SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The role of oxidative stress and autophagy in myocardium

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The Examination takes place at Department of Pharmacology, Faculty of Pharmacy, University of Debrecen, 9 a.m., June 3, 2019.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 1 p.m. June 3, 2019.

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I. Introduction and Objectives

Cardiovascular diseases (CVD) cause 3.9 million deaths in Europe and over 1.8 million deaths in the European Union (EU) each year. The total of CVD account for 45% of all deaths in Europe and 37% of deaths in the EU. Deaths caused by ischemic heart disease (IHD) and stroke are the most frequent among CVDs.

Nowadays, it is known that oxidative stress plays several roles within the cell. Under normal condition free radicals activate several signaling pathways; however, generation of excess amount of radicals leads to cellular damages. So far, a large amount of study aimed to investigate the connections between CVD and oxidative stress.

As a housekeeping process, autophagy is critical for the normal structure and function of the cardiac tissue. Autophagy at baseline level serves to maintain the structure and function of cardiomyocytes as well as to preserve physiological function of the heart during aging. In addition, autophagy is upregulated during stress conditions such as heart failure, pressure overload, nutrient starvation, ischemia-reperfusion injury, and proteotoxic diseases. Our main aim has been to investigate the autophagy process in different pathological conditions caused by oxidative stress.

The first study was designed to compare the responses of the heart to chronic moderate hypoxia and hyperoxia. We avoided extreme hypoxia/hyperoxia conditions for three reasons: (1) to prevent the involvement of non-specific, potentially confounding signals; (2) to simulate as closely as possible the clinical conditions of hypoxic patients and patients in O_2 therapy; and (3) to employ evenly spaced O_2 levels (10% - 21% - 30% O_2) to help the quantitative comparison. In addition, we selected a chronic (4 weeks) condition to assess the myocardial adaptation to altered O_2 levels. One difficulty encountered in this study was the effect of hypoxia/hyperoxia on the level of the housekeeping proteins. Normally, housekeeping proteins are employed as loading controls in Western blot analyses, under our experimental circumstances alteration of the level of

housekeeping proteins induce a considerable bias in the quantitative assessment of the target proteins. Therefore, we dedicated effort to get alternative reliable loading controls. The aim of the present study was to investigate whether altered O_2 levels in the inhaled atmosphere have divergent effects on key myocardial protein playing a role in the responses of the heart to stress given a special attention to apoptosis, autophagy and some other factors responsible for cardioprotection.

The second sets of experiments were planned to investigate the role of autophagy in heme toxicity. Heme is a “double edged sword”; in small amount, it acts as a functional group of heme proteins, and provides indispensable cellular functions. On the other hand, the accumulation of a large amount of heme was found to be toxic, which manifests as an extremely diverse process in large variety of cell types. In general, hem toxicity is accompanied by enhanced generation of iron-derived reactive oxygen species (ROS), DNA damage, with oxidation of lipids and proteins. It has been shown that overexpression of HO-1 prevented the diabetes related cardiac dysfunction by suppressing inflammation, oxidative stress, apoptosis and enhancing autophagy. Higdon *et al.* has used various concentrations of hemin to induce HO-1 and found that hemin caused mitochondrial dysfunction in endothelial cells by posttranslational modification of proteins induced by reactive lipid and oxygen species. Furthermore, hemin exposure also induced mitophagy, but it was not sufficient to prevent cell death. Dong and co-workers have reported a potential link between HO-1 and autophagy in podocytes. Their investigation revealed the importance of HO-1 in high glucose-mediated autophagy. Thus, several studies have investigated the association between HO-1 and autophagic pathways on different cells and tumors, however, it has not yet been fully elucidated, especially on cardiomyocytes.

II. Materials and Methods

II. 1. The effects of Moderate Chronic Hypoxia and Hyperoxia in Myocardial Tissue

II. 1.1. Animals and Treatment Protocol

The animals used in the present study (n=18) were male seven-week-old Foxn1 mice (Harlan Laboratory) with an average weight of 27-30 g. They were acclimatized to a 12-h light/12-h dark cycle and housed at an ambient temperature of 25 ± 2 °C. Food and water were freely accessible ad libitum. All animals were cared in accordance to the Guide for the Care and Use of Laboratory Animals as well as the use of the animals was approved by The University of Milan Committee for the Use of Laboratory Animals (OBPA).

Mice were randomly transferred into a gas chamber flushed. Animals were randomly segregated into three different groups as follows: 10% O_2 (hypoxia, n=6), 21% O_2 (normoxia, n=6) and 30% O_2 (hyperoxia, n=6). Animals were sacrificed and the hearts were harvested at the end of 28 days of exposure. Briefly, mice were transferred into the compensation chamber, anesthetized by i.p. Na-thiopental (10 mg/100 g body weight) and heparin (500 unites) by subcutaneous injection. Next, following induction of deep anesthesia, chest cavities were opened, hearts were excised, frozen in liquid nitrogen and stored at -80 °C for later analyses.

