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Quercetin inhibits the expression of *MYC* and *CYP2E1* and reduces oxidative stress in the myocardium of spontaneously hypertensive rats

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Oxidative stress is one of the most important pathological processes in chronic heart failure caused by hypertension. These processes involve MYC-regulated mechanisms, including the induction of CYP2E1 as a potent prooxidant factor. In this work, we used qPCR, Western blot analysis, and biochemical markers of oxidative stress to investigate the ability of quercetin to inhibit oxidative stress by modulating MYC expression. We studied spontaneously hypertensive rats (SHRs) in which the onset of cardiac pathology was observed at least at 4 months of age and the development of pathology occurred during life up to 22 months of age. Wistar rats were used as normotensive controls. We observed overexpression of the transcription factor MYC (p=0.0024) in the myocardium of SHRs compared to normotensive controls, and an increased expression of MYC-target gene, *CYP2E1*, (p=0.0001) in the old SHR group compared to young SHRs. This probably contributed significantly to the development of oxidative stress in the cardiac tissue of old SHRs. We demonstrated that long-term treatment of old SHRs with quercetin resulted in dramatic inhibition of *MYC* (p=0.0000), and a significant decrease in CYP2E1 (p=0.0001) expression and CYP2E1 protein levels (p=0.0136). This probably contributed significantly to the decrease in lipid peroxidation (p=0.0000). Quercetin was also able to activate antioxidant activity, resulting in a significant improvement in the prooxidant-antioxidant balance in the heart. In turn, the elimination of oxidative stress could contribute to a decrease in blood pressure (p=0.0000) and relative heart weight (p=0.0071) in quercetin-treated old SHRs compared to the untreated old SHR group.

Keywords: CYP2E1, hypertension, MYC, myocardium, oxidative stress, quercetin

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Abbreviations: cDNA, complementary deoxyribonucleic acid; CHF, chronic heart failure; CYP2E1 (*CYP2E1*), protein (gene) of cytochrome P450 2E1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; LPO, lipid peroxidation; MYC (*MYC*), protein (gene) of bHLH transcription factor; NADH, nicotinamide adenine dinucleotide (reduced form); qPCR, quantitative polymerase chain reaction; RNA, ribonucleic acid; ROS, reactive oxygen species; SHR, spontaneously hypertensive rat; SOD, superoxide dismutase

INTRODUCTION

Hypertension is one of the most common pathologies of the cardiovascular system, characterized by chronic high blood pressure. This is one of the main factors in the development of chronic heart failure (CHF). Hypertension leads to functional overload of the myocardium (Nolly *et al.*, 2015), which significantly increases the risk of arrhythmias, stroke, and myocardial infarction.

It is known that in cardiac pathologies, the expression of genes regulating energy metabolism in the myocardium changes significantly (Kodde *et al.,* 2007). This is favored by the strong activation of some transcription factors, including MYC (Ahuja *et al.*, 2010). MYC-mediated metabolic changes have been associated with the preservation of cardiac function under stressors (Ahuja *et al.*, 2010). MYC directly regulates glucose metabolism and mitochondrial biogenesis in cardiomyocytes and is an important regulator of energy metabolism in the heart. Activation of MYC has been shown to promote the development of myocardial hypertrophy and functional overload in response to pathological conditions (Ahuja *et al.*, 2010). In turn, increased expression of MYC causes the induction of transcription of many genes, including *CYP2E1* (cytochrome P450 2E1) (Guan *et al.*, 2019). CYP2E1 is one of the major sources of cellular (especially mitochondrial) reactive oxygen species (ROS). CYP2E1 is a potent prooxidant factor in the cell, an increase in its expression leads to an intensification of peroxide processes and the development of oxidative stress, resulting in oxidative damage to cellular structures, mainly biomembranes (Zhang *et al.,* 2011; Guan *et al.*, 2019). This affects the functionality of the plasma membrane (permeability, electrical conductivity, receptor function, etc.). Mitochondrial membrane damage contributes to the initiation of apoptosis (Zhang *et al.,* 2011). Moreover, *CYP2E1* is one of the genes that control mechanotransduction, mitochondrial energy metabolism, redox balance, and myocardial extracellular matrix function (Guan *et al.,* 2019). It has been shown that an increase in CYP2E1 contributes significantly to myocardial energy supply under overload and stress conditions. On the other hand, overexpression of CYP2E1 leads to the initiation of pathological mechanisms in the myocardium, including oxidative stress and stress-associated pathological processes (Guan *et al.,* 2019).

