with taurine, leading to ibuprofen taurate (IBP-TAU).

Ibuprofen as one of 2-arylpropionic acid derivatives holding a tetrahedral chiral carbon. The market formulation of IBP is frequently available in racemic form. As well studied that the *S*(+)-isomer shows more potent activity than the *R*(−)-isomer, which looks like an inactive form. In addition, the equal ratio of the prescribed racemate dose will be adjusted as the *R*(−)-enantiomer undergoes a unidirectional inversion to the S(+)-enantiomer (active form). The proportion of this configuration is not well clarified under abnormal physiological conditions such as hyperglycemia.

2. Aims

- To study the oxidative transformations of selective endogenous compounds – lipids, proteins, peptides (glutathione), and amino acids (phenylalanine) under the influence of STZ-induced experimental diabetes in the small intestine, the liver, and the kidney of rats.
- *In vitro* investigation of ibuprofen oxidation to explore the main oxidative products in two (Fenton and Udenfriend) non-enzyme catalyzed reactions.
- To study the *in vivo* oxidative transformations of ibuprofen in the small intestine and the liver under the experimental diabetes of the rat.

(a) Investigate the main oxidative and conjugated metabolites of ibuprofen in small intestine perfusates and bile of control and hyperglycemic rats.

(b) Investigate intestinal absorption and biliary excretion of ibuprofen and its oxidized metabolites in control and streptozotocin-induced rats.

(c) Investigate the ratio of *R*(-) and *S*(+)-ibuprofen in small intestinal perfusates and bile of control and hyperglycemic rats.

3. Materials and Methods

3.1. Animals and experimental procedure

Male Wistar rats (9-11 weeks old; weighing 250-300 g; TOXI-COOP, Hungary, Budapest) were separated into four groups. Group I was the control, and Group II, III, and IV were induced diabetic animals (n=4 each). They are subdivided into one-week (Group II), two-week (Group III), and four-week (Group IV) diabetic animals. Experimental diabetes was induced by intravenous injection of streptozotocin (STZ) (65 mg/kg bw). The experimental animals were provided standard chaw (Safe D40, Scientific Diets, Rosenberg, Germany) and water ad libitum. The chaw was withdrawn the day before the experiments (at 4 p.m.). Blood glucose levels were tested on the day of the experiments (at 8 a.m.) to confirm hyperglycemia. The animals were anesthetized with an intraperitoneal injection of urethane (1.2 g/kg bw). The abdomen was opened by a midline incision, and the selected organs were collected. The collected samples were stored in a deep freezer (-70 ^{o}C) until the analysis.

3.2. Sample preparation

A particular mass of the organ sample was weighted, then homogenized with the homogenizing buffer pH 7.4, in a proportion (1:3). The buffer was composed of KCl (0.154 M), Trizma (50 mM), and EDTA.Na_2 (5 mM). The matrix was completed with BHT (50 mg/ml in MeOH). The mixture was homogenized on ice by Witeg® homogenizer (HG-15A) for 90 seconds (50-speed rate). The homogenate was diluted with the buffer to reach the final mass/volume, in a proportion (1:4), sample preparation (homogenate 25%). The prepared samples were dispensed in Eppendorf vials and stored in a deep freezer (-70 ^{o}C) until the time of experiments.

3.3. UV-Vis determination of protein content (Biuret assay)

To 0.1 ml of organ homogenate (25%) in a 10 mL test tube, 1.0 mL of SDS (8.1%), 0.9 ml of NaCl (0.9%), and 8.0 ml of biuret reagent ($CuSO_4$•$5H_2O$, $KNaC_4H_4O_6$•$4H_2O$, KI, and NaOH in distilled water) were added. The mixture was incubated for 10 minutes at room temperature. Then, 1.0 ml of the sample was measured by UV-spectrophotometer (Jasco® V-750) at 550 nm against the blank. The protein concentration of samples was determined by the equation of calibration curve of bovine serum albumin (BSA) (y = 0.2583x +

0.1124, R^2 = 0.9997). The calibration curve plot was achieved with different concentrations of BSA in the same procedure of biuret assay designed and performed before sample measurements.