II. 1.2. Hemoglobin Level and D-ROMs Test

Blood hemoglobin concentration was measured by the Drabkin's method, assuming ε=11.05 cm^{-1} mM^{-1}. To evaluate the oxidative stress, we determined the overall level of oxidant chemical species produced, including ROS, H_2O_2, hypochlorous acid. By attacking organic molecules, these species generate stable Reactive O_2 Metabolites (ROMs), primarily composed by hydroperoxydes (ROOH). To determine oxidative stress in plasma, we used the photometric D-ROMs test (Diacron International srl, Grosseto, Italy) that evaluates the capacity of in vivo formed

ROOH to generate alkoxyl (•R-O) and peroxyl (•R-OO) radicals in the presence of iron released from plasma by an acidic buffer. Data are expressed as mg H_2O_2/dL.

II. 1.3. Immunofluorescent Cell Death Detection

Frozen samples were embedded in Optimum Cutting Temperature (OCT-Compound, Leica Instruments, Nussloch, Germany), and cut into 5 µm thick sections by cryomicrotome (Leica CM1510) at -22 °C. All tissue sections were placed on Superfrost Plus glass slides (Thermo Scientific, Rockford, IL) and dried at room temperature for 2 min. To detect apoptosis, we used the terminal deoxynucleotidyl transferase (TdT) nick end labelling test by the In Situ Cell Death Detection Kit, TMR (fluorescein-labeled cell markers) red (Roche, Mannheim, Germany). Apoptosis can be detected by labeling the free 3'-OH termini with modified nucleotides in an enzymatic reaction. The enzyme TdT catalyzes the template-independent polymerazation of deoxyribonucleotides to the 3'-end of single- and double-stranded DNA.

The sections were fixed in 4 % cold buffered formalin, at 4 °C for 45 min, then rinsed two times for 5 min in PBS (phosphate buffered saline) pH 7.4, at 4 °C. Next, they were post-fixed in ethanol-acetic acid (2:1, v/v) at -20 °C for 5 min, washed twice for 5 min in PBS. The sections were boiled in citrate buffer pH 6.0 for 12 min, then cooled at room temperature for 20 min, thereafter washed two times for 5 min in PBS. Finally, sections were incubated with TdT in a humidified box, at 37 °C for 1 hour. After washing, to identify nuclei, we used the blue karyophilic dye Hoechst 32258 (Sigma Aldrich, Schnelldorf, Germany). Glycerol+PBS pH 8.0 was used as mounting medium. Confocal microscopic images were obtained by confocal microscope (Leica SP2 confocal microscope with He/Kr and Ar lasers; Heidelberg, Germany). After merging the blue and red channels, purple spots were associated with apoptotic nucleus, while blue spots were identified as non-apoptotic nucleus (Adobe Photoshop CC 2017, San Jose, CA, USA). Apoptosis was quantified by ratio of TdT-positive nuclei / total nuclei in each section.

II. 1.4. Western Blot Analysis

Frozen heart tissue (50-80 mg) was lysed in a glass potter in a 1:3 ratio (w/v) buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF, 10 mM KCl and 10% Protease Inhibitor Cocktail (Roche, Mannheim, Germany), pH 7.9). Homogenates were kept on ice for 20 min and centrifuged at 14000 rpm at 4 °C for 20 min. The pellet was resuspended and centrifuged again for 10 min at 14000 rpm. Supernatants resulting from the two centrifugations were merged, transferred to a new tube and used as cytosolic extract. The resultant pellet was resuspended in isolating buffer B (20 mM HEPES, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 10% Protease Inhibitor Cocktail (Roche, Mannheim, Germany, pH 7.9), kept on ice for 20 min and centrifuged at 14000 rpm at 4 °C for 20 min. The protein concentration was determined by a Coomassie Protein Assay Kit (Thermo Scientific, Rockford, IL) using bovine serum albumin as the standard. Samples were mixed with loading buffer and boiled for 5 min.

