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

Investigation of different pharmacological interventions and cardiotoxicity in the myocardium: the importance of autophagy

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Supervisor: Prof. Dr. Arpad Tosaki

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The Examination takes place at Department of Pharmacology, Faculty of Pharmacy, University of Debrecen, 11 a.m. October 10, 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. October 10, 2019

1. Introduction and aims

Doxorubicin (DOX) is a highly effective and widely-used non-selective class I anthracycline antibiotic which is frequently incorporated in the treatment of acute leukemia, malignant lymphoma, and several solid tumors. However, the efficacy of DOX is hindered due to the cumulative and irreversible cardiotoxicity. The molecular mechanisms underlying DOX-induced cardiotoxicity are multifactorial and are still unclear, but mitochondrial dysfunction, oxidative stress, apoptosis, and dysregulation of autophagy are involved. Furthermore, the heart is very susceptible to DOX-induced lipid peroxidation and toxicity because of its high energy requirement and mitochondrial density. Autophagy is a highly conserved process which is aimed to maintain cell and tissue homeostasis, and involves the elimination of damaged and long-lived organelles under both physiological and pathological conditions, including energy and oxygen status, nutrient starvation, and modification in metabolism. Several studies have found that DOX treatment affects autophagy, however, it is still not clearly elucidated how DOX alters this process. Previous studies on this matter have shown many controversial results. Recently reported studies have demonstrated that DOX induces autophagy; however, it causes dysregulation in the autophagic flux and the autophagic process cannot be completed. In addition, Tokarska et al. reported that DOX can cause dysregulation in most processes of myocardial energy metabolism, such as the AMP-activated protein kinase (AMPK) signaling pathway. AMPK is a major sensor of cell energetic homeostasis. Low cellular energy levels and increased reactive oxygen species (ROS) result in the phosphorylation and activation of AMPK, which is able to induce autophagic processes.

Metformin (MET) is an orally used first-line anti-diabetic drug for the treatment of type 2 diabetes. Several studies reported that application of MET decreases mortality and cardiovascular end-points of type 2 diabetes and has protective effects in cardiac function.

Recently, several studies have found that MET activates AMPK, and through the AMPK signaling pathway it induces cardiac autophagy and improves cardiac functions. Various studies have revealed that boosting or restoring autophagy could help the cardiomyocytes to survive during DOX therapy. In the present study, we co-administered DOX and MET in order to investigate the role of MET in the autophagic process and its cardioprotective properties in DOX-induced cardiotoxicity. Thus, our investigation may offer further understanding of the role of cardiac autophagy in DOX-treated animal subjects. In addition, our hypothesis was that MET could activate AMPK, restore autophagy, and improve cardiac function, which may

consequently mean that DOX co-administered with MET help the cardiomyocytes to survive. To further study the importance of autophagy during cardiotoxicity we administered isoproterenol (ISO) at different doses (0.005 mg/kg ISO; 0.05 mg/kg ISO; 0.5 mg/kg ISO; 5 mg/kg ISO; 50 mg/kg ISO), and monitored the autophagic process and the two death pathways, including apoptosis and necrosis. ISO is a catecholamine and bearing a non-selective β-adrenergic agonist property. At low dose it can be used in heart block and cardiac arrest; however, at chronic or high doses, administration of ISO leads to the development of irreversible damage of the myocardium and ultimately causes infarct-like necrosis in heart muscle. β-adrenergic overstimulation by ISO upset the balance between the oxygen demand and supply of the myocardium leading to pathological alteration. The underlying mechanisms of ISO-induced cardiac injury are complex and multifactorial, but the major drawbacks of the ISO therapy are the generation of cytotoxic free radicals in myocytes followed by oxidative stress and lipid peroxidation, which leads to progressive mitochondrial damage, inflammatory cytokines production, ionic imbalance including intracellular Ca^{2+} overloading that result in cardiac injury. Previous studies on this matter showed that ISO-induced myocardial damage involves apoptosis, and necrosis, moreover apoptosis seems to be an important complicating factor of myocardial injuries increasing the degree of myocyte cell death, which eventually leads to irreversible damages. However, the role of autophagy, and its connection with apoptosis and necrosis under this condition remains to be elucidated. Taken together our aims were to investigate the autophagy and cell death pathways including apoptosis and necrosis in ISO-induced cardiac injury in a dose-dependent manner.