3.4. UV-Vis determination of protein carbonyl content (DNPH-protein adducts)

To 0.2 mL of organ homogenate (25%), 1.0 mL of 0.1 M phosphate buffer pH 7.2 and 0.8 mL of distilled water and 2.0 mL of 5mM 2,4-DNPH (in 2 M hydrochloric acid) were added. The mixture was incubated in the dark at room temperature for one hour by vortexing every 15 minutes. Then, 2.0 mL of 50% trichloroacetic acid was added, and the mixture was kept for another hour under the same condition. After that, the mixture was centrifuged for 15 minutes/5000 rpm. The supernatant was discarded, and the residue was washed with 1.0 mL of EtOH/EtOAc (1:1). The sample was vortexed and centrifuged for 15 minutes (5000 rpm), and the supernatant was discarded. This washing step was repeated three times, and the final residue was dissolved in 1 mL of 8 M guanidine hydrochloride. After the sample was completely dissolved, 0.1 mL of the solution was diluted in 0.9 mL of 8 M guanidine hydrochloride to be measured using a UV-spectrophotometer (Jasco® V-750). The absorbance was measured at 375 nm against 8 M guanidine hydrochloride.

3.5. UV-Vis determination of diene conjugates

To 1.0 mL of organ homogenate (25%) in a conical flask with a ground glass joint, 20 mL of chloroform: methanol (2:1 v/v) mixture was added. The mixture was incubated for 20 minutes in a water bath (50°C) with mechanical shaking. After that, it was washed into a separatory funnel using 10 mL of chloroform:methanol (2:1 v/v) mixture and 5 mL of distilled water. The mixture was allowed to stand for 20 minutes to allow separation. Then, the bottom phase was filtered into an Erlenmeyer flask and dried over anhydrous sodium sulfate. It was filtered into a weighted round-bottomed flask and evaporated by a rotary evaporator (Heidolph Hei-VAP) at 30°C (20 minutes). After evaporation, the mass of the flask mass was weighed, and the residue was dissolved in MeOH to obtain a 1 mg/mL solution. The absorbance of the obtained solution was measured by UV-spectrophotometer (VWR® U-1600PC) in a range of 400-200 nm against MeOH. After that, the solution was diluted ten times with MeOH, and the measurement was repeated.

3.6. UV-Vis determination of malondialdehyde (MDA) (TBARS assay)

To 0.1 ml of organ homogenate (25%), in a well-sealed ground-glass stoppered test tube, 0.2 mL of SDS (8.1%) and 1.5 ml of CH_3COOH (20%; pH 3.5) were added. After that, 1.5 ml of thiobarbituric acid (0.8%) and 0.7 ml of distilled water were pipetted into the mixture. The mixture was incubated in hot water (95ºC) for one hour to achieve the color reaction (TBARS). The sample was cooled down on the ice (5 minutes) and centrifuged (5 minutes, 3000 rpm). 1 mL of the clear supernatant was measured by UV-spectrophotometer (Jasco® V-750) at 532 nm against distilled water.

3.7. UV-Vis determination of non-protein thiols (NPSH)

To 1.0 ml of organ homogenate (25%), 0.25 mL of BHT (50 mg/ml in methanol) and 0.25 mL of trichloroacetic acid (25%) were added. The mixture was vortexed and then centrifuged for 5 minutes/5000 rpm. 0.1 mL of the collected supernatant was combined with 2.8 mL of Trizma buffer pH 8.9. The reaction was started after pipetting 0.1 mL of Ellman's reagent (DTNB) (0.01 M in MeOH). The sample was incubated at room temperature for 30 minutes, allowed the maximum reaction, and then measured at 412 nm against the blank.

3.8. HPLC determination of tyrosine isomers

To 50 mg organ sample, 400 µl of 6 N hydrochloric acid, 40 µl of 500 mM BHT in MeOH, and 4 µl of 400 mM desferrioxamine solution are added. The sample was vortexed for 1 minute and then hydrolyzed at 120°C for 18 hours. Then, the sample was allowed to cool, centrifuged at 3000 rpm for 10 minutes, and filtered through a syringe filter (0.2 µm) before analysis by HPLC-FLD.