A total of 70 µg of protein in each sample were loaded and separated on 8-15% SDS-PAGE gels (Sigma Aldrich, Schnelldorf, Germany) and then blotted onto a nitrocellulose membrane (PerkinElmer Life and Analytical Sciences, Boston, MA). After blocking the membranes with 5% of nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST), membranes were incubated overnight with primary antibody solution. Subsequently, the membranes were washed with TBST 3 times and incubated with the respective horseradish peroxidase (HRP)-conjugated secondary antibody solution for 1 h at room temperature. The following primary antibodies and dilutions were used: anti-Akt (Cell Signaling Technology, 1:1000), anti-phospho-Akt-Ser^{473} (Cell Signaling Technology, 1:1000), anti-Bcl-2 (Santa Cruz Biotechnology, 1:1000), anti-Bax (Santa Cruz Biotechnology, 1:500), anti-AMPK (Santa Cruz Biotechnology, 1:1000), anti-phospho-AMPK-Thr^{172} (Santa Cruz Biotechnology, 1:1000). The secondary antibodies were used: Peroxidase-

conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories Inc. 1:10000), Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories Inc. 1:10000). After washing, the membranes were incubated with Lite Ablot Chemilumisencence substrate (Lite Ablot, Euro Clone, EMPO10004) to visualize by enhanced chemiluminescence bands according to the recommended procedure (UVITEC LTD Cambridge, UK). The band intensities were measured by UVI-1D software.

II. 1.5. Western Blot Analysis with Stain-Free Gels

A total of 70 µg of protein in each sample were loaded and separated on TGX Stain-Free™ 7.5% acrylamide gels (Bio-Rad Laboratories, USA). Then, gels were exposed to UV light thereby trihalo compounds of the stain-free gels covalently bind to tryptophan residues of the proteins. Subsequently, the proteins were blotted onto a nitrocellulose membrane (PerkinElmer Life and Analytical Sciences, Boston, MA, USA). After transfer, the proteins of membrane were exposed by another brief irradiation and the resulting tryptophan adducts were emitted fluorescence signal. This picture was evaluated and used as total protein volume. The following primary antibodies and dilutions were used: anti-α-tubulin (Santa Cruz Biotechnology, 1:1000), anti-actin (Sigma Aldrich, St Louis, 1:2000), anti-GAPDH (Sigma Aldrich, St Louis, 1:15000), anti-Beclin-1 (Epitomics, Abcam Company, 1:3000), anti-LC3B (Cell Signaling Technology, 1:1000), anti-p62 (Abcam, 1:1000), anti-HIF-1α (Santa Cruz Biotechnology, 1:300), anti-HIF-2α (Abcam, 1:300), anti-NOX4 (Abcam, 1:5000). The further steps were performed in same way described under (II.1.4.). The chemiluminescent bands and each total protein lane intensities were measured by UVI-1D software. In this method, protein density and quantification is measured directly on the Western blot membranes with reference to total loaded proteins. This type of normalization totally eliminates the need to select an adequate panel of housekeeping proteins. The protein expression was quantified by the ratio of (band volume) / (total protein volume).

II. 1. 6. Statistical Analyses

All data passed the Kolmogorov-Smirnov normality test ($\alpha = 0.05$). Two types of analysis were performed to compare the groups. In the first, we considered three independent groups (hypoxia, normoxia, hyperoxia) and performed the one-way analysis of variance (ANOVA), followed by the Tukey's multiple comparison test if ANOVA $p<0.05$. In the second, data were considered as a continuous function of % O_2 (10, 21, 30) and we performed linear regressions analysis, followed by the generation of the best-fit equations. To assess the goodness of the fit, we calculated the squared correlation coefficient (r^2) to generate the p value of the regression (http://vassarstats.net/rsig.html). If $p<0.05$, we assessed whether the slope of the best-fit line was significantly different from zero, i.e., there is a statistical effect of the O_2 level in the inspired atmosphere on the variable under study. Statistical analyses were performed using Prism (Graph-Pad Software Inc).

II.2. The Effects of Toxicity of HO-1 Inducers on Autophagy Pathway

II.2.1. Cell Culture and Treatment

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% streptomycin-penicillin at 37 °C in a humidified incubator consisting of 5% CO_2 and 95% air. Cells were exposed to eight different conditions: only medium (untreated control group); medium containing 20 mM NaOH (vehicle treated control group); 3 μM hemin; 10 μM hemin; 30 μM hemin; 100 μM hemin; 2.5 μM $CoPP_{IX}$; 25 μM $CoPP_{IX}$ and 100 μM $CoPP_{IX}$ groups for 24 h.

II. 2.2. Cell Viability Assay

Cell viability was measured by MTT experiments on 96-well plates. After treatment, MTT solution (final concentration of 0.5 mg/ml) was added to each well and incubated for 3.5 hours at 37 °C. After that medium was replaced by isopropyl alcohol to dissolve formazan product and

incubated for 30 min at 37 °C. Absorbance was measured with a microplate reader (FLUOstar OPTIMA, BMG Labtech) at 570 and 690 nm. The values were expressed relative to untreated control, which was represented as 100% of viability. 1% H_2O_2 were used as positive control. Absorbance values were averaged across 7 replicate wells, and repeated minimum 3 times.