It has been shown that a diet enriched with quercetin has a beneficial effect on the cardiovascular system. Some molecular mechanisms of the cardioprotective effects of this bioflavonoid have already been described. In particular, quercetin may prevent the development

of cardiac hypertrophy, oxidative stress, etc. (Yan *et al.*, 2013; Ghafouri-Fard *et al.*, 2021). The positive cardioprotective properties of quercetin suggest that this bioflavonoid could be used for therapeutic purposes (Larson *et al.,* 2010; Patel *et al.,* 2018). Research into the detailed molecular mechanisms of action of quercetin remains important. This is necessary for the identification of new molecular targets for the treatment of various heart diseases. In this work, we investigated the effect of long-term consumption of quercetin on the expression levels of *MYC* (as an important transcription factor in cardiac pathology) and MYC-regulated *CYP2E1* (as a major factor for oxidative stress). Prooxidant and antioxidant processes were also investigated in the myocardium of spontaneously hypertensive rats (SHRs) as a model of hypertension in the elderly (Reckelhoff *et al.,* 2006).

MATERIAL AND METHODS

Animals

Adult male rats (Wistar – normotensive rats and SHRs – Spontaneously hypertensive rats) were provided by Bogomoletz Institute of Physiology NAS of Ukraine (Kyiv, Ukraine). Spontaneously hypertensive rats have been used as a well-established model for genetic hypertension and age-related left ventricle dysfunction. The study involved 20 animals, which were divided into four experimental groups: five young Wistar rats (aged 4 months), five young SHRs (aged 4 months), five old SHRs (aged 22 months), and five old SHRs (aged 22 months) treated with quercetin. The old SHRs were administered quercetin (≥95.0%, PhytoLab, Sigma-Aldrich, Germany) once daily in the morning via oral gavage at a dose of 50 mg/kg bw per day for 30 days. The group of young Wistar animals was used as a normotensive control. A noninvasive method of tail artery blood pressure measurement using the Sphygmomanometer S-2 ("HSE", Germany) was used. The experimental rats were decapitated at the end of the experiment under sodium pentobarbital anesthesia (60 mg/kg bw).

All manipulations of laboratory animals were performed in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986). The protocol was approved by the Local Committee on Bioethics (registration number: 0114U007233).

Oxidative stress markers

Levels of lipid peroxidation (LPO), catalase, and superoxide dismutase (SOD) in heart tissue were determined as previously described (Maksymchuk *et al.,* 2015). The level of LPO was determined in heart homogenates by assessing the level of malondialdehyde (MDA). Catalase activity in heart homogenates was measured by hydrogen peroxide (H_2O_2) degradation. SOD was determined using nicotinamide adenine dinucleotide and phenazine methosulfate reagents for the reduction of nitro-blue tetrazolium salt to blue-colored formazan. MDA values were expressed as μmol per milligram of protein. Enzyme activity values were expressed as U/mg protein (one unit of catalase activity means the amount of enzyme that degrades 1 μmol of H_2O_2 per minute, one unit of SOD activity – the amount of enzyme that oxidizes 1 nmol NADH per minute).