2. Materials and methods

2.1. Animals and study design

The experiments were accomplished using adult female rats (Charles River Laboratories), with a body weight range of 250–300 g during the doxorubicin experiment, meanwhile adult male rats (Charles River Laboratories) with a body weight range of 300–350 g were used in the other study. All animals were housed and treated according to the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication no. 86-23, revised in 1996). Maintenance and treatment of animals used in this study was additionally approved by the Institutional Animal Care and Use Committee of the University of Debrecen, Debrecen, Hungary. The animals were housed in wire-bottomed cages throughout the study and were maintained on 12:12-h light-dark cycle; and provided with laboratory rodent chew pellets and water ad libitum.

Sprague–Dawley rats were randomly divided into four groups as follows I., control group- animals received water per os, and saline intraperitoneally for a time period of 2 weeks; II., MET group- animals received MET at a dose of 250 mg/kg every day orally via gavage and were injected with saline intraperitoneally; III., DOX group- animals received DOX at the dose of 3 mg/kg every second day intraperitoneally (the cumulative dose was 18 mg/kg), and received water orally; IV., DOX+MET group- animals received DOX intraperitoneally every second day and MET each day orally at the above-mentioned dose. MET was dissolved in saline, and animals were sacrificed 24 h after the last dose of MET and DOX. Male Sprague-Dawley rats were randomly divided into six groups as follows I. control group- animals received saline intraperitoneally, II. 0,005 mg/kg ISO, III. 0,05 mg/kg ISO, IV. 0,5 mg/kg ISO, V. 5,0 mg/kg ISO, and VI. 50,0 mg/kg ISO. ISO was freshly prepared in saline, and rats were injected with a single intraperitoneal ISO injection (i.p.) in each group. Animals were sacrificed 24 h after the ISO injection.

2.2. Isolated working heart preparation and cardiac function assessment

Following the 2-week treatment with vehicle, MET, and DOX respectively rats were anesthetized with i.p. injection of pentobarbital (60 mg/kg), and heparin (1000 IU/kg) was intraperitoneally administered as an anticoagulant. Blood samples were collected from the left jugular vein. Then, thoracotomy was carried out under terminal anesthesia, followed by excision of the heart. The aorta was cannulated and perfused in Langendorff "non-working" mode. Oxygenated Krebs–Henseleit bicarbonate buffer (118.5 NaCl, 4.7 KCl, 2.5 CaCl2 _ 2H2O, 25 NaHCO3, 1.2 KH2PO4, 1.2 MgSO4, and 10.0 glucose (in mM) was the perfusion medium. In the meantime, the pulmonary vein was also cannulated, and the system was switched to the "working" mode, as previously described by Neely et al., modified by Yamamoto et al. and Tosaki and Hellegouarch. After 10 min of aerobic perfusion, the basic cardiac function was registered. Thus, aortic pressure (AOP) and heart rate (HR) were registered using a computer acquisition system (ADInstruments, PowerLab, Castle Hill, Australia). Coronary flow (CF) was obtained by timed collection of the coronary flow, and aortic flow (AF) was measured by a calibrated flow-meter. Cardiac output (CO) was calculated as the sum of AF and CF, and stroke volume (SV) was generated as the quotient of CO/HR.

2.3. Assessment of serum biomarkers

After the doxorubicin and isoproterenol studies blood samples were obtained from the left jugular vein, and the serum was separated for measurement of LDH, CK-MB and Troponin T (TrT). For the in vitro quantitative determination of the serum biomarkers electrochemiluminescence immunoassay "ECLIA" was used and measured by Roche/Hitachi cobas immunoassay analyzers.

