THE STUDY OF MOUSE MYOCARDIAL ISCHEMIC RESISTANCE AND RADICAL DAMAGE

L. Staněk[1], A. Fučíková[2], S. Lísová[1]

[1]Institute of Pathology, General Faculty Hospital, Charles University, Prague, Czech Republic
[2]Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Veterinary Sciences, Prague, Czech Republic

The heart is a strictly aerobic body with an energetically very demanding metabolism. Constant supply of oxygen is vital for its normal function. If the oxygen supply is stopped, such as coronary artery thrombotisation, or severely limited, such as stenosis, myocardial damage occurs due to hypoxia. The amount of hypoxic damage is dependent on the duration of ischemia and also on the resistance to myocardial oxygen deprivation. This resistance is conditioned by a number of mechanisms at both the biochemical and molecular levels. The aim of this work was to demonstrate UCP2 (-/-) animals resistant to ischemic damage – which we confirmed. Further evidence of free radical damage at the time and the expression of HIF ischemie. Free radical damage at the time of expression, we have demonstrated ischemia.

myocardium; ischemia; reperfusion; free radicals; uncoupling proteins

INTRODUCTION

Oxygen supply interruption (thrombotization) or limitation (stenosis) leads to myocardial damage from hypoxia. This hypoxic damage is directly dependent on the duration of the ischemia and indirectly on myocardium resistance to oxygen deficiency. This resistance is subject to a number of mechanisms at the biochemical and molecular levels. At the molecular level, the interactions of many genes that express proteins affect myocardial resistance during the attack. One of the genes playing a key role is a gene, which encodes uncoupling proteins (UCP) and Hypoxia inducible factor (HIF). Uncoupling proteins are a subgroup of mitochondrial ROS transport proteins and are probably derived from the proton/anion channels. In a large percentage, these channels, through protein complexes, are involved in the mechanism of cellular signaling and an antioxidant defense (Ishizawa et al., 2006). UCP are located on the inner mitochondrial membrane, where protons allow for the free passage in the direction of the electrochemical gradient back into the mitochondrial matrix (Sack, 2006). This reduces the mitochondrial membrane potential, which is used by the ATP (adenosinetriphosphate) synthase to phosphorylate ADP (adenosinediphosphate) to ATP. According to recent research, this depolarization could be the basis of increased coronary resistance due to the suppression of ROS (reactive oxygen species) and the inhibition of oxidative stress. This uncoupling can only be short term, because myocardium cannot get enough energy in another way. Five currently known subtypes of UCP 1-UCP 5, which, for example, regulate a body weight (Dulloo, Samec, 2000), also play a role in cardioprotection. The most important of them for radical protection and cardioprotection is UCP2. UCP2 belongs to the subfamily which affects the ROS signaling (Canon et al., 2006). Bodyak et al., (2006) argue that expression of this protein leads to a significant decrease of ATP and the development of acidosis, but has no effect on cell survival.

Hypoxia-induced factor is a transcription factor that responds to changes in the partial pressure of oxygen in the cellular environment, specifically the reduction of oxygen, or hypoxia. Transcriptional complex HIF-1, which is a hetero-dimer composed of alpha and beta subunits (alpha and beta subunit have a similar structure), promotes blood vessel formation and acts as an activation and transcription factor for a number of genes.

In terms of biochemistry, it is mostly the effects of free radicals (ROS) during ischemia and also the resulting reperfusion. Free radicals are atoms or molecules that contain one or more unpaired electrons in an electron shell. The main danger of free radicals' existence is that they are trying to get the missing electron from the surrounding molecules, converting these molecules into secondary free radicals, and a chain reaction spreads. The aim of this work was to confirm this hypothesis in a mouse model.

Table 1. The composition of Krebs-buffer Henseleit

$NaCl$	$NaHCO_3$	KCl	$MgSO_4$	KH_2PO_4	$CaCl_2$	Glucose
118 mM	25 mM	4.7 mM	1.2 mM	1.2 mM	1.25 mM	11 mM

MATERIALS AND METHODS

The animal model

We used adult male mice with a gene deletion in the UPC2 protein and the B6 strain (Anlab), which served as a control. The animals were kept under standard conditions in plastic containers, which correspond to the size of breeding standards. The room was maintained at a constant temperature (22 ± 2°C), and alternating dark and light in a twelve-hour interval was performed.