Determination of target gene expression levels by quantitative PCR (qPCR)

RNA isolation from rat heart tissue and cDNA synthesis were performed using the GeneJET RNA purification kit and the Maxima H Minus cDNA Synthesis master mix (ThermoSci), respectively. The qPCR was performed using 5x Hot FirePol EvaGreeen qPCR Supermix (Solis Biodynes) according to the manufacturer's protocol. The specific primers were used in qPCR to quantify gene expression of *MYC* (forward primer: CAACGTCTTGGAACGTCAGA, reverse primer: CTCGCCGTTTCCTCAGTAAG) (Barathidasan *et al.,* 2013), *CYP2E1* (forward primer: TGAAAAAGCCAAGGAACACC, reverse primer: TGTGCTGGTGGTCTCAGTTC) and *GAPDH* (forward primer: CTACCCACGGCAAGTTCAAC, reverse primer: CCAGTAGACTCCACGACATAC) (Cabiati *et al.,* 2012). *GAPDH* expression was used for normalization. Results are expressed in relative units.

Western blot analysis and protein measurement

Preparation of heart samples for Western blot analysis and measurement of CYP2E1 protein levels were performed as previously described (Maksymchuk *et al.,* 2015). CYP2E1 was detected using rabbit anti-CYP2E1 antibodies (Sigma-Aldrich, USA). GAPDH (loading control) was identified using mouse anti-GAPDH antibodies (Sigma-Aldrich, USA). After treatment of the membranes with secondary antibodies (Sigma-Aldrich, USA), chemiluminescence detection was performed according to the manufacturer's instructions. Western blots were visualized and calculated using the ChemiDoc XRS + system with Image Lab software (Bio-Rad, USA). Relative protein levels were calculated by comparing CYP2E1 levels with GAPDH levels and expressed as relative units.

Statistical analysis

Statistical analysis was performed using Status software (http://status-please.herokuapp.com). Data were tested for normal distribution using the Shapiro-Wilk test. The Tukey HSD test was used for multiple comparisons. Differences in means between groups were tested with the one-way ANOVA and the *t*-test. Statistically significant results were considered at $p<0.05$. Results are expressed as mean ± standard deviation (S.D.).

RESULTS

Blood pressure and body weight of animals

In this work, we studied the physiological parameters of adult male Wistar and SHR rats. We showed that the average blood pressure in the group of young Wistar rats (Table 1) corresponded to physiologically normal values for age (Novelli *et al.,* 2007). Therefore, we defined this group of rats as a normotensive control group. We found a significant increase in blood pressure in young SHRs at 4 months of age (22%, $p=0.0000$) compared to normotensive animals of the same age (Table 1). We also found an increase (42%, $p=0.0021$) in relative heart weight in young hypertensive rats compared to young normotensive rats (Table 1). This could be one of the signs of cardiac hypertrophy that may develop in chronic hypertension (Kahan & Bergfeldt, 2005). We demonstrated that the parameters studied in the hypertensive animals increased, even more, when they reached the

Table 1. Physiological parameters of experimental animals

Animal groups	Blood pressure, mmHg	Body weight, g	Heart weight, g	Relative heart weight, %
young Wistar	129±4.2	353.4±10.19	0.839±0.034	0.237±0.015
young SHR	158±5.7*	270.6±18.35	0.912±0.133	0.337±0.047*
old SHR	185±6.1#	391.0±26.39	1.628±0.362	0.414±0.079
old SHR/Quercetin	149±4.2•	444.4±29.16	1.262±0.095	0.284±0.015•

Values are means±S.D., n=5 rats in each group. *$p \leq 0.001$ compared to the Wistar group, #$p \leq 0.001$ compared to the young SHR group, •$p \leq 0.01$ compared to the old SHR group (One-way ANOVA)

Table 2. Oxidative stress markers in the heart of experimental animals

Animal groups	Catalase, U/mg	Superoxide dismutase, U/mg	Malondialdehyde, μmol/mg
young Wistar	96.37 ± 6.51	2.94 ± 0.27	5.9 ± 0.42
young SHR	68.94 ± 10.12*	2.07 ± 0.12*	8.64 ± 0.64*
old SHR	54.45 ± 9.34#	1.52 ± 0.14#	12.13 ± 0.87#
old SHR /Quercetin	107.32 ± 17.67•	2.93 ± 0.07•	8.68 ± 0.26•

Values are means ± S.D., n=5 rats in each group. *$p \leq 0.001$ compared to the Wistar group, #$p \leq 0.05$ compared to the young SHR group, •$p \leq 0.001$ compared to the old SHR group (One-way AOVA)

age of 22 months. We found an increase in blood pressure (17%, p=0.0001) and relative heart weight (23%, p=0.0995) in old SHRs compared to the group of young SHRs (Table 1).