II. 2.3. Protein Isolation

After the treatment, samples were lysed in 100 µl isolating buffer (in mM: 25 Tris-HCl, 25 NaCl, 1 orthovanadate, 10 NaF, 10 pyrophosphate, 10 okadaic acid, 0.5 EDTA, 1 PMSF, 1x protease inhibitor cocktail and TritonX-100). Subsequently, samples were centrifuged at 14000 rpm at 4 °C for 10 minutes in three times freezing-smelting cycles. The supernatant fraction were collected and protein concentration was determined using BCA kit (Thermo Scientific, Rockford, IL, USA).

II. 2.4. Western Blotting

A total of 25 µg of protein in each sample was separated on TGX Stain-Free™ 12% acrylamide gels. Then, gels were exposed to UV light thereby trihalo compounds contained in stain-free gels covalently bind to tryptophan residues in proteins allowing total protein quantification. After transferring the proteins to PVDF membranes for 1 hour at 100 V, membranes were exposed by another brief irradiation and the resulting fluorescence signals were recorded, and the signal intensity is proportional to the total protein volume. After blocking with 5% of non-fat dry milk in TBST, membranes were incubated with primary antibody solution at 4 °C, overnight. The membranes were washed with TBST and incubated with HRP-conjugated secondary antibody solution. After washing, the membranes were incubated with Clarity Western ECL substrate (Bio-Rad Laboratories) to visualize by enhanced chemiluminescence bands according to the recommended procedure (ChemiDoc Touch, Bio-Rad Laboratories). The chemiluminescent bands and each total protein lane intensity were measured by Image Lab software (Bio-Rad Laboratories).

During quantification, protein density is measured directly on the membranes and reflected to total loaded proteins. Thus, this type of normalization eliminates the need to select housekeeping protein.

II. 2.5. DCFDA Staining

Cells were seeded in black 96-well plates. After cells adhered, medium was removed and cells were washed with PBS. DCFDA dye was added for 1 hour and diffused into cells. At the end of incubation period excess amount of the dye was removed and fresh medium was added back. After 30 minutes cells were treated with hemin and $CoPP_{IX}$ (100 μM) for 24 hours. At the end of the treatment, medium was removed and washed with PBS. Then the intensity of fluorescent compound was detected by fluorescence spectroscopy with excitation and emission spectra of 485 nm and 528 nm respectively.

II. 2.6. MitoSOX Staining

Cells were seeded on coverslip. After the treatment with hemin and $CoPP_{IX}$ (100 μM) for 24 hours, medium was removed and cells were washed 3 times with PSB. MitoSOX™ Red was added for 10 minutes at 37 °C in dark. Then, dye was removed and cells were washed 3 times with PBS. Nucleus was stained by DAPI. Cells were fixed with 4% formaldehyde then washed again. The coverslips were placed to a slide and examined the staining by fluorescence microscopy. Images were captured by Zeiss Axio Scope.A1 fluorescent microscope and analyzed with ZEN 2011 v.1.0.1.0. Software (Carl Zeiss Microscopy GmbH, München, Germany). Mean intensity of red color was quantified by Image J software (NIH, Bethesda, Maryland, USA). 100 cells/group were measured in 3 different experiment.

II. 2.7. CYTO-ID Staining

CYTO-ID Autophagy Detection Kit 2.0 was performed to measure autophagic vacuoles and monitor autophagic living cells.

Cells were seeded overnight and treated with hemin and $CoPP_{IX}$ (100 μM) for 24 hours. Rapamycin (5 μM) was used as the positive control. Autophagic process was inhibited by chloroquine (10 μM, for 18 hours). After treatments, cells were collected by centrifugation and washed with 1x assay buffer. The cell pellets were resuspended in fresh 1x assay buffer. 250 μL of the diluted CYTO-ID Green stain solution was added to each sample and mixed, then incubated for 30 min at 37 °C in dark. Cells were washed with 1× assay buffer and fixed with 1% formaldehyde, then were immediately analyzed with a FC-500 flow cytometer (Beckman Coulter, Pasadena, CA, USA). The results were analyzed by CPX Analysis Software (Beckman Coulter). Autophagic flux was measured by ΔMFI in each group (ΔMFI: MFI with Chloroquine – MFI without Chloroquine).