The heart perfusion

The experiment was done on an oxygenated and tempered (37°C) perfused heart, bringing the crystalloid solution (Krebs-Henseleit solution) (Table 1) retrogradely into the aorta. The ischemia time was 55 min., when the inflow of solution was stopped. The reperfusion time was chosen to be 60 min.

Determination of an ischemia-reperfusion injury

To determine the extent of ischemia-reperfusion myocardial injury, we measured the size of the living heart tissue stained with tetrazol salts. Immediately after the experiment, the heart was flushed with 2 ml of 1% solution of TTC (2,3,5-trifenyltetrazolium) in a phosphate buffered saline (PBS) (Sigma). TTC solution was injected directely into the aortic cannula. TTC reacts efficiently with cellular dehydrogenase to form insoluble colored formazan compounds (Klein et al., 2004). Cells that do not have damaged enzyme machinery react with tetrazoliem and stain red. Damaged cells stay uncoloured. After staining, hearts were immersed for 24 hours in 10% formalin, which was used to highlight the necrotic areas. Formalin fixation also allowed for easier slicing of the heart tissue. Fixed hearts were deprived of the right ventricle and atriums, and the left ventricle was cut into 0.75 mm slices perpendicular to the long axis of theventricle. These sections were photographed from both sides using a digital camera (Olympus C4040 Zoom) and adjusted for photo processing software (Adobe Photoshop 7.0). The individual sections were analyzed using the planimetric Eclipse program.

Determination of 'ROS'

Levels of malondialdehyde (MDA) that react with primary amines to form LFP were monitored. The homogenized left myocardium ventricle was used for further experiments. As an important indicator, we determined the protein concentration according to Lowry, using a spectrophotometer (HP 8453). We have assumed that the amount of protein in the membrane influences the process of peroxidation. We determined the MDA using the thiobarbiturate acid according to the method of Asakawa and Matsushita. We then added 0.36% thiobarbiturovou acid (Penta) and 35% trichloroacetic acid (Penta) to the sample and incubated at 85°C for 60 min. After incubation, we added acetic acid (Penta) and chloroform (Fluka) and photometrically measured the concentration of the supernatant at a wavelength of 532 nm in triplicates (HP 8453) (standard solution for calibration curve LPO Oxis 586). To measure the LFP, we added a mixture of chloroform-isopropanol (Penta) (3:2) to the removed sample and shaked it for 1 hour. After centrifugation, we measured the excitation spectra of extracts from 250 to 400 nm and the emission spectra from 300 to 500 nm, increased by 10 nm on the fluorimeter (Perkin Elmer LS) again in triplicates. The concentration of fluorophores was expressed as relative fluorescence units expanded to the gram of a dry weight.

Table 2. Body weight, coronary flow (CF) and the size of myocardial infarction, expressed the percentage of left ventricular (LV), the UCP 2(-/-) and B6 controls, the number of animals

	UCP 2	B6
Number of animals	9	8
Animal weight (g)	34 ± 0.67	32 ± 0.25
CF default (ml/min)	2.3 ± 0.08	2.2 ± 0.07
CF at reperfusion (ml/min)	2.2 ± 0.08	2.0 ± 0.05
Infarction size (% LV)	18 ± 1.11	16 ± 0.53

Table 3. Body weight, coronary flow (CF) and the size of myocardial infarction, expressed the percentage of left ventricular (LV) in UCP 2 (-/-) and B6 controls after extension ischemia for 55 min, the number of animals

	UCP 2	B6
Number of animals	8	7
Animal weight (g)	34.5 ± 0.37	34 ± 0.29
CF default (ml/min)	2.3 ± 0.06	2.2 ± 0.03
CF at reperfusion (ml/min)	2.1 ± 0.06	2.1 ± 0.06
Infarction size (% LV)	44 ± 2.06	47 ± 1.22