Treatment of old hypertensive rats with quercetin resulted in a reduction in blood pressure (20%, p=0.0000) and relative heart weight (32%, p=0.0071) compared to untreated old SHRs. Thus, quercetin treatment contributed to the return of these indicators to the level of young SHRs (Table 1).

Oxidative stress markers in the heart of animals

It is well known that oxidative stress is one of the most important pathological processes in CHF caused by chronic hypertension (Rodrigo *et al.,* 2011). In this work, we found evidence of oxidative stress in the heart of SHRs. A significant decrease in the activities of the antioxidant enzymes catalase (29%, p=0.0009) and SOD (30%, p=0.0002) was observed in young SHRs compared to age-matched normotensive controls. At the same time, we found an increase in MDA content (45%, p=0.0000), which may indicate a dramatic intensification of peroxide processes (Table 2). It should be noted that significant changes in these parameters were observed in the heart of the old hypertensive rats in comparison with the young SHR group: a decrease in the activities of the antioxidant enzymes (catalase, 21%, p=0.0467; SOD, 27%, p=0.0002) and an increase in MDA content (40%, p=0.0001) (Table 2).

Treatment of old hypertensive rats with quercetin significantly improved the prooxidant-antioxidant balance and caused an increase in the activity of antioxidant enzymes: catalase (1.9-fold, p=0.0004) and SOD (2-fold, p=0.0000). At the same time, the MDA level decreased by 29% (p=0.0000) compared to untreated old hypertensive animals (Table 2). It should be noted that the prooxidant-antioxidant balance also improved compared to the young SHR group (Table 2).

MYC expression level in the myocardium

It is well known that MYC controls transcriptional regulation of cardiac metabolism and mitochondrial biogenesis in response to pathological stress (Ahuja *et al.,* 2010). In this work, a large increase (8-fold, p=0.0024) in the expression of *MYC* was observed in the myocardium of young SHRs compared to the age-matched normotensive control (Fig. 1a). It should be noted that the expression of this transcription factor in hypertensive rats contin-

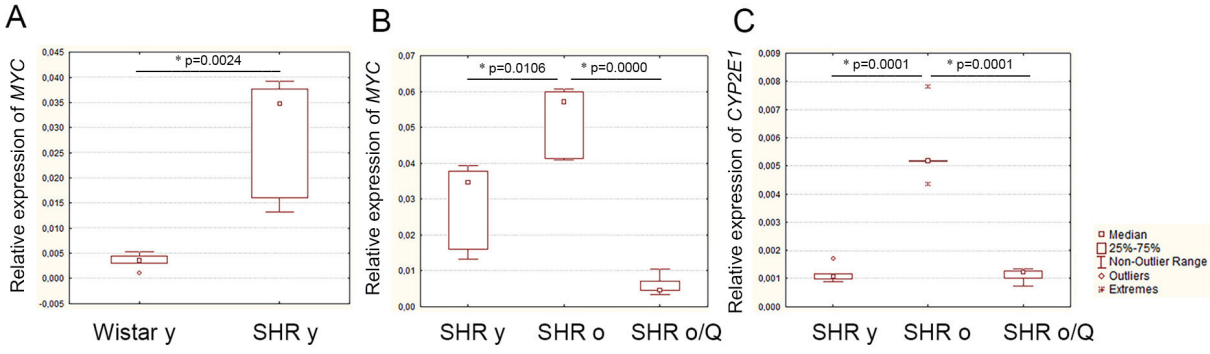

Figure 1. Gene expression in the myocardium of experimental rats.
(**A**) *MYC* expression in the myocardium of young rats. (**B**) *MYC* and (**C**) *CYP2E1*expression in the myocardium of spontaneously hypertensive rats. *Wistar y* – normotensive young rats, *SHR y* – spontaneously hypertensive young rats, *SHR o* – spontaneously hypertensive old rats, *SHR o/Q* – spontaneously hypertensive old rats treated with quercetin, *p-values <0.05 were considered statistically significant, (**A**) Student's t-test, (**B**) and (**C**) One-way ANOVA, n=5 in each group