Cells were seeded on coverslips and treated with hemin and $CoPP_{IX}$ (100 μM) for 24 hours. Rapamycin (5 μM) was used as the positive control. Autophagic process was inhibited by chloroquine (10 μM, 18 hours). After treatments, the medium was removed and cells were washed with 1x Assay Buffer. A total of 100 μL of Microscopy Dual Detection Reagent was added to each sample and incubated for 30 min at 37 °C. Cells were washed with 1× assay buffer and fixed with 4% formaldehyde then washed again. The coverslips were placed to a slide and examined the staining by fluorescence microscopy (Zeiss Axio Scope.A1 fluorescent microscope).

II. 2.8. Statistical Analysis

The data were expressed as mean ± SEM. Statistical analysis were performed with GraphPad Prism version 5 (La Jolla, CA, USA). One-way analysis of variance (ANOVA) test

followed by Dunnett multiple comparison tests, which identified the significant difference between control and treated groups. A probability value of $p<0.05$ was used as the criterion for statistical significance. When significant ($p<0.05$), *, **, and *** represent $p<0.05$, $p<0.01$, and $p<0.001$ at the Dunnett's post-test, respectively.

III. Results

III. 1. 1. Body Weight and Blood Hemoglobin

All mice survived the various treatments. The initial body weight (BW) of was no statistical differences among the groups. The Tukey test did not show statistical differences between 21% O_2 and 10% O_2, as well as between 21% O_2 and 30% O_2, the linear regression analysis shows that the slope was significantly greater than zero, indicating that the BW at the 28^{th} day values were positively related to % O_2.

Treatments with different % O_2 in breathed air differentially altered the blood hemoglobin level. Whereas breathing low % O_2 elevated the blood hemoglobin level, high % O_2 lowered it. This parameter was strictly dependent on % O_2, as shown by the inverse linear regression relationship between blood hemoglobin and % O_2 ($p<0.0001$).

III. 1.2. Oxidative Stress

The D-ROMs test enables the determination of the concentration of reactive O_2 metabolites in biological samples. The value of reactive O_2 metabolites found in the 21% O_2 group was considered as normal level. A significant increase was observed in samples obtained from animals kept in 10% O_2, indicating that the systemic redox balance under hypoxic condition is markedly impaired. However, hyperoxic environment did not alter the systemic pro-oxidant pool compared to 21% O_2. Nevertheless, we observed strong and statistically significant inverse association between the level of oxidant species and the O_2 concentration ($p=0.0002$).

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit 4 (NOX4) acts as an O_2 sensor, catalyzes the reduction of molecular O_2 to various reactive O_2 species (ROS), and plays an important role in cardiovascular pathophysiology. We found an elevated expression of NOX4 in 10% O_2 in comparison with 30% O_2, indicating increased oxidative stress. Moreover, we observed an inverse linear relationship between the level of NOX4 and % O_2 ($p=0.03$).

III. 1. 3. Hypoxia Signaling

We measured the expression of HIF-1α and HIF-2α in cardiac cytosolic extracts by Western blot. The HIF-1α protein level was upregulated in 10% O_2 compared to 30% O_2 as for the significant difference between 10% O_2 and 31 % O_2 at the ANOVA and Tukey test. Furthermore, a linear correlation is observed ($p=0.0075$) for HIF-1α. By contrast, there is no significant effect of neither 10% O_2 nor 30% O_2 on the expression patterns of HIF-2α.

III. 1.4. DNA Fragmentation and Apoptosis

To evaluate the effect of % O_2 in breathed air on cardiomyocytes apoptosis, we first analyzed the Bax/Bcl-2 ratio, which determines the cell susceptibility to apoptosis. The results show upregulated Bax/Bcl-2 ratio in 30% O_2 compared to both 10% O_2 ($p=0.05$) and 21% O_2 ($p=0.05$).

Direct detection of apoptotic nuclei by the immunofluorescent staining TUNEL technique, however, gave a different result. The percentage of apoptotic nuclei in 10% O_2, 21% O_2 and 30% O_2 indicating that, whereas very low in normoxia, moderate hypoxia markedly increases the degree of TdT-positive nuclei, but moderate hyperoxia induces a still higher degree of fragmented DNA. Therefore, the percentage of TdT-positive nuclei was not linearly related with % O_2, but rather a U-shaped relationship was observed.

III. 1.5. Effects of Different O_2 Concentration on Activation of Survival Pathways

Akt is a serine/threonine kinase that has been shown to play a central role in promoting cell survival and opposing apoptosis. Data reports that % O_2 was not related to this pathway. The ratio of total Akt expression and the phosphorylated form at Ser^{473} (p-Akt) remained unaltered in the groups.

We investigated another relevant defense mechanism which is often activated by stress. AMP-activated protein kinase (AMPK) is an energy sensor activated by increasing [AMP] or by

oxidant stress (reactive O_2 species [ROS]). Similarly, no alteration was observed in the ratio of p-AMPK/AMPK between the groups. There was no statistically significant correlation between the activation of investigated survival pathways and alteration of O_2 concentration.