3.9. Ibuprofen

3.9.1. Ibuprofen experiments *in vitro*

3.9.1.1. Fenton test

Iron (II) sulfate (100 µL of 30 mM) solution (in pH 3.0 sulfuric acid) was mixed with 700 µL of sulfuric acid (pH 3.0), and the mixture was vortexed for 30 seconds. Then, 100 µL of 10 mM IBP in phosphate buffer pH 7.2 was added and vortexed. The total volume was set to 1 mL by adding 100 µL of 10 mM hydrogen peroxide. The components were mixed in the respective order, and the reaction

mixtures were placed in a 37 °C water bath. The samples were analyzed after incubating 0, 10, 60, 80, and 120 minutes. "Blank" samples didn't contain IBP. At the end of each incubation period, the mixtures were acidified with 20 µL of 2 M sulfuric acid, and 50 µL of 10 mM salicylic acid as an internal standard was added (final concentration 0.467 mM). The samples were vortex-mixed and extracted twice with 2 ml of diethyl ether. The combined ether extracts were evaporated under N_2 gas. Before HPLC and HPLC-MS analysis, the dry residue was reconstructed in 100 µL of acetonitrile.

3.9.1.2. Udenfriend's assay

To a test tube, 3.0 mL of distilled water, 4.0 mL of 2.5 mM IBP solution in 0.1 M phosphate buffer (pH 7.2), 1.0 mL of 10 mM ascorbic acid, 1.0 mL of 2.4 mM EDTA, and 1.0 mL of 2.0 mM $Fe(NH_4)_2(SO_4)_2$ solution were added, in the listing order. The mixture was vortexed after adding each component. Then, it was incubated in a water bath at 37°C for 2 hours, with gently mechanical shaking, and 1.0 mL aliquot was taken from the mixture at 0, 10, 60, 80, and 120 min. To the 1.0 mL aliquot, 1 mL of 0.4 M ice-cold perchloric acid and 100 µL of 10 mM of salicylic acid (as an internal standard) were added (final concentration of 0.476 mM). The acidic solution was cooled in icy water and extracted twice with 3.0 mL of diethyl ether. The combined ether layers were evaporated under N_2 gas. Before analysis, the dry residue was reconstructed in 100 µL of acetonitrile.

3.9.2. Ibuprofen experiments *in vivo*

3.9.2.1. Intestinal perfusion

Male Wistar rats (9-11 weeks old; weighing 250-300) were separated into two groups. Group I was the control, and Group II was diabetic animals (n=5 per group). Experimental diabetes was induced by streptozotocin (STZ) one week before the intestinal perfusion, as stated in section (3.1.). The animals had fasted for 18–20 h before the experiments; then anesthetized with an intraperitoneal injection of urethane (1.2 g/kg bw). The abdomen was opened by a midline incision. A jejunal loop (length of the jejunal loop about 10 cm) was “in vivo” isolated and cannulated at its proximal and distal ends. Body temperature was maintained at 37°C using a heat lamp. Perfusion through the lumen of the jejunal loop with an isotonic medium containing 250 µM ibuprofen was carried out at a rate of 13 mL/min

in a recirculation mode. Perfusate samples (250 μL) were collected at selected time points (15, 30, 45, 60, 75, and 90 minutes) from the perfusion medium flowing out from the intestinal loop. The initial volume of the perfusate was 15.0 ml, and its temperature was maintained at 37°C. For parallel investigation of the biliary excretion, the bile duct was cannulated with PE-10 tubing. The bile outflow was collected in 15 minute-periods into tared Eppendorf tubes placed in ice. The collected samples were stored in a deep freezer (-70 °C) until analysis.

3.9.2.2. Sample preparation

To 0.1 mL of perfusate sample or 50 μL of bile sample, 20 μL of 2 M sulfuric acid and 10 μL of 10 mM salicylic acid (as an internal standard) were added. (The final concentration of salicylic acid in the perfusate and the bile samples was 0.77 mM and 1.25 mM, respectively). Then, the samples were vortex-mixed and extracted twice with 0.5 ml of diethyl ether. After vortexing (30 sec) and centrifugation (5 min, 5000 rpm), the ether layers were separated, and the combined ether extracts were evaporated under N_2 gas. Before HPLC-UV and HPLC-MS analysis, the evaporation residue was reconstructed in 100 μL of acetonitrile.