2.4. Lipidperoxidation assay

The level of MDA (malondialdehyde) in the heart tissue was detected by using a lipid peroxidation assay kit (Sigma-Aldrich, St. Louis, MO, USA). Lipid peroxidation was determined by the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric product, proportional to the MDA present. The intensity of the color was measured spectrophotometrically at 532 nm

2.5. Histopathological examination

For histopathological examination, cardiac tissues were collected from all studied groups and dissected and fixed in 4% formaldehyde solution. The heart samples were embedded in paraffin, and five micron-thick sections were sliced. Standard histological methods (xylol) were used in order to remove paraffin, and the samples were passed through a gradual alcohol series and hydrated. Trichrome staining was used to demonstrate the general histological structure; it is a three-color staining process, which differentially stains the nucleus, muscle tissue, and collagen. It was performed by using Trichrome Stain Kit (Abcam, Cambridge, UK). The sections were studied under Olympus CX-31 microscope and photomicrographs were taken using Olympus DP74 camera (Olympus Corporation, Tokyo, Japan) at 4X and 40X magnification.

2.6. Infarct size measurement

After 10 min of aerobic perfusion triphenyl tetrazolium chloride (TTC) staining was carried out to access the infarct size. Briefly, following 10 min of aerob perfusion the hearts were perfused with a 50 mL 1% (*w*/*v*) solution of TTC in a phosphate buffer (pH 7.4) and the samples were stored at −20 °C for subsequent analysis. The frozen sections were cut, subsequently scanned on an Epson J232D flat-bed scanner (Seiko Epson Corporation, Nagano Japan), blotted dry and weighted. The infarcted area (white coloration) and the risk area (entire scanned section) were analyzed using planimetry software (Image J, National Institute of Health, Bethesda, MD, USA). Estimates of the infarcted zone magnitude were subsequently obtained by multiplying the infarcted areas by the weight of each slice. The resulting numbers represent the weight of the risk zone and the infarcted zone. Outcomes were expressed as a percentage of the weight of the infarcted tissue and the weight of risk zone.

2.7. TUNEL assay

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). DNA fragmentation, sign of early stage of apoptosis, can be detected by labeling the free 3'-OH termin with modified nucleotides in an enzymatic reaction. The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes the template-independent

polymerazation of deoxyribonucleotides to the 3'-end of single- and double-stranded DNA. Harvested heart tissues were fixed in 4% formalin for 24 hours at 4 °C, embedded in paraffin, and cut into 4.5 micron thick sections. All tissue sections were placed on Superfrost Plus glass slides (Thermo Scientific, Rockford, IL), then sections were deparaffined in xylene and actone afterwards rehydrated in 70% ethanol and water. The sections were boiled in citrate buffer pH 6.0 for 6 min, then cooled at room temperature for 10 min, thereafter washed two times for 5 min in phosphate-buffer salin (PBS pH 7.4). Finally, sections were incubated with TdT (terminal deoxynucleotidyl transferase) in a humidified box, at 37 °C for 1 hour. After washing, to identify nuclei, we used DAPI (4′,6-diamidino-2-phenylindole), which emits blue fluorescence upon binding to AT regions of DNA (Thermo Fisher Scientific, Waltham, MA, USA). The slides were washed with PBS, after air-dried subsequently covered with mounting medium and glass slide covers. Moviol solution was used as mounting medium. Fluorescence microscopic images were obtained by a Zeiss Axioscope A1 microscope with HBO100 illuminator (Zeiss, Jena, Germany). After merging the blue and red channels, purple spots were associated with apoptotic nucleus, while blue spots were identified as non-apoptotic nucleus (ZEN 2012 software, Zeiss, Jena, Germany). Apoptosis was quantified by ratio of TdT-positive nuclei / total nuclei in each section..