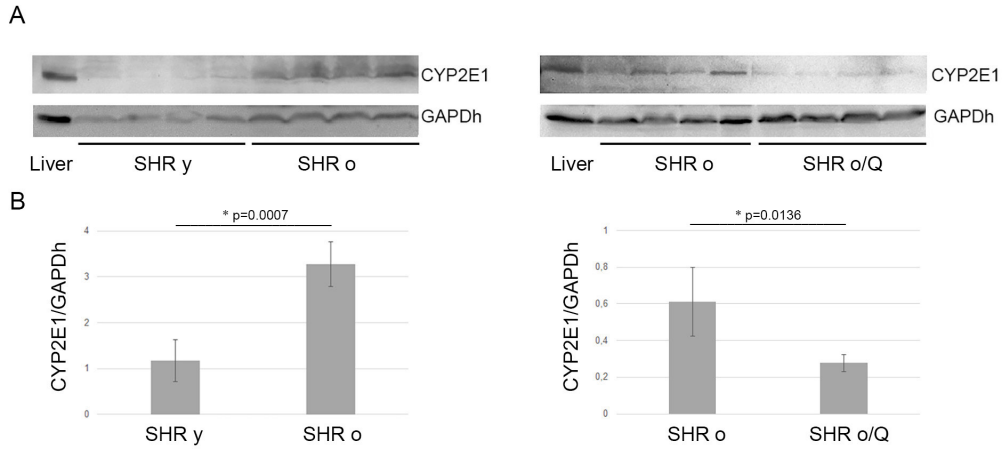

Figure 2. CYP2E1 protein levels in the heart of experimental rats.
(**A**) Western blot analysis of total heart lysates probed with specific anti-CYP2E1 antibodies. GAPDh is a loading control. **(B)** Quantification of Western blotting results. *SHR y* – spontaneously hypertensive young rats, *SHR o* – spontaneously hypertensive old rats, *SHR o/Q* – spontaneously hypertensive old rats treated with quercetin, *Liver* – a total liver lysate (as a reference control). **p*-values <0.05 were considered statistically significant, Student's *t*-test. Means ± S.D., n=4 in each group

ues to increase throughout life. We found that the expression of *MYC* was higher (1.9-fold, p=0.0106) in the myocardium of old SHRs than in the group of young SHRs (Fig. 1b).

Treatment of old SHRs with quercetin resulted in a significant decrease (8.7-fold, p=0.0000) in myocardial *MYC* expression compared to the group of untreated old SHRs (Fig. 1b). Thus, the expression of this transcription factor was much lower than in the group of young hypertensive rats (Fig. 1b).

CYP2E1 expression and protein levels in the myocardium

Since *CYP2E1* is under MYC-dependent transcriptional control, modulation of *MYC* could cause a change in CYP2E1 expression. We found a significant increase in *CYP2E1* gene expression (4.8-fold, p=0.0001) (Fig. 1c) as well as protein content (2.8-fold, p=0.0007) in the heart of old hypertensive rats compared to the young SHR group (Fig. 2).

Quercetin treatment resulted in a significant decrease in *CYP2E1* expression (4.9-fold, p=0.0001), which was accompanied by a decrease in protein content (2.2-fold, p=0.0136) in the heart of the old hypertensive rats compared to the group of untreated old SHRs (Fig. 1c and 2).