3.9.3. Analysis of racemic ibuprofen samples (Items 3.9.1. and 3.9.2.)

3.9.3.1. HPLC-UV

HPLC-UV analysis (Method I) of the Fenton and the Udenfriend's samples was performed on an integrated Agilent 1100 HPLC system equipped with a quaternary HPLC pump, a degasser, an autosampler, a thermostated column holder compartment, and a diode-array detector. Data were recorded and evaluated by Agilent ChemStation software (Rev.B.03.02-SR2).

HPLC-UV analysis (Method II) of the perfusate and bile extracts was performed on an integrated Jasco HPLC (LC-4000) system equipped with a quaternary HPLC pump, a degasser, an autosampler, a thermostated column holder compartment, and a PDA detector. Data were recorded and evaluated by ChromNAV Data System (Ver.2).

Method I. Separation of compounds was performed on a Teknokroma (NUCLEOSIL C18) (4.6 mm x 250 mm, 5μm particle

size) column with a Teknokroma (ODS cartridge, 1 cm x 0.32 cm) guard column at 40 ºC. The mobile phase consisted of 0.02 M phosphate buffer pH 2.5 (A) and acetonitrile (B) with a flow rate of 1.5 mL/min. The analyses (detection at 220 nm) were performed using the following gradient profile: 20% B for 15 min followed by a 5 min linear gradient to 60% B, a 4 min isocratic elution followed by a 2 min linear gradient to 20% B and a 4 min equilibration. The injected volume was 10 µL.

Method II. Separation of compounds was performed on a Zorbax SB C-18 (4.6 mm x 150 mm, 5µm particle size) column with a Teknokroma (ODS cartridge, 1 cm x 0.32 cm) guard column at 40 ºC. The mobile phase consisted of 0.02 M phosphoric acid pH 2.5 (A) and acetonitrile (B) with a flow rate of 1.6 mL/min. The analyses (detection at 220 nm) were performed using the following gradient profile: 20% B for 5 min followed by a 0.5 min linear gradient to 30% B, a 4.5 min isocratic elution followed by a 0.1 min linear gradient to 60% B, a 4.9 min isocratic elution followed by a 0.1 min linear gradient to 20% B and a 4.9 min equilibration. The injected volume was 10 µL.

3.9.3.2. HPLC-MS

To identify the metabolites, a Thermo Dionex UltiMate 3000 liquid chromatography (Dionex, Sunnyvale, USA) connected to a Thermo Q Exactive Focus quadrupole-Orbitrap hybrid mass spectrometer (Thermo Scientific) was used. Data acquisition and analysis were performed using the Q Exactive Focus 2.1, Xcalibur 4.2., and FreeStyle 1.8 software (Thermo Scientific). The HPLC separation was performed on an XTerra MS C18 column (150 mm x 2.1 mm, 3.5 µm) with XTerra MS C18 precolumn (5 mm x 2.1 mm, 3.5 µm) thermostatted at 40°C. The injection volume was 5 µl; the flow rate was 0.4 ml/min. A binary gradient of the eluents was as follows. Eluent: 5 mM ammonium-acetate/5 mM acetic acid in water (A) and methanol (B). Gradient: 10% B for 1 min, followed by a 9 min linear gradient to 90% B, a 2 min an isocratic elution followed by a 0.5 min linear gradient to 10% B, and a 2.5 min equilibration.

Analysis of the compounds was performed in HESI negative ionization modes with the following parameters: spray voltage 3.0 kV; probe heater temperature 300°C; capillary temperature 350°C; spray and auxiliary N_2 gas flows 20 and 5 arbitrary units, respectively; S-

Lens RF level 50%; automatic gain control 1e6; resolution (at m/z 200) 70000. Data acquisition range of m/z 100-1000, in full scan mode.