2.8. Western blot

The expression level of AMPK, p-AMPK, Beclin-1, LC3-II, p62 and Caspase-3 proteins in left ventricular tissue was evaluated using Western blot analysis. Approximately 300 mg of heart tissues were homogenized by using a polytron homogenizer in isolating buffer (25 mM Tris-HCL, 25 mM NaCl, 1 mM orthovanadate, 10 mM NaF, 10 mM pyrophosphate, 10 mM okadaic acid, 0.5 mM EDTA, 1 mM PMSF, and 1X protease inhibitor cocktail) and centrifuged at 2000 rpm at 4 °C for 10 min. The supernatant was transferred to a new tube and centrifuged at 10,000 rpm at 4 °C for 20 min, and then the supernatant was used as cytosolic fraction. Protein concentration was measured by a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). A total of 75 _g of protein in each sample was loaded and separated using electrophoresis on a polyacrylamide gel (TGX Stain-FreeTM FastCastTM Acrylamide Kit, 12%, Bio-Rad, Hercules, CA, USA) and transferred onto a PVDF membrane. Nonspecific binding sites were blocked with 7% skimmed milk in Tris-buffered saline solution with 0.5% Tween 20 for 1 hour at room temperature. Membranes were incubated overnight at 4 °C with primary antibodies directed against AMPK, p-AMPK, Beclin-1, LC3-II, p62 and Caspase-3 (Cell Signaling

Technology, MA, USA). After washing the membranes, they were incubated with corresponding horseradish-peroxidase-conjugated secondary antibodies for 1.5 h at room temperature and signal intensities for each protein band was detected using ClarityWestern ECL Substrate (Bio-Rad, California, USA). The optical density of bands was measured using the ChemiDoc Touch Imaging System (Bio-Rad, California USA). The level of the protein of interest was normalized against the total amount of protein in each lane with the Bio-Rad Image Lab 5.2.1 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.9. Statistical analysis

The data was analyzed by IBMSPSS Statistics 22.0 statistical software (IBMCorporation, New York, NY, USA) The data are expressed as mean ± standard error of the mean (mean ± SE). The significance of differences among groups was evaluated with one-way analysis of variance (ANOVA) followed by the Tukey comparison test. For the histopathological examination Mann–Whitney test was used to evaluate the data. In case of survival rate Chi-square and Fisher's exact test was performed. Henceforth, 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.

3. Results

3.1. DOX and MET effects on cardiac function in isolated hearts

The cardiac function in hearts isolated from the animals 24 h after the last dose of MET and/or DOX. The aortic flow (AF) was significantly lower ($P < 0.05$) in the DOX group compared to the control group. This value was significantly higher in DOX+MET group. The coronary flow (CF) showed no significant differences between the control and the treated groups. No significant difference in aortic pressure (AOP) among the four studied groups. We observed lower heart rate (HR) (not significant) in DOX-treated group compared to the drug-free control group, while in the DOX+MET group the values were almost at a same level than the control group. Furthermore, a significantly decrease ($P < 0.05$) in cardiac output (CO) and stroke volume (SV) in DOX group compared to the control group was detected. In the DOX+MET treated group, the CO was at a significantly higher ($P < 0.05$) level, while SV was slightly increased (not significant).

3.2. DOX and MET effects on serum biomarkers

The administration of 6 x 3 mg/kg of DOX alone resulted in a slight increase in serum enzyme activities such as LDH and CK-MB isotype compared to the control group, meanwhile in the DOX+MET group, these enzymes remained at a lower level compared to the DOX-treated group. In addition, we have found a significantly higher ($P < 0.05$) Troponin-T level in DOX group compared to the control, while 250 mg/kg MET treatment resulted in a significantly lower level of Troponin-T compared to the DOX group, indicating that MET is able to reduce the detrimental effect of DOX.

3.3. DOX and MET effects on lipidperoxidation

One of the major contributors to DOX toxicity is the oxidative stress. The result of the present study showed that injection of 6x3 mg/kg DOX was associated with a considerably elevated level of MDA compared to the control group. While in the DOX+MET treated group the MDA level was significantly lower ($P<0.05$) than in the DOX group.