III. 1.6. Consequence of Modified O_2 Tension on Autophagy

We assessed autophagy by examining the expression of several autophagy proteins. As part of a type III phosphoinositide-3 kinase complex, the autophagy gene Beclin-1 is required for the formation of the autophagic vesicles. The protein expression level of Beclin-1 significantly increased in 10% O_2 compared to 30% O_2. Besides this, a strong and inverse linear relationship between the level of Beclin-1 and % O_2 was detected ($p=0.012$). Interestingly, the average level of Beclin-1 autophagic protein was similar in 30% O_2 and 21% O_2, indicating that the autophagy pathway was not induced by O_2.

The level of LC3B-II and p62 proteins was not altered after the treatment of different % O_2 in breathed air. These proteins were not statistically associated with the modified O_2 tension.

III. 1.7. Housekeeping Proteins

In these experiments, the effect of varying % O_2 in inspired air on the tissue density of the level of the housekeeping proteins (α-tubulin, actin and GAPDH) were expressed with reference to the total protein volume. Surprisingly, the Tukey test shows, that 30% O_2 induced higher expression of α-tubulin and GAPDH (but not actin), than 10% O_2. However, the slope of the best-fit line was significantly less than zero for all the three housekeeping proteins, showing that higher % O_2 in inspired air corresponds to the decreased expression of these proteins ($p=0.006$, 0.03 and 0.0004, respectively).

III. 2.1. Effect of High Doses of HO-1 Inducers on Viability of H9c2 Cells and Hemeoxygenase-1 Expression

The treatment with hemin or $CoPP_{IX}$ decreased the viability of H9c2 cardiomyocytes in a dose-dependent manner. At hemin concentration of 25 and 100 μM the toxic effect was profound compared to untreated sample. Furthermore, similar alteration in cell viability was detected when $CoPP_{IX}$ was used. Western blot analysis using an antibody against HO-1 indicated a significant increase in HO-1 expression by both inducers in H9c2 cells.

2.2 Effect of Hemin or $COPP_{IX}$ Treatment on ROS Level

In order to study the role of ROS in the toxic effect induced by high dose of hemin or $CoPP_{IX}$ treatments were carried out in H9c2 cells MitoSOX and DCFDA staining. MitoSOX detect mitochondrial superoxide, whereas DCFDA offers general ROS detection. Data showed that both hemin and $CoPP_{IX}$ treatments enhanced ROS, which could play an important role in the toxic effect of the inducers and hem.

III.2.2. Effect of Hemin or $CoPP_{IX}$ Treatment on Autophagy

The expression level of Beclin-1 remained unchanged after HO-1 induction with hemin or $CoPP_{IX}$. Significantly increased LC3B-II and p62 expressions were measured by Western blotting. These results showed that hemin treatment markedly upregulated LC3B-II and p62 protein levels in cardiomyocytes, indicating that autophagy was induced, however, the level of p62 remained elevated suggesting the lack of its function. We further investigated these autophagic markers after HO-1 induction by $CoPP_{IX}$. The results supported the upregulation of autophagy pathway. Similarly, we found significantly increased expression level of LC3B-II and p62 in $CoPP_{IX}$ 100 group compared to untreated cells.

To confirm our Western blot results, Cyto-ID Green staining was carried out and samples were analyzed by microscopy and flow cytometry. Monitoring autophagic flux, cells were treated

with chloroquine, which is a known autophagic flux inhibitor.

The number of autophagic vacuoles were elevated in untreated and 20 mM NaOH cells in the presence of chloroquine indicating a functioning autophagic flux. Rapamycin was used as positive control, these pictures show numerous vacuoles in the presence or absence of chloroquine. By Cyto-ID Green staining, green fluorescent signals and punctate structures were detected. However, after the $CoPP_{IX}$ treatment we found some autophagic vacuoles with decreased number compared to the untreated group. Comparing hemin and Hemin + Q groups no significant alteration was observed. Same results were seen in $CoPP_{IX}$ treated groups in the presence or absence of chloroquine. To quantify the autophagic flux, flow cytometric analysis was carried out. The ΔMFI in each group was assessed. The results from this measurement supported the microscopic data. In untreated and 20 mM NaOH treated group supporting the existence of normal autophagic flux. However, after hemin treatment ΔMFI was actually zero. Additionally, this value also significantly decreased in the $CoPP_{IX}$ treated group. As expected, a significant increase was found in ΔMFI in rapamycin treated cells. The findings indicate that autophagic process is incomplete when high amount of hemin or $CoPP_{IX}$ was used for HO-1 induction. Thus, our finding shows that both hemin and $CoPP_{IX}$ induced Beclin-1 independent or non-canonical autophagy; however, it was not functioning.