3.9.4. Chiral analysis

3.9.4.1. Sample preparation

The animal experiment and intestinal perfusion were identical to the section (3.9.2.). Perfusate and bile samples were kept at room temperature for a short time to become defrost. The sample volume (50 µL) was placed in an Eppendorf tube, and then 10 µL of naproxen sodium salt (250 µM) was added as an internal standard. The mixture was acidified by 40 µL of hydrochloric acid (2 M) and extracted with 1.5 ml (3-times 0.5 ml) of a mixture of n-hexane and ethyl acetate (8:2 v/v). The mixture was vortexed for 30 seconds, centrifuged for 5 minutes at 3500 rpm, and the transparent upper layer was collected. The combined extracts were evaporated under nitrogen at 40ºC. The evaporated residue was reconstituted in 50 µL of the mobile phase.

3.9.4.2. HPLC-UV analysis

HPLC-UV analysis of the perfusate and bile samples was performed on an integrated Jasco HPLC (LC-4000) system equipped with a quaternary HPLC pump, a degasser, an autosampler, a thermostated column holder compartment, and a PDA detector. Data were recorded and evaluated by ChromNAV Data System (Ver.2).

Ibuprofen enantiomers were separated on a Kromasil 3-amylCoat RP (4.6 mm x 150 mm, 3 µm) chiral column with a Teknokroma (ODS cartridge, 1 cm x 0.32 cm) guard column. The mobile phase consisted of methanol/water/acetic acid (70/30/0.1). The flow rate was 0.5 ml/min. The temperature of the column holder compartment was thermostated at 22 °C. Detection was performed at a wavelength of 220 nm; the injected volume was 10 µL. The analytical method is based on the Kromasil application note. Identification of the separated peaks was made by determination of the retention time of (*S*)-(+)-ibuprofen.

3.9.4.3. HPLC-UV analysis - Validation data

The analytical method was evaluated for a number of validation characteristics (specificity, system suitability, accuracy, repeatability and intermediate precision, limit of detection, limit of

quantification, and calibration range). The limit of quantitation (LOQ) was considered the lowest concentration (50 μM) of the calibration curve contracted by using racemic ibuprofen. Regarding the exact concentration of the two enantiomers in the investigated sample (R-IBP: 49.81%; S-IBP: 50.019%), the LOQ of the R-IBP and S-IBP was 24.91 μM and 25.09 μM, respectively

4. Results

4.1. Oxidative transformations of selective endogenous compounds under the influence of STZ-induced experimental diabetes

4.1.1. Determination of protein content

The results showed that the protein content of each organ was affected by the hyperglycemic conditions. The protein content of the small intestine in all groups is lowered, showing similar reduced values over the four-week period. In the kidney, the values were continuously lowered. The protein content of the liver showed the least reduction; the two-week STZ-treated samples had the lowest values.

4.1.2. Determination of protein carbonyl (Prot-DNPH) content

The carbonyl content of oxidized proteins was determined after derivatization with 2,4-dinitrophenylhydrazine (DNPH). Protein-DNPH content of the STZ- treated samples showed a distinct continuous increase compared to the controls in each tissue homogenate. Each two-week STZ-treated organ level was significantly different from the control. The protein-DNPH level in the four-week STZ-treated liver samples decreased. In the kidney, the protein carbonyl content showed a moderate continuous increase over the four-weeks.

4.1.3. Determination of diene conjugates

In the liver, the conjugated diene level slightly increased in the hyperglycemic rats at each timepoint. In the small intestine, the conjugated diene level significantly increased at each timepoint. However, the relative increase, specifically in the two-week and four-week hyperglycemic samples, was much higher than in the liver. In the kidney, the diene level was significantly higher in the four-week STZ-treated samples. The one-week and two-week STZ treatment

didn't significantly affect the conjugated diene level. The four-week samples showed a statistically higher value than the control.