3.4. DOX and MET effects on histopathological features

DOX-induced cardiotoxicity was further assessed using trichrome staining. Cardiac tissues were stained with aniline blue for detection of fibrillar collagen and hence fibrosis. Two weeks of treatment with DOX and/or MET did not show any visible differences in the amount of collagen deposit in the treated groups. However, it is important to highlight that the myofibrillum in DOX-treated groups were significantly thinner ($P < 0.05$) than in the control group. Furthermore, the diameter of myocytes in DOX+MET group was almost at the same than in the control group.

3.5. DOX and MET effects on AMPK and autophagic markers

Left ventricular tissue levels of AMPK were elevated in all three groups in comparison with the control value, but these changes were not at a significant level. Beclin-1 showed a significantly increased ($P<0.05$) expression level in the MET group compared to the control group, while the DOX and DOX+MET groups did not show any changes in comparison with the control group. LC3-II was significantly decreased ($P<0.05$) in DOX group compared to the control group, while in the DOX+MET group its expression was increased (none significant) compared to the DOX group. P62, a protein that recognizes toxic cellular waste, which is consequently scavenged by a sequestration process, we experienced the exact opposite changes. A significantly increased ($P<0.05$) p62 level was detected in the DOX group compared to the control group, meanwhile DOX co-administered with MET (DOX+MET), the expression of p62 was considerably lower, indicating that DOX treatment impaired autophagic protein clearance and it is restored via MET treatment.

3.6. ISO effects on the survival rate

The survival rate of animals was dose-dependently decreased with the increase of intraperitoneal administration of ISO. Thus, at the highest doses of 0,5 mg/kg, 5,0 mg/kg and 50,0 mg/kg of ISO, the survival rate was significantly decreased in comparison with the control value, respectively. ISO 50 reached the median lethal dose (LD_{50}) value.

3.7. ISO effects on the serum biomarkers

The 0,005 mg/kg, 0,05 mg/kg and 0,5 mg/kg of ISO administration resulted in a gradual slight increase in serum enzyme activities of Troponin T, creatine kinase isoenzyme MB (CK-MB) and lactate dehydrogenase (LDH) in comparison with the vehicle-treated control following 24 hours of ISO administration. In addition, we have found a significantly higher (p<0.05) Troponin T and CK-MB level at 5,0 mg/kg of ISO and 50,0 mg/kg of ISO groups, and a significantly (p<0.05) elevated LDH level at 50,0 mg/kg of ISO group compared to the vehicle treatment, indicating the presence of acute myocardial damage.

3.8. TTC staining (necrosis)

To further confirm myocardial damage, TTC staining was carried out to measure infarct size. In line with the serum biomarkers, in hearts originated from animals challenged by smaller doses of ISO (0,005 mg/kg; 0,05 mg/kg) no alterations were observed in comparison with hearts obtained from the drug-free control animals. However, starting from a dose of 0,5 mg/kg of ISO, a gradually increasing infarct size was observed. Infarct size at higher doses including ISO 5,0 and ISO 50,0 was significantly enlarged compared to the vehicle-treated group.

3.9. TUNEL assay and Caspase-3 (apoptosis)

To evaluate the effect of ISO on cardiomyocyte apoptosis, we first analyzed DNA fragmentation by TUNEL assay, which is the hallmark of the early stage of apoptosis. We found a significantly elevated level of TdT+ cells at 0,05 mg/kg of ISO; 0,5 mg/kg of ISO; 5,0 mg/kg of ISO and 50,0 mg/kg of ISO groups compared to vehicle. In agreement with Western blot analyses of Caspase-3, results demonstrated that the level of apoptosis was increased depending on ISO concentration. It reached the highest level in ISO 5,0 group and it slightly decreased in ISO 50,0 group.