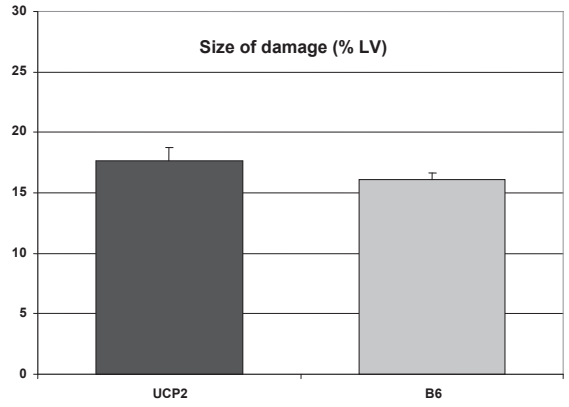

Fig. 1. The size of myocardial infarction, expressed as a percentage of left ventricular (LV) of UCP 2 (-/-) and B6 controls

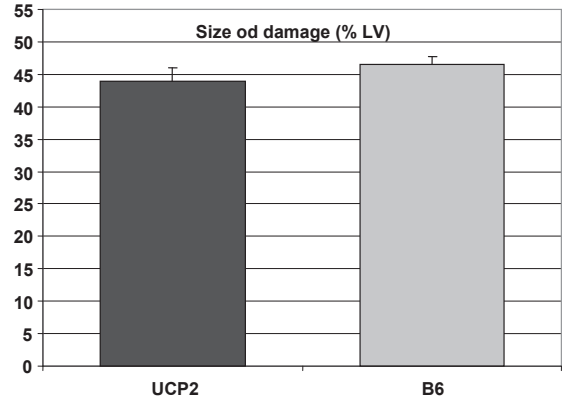

Fig. 2. The size of myocardial infarction, expressed as a percentage of left ventricular (LV) of UCP 2 (-/-) B6 and controls the extension of ischemia to 55 min

Determination of HIF-1α and 1ß expression

The detection was performed on 5–7 um fixed and paraffin embedded sections, which were cut on conventional microtome (Leova).

To determine the expression of HIF-1α 'Hipoxia induciblefactor-1α', we used the monoclonal antibodies Monoclonal anti-HIF-1α (Dako), (mouse IgG2b isotope) 120 kDa, diluted in PBS (Sigma) 'phosphate buffer saline', pH 7.4. Concentrations 0.5 to 1.0 µg/ml were used.

To determine the expression of HIF-1ß, 'Hipoxia inducibleFactor-1ß', we used the monoclonal antibodies Monoclonal anti-HIF-1ß (Dako), (mouse IgG1b isotope) 92 kDa, diluted in PBS (Sigma), pH 7.4. Concentrations of 0.5 to 1.0 µg/ml were used. An evaluation was performed using a light microscope (Leica), and the positivity was marked in the range 0–3+.

Statistics

Results are presented as a ± average error of mean (SEM). The t-test was used. Statistically significant differences were seen at the level of $P < 0.05$. All results are processed in Excel (MSOffice).

RESULTS

Ischemic myocardial resistance in mice with UPC2 deletion

Given the role of UCP in the production of oxygen radicals, we hypothesized that mice with a deletion of this gene are more sensitive to ischemic damage. However, this assumption was not confirmed (Table 2 and Figure 1), and the damage was almost identical. In both groups, the range of ischemic damage ranged from 18% of UCP2 and 16% B6 of the size of the left ventricle. Because of the fact that the extent of the damage was relatively small, we have extended the ischemic period from 45 min to 55 min (Table 3 and Figure 2); a 10 min extension of ischemia resulted in a 45% increase in heart attacks (44% of UCP2 and 47% B6). Even in this situation, we have not seen any difference in the size of myocardial infarction between the UCP 2 (-/-) and B6 controls.

ROS production

An ROS increase was demonstrated in the extended duration of ischemia and then reperfusion. We showed increased MDA and LFP production in both ischemia and subsequent reperfusion, which suggests the release of free radicals from degraded FA (fatty acids).

ROS production during ischemia

The increase in MDA as a degradation product of the initial MC during the duration of ischemia was most pronounced in the 30th minute of the ischemia's duration. Then the production of MDA started to decline slightly (Table 4).

As a final product of the degradation of MC, LFP-production (Table 5) was analyzed from sample No. 1, where we detected the largest increase in MDA again in 30th minute of the duration of ischemia at the (emission/excitation) 290/350 nm spectrum.

ROS production during reperfusion

The increase in MDA during reperfusion was most pronounced in the 30th minute. Thereafter, the MDA production started to decline slightly (Table 6).