4.1.4. TBARS determination

MDA is frequently determined as an indicative endogenous metabolite resulting in lipids oxidation by reactive oxygen species. It is a reactive carbonyl compound, readily reacting with nucleophilic sites of proteins and other cellular macromolecules. Accordingly, the MDA test results indicate the steady-state lipid-peroxide levels, which can undergo rearrangements to form MDA under the test conditions. Our results showed a continuous slight increase in the MDA (TBARS) levels in each organ. The TBARS levels of the four-week STZ-treated small intestine and kidney samples significantly differ from the control.

4.1.5. Determination of non-protein thiols (NPSH)

STZ-treatment of the experimental animals decreased the NPSH levels in the liver at the one-week and four-week timepoints. In contrast, the two-week STZ liver samples had somewhat increased values compared to the control. In the kidney, the NPSH content marginally raised at each timepoint. In the small intestine, the NPSH levels in the STZ-treated samples increased at each timepoint compared to the control. The lowest values could be measured in the two-week STZ-treated samples.

4.1.6. Determination of hydroxylated phenylalanine derivatives

Phenylalanine (Phe) is an essential amino acid that is the physiological precursor of *para*-tyrosine (*p*-Tyr), dihydroxy-phenylalanine (DOPA), catecholamines, melanine, and thyroid hormones. Beyond these enzymatic reactions, Phe, due to the nucleophilic character of its aromatic ring, is a subject of non-enzymatic oxidation processes, i.e., the attack of ROS. In such reactions, all three hydroxylated Phe isomers (*p*-Tyr, *m*-Tyr, and *o*-Tyr) are formed. Under our experimental conditions, the relative amount of the *m*-Tyr, and *o*-Tyr isomers, formed in non-enzyme-catalyzed hydroxylation reactions, increased at the two- and four-week timepoints in each investigated organ.

4.2. *In vitro* investigation of non-enzyme catalyzed (Fenton and Udenfriend) oxidation of ibuprofen

4.2.1. Fenton tests

HPLC-UV analysis (Method I) of the Fenton extracts indicated the formation of 1-HO-IBP (**2**), 2-OH-IBP (**3**), and several other products. To verify the structures of **2** and **3** and identify other oxidation products, a high-performance liquid chromatographic analysis using mass spectrometric detection (HPLC-MS) of the extracts was performed. HPLC-MS investigations of the extracts confirmed the presence of **2** (m/z 221.1176), **3** (m/z 221.1176), and HOOC-IBP (**5**) (m/z 235.0969). The derivative X-OH-IBP (**8**) with the highest HPLC-UV integrated peak area (t_R=22.05 min) couldn't be unambiguously identified. The formed product (X-OH-IBP) has a mass of m/z 221.1175, and its fragmentation makes no distinction between the 2'-OH-IBP and OH(Ar)-IBP structures. Another IBP-derived HPLC-UV peak (t_R=21.09 min) was identified as a dihydroxibuprofen (**10**) (OH-IBP-OH) derivative (m/z 237.1125). Determination of the exact structures needs further investigation.

4.2.2. Udenfriend's test

Using the *in vitro* non-enzyme-catalyzed hydroxylation test developed by Udenfriend et al., a similar oxidative metabolic pattern of ibuprofen could be observed. Like the Fenton incubations, the derivatives with the t_R values of 22.03 min (**8**) and 21.07 min (**10**) were those with the highest HPLC-UV peak areas. 2-HO-IBP (**3**) was formed in lower amounts than in the respective Fenton-samples. 1-OH-IBU (**2**) couldn't be detected. HPLC-MS investigations of the samples confirmed the presence of the **2**, **3, 5**, **8**, and **10** derivatives.

4.3. Investigation of the main oxidative and conjugated metabolites of ibuprofen in small intestine perfusates and bile of control and hyperglycemic rats

HPLC-UV analysis of the ether extract of the intestinal perfusate samples of the control and the hyperglycaemic animals did not indicate either hydroxy- or carboxyibuprofen metabolites. However, ibuprofen-β-D-glucuronide (**6**) (m/z 381.1549) could be identified by HPLC-MS in the perfusate of both the control and the STZ-treated animals. HPLC-UV analysis of the ether extract of bile samples showed the presence of IBP (**1**), IBP-GLU (**6**), and 2-OH-IBP