3.10. Autophagic markers

In order to monitor autophagic pathway, the levels of Beclin-1, LC3B-II and p62 were measured by Western blot. Left ventricular tissue levels of Beclin-1, which is an initiator in the macro-autophagic pathway, were significantly (p<0.05) elevated at 50,0 mg/kg of ISO group in comparison with vehicle. A significantly increased LC3B-II and p62 expression levels were detected in ISO 5,0 and ISO 50,0 groups compared to the vehicle-treated group, indicating that ISO treatment impaired the autophagic process in a dose-dependent manner.

4. Discussion

The major limitation of DOX treatment is the acute or chronic cardiac toxicity, which may manifest in heart failure. Acute toxicity could be reversible with adequate treatment; however, life expectancy of patient possessing DOX-induced heart failure is currently unclear. The underlying mechanism appears to be multifactorial; however, enhanced oxidative stress is one of the major contributor. Indeed, cells fail to cope with enhanced amount of reactive oxygen, and nitrogen species and enhanced oxidative stress leads to DNA and protein damage and mitochondrial dysfunction. In line with the literature, our results also show elevated oxidative stress evidenced by enhanced level of MDA in DOX-treated animals. Increased oxidative stress was accompanied by impaired left ventricular functions including AF, CO and SV in DOX-treated animals compared to the control group. MET-alone treatment did not alter significantly the heart function and MDA levels. However, our results revealed that MET co-treatment decreased oxidative stress and improved myocardial function. In the present study, cardiac damage induced by DOX, serum levels of LDH, CK-MB and Troponin T were measured. Elevations in LDH and CK-MB levels represent their leakage from the damaged membranes of cardiomyocytes into the circulation, and were previously shown to be an indicator for cardiotoxicity. The administration of 6 x 3 mg/kg DOX alone resulted in a slight elevation in serum enzyme levels in LDH and CK-MB, and serum Troponin T levels, which refer to the development of cardiotoxicity of DOX. Troponin T molecule is consisting of amino acid sequences that are found only in cardiac tissue, making it highly specific for detecting cardiac damage. Meanwhile, in the DOX+MET group, the levels of LDH and CK-MB remained at a lower level, and a significantly lower level of Troponin T was also detected. Our results revealed that the daily administration of MET significantly attenuated the rise of LDH, CK-MB and Troponin T enzyme levels during the DOX treatment, while the administration of MET alone did not show any significant changes in serum enzyme levels. Confirming the cardioprotective effect of MET in DOX-induced cardiac toxicity, histopathological analysis revealed that DOX treatment altered the size of myocytes, which was restored in the presence of MET treatment. Earlier, Ashour and co-workers reported myofibrillar loss and derangement with abnormal mitochondria in DOX-treated myocardium, which was partially normalized when MET was administered to animals.

Considering autophagy as a mechanism to restore energy status and to clear damaged macromolecules, the expression of LC3B-II was studied, which is considered as a major marker of the macroautophagy. Upon DOX treatment a significantly reduced level of LC3B-II protein expression was found, suggesting that suppression of autophagy contributes to the detrimental effect of DOX. Results with p62 protein strongly supports this hypothesis. One function of p62 is directing ubiquitinated protein to autophagosome for degradation. Enhanced level of p62 indicates impaired autophagic flux, and an enhanced level of p62 in DOX-treated group suggests that autophagy is malfunctioning. Our results revealed that co-administration of MET restored autophagy since level of LC3B-II and p62 was normalized comparing to controls. Our results support the suggestion that induction of autophagy before and during DOX treatment serves as a protective mechanisms against cardiovascular complications.