III.2.3. Apoptosis Activation by High Concentration of Hemin and $CoPP_{IX}$

A diminished level of pro-caspase-3 and enhanced level of cleaved caspase-3 after the hemin or CoPPIX treatment was detected in comparison with controls, suggesting that the apoptotic machinery is activated after high dose of hemin or $CoPP_{IX}$ treatment.

IV. Discussion

IV. 1. The Effects of Moderate Chronic Hypoxia and Hyperoxia in Myocardial Tissue

In this study, the myocardial response to 28-day normobaric hypoxia, normoxia or hyperoxia were studied in a mouse model. Under our experimental circumstances alteration related to reoxygenation or deoxygenation events were prevented. The O_2 tension were chosen to simulate clinically relevant O_2 levels avoiding poorly significant extremely hypoxic or hyperoxic situations. The % O_2 selected for hypoxia is known to induce sub-lethal metabolic and signaling alterations in myocardial tissue. In hyperoxic grouop we aimed to mimics the situation when breathing of pulmonic patients are supported portable O_2 bottles. Furthermore, evenly spaced % O_2 tension were chosen enabling to test linear correlation between % O_2 and the studied signaling molecules.

The redox imbalance did not appear pronounced in hyperoxia but was greater in hypoxia. In line with our results earlier, it has been shown that >40% oxygen tension disturbs the redox balance. Our results show that chronic hypoxia (10% O_2) induces the expression of HIF-1α in heart. Furthermore, in accordance with the literature and our HIF-1α results, as an underlying mechanism an elevated oxidative stress were found in hypoxic group. In contrast to literature, after considering the experimental conditions used in this study, where we did not find any relevant hyperoxia-induced increase in neither HIF-1α nor HIF-2α, it is plausible to assume that 30% O_2 is not a sufficient trigger for HIFs in hearts, or after a transient increase, as it was shown in the brain, it returns to the baseline value.

Our TUNEL assay indicated an enhanced DNA fragmentation under hypoxic and hyperoxic condition, the number of TdT positive nuclei were more prevalent under hyperoxia. The hypoxic results are in correlation with HIF-1α expression. On the other hand, Bax/Bcl-2 ratio was significantly increased after hyperoxia. This may reflect the fact that DNA fragmentation and apoptosis were more pronounced under hyperoxia. Moreover, a significant increase in, expression

of Beclin-1 were detected under hypoxia. However, we failed to observe any significant alteration in the levels of LC3B and p62. It has to be noted that, autophagic flux was not measured, thus, we can not draw any definitive conclusion on the relation between autophagy and high % O_2 in the hearts, mainly in hyperoxia. The question whether a possible crosstalk between apoptosis and autophagy exists may be raised. Based on previous findings, it is attractive to speculate that Beclin-1 may be induced by the oxidative stress upon prolonged moderate hypoxia, and might alleviate the elevation of Bax/Blc_2 ratio, which could give a room for adaptation. Nevertheless, under prolonged hyperoxia, relative lack of oxidative stress might fail to increase Beclin-1 leading to enhanced DNA fragmentation.

Moreover, at the beginning of the present investigation, traditional Western Blot were employed. Unexpectedly, the level of the reference proteins, that are generally used to normalize densitometry values, were exhibited considerable fluctuations. Employing total protein normalization methods confirmed the suspicion that the level of this commonly used housekeeping proteins fluctuated under our experimental circumstances. Our results suggested that the expression of housekeeping proteins was remarkably increased by hypoxia (10% O_2) treatment. Taken together, our unexpected findings reported here suggest that data normalized against housekeeping proteins should be handled with caution when the inhaled O_2 tension is altered.

IV. 2. The Effects of Toxicity of HO-1 Inducers on Autophagy Pathway

The current study shows that high doses of different HO-1 inducers possess a toxic effect on H9c2 cells. The toxic effect is accompanied by malfunctioning autophagy. Several lines of evidence indicated that different concentrations (0.1-1000 μM) of hemin could increase the level of HO-1 expression; however, it may have positive outcome or cytotoxic effects depending on the dose used. Recently, it has been shown that induction of HO-1 by 20 μM $CoPP_{IX}$ protected H9c2 cells against H/R evidenced by decreased apoptosis. However, in the present study, a higher dose

of $CoPP_{IX}$ was used, which exhibited a toxic effect.