(**3**). HPLC-MS analysis confirmed the presence of **1** (m/z 205.1229), **6** (m/z 381.1549), and **3** (m/z 221.1178) in both the control and the hyperglycemic samples. The areas were lower in the bile samples of the STZ-treated animals at each investigated time point. Considering the respective bile outflows, the relative amounts of the cumulative excretions of the IBP (**1**), IBP-GLU (**6**), and 2-HO-IBP (**3**) were calculated. The result showed depression in the biliary excretion of all three compounds in the STZ-treated animals. Besides, several other peaks appeared in the extracts. Based on the HPLC-MS analysis of the samples, these peaks are bile acids and conjugated bile acid derivatives. Furthermore, HPLC-MS analysis of the bile samples confirmed the presence of the glucuronide conjugate of a hydroxylated ibuprofen (**9**) (m/z 397.1501) (X-HO-IBP-GLU), and the taurine conjugate of ibuprofen (**7**) (m/z 312.1274) (IBP-TAU). The excreted X-OH-IBP-GLU and IBP-TAU were depressed.

4.4. Investigation of the ratio of *R*(-) and *S*(+)-ibuprofen in small intestinal perfusates and bile of control and hyperglycemic rats

The identification of the ibuprofen enantiomers was confirmed based on the available S-IBP standard. The cumulative peak areas of the intestinal perfusates of the *R*(-)- and *S*(+)-ibuprofen (relative to the IS)enantiomers were depressed in the hyperglycemic rats. The relative amount of the (*S*) enantiomer in the control animals was slightly higher but not significant. Such a change couldn't be observed in the samples of hyperglycemic rats. In the bile samples collected during the intestinal perfusion of racemic ibuprofen, only the (S)-IBP enantiomer could be detected. In the chromatograms, two unknown peaks also appear. Based on their relative retention times (compared to the IS), none of them could be identified by the available standard (IBP, 1-, 2-, and 3-OH-IBP, and IBP-GLU). The excreted (S)-IBP (relative to the IS) was statistically lower in the hyperglycemic (STZ-treated) animals.

5. Summary

This study investigated the oxidative transformation of lipids, proteins, non-protein thiols (NPSH), and phenylalanine (Phe) in the liver, small intestine, and kidney in STZ-treated rats at the one-, two-, and four-week timepoints after the STZ-administration. The MDA,

Prot-DNPH, and the *o*-Tyr+*m*-Tyr levels followed a similar pattern: the highest values were recorded after two weeks of the STZ treatment. The compensation of oxidative stress is parallel with the change in the NPSH content of the organs. Such compensation was the least effective in the kidney, where the above three parameters continuously increased over the time of investigations. Accordingly, this organ is the most vulnerable to the hyperglycemia-induced oxidative stress.

The results indicate that reactions of hydroxyl radicals (ROS) with the aromatic ring of Phe are less favored than those with sensitive amino acid/protein and lipid molecules. This observation could be one of the reasons they are only limited data on increased non-enzyme catalyzed oxidized/hydroxylated aromatic xenobiotics under the present experimental conditions. However, modulation (mostly depression) of enzyme and transporter activities (oxidative stress-induced damage of proteins/lipids) is well documented in the literature.

The present results show that one of the main hydroxylated IBP-derivatives formed in the non-enzyme-catalyzed oxidation reactions (Fenton-test and Udenfriend's test) is a hydroxylated IBP (**8**). Since the formation of neither 2'-OH-IBP nor OH(Ar)-IBP (**8)** – the possible structures of the product - hasn't been reported in CYP-catalyzed reactions, the formation of this derivative might be used as a biomarker of oxidative stress in living organisms.

Contrary to 4-nitrophenol and capsaicin - both are phenolic derivatives - the glucuronide conjugate of ibuprofen could be detected only in a trace amount in the small intestinal perfusates. On the other hand, IBP (**1**), IBP-GLU (**6**), and IBP-TAU (**7**) were excreted in the bile. However, no specific non-enzymatic hydroxylation product could be detected. The results agree with the previous experimental findings demonstrating decreased expression of the organic anion transporters P-gp (MDR1), MRP2, and BCRP in the liver of the STZ-treated (hyperglycemic) animals. Such changes impact the pharmacokinetics of drugs administered in hyperglycemic individuals.