In order to study the underlying mechanisms of autophagy induction, the phosphorylation level of AMPK was studied in different groups. AMPK is an energy sensor of the cells, and MET induces a mild inhibition of respiratory-chain complex I in the mitochondria, thereby influencing AMP/ATP ratio leading to AMPK activation. As expected, an enhanced level of p-AMPK in MET group was detected, since AMPK is one of the target molecules of MET medication. Literature is more complex in the effect of DOX treatment on the mechanism of AMPK activation/deactivation. The same level of p-AMPK, like in samples originated from DOX-treated group, was observed, however, it was not accompanied by enhanced autophagy. It is to be speculated that enhanced phosphorylation of AMPK is not sufficient trigger to induce functioning autophagy if DOX is presented, or even if autophagy is being initiated the whole process is not terminated. Moreover, a significantly elevated level of p-AMPK in animals treated with DOX and MET at the same time was measured, which was accompanied by a decreased level of p62 indicating that autophagy is completed and damaged macromolecules are cleared.

Taken together the results presented herein, confirmed the cardioprotective ability of MET in DOX-induced cardiac complications. The obtained results suggest that DOX treatment impairs the autophagic processes, and damaged macromolecules cannot be degraded in the cells, and co-administration of MET with DOX restores the autophagic activity and confers cardioprotection.

To further highlight the importance of autophagy, we investigated the autophagic processes and the the death pathways, including apoptosis and necrosis in ISO induced cardiotoxicity. The major clinical limitation of ISO therapy, in a long term period, is cardiac toxicity, which consequently leads to irreversible injuries in the myocardium. The underlying mechanisms are

multifactorial and still not well-understood. The injection of ISO in animals provides a rapid and simple method, developing myocardial damages similar to that seen in acute myocardial infarction in patients. In the present study, we investigated the role of autophagy, and two different cell death pathways; apoptosis and necrosis following the administration of ISO at different doses. In line with the literature, the dose-dependent increase was seen in the serum level of cardiac- specific Troponin T, CK-MB and LDH. Furthermore, enlarged infarct size at the higher dose indicates the infarct-like cardiac damage.

Autophagy and apoptosis often appear in the same cell and control the turnover of damaged or long-lived organelles in cells. In most cases autophagy precedes apoptosis and the autophagy could block the apoptosis, although in some cases autophagy may help to induce apoptosis together with necrosis, however, it has important consequences when the interruption occurs between autophagy and apoptosis. To analyse the autophagic and apoptotic pathways after ISO injection, Beclin-1, LC3B-II, p62 and Caspase-3 expression levels were measured in left ventricular tissue. LC3B-II and p62 are considered as major markers of autophagy processes. Enhanced level of LC3B-II indicates enhanced autophagy in a dose-dependent manner by ISO treatment. p62 is an autophagy substrate that is used as a reporter of autophagy activity, and p62 is widely used as a predictor of autophagic flux. The administration of 5,0 mg/kg and 50,0 mg/kg ISO resulted in a significant p62 protein levels indicating the accumulation of damaged macromolecules and suggests that the autophagy process is malfunctioning. Based on these findings we may assume that after the administration of ISO at lower doses the autophagy may serve as a survival pathway, however treating with a high dose of ISO the cells fail to adapt and cope with the increasing stress. DNA fragmentation represents a characteristic hallmark of apoptotic cell death, and TUNEL is a well-established method for its detection. Treatment of ISO 0,5; ISO 5,0 and ISO 50,0 enhanced the level of TdT+ nuclei, which was expertly correlated with the expression level of Caspase-3. These data suggest that autophagy is malfunctioning, which leads to the accumulation of removal of damaged proteins and organelles. This impairment of the autophagic pathway was detrimental for the cardiomyocytes, which eventually induced cell death and triggered the apoptotic pathway. During the administration of the highest doses of isoproterenol, autophagy could have been a survival mechanism for cells, however, it failed to be completed and promoted the interplay of autophagy and apoptosis. Interestingly, we observed a diminished level of apoptosis in the ISO 50,0 group, moreover, in this group, the level of lysosomal degradation during the autophagic process was extremely damaged. Based on these results, we may assume that under this experimental condition there was a disruption of the cross-talk between autophagy and

apoptosis, and the extremely high unsupplied oxygen demand lead to an acute and significant increment of myocardial necrosis. Our hypothesis is further supported by the results of survival rate and serum biomarkers, which were significantly evaluated in ISO 50 group. In conclusion, our results indicate that ISO treatment stimulated autophagy in a dose-dependent manner, although ISO treatment at high doses including ISO 5,0 and ISO 50,0 this pathway was impaired, and apoptosis and necrosis take place in the heart tissue. Thus, in such cases, understanding the reason for the incomplete degradation and the excessive cell death, and then specific stimulation and boosting of the autophagic pathway might be a novel strategy to enhance the survival rate. Under conditions, such as tachycardia and/or myocardial injury induced by ISO, this could be a protective mechanism against the extreme activation of death