DISCUSSION

Despite modern optimal therapies, mortality of patients with cardiovascular pathology (coronary heart diseases, myocardial infarction etc.) caused by hypertension remains high. Detailed study of the pathogenesis of heart disease is necessary to prevent and correct pathological changes in the myocardium, improve the quality of life of patients, and prolong their lives. It is known that in a normal physiological state, mitochondrial fatty acid oxidation is the main source of energy production in the myocardium. In heart failure, the level of this oxidation often decreases, while glycolytic pathways become more prominent, ultimately leading to contractile dysfunction. Chronic hypertension has been shown to be one of the key factors in such pathological processes (Oh & Cho, 2020). In this work, we investigated the development of cardiac pathology using a model of spontaneously hypertensive rats. We found a steady increase in blood pressure even in young SHRs compared with normotensive animals of the same age and sex. An increase in relative heart weight was also noted in young SHRs, which may be a sign of cardiac hypertrophy. It is well known that chronic hypertension may contribute to the development of oxidative stress, one of the major pathological processes leading to structural and functional damage of the myocardium in the development of CHF (Rodrigo *et al.,* 2011; Mei *et al.*, 2015). We also found evidence of an imbalance between prooxidants and antioxidants, which may indicate the development of oxidative stress in the heart of young SHRs. Thus, the data obtained may indicate the onset of pathological processes in the heart of young hypertensive animals. These processes continued and are exacerbated during the life of the animals.

Significant metabolic changes have been shown to occur in the myocardium during hypertension-induced cardiac overload. Changes in the expression of key transcription factors (including MYC) that regulate metabolic genes in cardiomyocytes lead to transient adaptation of the myocardium to stress and also initiate pathological processes (Ahuja *et al.,* 2010; Guan *et al.*, 2019). We found strong activation of *MYC* expression in the myocardium of young hypertensive animals compared to normotensive controls. It should be noted that this activation further increased during life in hypertensive rats. It has been shown that *MYC* is expressed at a very low level in the adult heart under normal physiological conditions. However, in response to cardiac overload, the expression of *MYC* increases sharply (Xiao *et al.,* 2001). In particular, it has been shown that the expression of *MYC* was significantly increased in the myocardium of spontaneously hypertensive animals and was associated with cardiac hypertrophy and atherosclerosis (Negoro *et al.,* 1988). In our work, we also demonstrated an increase in *MYC* expression that was accompanied by an increase in relative heart weight. Such activation of MYC in adult myocardium was found to increase glucose uptake and utilization, reduce fatty acid oxidation by downregulat-

ing PPARα, and induce mitochondrial biogenesis. As a result, the function of the left ventricle remains normal despite hypertrophic changes (Ahuja *et al.,* 2010). Thus, in young hypertensive rats, we found evidence of pathological processes and a sharp increase in the expression of *MYC*, which persisted and increased until 22 months of age.

In turn, the increased expression of *MYC* may be a key factor in the induction of transcription of many genes, including *CYP2E1* in the myocardium (Guan *et al.,* 2019). We have demonstrated a significant increase in *CYP2E1* expression as well as protein content in the myocardium of old hypertensive animals compared to young SHRs. It has been reported that CYP2E1 expression levels may change significantly during the development of myocardial hypertrophy in various models of heart failure. Such an increase in CYP2E1 expression has previously been demonstrated in hypertension and acute myocardial injury, and a key factor might be the upregulation of MYC (Guan *et al.,* 2019). Clearly, increased expression of CYP2E1, a potent prooxidant factor, may be one of the most important reasons for the development of oxidative stress (Pang *et al.,* 2021) in the myocardium, for which we found evidence in our experiment. It has been shown previously that altered expression of CYP2E1 may be a marker of various pathophysiological factors and conditions in the myocardium. Increased expression of CYP2E1 plays several pathophysiological roles in the heart, including oxidative stress and apoptosis, as well as involvement in energy production and supply pathways to meet the increased energy demands of the heart under pathological conditions (Zhang *et al.,* 2011; Guan *et al.*, 2019). Therefore, an increase in CYP2E1 expression in the early stages of heart failure development may be an adaptation of the myocardium to the conditions of altered energy exchange pathways, ensuring the preservation of the contractile function of the heart. On the other hand, oxidative stress, which may be caused by increased CYP2E1 expression, is one of the crucial pathological mechanisms in the development of heart disease (Guan *et al.,* 2019; Pang *et al.,* 2021).