The LFP-production (Table 7) was analyzed from sample No. 2, where we detected the largest increase in MDA in the 40th minute of reperfusion's duration at the (emission/excitation) 290/350 nm spectrum.

Table 4. Growth of MDA in ischemia

Duration of ischemia/min	Sample 1	Sample 2	Sample 3
0	2.6 ± 0.16	1.4 ± 0.05	1.8 ± 0.6
20	4.1 ± 0.08	2.1 ± 0.3	2.1 ± 0.02
30	4.5 ± 0.05	3.1 ± 0.56	2.7 ± 0.34
45	3.1 ± 0.11	2.3 ± 0.32	2.1 ± 0.54
Growth MDA (%)	73	121	50

Table 5. The concentration of fluorophores in ischemia-sample 1

Duration of ischemia/min	290/350 nm	340/410 nm	395/470 nm
0	1.17 ± 0.4	1.33 ± 0.08	0.88 ± 0.25
20	1.19 ± 0.12	1.31 ± 0.03	0.67 ± 0.48
30	3.3 ± 0.19	2.14 ± 0.06	0.89 ± 0.08
45	1.86 ± 0.34	3.09 ± 0.07	0.84 ± 0.14

Table 6. The increase in MDA duration of reperfusion

Duration of reperfusion/min	Sample 1	Sample 2	Sample 3
0	8 ± 0.05	5.8 ± 0.12	6.9 ± 0.21
20	8.2 ± 0.08	10.6 ± 0.18	9.4 ± 0.19
30	11.2 ± 0.07	12.8 ± 0.20	11.9 ± 0.14
40	6.7 ± 0.03	5.2 ± 0.09	7.1 ± 0.34
60	6.3 ± 0.11	7.6 ± 0.18	5.4 ± 0.27
Growth MDA (%)	40	120	73

Table 7. The concentration of fluorophores in the duration of reperfusion-sample 1

Duration of reperfusion/min	290/350 nm	340/410 nm	395/470 nm
0	1.6 ± 0.08	1.4 ± 0.09	1.8 ± 0.58
20	0.7 ± 0.04	1.2 ± 0.52	1.5 ± 0.15
30	5.7 ± 0.05	2.3 ± 0.43	2.5 ± 0.17
40	6.6 ± 0.06	2 ± 0.27	2.3 ± 0.08
60	0.5 ± 0.12	1 ± 0.63	0.7 ± 0.72

HIF-1α and ß protein expression

The results confirmed the expression of HIF-1-ß throughout the myocardium, both in hypoxic and normoxic.

HIF-1-α has been demonstrated only with myocardial hypoxia, and classification corresponded to grade 3 +, (results not shown).

DISCUSSION

UCP and coronary resistance

Uncoupling proteins, which are located on the inner mitochondrial membrane, are causing partial dissipation of the electrochemical proton gradient and the membrane's potential decline. This function is best documented in the case of UCP1, which is expressed exclusively in brown adipose tissue, its main role being to create heat (Ricquier et al., 2000). UPC 2 is located in the myocardium. Its role is, in comparison with UCP1, much less studied. Recently some studies have dealt with the UCP (including UCP2) in connection with the protection of tissues and cells from oxidative stress. It turns out that UCP2 is induced and activated by oxidative stress (Echtay et al., 2002), and its function leads to the inhibition of ROS production (Teshima et al., 2003, Ishizawa et al., 2006). So, there is feedback suggesting that UCP regulate the production of ROS. ROS production depends on the value of the mitochondrial membrane's potential – an increase stimulates the production of ROS, and a decrease due to a slight uncoupling reduces ROS production. The inner mitochondrial membrane's hyperpolarization occurs in reperfusion tissue that was exposed to an ischemic insult, and this situation is associated with a large increase in ROS, which, at this stage, has harmful effects and contributes to ischemic damage. The slight uncoupling of oxidative phosphorylation and the UPC-induced decrease in the low membrane's potential can thus have a protective effect, which provides an explanation for the reduction of oxidative stress in reperfusion. There are several articles which, in accordance with this notion that UCP may protect the heart against ischemia-reperfusion damage, such as Hoerter et al. (2004), found that the hearts of transgenic mice expressing a large amount of UCP1, were more resistant to damage than control mice hearts, which showed a much better resistance to functional contractility during reperfusion. An increased expression of UCP2 in cardiomyocytes inactivates the formation of ROS, preventing calcium overload and delaying cell death (Teshima et al., 2003). Furthermore, it was shown that the late phase of ischemic preconditioning is associated with the induction of UCP2 and UCP3 in the myocardium (McLeod et al., 2005).