5. Summery

DOX is a widely-used antineoplastic agent, however one of the most prominent side effects of DOX is its cumulative and irreversible cardiotoxicity. The molecular mechanisms are still poorly understood, but oxidative stress, mitochondrial dysfunction, and the dysregulation of autophagy are involved. The objective of the current study was to investigate the protective role of MET and its effect on autophagy in DOX-induced cardiotoxicity. Sprague–Dawley rats were segregated into four groups at random. The DOX-group received DOX (3 mg/kg every second day) intraperitoneally. The MET-group received 250 mg/kg/day MET via gavage. The DOX+MET-group received both at the aforementioned doses. Based on our findings MET conferred increased cardioprotection against the development of cardiotoxicity manifested by a significant decrease in serum Troponin T and cardiac MDA levels, and improvement in heart function in connection with histopathological features. Moreover, by focusing on the contribution of autophagic proteins, we have found that MET restored autophagy, which could be survival pathway for cardiomyocytes in DOX-induced toxicity. These results indicate that the administration MET, might be a preferable drug for patients receiving DOX.

To further study the importance of autophagy, during cardiotoxicity we administered isoproterenol (ISO) at different doses (0,005 mg/kg ISO: 0,05 mg/kg ISO: 0,5 mg/kg ISO: 5,0 mg/kg ISO: 50,0 mg/kg ISO), ISO is a catecholamine and bearing a non-selective β-adrenergic agonist property. However, the major clinical limitation of ISO administration is its cardiotoxicity, which is complex and multifactorial, including the generation of cytotoxic free radicals in myocytes followed by oxidative stress and lipid peroxidation. We investigated autophagic processes and the two death pathways, including apoptosis and necrosis. Based on our results, we may assume that under this experimental condition the autophagy is malfunctioning in dose-dependent manner, thus at high doses including ISO 5, and ISO 50,0, apoptosis and necrosis take place in the heart tissue.

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List of publications related to the dissertation

1. Gyöngyösi, A., **Zilinyi, R.**, Czeglédi, A., Tósaki, Á., Tósaki, Á., Lekli, I.: The role of autophagy and death pathways in dose-dependent isoproterenol-induced cardiotoxicity.
Curr. Pharm. Design. [Epub ahead of print], 2019.
DOI: http://dx.doi.org/10.2174/1381612825666190619145025
IF: 2.412 (2018)

2. **Zilinyi, R.**, Czompa, A., Czeglédi, A., Gajtkó, A., Pituk, D., Lekli, I., Tósaki, Á.: The Cardioprotective Effect of Metformin in Doxorubicin-Induced Cardiotoxicity: the Role of Autophagy.
Molecules. 23 (5), 1-12, 2018.
DOI: http://dx.doi.org/10.3390/molecules23051184
IF: 3.06

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List of other publications

3. Czeglédi, A., Tósaki, Á., Gyöngyösi, A., **Zilinyi, R.**, Tósaki, Á., Lekli, I.: Electrically-Induced Ventricular Fibrillation Alters Cardiovascular Function and Expression of Apoptotic and Autophagic Proteins in Rat Hearts.
Int. J. Mol. Sci. 20 (7), 1628-, 2019.
DOI: http://dx.doi.org/10.3390/ijms20071628
IF: 4.183 (2018)

Total IF of journals (all publications): 9,655
Total IF of journals (publications related to the dissertation): 5,472

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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