Thus, increased expression of *MYC* could lead to activation of CYP2E1 expression, which is associated with the development of oxidative stress and other pathological processes in the myocardium. We hypothesize that modulation of *MYC* expression level in the myocardium may help to inhibit the development of the above pathological processes (including oxidative stress) caused by hypertension. This conjecture was tested in our study. A natural agent such as quercetin was chosen as a modulatory agent because this agent has cardioprotective properties and can prevent the development of CYP2E1-induced oxidative stress (Larson *et al.,* 2010; Yan *et al.,* 2013; Maksymchuk *et al.,* 2017; Ghafouri-Fard *et al.*, 2021).

We found that treatment with quercetin resulted in a significant decrease in *MYC* expression in old SHRs compared to untreated old SHRs and young SHRs. This bioflavonoid has been shown to modulate multiple signaling pathways, including activation of PPAR (Yan *et al.*, 2013), resulting in downregulated MYC expression. In addition, quercetin has been found to directly inhibit the expression of MYC by binding to the regulatory region of the corresponding gene (Tawani *et al.,* 2017). It has been shown that inhibition of MYC can significantly inhibit myocardial hypertrophic growth (Ahuja *et al.,* 2010). In this work, we also showed a significant decrease in relative heart weight, which may be a consequence of the downregulation of this transcription factor. Since MYC induces *CYP2E1* (Guan *et al.,* 2019), suppression of *MYC* could lead to a decrease in *CYP2E1* expression, which contributes to a decrease in CYP2E1 protein content in the heart. This is exactly the effect we observed in the myocardium of animals treated with quercetin. We also noted some normalization of the balance between pro- and antioxidants in the heart, i.e., a decrease in the level of LPO and an increase in the activity of antioxidant enzymes. A decrease in the expression of CYP2E1 (as a potent prooxidant factor) could lead to a decrease in LPO levels (Pang *et al.*, 2021). Moreover, the quercetin molecule can directly inhibit the activity of CYP2E1 by blocking the active site of the enzyme (Östlund *et al.,* 2017). At the same time, this bioflavonoid can scavenge radicals and activate the expression of antioxidants (de Lacerda Alexandre *et al.*, 2021). It also contributes to the antioxidant effect of quercetin in the myocardium. It is known that quercetin can lower blood pressure and hypertrophy by reducing the manifestations of oxidative stress (Larson *et al.,* 2010; de Lacerda Alexandre *et al.*, 2021). We observed a similar effect in old-treated hypertensive rats.

CONCLUSION

Oxidative stress is one of the most important pathological processes in chronic heart failure caused by hypertension. These processes involve MYC-regulated mechanisms, including the induction of CYP2E1 as a potent prooxidant factor. We observed overexpression of the transcription factor MYC in the myocardium of SHRs compared to normotensive controls, and an increased expression of the MYC-target gene, *CYP2E1*, in the old SHR group compared to young SHRs. This probably contributed significantly to the development of oxidative stress in the cardiac tissue of old SHRs. We demonstrated that long-term treatment of old SHRs with quercetin resulted in dramatic inhibition of *MYC,* and a significant decrease in CYP2E1 expression and CYP2E1 protein levels. This probably contributed significantly to the decrease in lipid peroxidation. Quercetin was also able to activate antioxidant activity, resulting in a significant improvement in the prooxidant-antioxidant balance in the heart. In turn, the elimination of oxidative stress could contribute to a decrease in blood pressure and relative heart weight in quercetin-treated old SHRs compared to the untreated old SHR group.

Statements and Declarations

Conflict of Interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions. OM and AS contributed equally to this work and share the first authorship. OM and AS made substantial contributions to the conception and design, acquisition, and analysis of the data, and participated in the critical review of the manuscript for important intellectual content. OM performed the Western-blot analysis and RT-PCR. AS and AK examined markers of oxidative stress and performed a statistical analysis. The first draft of the manuscript was written by OM. All authors contributed to the revision of the manuscript, read and approved the submitted version.

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Data Availability Statement. All relevant data is contained within the article. The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Ethical Approval. All manipulations with laboratory animals were performed in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986). The protocol was approved by the Local Committee on Bioethics (registration number: 0114U007233).

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