Our results, however, did not confirm the above studies. We were using mice with an inactivated UCP2 gene, whose hearts showed reduced tolerance to ischemia-reperfusion damage compared to the control B6 strain. Based on the results of the above studies, we expected that the lack of UCP2 would show increased susceptibility to the damage, which did not happen. The size of the infarction induced by a global ischemia and reperfusion *in vitro* did not significantly differ in both groups, not even after the extension of ischemia and an increase in the proportion of necrotic tissue.

We do not have an explanation for this finding, but we can speculate several possible reasons.

One of the possibilities is, that although UCP2 is important for ischemic resistance, its absence in transgenic mice is compensated by another mechanism. In these hearts, there could be, for example, increased expression of UCP3, which may also participate in cardioprotection (McLeod et al., 2005) and could fulfill the function of UCP2. However, we cannot prove this option, because we have not measured the expression levels of UCP3. Another theoretical possibility is that the UCP2 (-/-) mice have an increase in the expression of or the activity of mitoK ATP channels, which can also cause mild uncoupling and decrease mitochondrial membrane potential (Huppertz et al., 2001), thus masking the adverse effects of the loss of UCP2. As a result of the activation of the mitoK ATP channels, coronary resistance would increase, which would just offset the deterioration in resistance caused by the absence of UCP2.

We cannot exclude the possibility that UCP2 plays a significant role in terms of durability in ischemic mouse hearts under the given conditions. There are currently no other studies of this model which deal with the heart, but we must take into account studies dealing with the possible neuroprotective role of UCP2. Most of them showed an increased expression of UCP2 in the brain under conditions of oxidative stress, which may not be directly related to resistance to damage (Cannon et al., 2006).

The current findings do not clearly interpret the role of UCP2 in coronary resistance, and it is necessary to conduct further studies.

ROS during ischemia and subsequent reperfusion

The production of ROS occurs during ischemic attacks, but also during subsequent reperfusion. The question is to what extent can the production of ROS damage our own sarkolemu cardiomyocytes. From our achievements, we have demonstrated a significant incidence of subsequent reperfusion. At the same time, however, it is evident, as demonstrated experimentally in the growth of tumors, that the increased incidence of ROS stimulates the expression of HIF-1α, resulting in increased tumor vascularization (Stanek et al., 2010). This aspect could be, in contrast to tumors, highly cardioprotective.

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STANĚK, L. – FUČÍKOVÁ, A. – LÍSOVÁ, S. (Univerzita Karlova v Praze, 1. lékařská fakulta, Praha, Česká republika; Česká zemědělská univerzita v Praze, Fakulta agrobiologie, potravinových a přírodních zdrojů, Praha, Česká republika)

Studie ischemické odolnosti myšího myokardu a radikálového poškození

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Srdce je striktně aerobní orgán s náročným enerpatologie, getickým metabolizmem. Pro jeho fyziologickou funkci je nezbytný stálý přísun kyslíku. Při trombotizaci koronární tepny nebo stenóze, dojde k poškození myokardu v důsledku hypoxie. Závažnost poškození je závislá jednak na době trvání ischemie, tak na odolnosti myokardu vůči nedostatku kyslíku. Odolnost myokardu je podmíněna celou řadou mechanizmů a to jak na biochemické tak molekulární úrovni. Cílem této práce bylo prokázat odolnost zvířat UCP2(-/-) k ischemickému poškození – což se nám nepotvrdilo. A dále prokázat poškození volnými radikály a expresi HIF v době trvání ischemie. Radikálové poškození i exprese HIF byla plně prokázána v době trvání ischemie.

myokard; ischemie; reperfuze; volné radikály; odpřahovací proteiny

Contact Address:

RNDr. Ing. Bc. Libor Staněk, Univerzita Karlova v Praze, 1. lékařská fakulta, Ústav patologie, Studničkova 2, 128 00 Praha 2, Česká republika, tel:+420 732 760 265, e-mail: stanek.libor@seznam.cz