A chromatographic study using a chiral column provided experimental data on intestinal elimination and hepatic excretion of the IBP enantiomers in control and hyperglycemic experimental animals. The results demonstrated that the elimination of IBP from the small intestine is not enantioselective. Analysis of the bile showed the

presence of only the pharmacologically more active (*S*)-IBP enantiomer. Since the pharmacological activity of (*S*)-IBP is one order of magnitude higher than that of the (*R*)-IBP, the asymmetric appearance of the enantiomers in the bile could determine the pharmacokinetics and pharmacodynamic action of the drug.

6. Publication and presentation

Number of publications related to the subject of the thesis: 4
Number of publications not related to the subject of the thesis: 3
Number of oral and poster presentations related to the subject: 3
Sum of impact factors from publications related to the topic of Ph.D. thesis: 4.927

Publications related to the topic of the Ph.D. thesis:

- Perjesi P, Napolitano C, Napolitano HB, **Mohammed HO**. Physiochemical Characterization of Glutathione (GSH). In: Perjesi P, editor. Glutathione: Biosynthesis, Functions and Biological Implications. New York: Nova Science Publisher; 2019. p. 3-53.
- **Mohammed HO**, Almási A, Molnár S, Perjési P. The Intestinal and Biliary Metabolites of Ibuprofen in the Rat with Experimental Hyperglycemia. Molecules. 2022;27(13):4000. doi: 10.3390/molecules27134000.
- **Mohammed HO**, Almasi A, Perjesi P. Effect of experimental hyperglycemia on intestinal elimination and biliary excretion of ibuprofen enantiomers in hyperglycemic rats. (Submitted, under review).
- **Mohammed HO**, Almasi A, Kun Sz, Wittmann I, Perjesi P. Study on oxidative transformations of endogenous lipids, proteins, and amino acids in hyperglycemic rats. Preference of oxidation of non-aromatic sites. (Submitted, under review).

Other publications not related to the topic of the Ph.D. thesis:

- Ali KN, Marif HF, Kakarash NA, **Mohammed HO**. Liverfluke Coprological Cross-Sectional Survey in Cattle, Sheep and Goats in Sharazur District Kurdistan-Iraq. Al-Anbar Vet Sci. 14 (1), (2021).
- Hardi FM, Rashid ZM, **Mohammed HO**, Dyary HO. Resistance of Fasciola hepatica to triclabendazole, closantel, and rafoxanide in a sheep farm in Sharazor district, Kurdistan-Iraq. Basrah J Vet Res.18 (2), 16-26 **(**2019).

- Rahman HS, **Mohammed HO**, Fattah H, Othman HH, Amin KM, Abdullah R. (2019). Phytochemical analysis and augmentation of pulmonary adenocarcinoma in BALB/c mice model treated with flaxseed oil. Front Pharmacol. Conference Abstract: International Conference on Drug Discovery and Translational Medicine 2018 (ICDDTM '18) "Seizing Opportunities and Addressing Challenges of Precision Medicine." doi: 10.3389/conf.fphar.2018.63.00110. (Conference paper).

Oral and poster presentations:

- **Mohammed HO,** Almasi A, Molnar S, Perjesi P. Oxidative transformations of lipids and ibuprofen in hyperglycemic rats. XVI Congress Pharmaceuticus Hungaricus (CPH 2020) at the University of Debrecen (2020). "Poster"
- **Mohammed HO,** Almasi A, Molnar S, Perjesi P. Oxidative transformations of lipids and ibuprofen in hyperglycemic rats. 9th Medical Conference for Ph.D. Students and Experts of Clinical Sciences (MEDPE CS2019) at the University of Pecs (2019). "Poster"
- **Mohammed HO,** Almasi A, Molnar S, Perjesi P. A study of the incidence of lipid peroxidation in liver and small intestine of hyperglycemic rats by HPLC, LC-MS, and UV-VIS analysis. 8th International Doctoral Conference (IDK 2019) at the University of Pecs (2019). "Oral presentation"

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