When the amount of free heme exceeds the capacity of heme detoxifying enzymes, free heme exerts its toxic effect, which could be mediated via enhanced ROS generation and inflammation. As expected, in our experiments, an enhanced level of HO-1 was detected upon treatment with HO-1 inducer in a dose-dependent manner. However, our cell viability assay indicated an increased toxic effect. In line with the literature an enhanced level of ROS was detected in both treated groups, indicating that under the experimental circumstances used in this study the elevated ROS level contributes to the cell death.

Several studies investigated the connection between HO-1 and autophagy in different tissues. The precise role of autophagy in cardiovascular system has always elicit controversy; there are evidence to support its role as a saviour and also as a killer. Our results show that autophagy is induced since the level of LC3B-II was significantly higher in the presence of HO-1 inducers. p62 is another widely used marker, which physically links autophagic cargo to the autophagic membrane. p62 binds directly to LC3 and GABARAP family proteins and is selectively degraded by autophagy processes. Since p62 accumulates when autophagy is inhibited/impaired, decreased levels can be observed when autophagy is induced by oxidative stress. Thus, p62 serves as an indicator of autophagic degradation and used as a marker to study autophagic flux. Impaired autophagic flux by HO-1 dependent autophagy was previously found. In line with this study an enhanced level of p62 was found upon HO-1 induction, indicating that the autophagy is malfunctioning.

Autophagy flux is a dynamic process that includes initiation, elongation, maturation and degradation. Interestingly, autophagosome formation was independent of Beclin-1, indicating non-canonical autophagy activity in hemin and $CoPP_{IX}$-treated cells. Recent findings suggest that autophagosome biogenesis occurs also in the absence of Beclin-1. To further study the autophagic

flux, cells were treated with chloroquine, which induces the accumulation of autophagic vacuoles. The fluorescent microscopy images and flow cytometry results revealed an enhanced number of autophagic vacuoles in control and rapamycin treated cells. However, in line with our Western blot data, we failed to find any differences between signals in HO-1 inducers treated cells in the presence or absence of chloroquine, further supporting that malfunctioning autophagy contributes to heme toxicity. Several studies have published that autophagy is necessary process to remove damaged organelles. If this process fails or overwhelmed, these damaged organelles trigger an apoptotic cell death.

Our Western-blot results show a lower level of pro-caspase-3 and enhanced level of cleaved-caspase-3 indicating the activation of apoptosis. Taken together, we demonstrated that overexpression of HO-1 by high dose of hemin and $CoPP_{IX}$ induces cell toxicity in H9c2 cells, in which malfunctioning autophagy and enhanced ROS level plays a role.

V. Summary

In the first part of our experiment, we investigated the effects of different inhaled O_2 tensions (10-21-30) on various pathways in myocardium. 10% O_2 appears to cause a considerable level of oxidative stress in heart tissue, which increases the expression of HIF-1α, while 30% O_2 does not cause significant oxidative stress as evidenced by unaltered level of derivatives of ROMs. However, both hypoxia and hyperoxia elevate TUNEL positivity. Alterations in Beclin-1 protein level suggest that altered O_2 tensions may have an impact on autophagy. Although a definitive conclusion can not be drawn because the autophagy flux was not measured, a crosstalk between apoptosis and autophagy may have been established under these circumstances. Furthermore, we found, that the level of the commonly used housekeeping proteins such as α-tubulin, actin and GAPDH are altered. Thus, these proteins should be used with caution as loading controls when oxygen tension of the inhaled air is a variable.

In the other study we also investigated the influence of toxic doses of HO-1 inducers (hemin and $CoPP_{IX}$) on level of oxidative stress, the pathway of autophagy and apoptosis in H9c2 cells. Our results showed decreased cell viability at high dose of hemin and $CoPP_{IX}$ treated H9c2 cells in a dose-dependent manner. As it was expected both treatments induced a robust elevation of HO-1 level. Furthermore, at concentration of the inducers used in the present study a significantly enhanced level of ROS was detected. In addition, the Beclin-1-independent autophagy was significantly increased, but caused a defective autophagic flux with triggered activation of apoptosis.

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List of publications related to the dissertation

1. **Gyöngyösi, A.**, Szőke, K., Fenyvesi, F., Fejes, Z., Bekéné Debreceni, I., Nagy, B. J., Tósaki, Á., Lekli, I.: Inhibited autophagy may contribute to heme toxicity in cardiomyoblast cells.
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List of other publications

3. Czompa, A., Szőke, K., Prokisch, J., **Gyöngyösi, A.**, Bak, I., Balla, G., Tósaki, Á., Lekli, I.: Aged (Black) versus Raw Garlic against Ischemia/Reperfusion-Induced Cardiac Complications. *Int. J. Mol. Sci. 19* (4), 1-13, 2018.
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Total IF of journals (all publications): 16,415
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